ABSTRACTS SUBMITTED FOR THE 2005 MEETING OF THE SOCIETY FOR GLYCOBIOLOGY

November 9–12, 2005
Boston, Massachusetts

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PROGRAM OVERVIEW

Wednesday, November 9	8:30 am – 5:00 pm	**SATELLITE MEETING I** **Bioinformatics in Glycomics: An Integrated Informatics Approach to Glycans.** Organized by Ram Sasisekharan and Rahul Raman, *MIT, Cambridge, MA*
	8:30 – 8:40 am	Welcome and Introduction by Organizers
	8:40 – 9:10 am	**SESSION I: GLYCAN ANALYSIS: FROM TISSUE TO FINE STRUCTURE ON GLYCOPROTEINS AND GLYCOLIPIDS** Chromatographic Methods for Glycan Sequencing Pauline Rudd, *University of Oxford, Oxford, UK*
	9:10 – 9:40 am	Structural Determination of Oligosaccharide Disease Markers by Infrared Multiphoton Dissociation Carlito Lebrilla, *University of California, Davis, CA*
	9:40 – 10:10 am	Glycomics Profiling with Isotopic Tags Using an LC/MS/MS Platform Joe Zaia, *Boston University School of Medicine Boston, MA*
	10:10 – 10:25 am	Break and Exhibits
	10:25 – 10:55 am	Nano-NMR Analysis of Glycans Isolated from Tissues Herman van Halbeek, *University of California, Berkeley, CA*
	10:55 – 11:25 am	FT-ICR MS/MS Techniques for Glycomics Carol Nilsson, *Florida State University, Tallahassee, FL*
	11:25 – 11:55 am	Carbohydrate Sequencing: Fracturing, Filing, Fusion Vern Rienhold, *University of New Hampshire, Durham, NH*
	11:55 – 12:10 am	Closing Session I [additional questions]
	12:10 – 1:30 pm	Lunch
		SESSION II: DATA INTEGRATION IN GLYCOMICS: CHALLENGES AND IMPLEMENTATION STRATEGIES
	1:30 – 2:00 pm	Informatics Approach to Glycomics: Strategies of the Consortium for Functional Glycomics Rahul Raman, *MIT, Cambridge, MA*
	2:00 – 2:30 pm	SWEET-DB2 Willi von der Lieth, *German Cancer Research Center, Heidelberg, Germany*
	2:30 – 3:00pm	KEGG Bioinformatics Tools for Glycomics Minoru Kanehisa, *Kyoto University, Kyoto, Japan*
	3:00 – 3:30 pm	Semantic Knowledge Integration in Glycomics William York, *University of Georgia, Athens, GA*
	3:30 – 3:45 pm	Break and Exhibits
	3:45 – 4:45 pm	**LARGE SCALE RESEARCH INITIATIVES: PANEL DISCUSSION:** (James Paulson, *The Scripps Research Institute, La Jolla, CA*; Naoyuki Taniguchi, *Osaka University, Osaka, Japan*; Michael Pierce, *University of Georgia, Athens, GA*) Each panel member makes a 10 minute presentation on the key aspects of their initiatives in the context of glycan analysis and data integration.
	4:45 – 5:00 pm	**MEETING SUMMARY** Ram Sasisekharan and Rahul Raman, *MIT, Cambridge, MA*

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Wednesday, November 9	8:30 am – 4:30 pm	**SATELLITE MEETING II** **Therapeutic Recombinant Glycoproteins – Production, Purification and Analytical Methods.** Organized by Shekar Ganesa, *Genzyme, Framingham, MA* and Joesph Siemiatkoski, *Biogen Idec, Cambridge, MA*
	8:30 – 8:40 am	Welcome and Introduction by Organizers
	8:40 – 9:10 am	**SESSION I: SYSTEMS FOR THERAPEUTIC RECOMBINANT GLYCOPROTEIN PRODUCTION** A Comparison Between the Glycosylation of Monoclonal Antibodies Produced in Tissue Culture and in the Milk of Transgenic Animals Harry Meade, Senior V.P. R&D, *GTC Biotherapeutics, Framingham, MA*
	9:10 – 9:40 am	Fungal Protein Expression Systems with Humanized Secretory Pathways: The Answer to Therapeutic Protein Production? Tillman U. Gerngross, Chief Scientific Officer, *GlycoFi Inc., Lebanon, NH*
	9:40 – 10:10 am	Engineering Protein Glycosylation Pathways in Baculovirus-Insect Cell System Don Jarvis, Professor, *Department of Molecular Biology, University of Wyoming, Laramie, WY*
	10:10 – 10:30 am	Break and Exhibits
	10:30 – 11:00 am	**SESSION II: CONSIDERATIONS FOR THERAPEUTIC RECOMBINANT GLYCOPROTEIN ANALYSIS** USP's Glycoprotein and Glycan Analysis Procedural Standards Initiative Tina Morris, Team Leader Biotechnology & Biologics Complex Actives Division, *Department of Standards Development, U.S. Pharmacopeia, Rockville, MD*
	11:00 – 11:30 am	Validation of Carbohydrate Profiling Assays for Glycoproteins: Lessons Learned About Design of Experiments to Assess Accuracy and Linearity Donnie Pulliam, Research Scientist, Analytical Technology Product Quality Management, *Biogen Idec, Cambridge, MA*
	11:30 – 12:00 am	PNGase F Treatment of Glycoproteins: Evidence for Selective Release of Glycans Samnang Tep, Research Scientist, Analytical Technology Product Quality Management, *Biogen Idec, Cambridge, MA*
	12:00 – 1:30 pm	Lunch
	1:30 – 2:00 pm	**SESSION III: METHODS FOR THERAPEUTIC GLYCOPROTEIN GLYCAN ANALYSIS** Mapping Sites of O-linked and N-linked Glycosylation on Proteins Lance Wells, Assistant Professor of Biochemistry & Molecular Biology, *Complex Carbohydrate Research Center, University of Georgia, Athens, GA*
	2:00 – 2:30 pm	Oligosaccharide Structure Determination using Sequential Mass Spectrometry (MS^n): Fragment Analysis and Total Structure Andy Hanneman, Research Scientist, Reinhold Structural Glycomics Group, *University of New Hampshire, Durham, NH*
	2:30 – 3:00 pm	Profiling of N-linked Oligosaccharides on Monoclonal Antibodies with an Integrated Top–Down and Bottom–Up Mass Spectrometric Approach Kelly N. Toler, Research Scientist, *Wyeth BioPharma, Andover, MA*
	3:00 – 3:30 pm	Break
	3:30 – 4:00 pm	**SESSION IV: EFFECTS OF GLYCOSYLATION ON THERAPEUTIC GLYCOPROTEIN FUNCTION** Bioactivity of Cytokines by Altering Carbohydrate Content Angus M. Sinclair, Senior Scientist, *Amgen, Inc., Thousand Oaks, CA*
	4:00 – 4:30 pm	High Mannose Type *N*-linked Oligosaccharide Does Not Affect the Biological Function of a Monoclonal Antibody Wesley Wang, Senior Principal Scientist, Analytical Sciences, *Amgen, Inc., Seattle, WA*

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Wednesday, November 9, 2004
7:00 – 8:45 PM
NEW TECHNOLOGIES FOR GLYCOBIOLOGY
Anne Dell, Chair

Time *Abstract Number*

7:05 PM **New Technologies to Simplify Glycomics;** Anders Lohse, Rita Martins, Malene R. Jorgensen, Mads D. Sorensen and Ole Hindsgaul; *Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Valby-Copenhagen, Denmark* 1

7:30 PM **New Strategies for Glycan Modification and Derivatization and Enhancement of Glycan Arrays;** Baoyun Xia[1], Ziad S. Kawar[1], Tongzhong Ju[1], Richard A. Alvarez[1], Goverdhan P. Sachdev[2] and Richard D. Cummings[1]; *[1] Department of Biochemistry & Molecular Biology and the Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, [2] College of Pharmacy and the Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104* 2

7:55 PM **Understanding Carbohydrate Antigenicity: *Streptococcus agalactiae* (Type III) Versus *Streptococcus pneumoniae* (Type 14);** Renuka Kadirvelraj[1], Jorge Gonzalez-Outeiriño[1], Harold J. Jennings[2], Simon Foote[2] and Robert J. Woods[1]; *[1] Complex Carbohydrate Research Center, University of Georgia, 315 Riverbend Road, Athens, GA 30602, [2] Institute for Biological Sciences, National Research Council of Canada, 100 Sussex Drive, Ottawa, Ontario, Canada K1A 0R6* 3

8:20 PM **Noninvasive Imaging of Glycosylation *in vivo*;** Jennifer A. Prescher[1], Danielle H. Dube[1], Anderson Lo[1] and Carolyn R. Bertozzi[1,2,3]; *[1] Department of Chemistry, University of California, Berkeley, CA 94720, [2] Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, [3] Howard Hughes Medical Institute, University of California, Berkeley, CA 94720* 4

8:25 PM **A Profile HMM for Tree Structures to Locate Glycan Structure Profiles;** Kiyoko F. Aoki-Kinoshita, Nobuhisa Ueda, Hiroshi Mamitsuka, Susumu Goto and Minoru Kanehisa; *Bioinformatics Center, Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan* 5

8:30 PM **Analyses of Carbohydrate Recognition by Mammalian Sialic Acid-Binding Proteins of the Immune System, Siglecs, Using Microarrays of Lipid-Linked Oligosaccharide Probes;** Maria-Asuncion Campanero-Rhodes[1], Paul Crocker[2], Robert A. Childs[1], Wengang Chai[1] and Ten Feizi[1]; *[1] The Glycosciences Laboratory, Imperial College, Northwick Park and St. Mark's Campus, Harrow HA1 3UJ, UK, [2] Wellcome Trust Biocentre, School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK* 6

Thursday, November 10, 2004
8:30 – 10:00 AM
PROTEOGLYCAN FUNCTIONS
Jeffrey D. Esko, Chair

Time *Abstract Number*

8:30 AM **Sulfotransferases: Tuning Heparan Sulfate Functions in Neural Cell Migration and Development;** Jeremy Turnbull, Scott Guimond and Tarja Kinnunen; *School of Biological Sciences, University of Liverpool, Crown Street, Liverpool L69 7ZB, UK* 7

8:55 AM **HSPGs and Sorting of Retinal Axons in the Zebrafish Optic Tract;** Chi-Bin Chien; *Department of Neurobiology & Anatomy, University of Utah, 401 MREB, 20 North 1900 East, Salt Lake City, UT 84132* 8

9:20 AM **Proteoglycans in Axon Regeneration and Plasticity in the Adult CNS;** James W Fawcett; *Centre for Brain Repair, Cambridge University, Robinson Way, Cambridge CB2 2PY, UK* 9

9:45 AM **A Large Panel of Phage Display-Derived Human Antibodies Against Specific Glycosaminoglycan Epitopes: Versatile Tools for the Glycobiologist;** Guido J. Jenniskens and Toin H. van Kuppevelt; *Department of Matrix Biochemistry, University Medical Center Nijmegen, Nijmegen Center for Molecular Life Sciences, P.O. Box 9101, 6500 HB, Nijmegen, The Netherlands* 10

9:50 AM **Msulf1 and Msulf2 Differentially Modify Heparan Sulphate 6-O-Sulphation Patternin;** William Christopher Lamanna[1], Rebecca Baldwin[2], Cathy Merry[2] and Thomas Dierks[1]; *[1] Department of Biochemistry, University of Bielefeld, 33615 Bielefeld, Germany, [2] Department of Medical Oncology, University of Manchester, Christie Hospital NHS Trus, Wilmslow Road, Manchester 20 4BX, UK* 11

9:55 AM **Functions of Heparan Sulfate Proteoglycans and the Kallman Syndrome Protein KAL-1 in *Caenorhabditis elegans* Embryogenesis;** Martin L. Hudson[1], Tarja Kinnunen[2], Jeremy E. Turnbull[2] and Andrew D. Chisholm[1]; *[1] Department of Molecular, Cellular and Developmental Biology, University of California, Santa Cruz, CA 95064, [2] School of Biological Sciences, University of Liverpool, Crown Street, Liverpool L69 7ZB, UK* 12

Thursday, November 10, 2004
10:30 AM – 12:30 PM
EVOLUTION OF GLYCANS AND GLYCAN FUNCTION
Christopher West, Chair

Time *Abstract Number*

10:30 AM **The Basic Principles of N-Linked Protein Glycosylation;** Markus Aebi; *Institute of Microbiology, Department of Biology, Swiss Federal Institute of Technology (ETH), CH-8093 Zürich, Switzerland* 13

10:55 AM **Egghead and Brainiac are Essential for Glycosphingolipid Biosynthesis in vivo;** Hans H. Wandall[1], Sandrine Pizette[2], Johannes W. Pedersen[1], Heather Eichert[3], Steven B. Levery[3], Ulla Mandel[1], Stephen M. Cohen[2] and Henrik Clausen[1]; *[1] Faculty of Health Sciences, University of Copenhagen, Nørre Allé 20, 2200 Copenhagen N, Denmark, [2] European Molecular Biology Laboratory, Meyerhofstr 1, 69117 Heidelberg, Germany, [3] Department of Chemistry, University of New Hampshire, Durham, NH 03824* 14

11:20 AM **Structural and Evolutionary Aspects of Animal Lectins: Diversity in Glycan Recognition;** Gerardo R. Vasta; *Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Columbus Center Suite 236, 701 E Pratt Street, Baltimore, MD 21202* 15

11:45 AM **Regulation of Notch Signaling by Glycosylation;** Kenneth D. Irvine, Nicola Haines, Liang Lei, Tetsuya Okajima and Aiguo Xu; *Waksman Institute, Rutgers University, 190 Frelinghuysen Road, Piscataway, NJ 08904* 16

12:10 PM ***N*-Acetylglucosaminyltransferase I-Dependent *N*-Glycans are Involved in the Response of *Caenorhabditis elegans* to Bacterial Pathogens;** Harry Schachter[1,2], Hui Shi[1] and

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Andrew M. Spence[3]; *[1] Program in Structural Biology and Biochemistry, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8, [2] Department of Biochemistry, University of Toronto, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8, [3] Department of Molecular and Medical Genetics, University of Toronto, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8* 17

12:15 PM **O-GlcNAc Cycling Enzymes Modulate Life Span in *Caenorhabditis elegans*;** Olga Stuchlik[1], Mohammad M. Rahman[2], Edward T. Kipreos[2] and Lance Wells[1]; *[1] Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, [2] Department of Cellular Biology, University of Georgia, Athens, GA 30602* 18

12:20 PM **O-Glucosylation of Notch1 and its Significance in Notch Signaling;** Aleksandra Nita-Lazar, Rosemary Orhue and Robert S. Haltiwanger; *Department of Biochemistry and Cell Biology, Institute for Cell and Developmental Biology, Stony Brook University, Stony Brook, NY 11794-5215* 19

Thursday, November 10, 2004
4:00 – 6:00 PM
NEUROGLYCOBIOLOGY
Karen Colley, Chair

Time *Abstract Number*

4:00 PM **Polysialic Acid-Dependent Cell Migration is Essential for Mammalian Brain Development;** Minoru Fukuda; *The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037* 20

4:25 PM **Polysialic Acid is Essential to Control NCAM Functions During Mouse Development;** Birgit Weinhold[1], Iris Röckle[2], Martina Mühlenhoff[1], Ralph Seidenfaden[3], Herbert Hildebrandt[2] and Rita Gerardy-Schahn[1]; *[1] Zelluläre Chemie, Zentrum Biochemie, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, 30625 Hannover, Germany, [2] Institut für Zoologie, Universität Hohenheim, Garbenstr. 30, 70593 Stuttgart, Germany, [3] Institut de Biologie du Développement de Marseille, Campus de Luminy, 13288 Marseille 9, France* 21

4:50 PM **Glycosphingolipids in Nervous System Development, Stability, and Disease;** Richard L. Proia; *NIDDK, NIH, Bethesda, MD 20892* 22

5:15 PM **Human GM3 Synthase Deficiency: A Novel Form of Hereditary Childhood Epilepsy;** David A. Priestman[1], David C.A. Neville[1], Gabriele Reinkensmeier[1], Michael A. Simpson[2], Christos Proukakis[2], Michael Patten[2], Raymond A. Dwek[1], Terry D. Butters[1], Frances M. Platt[1] and Andrew H. Crosby[2]; *[1] Glycobiology Institute, Department of Biochemistry, South Parks Road, University of Oxford, Oxford OX1 3QU, UK, [2] Department of Medical Genetics, St. George's Hospital Medical School, University of London, Cranmer Terrace, London SW17 0RE, UK* 23

5:40 PM **Substrate Reduction Therapy Reduces Brain Ganglioside GM2 in Neonatal Sandhoff Disease Mice;** Rena C. Baek[1], Julie L. Kasperzyk[1], Frances M. Platt[2] and Thomas N. Seyfried[1]; *[1] Department of Biology, Boston College, Chestnut Hill, MA 02467, [2] Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, UK* 24

5:45 PM **Protein-Specific Polysialylation of NCAM by Polysialyltransferases;** Shalu Shiv Mendiratta, Nikolina Sekulic, Arnon Lavie and Karen J. Colley; *Department of Biochemistry and Molecular Genetics, University of Illinois at Chicago, College of Medicine, Chicago, IL 60607* 25

5:50 PM **Role of Sialyltransferase in the Nervous System Development of *Drosophila*;** Elena A. Repnikova[1], Kate Koles[1], Jarred Pitts[1], Christina Ramos[1], Eduardo J. Garza[1], Stylianos Kosmidis[2], Efthimios M.C. Skoulakis[2] and Vlad M. Panin[1]; *[1] Department of Biochemistry/Biophysics, Texas A&M University, College Station, TX 77843-2128, [2] Alexander Fleming Biomedical Research Center, Vari 16602, Greece* 26

Friday, November 11, 2004
8:15 – 9:45 AM
GLYCANS AND LECTINS IN PATHOGEN RECOGNITION
Tamara L. Doering, Chair

Time *Abstract Number*

8:15 AM **Genetic Analysis of Pathways Required for the Assembly of the Surface Glycocalyx Coat of the Protozoan Parasite *Leishmania* and Their Roles in the Infectious Cycle;** Stephen M. Beverley[1], Althea Capul[1], Kai Zhang[1] and Salvatore J. Turco[2]; *[1] Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63105, [2] Department of Biochemistry, University of Kentucky Medical School, Lexington, KY 40536* 27

8:40 AM **Lipophosphoglycan–Galectin Interactions Controlling Sand Fly Vector Competence for *Leishmania major*;** David Sacks[1], Shaden Kamhawi[1], Marcelo Ramalho-Ortigao[1], Phillip Lawyer[1] and Jesus Valenzuela[2]; *[1] Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, MD 20892, [2] Laboratory of Malaria and Vector Research, NIAID, NIH, Bethesda, MD 20892* 28

9:05 AM **Mannose 6-Phosphate Receptors and the Pathogenesis of Infections due to Varicella Zoster Virus;** Michael D. Gershon; *Department of Anatomy & Cell Biology, XXXX* 29

9:30 AM **Identification and Functional Characterization of a UDP-Glucose Pyrophosphorylase from *Leishmania major*;** Anne-Christin Lamerz[1], Barbara Kleczka[1], Martin Wiese[2], Francoise Routier[1], Ger van Zandbergen[3], Tamas Laskay[3], Werner Solbach[3] and Rita Gerardy-Schahn[1]; *[1] Department of Cellular Chemistry, Medizinische Hochschule Hannover, Hannover, Germany, [2] Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany, [3] Institute for Medical Microbiology and Hygiene, Innovations Campus Lübeck, Lübeck, Germany* 30

9:35 AM **Origin of the Galacturonic Acid Modifications to the Inner Core of *Rhizobium leguminosarum* Lipopolysaccharides;** Suparna Kanjilal, Shib S. Basu, Margaret I. Kanipes and C.R.H. Raetz; *Department of Biochemistry, Duke University Medical Center, Durham, NC 27710* 31

9:40 AM **Pathogen Capture in Water Using Glycoprotein Micelles;** Elaine H. Mullen[1], Baddr A. Shakhsheer[1], Jason J. Quizon[2], James C. Crookston[2], Miquel D. Antoine[2] and Juan Arroyo[1]; *[1] The MITRE Corporation, 7515 Colshire Drive, McLean, VA 22102, [2] Johns Hopkins*

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University Applied Physics Lab, 11000 Johns Hopkins Road, Laurel, MD 20723 32

Friday, November 11, 2004
10:15 AM – 12:15 PM
GLYCAN IMMUNOLOGY
Richard D. Cummings, Chair

Time	Abstract Number
10:15 AM	**Development of a Conjugate Vaccine Against *Haemophilus influenzae* Type B Based on Synthetic Antigens;** Vicente Verez-Bencomo[1], Violeta Fernandez-Santana[1], Eugenio Hardy[2], Maria Eugenia Toledo[3], Rene Roy[4], Maria C. Rodriguez[1], Arlene Rodriguez[2], Lazaro Heynngnezz[2], Alberto Baly[3], Mabel Izquierdo[2], Annette Villar[1], Yury Valdes[1], Karelia Kosme[2], Mercedes Deler[1], Manuel Montane[2], Ernesto Garcia[1], Alexis Ramos[1], Aristides Aguilar[2], Ernesto Medina[2], Gilda Toraño[3], Ivan Sosa[2], Ibis Hernandez[3], Raydel Martinez[3], Alexis Mussachio[2], Ania Carmenate[5], Lourdes Costa[2], Olga L. Garcia[2] and Luis Herrera[2]; *[1] Center for the Study of Synthetic Antigens, University of Havana, [2] Center for Genetic Engineering and Biotechnology, Havana, Cuba, [3] Institute of Tropical Medicine Pedro Kouri, Havana, Cuba, [4] Department of Chemistry, Université du Québec à Montréal, [5] Camaguey Public Health Center* 33
10:40 AM	**C-Type Lectins on Dendritic Cells: Antigen Receptors and Modulators of Immune Responses;** Y. van Kooyk, S. van Vliet, I. van Die and T.B.H. Geijtenbeek; *Department of Molecular Cell Biology and Immunology, VU University Medical Center Amsterdam, v.d. Boechorststraat 7, 1081 BT Amsterdam, The Netherlands* 34
11:05 AM	**Structural Basis of DC-SIGN Ligand Specificity;** Hadar Feinberg[1], Yuan Guo[2], Edward Conroy[2], Daniel Mitchell[2], Richard Alvarez[3], Ola Blixt[4], Maureen Taylor[2], Kurt Drickamer[2] and William Weis[1]; *[1] Department of Structural Biology and Molecular & Cellular Physiology, Stanford University School of Medicine, 299 Campus Drive West, Stanford, CA 94305, [2] Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, UK, [3] Department of Biochemistry & Molecular Biology, University of Oklahoma Health Science Center, Oklahoma City, OK 73104, [4] Department of Molecular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037* 35
11:30 AM	**Glycan Processing and Presentation: The New MHC Class II Pathway;** Brian A. Cobb; *10900 Euclid Avenue, Cleveland, OH 44106-7288* 36
11:55 AM	**Exogenous and Endogenous Glycolipid Antigens Activate NKT Cells During Microbial Infections;** Albert Bendelac; *5841 South Maryland Avenue, MC 1089, Chicago, IL 60637* 37
12:30 – 2 PM	SPECIAL LUNCHTIME DISCUSSION: **Assignment and Review of Glycobiology-Related Grant Applications by the National Institutes of Health;** Donald Schneider; *Division of Molecular and Cellular Mechanisms, Center for Scientific Review, NIH, Rockville, MD*
4:00 PM	SOCIETY FOR GLYCOBIOLOGY BUSINESS MEETING
4:30 PM	KARL MEYER AWARD LECTURE
7:00 PM	CONFERENCE BANQUET

Saturday, November 12
8:30 – 10:00 AM
N-LINKED GLYCAN FUNCTIONS
Michael Pierce, Chair

Time	Abstract Number
8:30 AM	**Gains of Glycosylation Comprise an Unexpectedly Large Group of Pathogenic Mutations;** Jean-Laurent Casanova; *INSERM U550, Faculté de Médecine Necker, 156 rue de Vaugirard, 75015 Paris, France* 38
8:55 AM	**Hexosamine, *N*-Glycans, and Cytokine Signaling—A Regulatory Network;** Ken Lau[1], Emily A. Partridge[1], Pam Cheung[1], Rick Mendelsohn[1], Cristina I. Silvescu[2], Vern N. Reinhold[2] and James W. Dennis[1]; *[1] Samuel Lunenfeld Research Institute, Mount Sinai Hospital, University of Toronto, 600 University Avenue, Toronto, Ontario, Canada M5G 1X5, [2] Department of Chemistry, University of New Hampshire, Durham, NH 03824* 39
9:20 AM	**Dietary and Genetic Control of Pancreatic Beta Cell Glucose Transporter-2 Glycosylation Promotes Insulin Secretion in Suppressing the Pathogenesis of Type 2 Diabetes;** Kazuaki Ohtsubo[1], Shinji Takamatsu[2,3], Mari T. Minowa[2], Aruto Yoshida[2], Makoto Takeuchi[2] and Jamey D. Marth[1]; *[1] Howard Hughes Medical Institute and Department of Cellular and Molecular Medicine, 9500 Gilman Drive, University of California at San Diego, La Jolla, CA 92093, [2] Central Laboratories for Key Technology, Kirin Brewery Co. Ltd., 1-13-5, Fuku-ura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan, [3] Biomedical Imaging Research Center, University of Fukui, 23-3 Shimoaizuki, Matsuoka, Yoshida, Fukui 910-1193, Japan* 40
9:45 AM	**N-Glycosylation-Dependent Apical Trafficking of the Sialomucin Endolyn in Polarized Epithelial Cells;** Beth A. Potter[1], Kelly M. Weixel[1], Jennifer R. Bruns[1], Gudrun Ihrke[2] and Ora A. Weisz[1]; *[1] Renal-Electrolyte Division, Department of Medicine, University of Pittsburgh, Pittsburgh, PA, [2] Clinical Biochemistry, Cambridge Institute for Medical Research, University of Cambridge, Cambridge CB2 1TN, UK* 41
9:50 AM	**HIV Envelope Glycoproteins: Modification of Glycans and Glycan-Dependent Folding Pathways Provide New Targets for Vaccine Design and Anti-Viral Therapies;** Pauline M. Rudd, Christopher S. Scanlan, Stephanie Pollock and Raymond A. Dwek; *Glycobiology Institute, University of Oxford, South Parks Road, Oxford OX1 3QU, UK* 42
9:55 AM	**Characterization of a Human Core-Specific Lysosomal α1-6Mannosidase Involved in *N*-Glycan Catabolism;** Kelley W. Moremen[1,2], Chaeho Park[1,2], Lu Meng[2], Leslie Stanton[1], Robert E. Collins[1], Steven Mast[1,2], Yaiobing Yi[2] and Heather Strachan[1,2]; *[1] Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, [2] Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602* 43

Saturday, November 12, 2004
10:30 AM – 12:30 PM
GLYCANS IN IMMUNE SYSTEM REGULATION
Jamey D. Marth, Chair

Time	Abstract Number
10:30 AM	**CD22: A Multifunctional Lectin that Regulates B Lymphocyte Survival and Signal Transduction;**

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Thomas F. Tedder, Jonathan C. Poe and Karen M. Haas; *Department of Immunology, Duke University Medical Center, P.O. Box 3010, Durham, NC 27710* 44

10:55 AM **CD22-Ligand Interactions in BCR Signaling;** Brian E. Collins[1], Shoufa Han[1], Brian A. Smith[2], Per Bengtson[1], Hiroaki Tateno[1], Nicolai Bovin[3], Ola Blixt[1] and James C. Paulson[1]; *[1] Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037, [2] The Sidney Kimmel Cancer Center, La Jolla, CA 92037, [3] Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Ul. Miklukho-Maklaya, 16/10, 117871 GSP-7 Moscow V-437, Russia* 45

11:20 AM **Siglec-8: An Inhibitory Receptor on Eosinophils and Mast Cells;** Bruce Bochner[1], Hidenori Yokoi[1], Esra Nutku[1], Paul Crocker[2], Nicholai V. Bovin[3], Ronald L. Schnaar[4] and Nives Zimmermann[5]; *[1] Division of Allergy and Clinical Immunology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, [2] Division of Cell Biology and Immunology, The Wellcome Trust Biocentre, University of Dundee, Dundee, UK, [3] Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia, [4] Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD, [5] Division of Allergy and Immunology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH* 46

11:45 AM **Probing the Functions of Siglecs Expressed on Myeloid Cells;** Paul R. Crocker, Cornelia Oetke and Tony Avril; *Division of Cell Biology and Immunology, The Wellcome Trust Biocentre, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK* 47

12:10 AM **Regulation of Intracellular Immune Signal Transduction by Protein Glycosylation: Setting Thresholds for B Lymphocyte Activation and Immunoglobulin Homeostasis;** Pam Grewal, Mark Boton, Kevin Ramirez, Akira Saito, Ryan Green, Kazuaki Ohtsubo, Daniel Chui and Jamey Marth; *Department of Cellular and Molecular Medicine, Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA 92093* 48

12:15 AM **P-Selectin Expression in the Thymus is Required for Importation of T-Cell Progenitors and is Modulated by Thymic T-Cell Production;** Klaus Gossens, Stephane Y. Corbel, Fabio M. Rossi and Hermann J. Ziltener; *The Biomedical Research, Centre University of British Columbia, 2222 Health Sciences Mall, Vancouver, British Columbia, Canada V6T 1Z3* 49

12:20 AM **Lymphocyte Trafficking in Mice Deficient in MECA-79 Antigen: Analysis of Core 1 Extension Enzyme (β1,3-*N*-Acetylglucosaminyltransferase-3) Knockout Mice;** Junya Mitoma[1], Jean-Marc Gauguet[2], Bronislawa Petryniak[3], Hiroto Kawashima[1], Patrick Schaerli[2], John B. Lowe[3], Ulrich H. von Andrian[2] and Minoru Fukuda[1]; *[1] Glycobiology Program, Cancer Research Center, The Burnham Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037, [2] Department of Pathology, Howard Hughes Medical Institute, The University of Michigan Medical School, Ann Arbor, MI 48109, [3] CBR Institute for Biomedical Research, Harvard Medical School, Boston, MA 02115* 50

Saturday, November 12, 2004
4:00 – 6:00 PM
GLYCANS IN DISEASE
Linda G. Baum, Chair

Time *Abstract Number*

4:00 PM **Inactivation of the Golgi CMP-Sialic Acid Transporter Gene Reveals a New Human Typeii Congenital Disorder of Glycosylation (CDGIIf);** Rosella Mollicone[1], Thierry Dupre [2], Jean-Jacques Candelier [1], Ivan Martinez-Duncker [1], Gil Tchernia[3] and Rafael Oriol[1]; *[1] INSERM U504, Hospital Paul Brousse, University of Paris-Sud XI, Villejuif Cedex 94807, France, [2] Laboratoire Biochimie A, CHU Xavier Bichat, Paris Cedex 75877, France, [3] Laboratoire d'hematologie et Immunologie, CHU Kremlin-Bicetre, 94275 Cedex, France* 51

4:25 PM **Functional Domains in Dystroglycan Processing and Laminin Binding;** Kevin P Campbell; *HHMI, Department of Physiology and Biophysics, University of Iowa, Iowa City, IA 52242* 52

4:50 PM **Galectins and the Inflammatory Response;** Fu-Tong Liu; *Department of Dermatology, University of California Davis, 4860 Y Street, Sacramento, CA 95817* 53

Saturday, November 12, 2004
5:15 – 00 PM
PRESIDENT'S LECTURE
Ronald L. Schnaar, Chair

Time *Abstract Number*

5:15 PM **Hepatic Clearance of Triglyceride Rich Lipoproteins Depends on Heparan Sulfate;** Jennifer M. MacArthur[1], Lianchun Wang[1], Joseph R. Bishop[1], Andre Bensadoun[2], Joseph L. Witzum[3] and Jeffrey D. Esko[1]; *[1] Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California at San Diego, La Jolla, CA 92093, [2] Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853, [3] Department of Medicine, University of California at San Diego, La Jolla, CA 92093* 54

Thursday, November 10
2:00 – 4:00 PM
POSTER SESSION 1
***Topics:* New Technologies for Glycobiology and Evolution of Glycans and Glycan Function**

Poster Number *Abstract Number*

1 **Noninvasive Imaging of Glycosylation *in vivo*;** Jennifer A. Prescher[1], Danielle H. Dube[1], Anderson Lo[1] and Carolyn R. Bertozzi[1,2,3]; *[1] Department of Chemistry, University of California, Berkeley, CA 94720, [2] Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, [3] Howard Hughes Medical Institute, University of California, Berkeley, CA 94720* 4

2 **A Profile HMM for Tree Structures to Locate Glycan Structure Profiles;** Kiyoko F. Aoki-Kinoshita, Nobuhisa Ueda, Hiroshi Mamitsuka, Susumu Goto and Minoru Kanehisa; *Bioinformatics Center, Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan* 5

3 **Analyses of Carbohydrate Recognition by Mammalian Sialic Acid-Binding Proteins of the Immune System, Siglecs, Using Microarrays of Lipid-Linked Oligosaccharide Probes;**

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Maria-Asuncion Campanero-Rhodes[1], Paul Crocker[2], Robert A. Childs[1], Wengang Chai[1] and Ten Feizi[1]; *[1] The Glycosciences Laboratory, Imperial College, Northwick Park and St. Mark's Campus, Harrow HA1 3UJ, UK, [2] Wellcome Trust Biocentre, School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK* 6

4 **Effect of *N*-Acetylmannosamine Kinase Gene Deletion for the Synthesis of Cytidine 5′-Monophosphate *N*-Acetylneuraminic Acid in *Escherichia coli* K12;** Hyun Kim, Woo Jong Ju, Yeo Jin Son and Nam Soo Han; *Department of Food Science and Technology, Research Center for Bioresource and Health, Chungbuk National University, Cheongju, Chungbuk 463-763, South Korea* 55

5 **Synthesis of New Nonnatural Carbohydrates and Analogs Proposed as Glycomimetics;** Elena Vismara[1], Annamaria Naggi[2], Roberto Santarsiero[2] and Cristina Carpanese[1]; *[1] Dipartimento di Chimica, Ing. Chimica e Materiali "G.Natta" Politecnico,Via Mancinelli 7, I-20131 Milan, Italy, [2] Istituto di Ricerche Chimiche e Biochimiche "G. Ronzoni", Via G. Colombo 81, Milan, Italy* 56

6 **O-Linked Glycosylation in Maize-Expressed Human IgA1;** Anton S. Karnoup, Virgil Turkelson and W.H. Kerr Anderson; *The Dow Chemical Company, Midland, MI 48667* 57

7 **Developing of Lectin-Enhanced Laser Adsorption/Ionization Microarray;** Yaron Tzur and Rachel Glicklis; *Department of Biotechnology and the Center for Glycobiology, National Institute of Biotechnology, Ben-Gurion University of the Negev, Beer-Sheva, Israel* 58

8 **Specificity and Inhibition of Beta1,4-Galactosyltransferase;** Inka Brockhausen[1], Melinda Benn[1], John Schutzbach[1], Hans Paulsen[2] and Walter Szarek[3]; *[1] Department of Medicine, Department of Biochemistry, Queen's University, Kingston General Hospital, Angada 1, Kingston, Ontario, Canada K7L 2V7, [2] Department of Organic Chemistry, Edmund-Siemers-Allee 1, D-20146 Hamburg, Germany, [3] Department of Chemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6* 59

9 **Development of New Chemistries for Labeling Glycans *in vivo*;** Jeremy M. Baskin[1], Nicholas J. Agard[1], Jennifer A. Prescher[1], Anderson Lo[1] and Carolyn R. Bertozzi[1,2,3]; *[1] Department of Chemistry, University of California, Berkeley, CA 94720, [2] Department of Molecular and Cell Biology, CA 94720, [3] Howard Hughes Medical Institute, University of California, Berkeley, CA 94720* 60

10 **Chemical Synthesis of Fluorinated UDPGalNAc Analogs as Probes for the Study of the Retaining Polypeptidyl GalNAc Transferases;** Carlos A. Valdez[1] and Carolyn R. Bertozzi[1,2,3]; *[1] Department of Chemistry, University of California, Berkeley, CA 94720, [2] Department of Molecular and Cell Biology, CA 94720, [3] Howard Hughes Medical Institute, University of California, Berkeley, CA 94720* 61

11 **Novel Assays for Cell-Binding Studies as well as for Identification of Compounds that Inhibit or Enhance Cell Attachment;** Smita Yadav, Lothar Goretzki, Jue Wang and Mo Saedi; *EMD Biosciences Inc., 10394 Pacific Center Court, San Diego, CA 92121* 62

12 **Immobilization of 2-Aminopyridine-Oligosaccharides: Bridging Structural Analysis and Glycoarrays;** Nadezhda V. Shilova, Tatiana V. Pochechueva and Nicolai V. Bovin; *Shemyakin&Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya 16/10, 117997 Moscow, Russia* 63

13 **Oligosaccharide Preferences of β1,4-Galactosyltransferase-I: Crystal Structures of Met340His Mutant of Human β1,4-Galactosyltransferase-I with a Pentasaccharide and Trisaccharides of the *N*-Glycan Moiety;** Velavan Ramasamy[1], Boopathy Ramakrishnan[1,2], Elizabeth Boeggeman[1,2], Daniel M. Ratner[3], Peter H. Seeberger[3,4] and Pradman K. Qasba[1]; *[1] Structural Glycobiology Section, CCR Nanobiology Program, NCI-NIH, Frederick, MD 21702, [2] Basic Research Program, SAIC Frederick Inc., CCR Nanobiology Program, NCI-NIH, Frederick, MD 21702, [3] Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, [4] Laboratorium fur Organische Chemie, ETH Honggerberg/HCI F 315, Wolfgang-Pauli-Strasse, 10 CH-8093, Zurich, Switzerland* 64

14 **Synthesis and Analytical Evaluation of New Oligosaccharide Tags;** Nandkishor Chindarkar and Andreas H. Franz; *Department of Chemistry, University of the Pacific, College of the Pacific, 3601 Pacific Avenue, Stockton, CA 95211* 65

15 **Biodegradation of Xanthan by Newly Isolated *Sphingomonas* sp. XT-11 and Biological Activity of Xantho Oligosaccharides;** Liu Han, Huang Chengdong, Bai Xuefang and Du Yuguang; *Dalian Institute of Chemical Physics 1805 Group, Chinese Academy of Science, Liaoning Dalian 116023, People's Republic of China* 66

16 **The Effects of Elevated Ammonium on Gene Expression in CHO Cells Producing a Glycoprotein;** Peifeng Chen[1] and Sarah W. Harcum[2]; *[1] Department of Chemical and Biomolecular Engineering, [2] Department of Bioengineering* 67

17 **Heteronuclear NMR Methods for Structural Studies of Intact Asparagine-Linked Glycoproteins;** Timothy M. Logan[1,2], Wendy J. Walton[1] and Agnieska Kasprzak[2]; *[1] Institute of Molecular Biophysics, Kasha Laboratory, Florida State University, Tallahassee, FL 32306, [2] Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL 32306* 68

18 **Monitoring Tunicamycin-Induced Apoptosis by Fourier Transform Infrared Spectroscopy;** Maria O. Longas[1], Dipak K. Banerjee[2] and William Xie[1]; *[1] Department of Chemistry and Physics, Purdue University Calumet, 2200 169th Street Hammond, IN 46323, [2] Department of Biochemistry, University of Puerto Rico School of Medicine, San Juan, PR 00936* 69

19 **MS^n Fragmentation of Glycosaminoglycan Oligosaccharides: Identification of Sequence- and Isomerism-Informing Fragment Ions;** Toshikazu Minamisawa[1,2], Kiyoshi Suzuki[2], Hiroshi Maeda[2], Satoshi Shimokata[2,3], Nobuo Sugiura[2,3], Koji Kimata[3] and Jun Hirabayashi[1]; *[1] Glycostructure Analysis Team, Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), Central-2-12, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan, [2] Central Research Laboratories, Seikagaku Corporation, 3-1253 Tateno, Higashi-Yamato, Tokyo 207-0021, Japan, [3] Institute for the Molecular Science of Medicine, Aichi Medical University, Yazako, Nagakute, Aichi 480-1195, Japan* 70

20 **Construction of Advanced gLectin Map h by Comprehensive Interaction Analysis Between Lectins and PA Oligosaccharides Using Frontal Affinity Chromatography;** Sachiko Nakamura[1], Noboru Uchiyama[1], Junko Kominami[1,2], Masugu Kamei[2], Yoriko Takahashi[3], Yusuke Osaka[4], Shuzo Maruyama[4] and Jun Hirabayashi[1]; *[1] Research Center for Glycoscience, AIST, Tsukuba 305-8568, Japan, [2] J-OIL MILLS, Yokohama 245-0064, Japan, [3] Mitsui Knowledge Industry,*

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Tokyo 164-8721, Japan, [4] SHIMADZU Corp., Kyoto 604-8511, Japan 71

21 **Alignment of Low-Complexity Glycoprotein Sequences: Composition-Modified Scoring Matrices Allow Alignment of Yeast Cell Wall Proteins;** Juan Coronado[1], Oliver Attie[1], Susan L. Epstein[2], Wei-Gang Qiu[1] and Peter N. Lipke[1]; *[1] Department of Biology, Hunter College of CUNY, New York, NY 10021, [2] Department of Computer Science, Hunter College of CUNY, New York, NY 10021* 72

22 **Further Developments in a Lectin Microarray: Complex Systems and Quality Control;** Kanoelani T. Pilobello and Lara K. Mahal; *Department of Chemistry and Biochemistry, University of Texas – Austin, 1 University Station A5300, Austin, TX 78712* 73

23 **Conformational Aspects of GalNAc Transferase Glycopeptide Substrates;** Andrew Borgert[1], Mian Liu[1,2], George Barany[2] and David Live[1]; *[1] Department of Biochemistry, Molecular Biology and Biophysics, Universty of Minnesota, Minneapolis, MN 55455, [2] Department of Chemistry, University of Minnesota, Minneapolis, MN 55455* 74

24 **Application of 2Dical, a New Platform for Large-Scale Proteomics, to Glycoprotein Analysis;** Ono Masaya, Setsuo Hirohashi and Tesshi Yamada; *Chemotherapy Division and Cancer Proteomics Project, National Cancer Center Research Institute, Tokyo 104-0045, Japan* 75

25 **High-Efficiency Production and Enzyme Properties of a Soluble Recombinant α2,6-Sialyltransferase**; Takeshi Yamamoto, Yoshimitsu Takakura and Hiroshi Tsukamoto; *Plant Innovation Center, Japan Tobacco Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, Japan* 76

26 **Molecular Cloning and Production of a β-Galactoside α2,3-Sialyltransferase of *Vibrio* sp. JT-FAJ-16;** Yoshimitsu Takakura, Hiroshi Tsukamoto and Takeshi Yamamoto; *Plant Innovation Center, Japan Tobacco Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, Japan* 77

27 **Purification, Cloning, and Production of an α2,3-Sialyltransferase from *Photobacterium phosphoreum* JT-ISH-467;** Hiroshi Tsukamoto, Yoshimitsu Takakura and Takeshi Yamamoto; *Plant Innovation Center, Japan Tobacco Inc., 700 Higashibara, Iwata, Shizuoka 438-0802, Japan* 78

28 **Prediction of Glycan Structures by Combining DNA Expression and Mass Data;** Shin Kawano, Kosuke hashimoto, Kiyoko F. Aoki-Kinoshita, Susumu Goto and Minoru Kanehisa; *Bioinformatics Center, Institute for Chemical Research, Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan* 79

29 **Tissue-Specific Expression and Function of a Novel Human pp-GalNAc-T, O-21;** Kouichi Tachibana[1], Tokiko Sakai[2], Kahori Tachibana[1], Satoshi Ogasawara[1], Tosiaki Noce[3] and Hisashi Narimatsu[1]; *[1] Glycogene Function Team, Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology, 1-1-1 Umezono, Central 2, Tsukuba, Ibaraki 305-8568, Japan, [2] Central Research Laboratories, Seikagaku Corporation, 1253, Tateno 3-shome, Higashiyamatoshi, Tokyo 207-0021, Japan, [3] Mitsubishi-Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194-8511, Japan* 80

30 **Binding Specificity of Jacalin Towards O-Glycosylated Peptides; Exclusion of the Binding to 6-Glycosylated GalNAc;** Satoshi Ogasawara[1], Kouichi Tachibana[1], Sachiko Nakamura[2], Kahori Tachibana[1], Jun Hirabayashi[2] and Hisashi Narimatsu[1]; *[1] Glycogene Function Team, Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), Central-2, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan, [2] Glycostructure Analysis Team, Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology (AIST), Central-2, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan* 81

31 **Rapid Mass Spectrometric Screening Methodology for the Glycome of Glycolipids;** Simon Parry, Stuart Haslam, Howard R. Morris and Anne Dell; *Division of Molecular Biosciences, Imperial College London, South Kensington, London SW7 2AZ, UK* 82

32 **Structural Analysis of UDP-GlcNAc 2-Epimerase/ManNAc Kinase, the Key Enzyme of Sialic Acid Biosynthesis, by Biophysical Methods;** Darius Ghaderi[1], Holger Strauss[2], Sebahattin Cirak[3], Werner Reutter[1], Lothar Lucka[1] and Stephan Hinderlich[1]; *[1] Charité – Universitätsmedizin Berlin, Campus Benjamin Franklin, Institut für Biochemie und Molekularbiologie, Arnimallee 22, 14195 Berlin-Dahlem, Germany, [2] Max Delbrück Center for Molecular Medicine, Robert-Rössle-Strasse 10, 13125 Berlin, Germany, [3] Department of Pediatrics and Pediatric Neurology, University of Essen, Hufelandstr. 55, 45122 Essen, Germany* 83

33 **Defining the Binding Specificity of Commercially Available Plant Lectins Using a Printed Glycan Array;** Richard A. Alvarez[1], Angela Lee[1], Carole Davis[1], Julia Hoffmann[2] and Ola Blixt[2]; *[1] Protein-Carbohydrate Interaction Core H, Consortium for Functional Glycomics and the Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, [2] Carbohydrate Synthesis and Protein Expression Core D, Consortium for Functional Glycomics and the Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037* 84

34 **Prominent Role of Tryptophan in Carbohydrate–Protein Interactions;** Thomas Luetteke and Claus-W. von der Lieth; *German Cancer Research Centre, Central Spectroscopic Department–B090, Molecular Modelling Group, Im Neuenheimer Feld 280, D-69210 Heidelberg, Germany* 85

35 **Development of a High-Throughput Transcript Analysis of Glycan-Related Genes and a Cross-Platform Comparison with Microarray Expression Analysis and Correlation with Relative Quantitation of Glycan Mass Spectral Analysis;** Alison V. Nairn[1], William S. York[1], Timothy J. Gilmartin[2], Steven R. Head[2], Rodney J. Nash[3], Stephen Dalton[3], Simon J. North[4], Stuart M. Haslam[4], Anne Dell[4] and Kelley W. Moremen[1]; *[1] Complex Carbohydrate Research Center and Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, [2] The Scripps Research Institute, DNA Array Core Facility, La Jolla, CA 92037, [3] Department of Animal and Dairy Sciences, University of Georgia, Athens, GA 30602, [4] Division of Molecular Biosciences, Imperial College London, London SW7 2AZ, UK* 86

36 **A Metabolic-Labeling Approach to Proteomic Analysis;** Anjali S. Ganguli[1], Isaac S. Carrico[1] and Carolyn R. Bertozzi[1,2,3]; *[1] Department of Chemistry, University of California, Berkeley, CA 94720, [2] Department of Molecular and Cellular Biology, University of California, Berkeley, CA 94720, [3] Howard Hughes Medical Institue, University of California, Berkeley, CA 94720* 87

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37 ***N*- and *O*-Glycans of CHO Glycosylation Mutants Determined by High-Throughput Glycomic Screening Using MALDI-TOF Mass Spectrometry;** Hung-Hsiang Huang[1], Subha Sundaram[1], Simon J. North[2], Anne Dell[2], Pamela Stanley[1] and Stuart M. Haslam[2]; *[1] Department of Cell Biology, Albert Einstein College Medicine, New York, NY 10461, [2] Division of Molecular Biosciences, Imperial College London, London SW7 2AZ, UK* 88

38 **Tools for Glycoproteomic Analysis: Size Exclusion Chromatography Facilitates Identification of Tryptic Glycopeptides with N-Linked Glycosylation Sites;** Gerardo Alvarez-Manilla, James Atwood III, Satya S. Sahoo, Yan Guo, Nicole L. Warren, William S. York, Ron Orlando and Michael Pierce; Complex Carbohydrate Research Center, *University of Georgia, Athens, GA 30602* 89

39 **Tandem Mass Spectrometry Approaches for the Mapping of O-Glycosylation Sites;** Lance Wells[1], Bryan Woosley[1], Jae-Min Lim[1], Dan Sherling[1], Gerardo Alvarez-Manilla[1], Michael Tieymer[1], Michael Pierce[1], Ron Orlando[1], Frances I. Smith[2] and Carl Bergmann[1]; *[1] Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, [2] Shriver Center for Mental Retardation, University of Massachusetts Medical School, Waltham, MA 02452* 90

40 **Glycomics Using Negative Ion Tandem Mass Spectrometry of Native Glycans;** Joseph Zaia, Jennifer L. Seymour and Catherine E. Costello; *Department of Biochemistry, Boston University School of Medicine, 715 Albany Street, R-806, Boston, MA 02118*..... 91

41 **Discrimination of Isomeric/Isobaric Glycosphingolipid Glycan Structures Using Ion Trap MS^n in Combination with Glycan Fragment Library and Decomposition Pathway Constraint Strategies;** Steven B. Levery, David J. Ashline, Andy J. Hanneman, Suddham Singh, Hailong Zhang, Anthony J. Lapadula and Vernon N. Reinhold; *University of New Hampshire, Durham, NH 03824*..... 92

42 **A Universal High Throughput Screening Assay for Glycosyltransferases;** Matt Staeben, Karen Kleman-Leyer, Thane Westermeyer and Robert G. Lowery; *BellBrook Labs, 525 Science Drive, Suite 110, Madison, WI 53711*..... 93

43 **MonoSaccharideDB: A Reference Resource to Unify the Notation of Carbohydrate Residues;** Thomas Lütteke and Claus-W. von der Lieth; *German Cancer Research Center, Central Spectroscopic Department (B090), Molecular Modeling Group, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany* 94

44 **A New Tandem LC-MS-Based Method for Glycolipids;** Ulf Sommer and Catherine E. Costello; *Mass Spectrometry Resource, Boston University School of Medicine, Boston, MA 02118* 95

45 **Automated Analysis and High-Throughput Mass Spectrometric Glycomics Profiling of Mammalian Cells and Tissues;** Simon J. North[1], David Goldberg[2], Mark Sutton-Smith[1], Stuart M. Haslam[1], Sara Chalabi[1], Howard R. Morris[1,3] and Anne Dell[1]; *[1] Division of Molecular Biosciences, Imperial College London, London SW7 2AZ, UK, [2] Scripps-PARC Institute for Advanced Biomedical Sciences, 3333 Coyote Hill Road, Palo Alto, CA, [3] M-Scan Mass Spectrometry Research and Training Centre, Silwood Park, Ascot SL5 7PZ, UK*..... 96

46 **Modulation of Cell Adhesion or Attachment Using Sialic Acid Engineering Methods;** S.-Gopalan Sampathkumar, Christopher T. Campbell, Adrienne V. Li, Mark B. Jones, Zhonghui Sun, Anshu Sarje, Nitish V. Thakor and Kevin J. Yarema; *Department of Biomedical Engineering, Johns Hopkins University, Baltimore, MD 21218* 97

47 ***N*-Glycan Chemoenzymatic Synthesis Using Oligosaccharide Oxazolines as Donor Substrates;** Ying Zeng, Steven Hauser, Haijing Song and Lai-xi Wang; Institute of Human Virology, *University of Maryland Biotechnology Institute, University of Maryland, 725 West Lombard Street, Baltimore, MD 21201* 98

48 **A Simple Method for Separation of N- and O-Linked Oligosaccharides from Glycoproteins and Analysis by HPLC and Electrospray Mass Spectrometry;** Mike Madson, Srinivasa Rao and Chris Pohl; *Dionex Corporation, 445 Lakeside Drive, Sunnyvale, CA 94088* 99

49 **Synthesis of Oligo-β-(1-6)-*N*-Acetylglucosamines, Fragments of the Polysaccharide Intercellular Adhesin of Staphylococci;** Marina L. Gening[1], Yury E. Tsvetkov[1], Olga N. Yudina[1], Gerald B. Pier[2] and Nikolay E. Nifantiev[1]; *[1] N.D. Zelinsky Institute of Organic Chemistry, Leninsky Prospect 47, 119991 Moscow, Russia, [2] Harvard Medical School, Brigham and Women's Hospital, Boston, MA 02115* 100

50 ***N*-Acetylglucosaminyltransferase I-Dependent *N*-Glycans are Involved in the Response of *Caenorhabditis elegans* to Bacterial Pathogens;** Harry Schachter[1,2], Hui Shi[1] and Andrew M. Spence[3]; *[1] Program in Structural Biology and Biochemistry, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8, [2] Department of Biochemistry, University of Toronto, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8, [3] Department of Molecular and Medical Genetics, University of Toronto, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8*..... 17

51 ***O*-GlcNAc Cycling Enzymes Modulate Life Span in *Caenorhabditis elegans*;** Olga Stuchlik[1], Mohammad M. Rahman[2], Edward T. Kipreos[2] and Lance Wells[1]; *[1] Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, [2] Department of Cellular Biology, University of Georgia, Athens, GA 30602* 18

52 **O-Glucosylation of Notch1 and its Significance in Notch Signaling;** Aleksandra Nita-Lazar, Rosemary Orhue and Robert S. Haltiwanger; *Department of Biochemistry and Cell Biology, Institute for Cell and Developmental Biology, Stony Brook University, Stony Brook, NY 11794-5215* 19

53 ***N*-Acetylglucosaminyltransferase I-Null *Drosophila melanogaster* is Unable to Compete for Survival in the Presence of Wild-Type Flies;** Mohan Sarkar[1], Cristina I. Silvescu[2], Vern N. Reinhold[2], Harry Schachter[1,3] and Gabrielle Boulianne[4]; *[1] Program in Structural Biology and Biochemistry, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8, [2] Department of Chemistry, University of New Hampshire, Durham, NH 03824, [3] Department of Biochemistry, University of Toronto, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8, [4] Program in Developmental Biology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8* 115

54 **Purification and Characterization of *Helicobacter pylori* α1,3/4Fucosyltransferases;** Bing Ma[1], Gerald F. Audette[1], Shuangjun Lin[2], Monica M. Palcic[2], Bart Hazes[1] and Diane E. Taylor[1]; *[1] Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7, [2] Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2* 116

55 **Acceptor Protein Requirements for Recombinant N-Glycosylation in *Escherichia coli*;** Michael Kowarik[1], Isabelle Hug[1], Nico Callewaert[2], Marcela Hernandez[3],

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Michael Wacker[4] and Markus Aebi[1]; *[1] Departement of Biology, Institute of Microbiology, Swiss Federal Institute of Technology Zuerich, ETH Hoenggerberg, CH-8093 Zuerich, Switzerland, [2] Department for Molecular Biomedical Research, Unit for Molecular Glycobiology, Ghent University and VIB, B-9052 Ghent-Zwijnaarde, Belgium, [3] Cytos Biotechnology AG, Wagistrasse 25, CH-8952 Schlieren, Switzerland, [4] GlycoVaxyn AG, Zollikerstrasse 44, CH-8008 Zuerich, Switzerland* 117

56 **Galectins Bind to the Multivalent Glycoprotein Asialofetuin with Enhanced Affinities and a Gradient of Decreasing Binding Constants;** Tarun K. Dam[1,2], Hans-J. Gabius[3], Sabine André[3], Herbert Kaltner[3], Martin Lensch[3] and Curtis F. Brewer[1,2]; *[1] Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York, NY 10461, [2] Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, NY 10461, [3] Institut für Physiologische Chemie, Tierärztliche Fakultät, Ludwig-Maximilians Universität Munchen, 80539 München, Germany* 118

57 **Role of the Low-Affinity Mannose 6-Phosphate Binding Site in the Cation-Independent Mannose 6-Phosphate Receptor;** Carrie A. Chavez, Susanna G. Driscoll and Nancy M. Dahms; *Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226* 119

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59 **Human Salivary Mucins: MG2 (MUC7) Glycosylation is Consistent, Whereas MG1 (MUC5B) Glycosylation Varies Extensively Between Healthy Individuals;** Kristina A. Thomsson[1], Benjamin Schulz[2], Nicolle H. Packer[2] and Niclas G. Karlsson[2]; *[1] Department of Medical Biochemistry, Göteborg University, Box 440, 405 30 Göteborg, Sweden, [2] Proteome Systems Limited, Locked Bag 2073, North Ryde, Sydney NSW 1670, Australia* 121

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84 **Terminal Differentiation and Morphogenesis in *Dictyostelium* Depends on its Skp1 Prolyl 4-Hydroxylase;** Christopher M. West and Hanke van der Wel;*Department of Biochemistry & Molecular Biology and the Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104* 148

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POSTER SESSION 2
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1 **A Large Panel of Phage Display-Derived Human Antibodies Against Specific Glycosaminoglycan Epitopes: Versatile Tools for the Glycobiologist;** Guido J. Jenniskens and Toin H. van Kuppevelt; *Department of Matrix Biochemistry, University Medical Center Nijmegen, Nijmegen Center for Molecular Life Sciences, P.O. Box 9101, 6500 HB, Nijmegen, The Netherlands* 10

2 **Msulf1 and Msulf2 Differentially Modify Heparan Sulphate 6-O-Sulphation Patternin;** William Christopher Lamanna[1], Rebecca Baldwin[2], Cathy Merry[2] and Thomas Dierks[1]; *[1] Department of Biochemistry, University of Bielefeld, 33615 Bielefeld, Germany, [2] Department of Medical Oncology, University of Manchester, Christie Hospital NHS Trus, Wilmslow Road, Manchester 20 4BX, UK* 11

3 **Functions of Heparan Sulfate Proteoglycans and the Kallman Syndrome Protein KAL-1 in *Caenorhabditis elegans* Embryogenesis;** Martin L. Hudson[1], Tarja Kinnunen[2], Jeremy E. Turnbull[2] and Andrew D. Chisholm[1]; *[1] Department of Molecular, Cellular and Developmental Biology, University of California, Santa Cruz, CA 95064, [2] School of Biological*

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7 **Unraveling the Molecular Basis of the Role of Pectins in Human Health;** Carl W. Bergmann and Stephanie C. Yarnell; *Complex Carbohydrate Research Center, University of Georgia, 315 Riverbend Road, Athens, GA 30602-4712* 104

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33 **Tandem Mass Spectrometric Analysis of Heparan Sulfate Structure in the Trigeminal Ganglion and in a Genetic Cell Model Demonstrating the Importance of *N*-Deacetylase/*N*-Sulfotransferase Isoforms on the Generation of Biologically Active Heparan Sulfate;** Roger Lawrence[1], Tomio Yabe[2], Sassan HajMohammadi[3], John Rhodes[3], Melissa McNeely[4], Edward D. Lamperti[5], Paul A. Toselli[5], Miroslaw Lech[2], Patricia G. Spear[4], Robert D. Rosenberg[2], Nicholas W. Shworak[3] and Jeffrey D. Esko[1]; *[1] Department of Cellular and Molecular Medicine, University of California at San Diego, La Jolla, CA 92093, [2] Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, [3] Section of Cardiology, Department of Medicine, Dartmouth Medical School, Hanover, NH 03755, [4] Department of Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, [5] Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118* 159

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Session Topic: New Technologies for Glycobiology

(1) New Technologies to Simplify Glycomics

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Glycan chains can be released from glycoproteins or glycolipids yielding reducing sugars that have unique chemical reactivity. Solution labeling of their reducing-end aldehydes has long been a key tool in the area of oligosaccharide analysis. We report here that the same type of chemistry can be carried out on the solid phase resulting in the transient covalent immobilization of oligosaccharides onto particles, a process termed glycoblotting by Nishimura *et al.* (2005). The clear advantages of manipulating an oligosaccharide that has been covalently captured from solution are that it becomes and stays concentrated in a small volume of insoluble matrix, reagents for tagging, and detection can be used at high concentrations as the excess can easily be washed away, and that there can be no selective losses during the manipulations. Installation of a cleavable linker further allows for the oligosaccharide to be released back into solution in a small and controlled volume, for example, after it has been fluorescently tagged. For convenience, we term this general process solid phase oligosaccharide capture-analysis (SPOC-A). In the process described here, hydroxylamine groups are attached to controlled pore glass (CPG) or polyethylene glycol-polyacrylamide (PEGA) beads through optional PEG spacer chains containing a base-labile ester linker. After covalent capture of a reducing sugar and washing, the excess unreacted hydroxylamine groups can be capped with acetic anhydride, the oxime double bond can be reduced, the resulting sugar-NH-group can be reacted with a large excess of derivatizing agent, and the unreacted excess being removed by simple washing. The net result is that despite using a large excess of both capture groups and tagging agent, no product manipulation/purification is required before analysis of the product oligosaccharide that contains a single label. The SPOC-A process was optimized using lacto-*N*-tetraose. Fluorescent tags were added using aryl isothiocyanates (FITC and TRITC), permitting CE analysis after cleavage. Incorporation of bromine-containing labels facilitates analysis by mass spectrometry (MS). Use of stable isotope-coded reagents permits differential glycomics analysis by MS. The immobilized-labeled glycans can also be made fully accessible to glycosidases when PEG spacers are included, permitting enzyme-assisted sequencing on CPG. Captured oligosaccharides or monosaccharides released from them by exoglycosidase digestion can be detected on glass surfaces by either lectins or tetramethylrhodamine–arylboronate conjugates. SPOC-A provides the opportunity to gain structural information on a glycan using only the simplest of manipulations: pipetting. It can also yield a panel of derivatives of a given immobilized glycan within a few hours.

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(2) New Strategies for Glycan Modification and Derivatization and Enhancement of Glycan Arrays

Baoyun Xia[1], Ziad S. Kawar[1], Tongzhong Ju[1], Richard A. Alvarez[1], Goverdhan P. Sachdev[2] and Richard D. Cummings[1]

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The new field of functional glycomics encompasses information about both glycan structure and recognition by carbohydrate-binding proteins (CBPs), which is currently being explored through glycan array technology. However, glycan array construction is limited by the complexity of efficiently generating derivatives of free, reducing glycans with primary amines for conjugation. This presentation will high new developments in our laboratory that have resulted in novel and straightforward methods to derivatize glycans with fluorescent amine reagents to generate glycan conjugates that contain a primary amine for further conjugation (glycan-fluorescent amines [GFAs]). A wide variety of glycans, including milk sugars, *N*-glycans, glycosaminoglycans, and chitin-derived glycans, have been converted to GFAs. These GFA derivatives have been covalently conjugated in high yield to NHS-activated glass slides, maleimide-activated protein, carboxylated microspheres, and *N*-hydroxysuccinimide biotin. An advantage of GFA derivatization is that all of the immobilized GFAs can be easily visualized and directly quantified. All of the immobilized GFAs studied are well recognized by appropriate CBPs. Importantly, we have also used immobilized GFAs to purify novel carbohydrate-binding proteins. Thus, GFA derivatives provide versatile new tools for biologists to quantify and covalently capture minute quantities of glycans for generating novel glycan arrays from naturally occurring glycans and provide new approaches for exploring CBP functions in biology and pathogenesis.

(3) Understanding Carbohydrate Antigenicity: *Streptococcus agalactiae* (Type III) Versus *Streptococcus pneumoniae* (Type 14)

Renuka Kadirvelraj[1], Jorge Gonzalez-Outeiriño[1], Harold J. Jennings[2], Simon Foote[2] and Robert J. Woods[1]

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Gram-positive bacteria *Streptococcus agalactiae* (group B streptococcus [GBS]) and pneumoniae (Pn) are leading causes of neonatal sepsis, meningitis, and pneumonia. To establish the structural origin of the variation in antigenicity with capsular polysaccharide (CPS) sequence, models for the immune complexes of GBS Type III and Pn Type 14 CPS with the variable fragment (Fv) of monoclonal antibody 1B1 are presented. The structures were generated through a combination of comparative modeling, molecular docking, and molecular dynamics simulation. The relationship between carbohydrate sequence and antigenicity is quantified, and the mechanism whereby the neuraminic acid residues mediate affinity established. The similarity of the solution conformation with that in the theoretical Fv–CPS complex establishes the origin of the conformational epitope in GBS III.

(4) Noninvasive Imaging of Glycosylation *in vivo*

Jennifer A. Prescher[1], Danielle H. Dube[1], Anderson Lo[1] and Carolyn R. Bertozzi[1,2,3]

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A fundamental goal in the field of molecular imaging is the identification of tissue-specific biomarkers that can be targeted with probes for visualization. Aberrant glycosylation is a hallmark of malignancy and a feature of tumor cell surfaces that could, in principle, be exploited for targeted diagnostics. Numerous cancers have been shown to express elevated levels of glycan structures bearing the monosaccharide sialic acid. Therefore, an imaging strategy that targets sialic acid could potentially be used for the noninvasive tracking of disease progression. We have previously shown that *unnatural* sialic acids can be introduced into cell surface glycans by the metabolism of precursor sugar analogs. The unnatural analogs can be endowed with bioorthogonal chemical reporters capable of covalent reaction with exogenous probes. For example, an azide-functionalized analog of *N*-acetylmannosamine termed ManNAz is converted by cells to the corresponding sialic acid (SiaNAz) *in vivo*. The sialic acid-resident azides can be covalently tagged within living animals using phosphine probes via the Staudinger ligation, enabling the delivery of reagents to cell surfaces that are rich in sialic acid. Here we present a noninvasive imaging strategy that exploits azidosugars as metabolic markers for the covalent targeting of tissues and tumor cells with diagnostic agents. We synthesized an assortment of probes for three imaging modalities and are currently employing these reagents for glycan-specific imaging in healthy and tumor-bearing mice. The ability to chemically tag cell surface glycans in living animals provides a means to track changes in glycosylation in a physiologically relevant context.

(5) A Profile HMM for Tree Structures to Locate Glycan Structure Profiles

Kiyoko F. Aoki-Kinoshita, Nobuhisa Ueda, Hiroshi Mamitsuka, Susumu Goto and Minoru Kanehisa

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Probabilistic models are often used in bioinformatics for profiling families of sequences to characterize groups of structures. Similarly, glycan structures may also be characterized with more advanced probabilistic models in the form of tree structures that take into consideration the nature of their structures. Specifically, models that incorporate the dependencies inherent in glycans, such as ordered glycosidic linkages, can and should be used. Previously, a tree Markov model was developed to find patterns in glycan structures (Aoki *et al.*, 2004) by considering the ordering of linkages to any particular monosaccharide. This model was called PSTMM, for probabilistic sibling-dependent tree Markov model. Parameter estimation algorithms were developed, and this model was evaluated on glycan data from the KEGG GLYCAN database. In our results, PSTMM was able to find patterns among the *N*-glycans, in particular, the three known subtypes of hybrid, complex, and high mannose. With such promising results, in this work, this model was further extended to profile groups of glycan

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structures, similar to sequence profiles for protein sequence families. That is, a profile PSTMM model was developed to capture glycan structure profiles of sets of glycans. Because glycan families are not so clearly defined as of yet, once such glycan structure profiles are found, they should lead the way to define glycan structure families. With this, prediction of possibly recognized glycan structures may be performed to enable more efficient analyses of lectins and other glycan-binding biomolecules, in addition to many other similar analyses. Preliminary results will be presented on glycan structures recognized by various lectins.

(6) Analyses of Carbohydrate Recognition by Mammalian Sialic Acid-Binding Proteins of the Immune System, Siglecs, Using Microarrays of Lipid-Linked Oligosaccharide Probes

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To investigate biological systems that operate through carbohydrate recognition, a microarray system has been established using lipid-linked oligosaccharide probes (Fukui *et al*., 2002; Feizi and Chai, 2004). These encompass naturally occurring oligosaccharide sequences of glycoproteins, glycolipids, proteoglycans, polysaccharides, as well as chemically synthesized sequences. Over 200 sequence-defined oligosaccharide, probes have been synthesized and arrayed for evaluating recognition by receptors of the innate immune system. Among them are numerous mammalian-type carbohydrate sequences: *N*-glycans (neutral and acidic, high mannose, and complex types), major blood-group types (A, B, H, Lewis a, Lewis b, Lewis x, and Lewis y) on linear or branched backbones and their sialylated and sulfated analogs, major gangliosides, glycosaminoglycans (chondroitin sulfates A, B, and C), homo-oligomers of sialic acid and fragments of other polysaccharides; they range in size from two to twenty monosaccharides. This communication will be focused on oligosaccharide recognition by several sialic acid-binding proteins: human and murine Siglecs (examined as recombinant soluble IgG-Fc chimeras). We validate the system and derive new information on the specificities of members of this family of proteins.

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Session Topic: Proteoglycan Functions

(7) Sulfotransferases: Tuning Heparan Sulfate Functions in Neural Cell Migration and Development

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Heparan sulfate (HS) biosynthesis involves the action of a complex set of enzymes with polymerase, epimerase, and sulfotransferase (ST) activities. Multiple isoforms of *N*- and *O*-STs decorate the nascent HS chains with specific sulfation patterns which confer selective biological functions. We have been studying HSSTs in model organisms since they provide opportunities to study the expression of these enzymes in relation to the structure and activities of the HS produced. In mice, we find that there are stage-specific combinations of HSST isozymes that underlie the synthesis of HS species in developing neural cells that differ in structure and activity (ability to activate signaling by specific FGF–FGF receptor combinations). In addition, we find distinct spatiotemporal expression patterns of the three isoforms of 6OSTs in developing brain tissue. Knocking out the single-specific OST which sulfates the 2 position of uronic acids (2OST) results in intriguing changes in expression of other STs (6OSTs and NDSTs) and in turn produce altered HS structures with modified abilities to regulate FGF signaling. Our data indicate that differential expression of HSSTs is a dynamic process which results in the synthesis of structurally and functionally variant HS species. Regulated synthesis of specific HS species could be an important mechanism for modulating proliferation and differentiation of neural cells in the developing mouse brain. To gain insight into the functions of STs at the whole organism level, we have also used the nematode *Caenorhabditis elegans* as a model. We have found that STs have partially overlapping yet partially specific roles for cell and neuron migration and axon outgrowth. We have, in particular, characterized the *C. elegans* homolog of heparan 2OST, hst-2, and shown that it encodes the enzyme activity responsible for modifying HS with 2-*O*-sulfates. In addition, a deletion mutant of hst-2, ok595, lacks 2OST activity. hst-2 is widely expressed in neurons, in the hypodermis (epidermis) and pharyngeal muscle. Lack of heparan 2-O-sulfation in the null mutant ok595 leads to specific cell and neuron migration and axon guidance defects. Taken together, these studies in both mice and *C. elegans* emphasize the critical role of STs in the biosynthesis of functionally specific HS proteoglycans—by tuning the structure and thereby the function of these molecules, they can fulfill specific regulatory biological functions in the developing nervous system.

(8) HSPGs and Sorting of Retinal Axons in the Zebrafish Optic Tract

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Retinal ganglion cell (RGC) axons are topographically ordered in the optic tract according to where they originate in the retina. In the zebrafish, mutants *dackel* (*dak*), *boxer* (*box*), and *pinscher (pic)*, a subset of dorsal RGC axons missort in the optic tract but nevertheless innervate the tectum topographically. The *dak* and *box* genes encode *exostosin 2* (*ext2*) and *exostosin-like 3* (*extl3*), glycosyltransferases implicated in heparan sulfate (HS) biosynthesis. Biochemical and immunohistochemical analysis shows that *dak* and *box* are required for HS synthesis *in vivo*. Both genes are expressed maternally and then ubiquitously and so likely play permissive roles. The *box* missorting defect can be rescued by overexpression of *extl3* mRNA. *dak; box* double mutants show synthetic pathfinding phenotypes that phenocopy *robo2* mutants, suggesting that Robo2 function requires HS *in vivo*; however, tract sorting does not require Robo function, since it is normal in *robo2* null mutants. Our genetic evidence that heparan sulfate proteoglycan (HSPG) function is required for optic tract sorting provides the first clues for understanding the molecular mechanisms underlying this process. We are continuing to analyze the mechanisms by which HSPGs control optic tract sorting, by analyzing *pic* and other mutants.

(9) Proteoglycans in Axon Regeneration and Plasticity in the Adult CNS

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Whenever the CNS is injured, a reactive process is initiated known as glial scar formation which acts as a barrier to the regeneration of damaged axons. Various lines of evidence suggest that the main inhibitory molecules in the glial scar are chondrointin sulphate proteoglycans (CSPGs), most of which have axon growth inhibitory properties, and are up-regulated after CNS injury. Not only are the protein cores up-regulated, but also there is more glycosaminoglycan (GAG) attached to them. The final stage of GAG synthesis is sulfation, which can occur in three positions. The sulfotransferase that sulfates *n*-acetlygalactosamine in the 6 position is specifically up-regulated. All the CSPGs possess GAG chains of similar structure produced by the same enzymes, and removal of GAG chains by digestion with chondroitinase or inhibition of GAG synthesis with chlorate or beta d xylosides removes much of the inhibition from CSPGs *in vitro*. We therefore tested to see whether GAG digestion by chondroitinase would promote axon regeneration *in vivo*. We first treated mechanical lesions of the nigrostriatal tract and saw regeneration of about 4% of axons back to their target. Next dorsal column lesions of the spinal cord at C4 were treated. Both sensory and corticospinal axons regenerated in treated cords, and there was rapid return of function in beam and grid walking tests. The return of function after chondroitinase treatments is so rapid that we hypothesized that some of it might be because of enhanced plasticity. Many neuronal cell bodies and dendrites are coated in thick perineuronal nets of inhibitory CSPGs and tenascin R which would certainly be expected to prevent the formation of new synapses. We therefore tested the effects of chondroitinase treatment in a well-established plasticity model, ocular dominance shift in the visual cortex following monocular deprivation. In adult animals, in which the cortex was treated with chondroitinase ocular, dominance plasticity was reactivated to almost the same extent as is seen during the critical period. We have also been able to reactivate plasticity in the spinal cord allowing recovery of function after peripheral nerve repair. The CSPG structures that turn off plasticity are probably the perineuronal nets, which contain one or several CSPGs. The components of these may be produced either by the neurons themselves or by the surrounding glial cells. Neurons must produce molecules capable of anchoring the matrix to their surface. Both hyaluronan synthase and link proteins are produced by neurons with perineuronal nets at the time that these structures are forming.

(10) A Large Panel of Phage Display-Derived Human Antibodies Against Specific Glycosaminoglycan Epitopes: Versatile Tools for the Glycobiologist

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Glycosaminoglycans (GAGs) are long, unbranched polysaccharides, most of which are covalently linked to a protein core to form proteoglycans. Depending

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on the nature of their backbone, one can discern the class of galactosaminoglycans: chondroitin sulfate (CS) and dermatan sulfate (DS), and glucosaminoglycans: heparan sulfate (HS) and heparin. Superimposed upon the backbone is a pattern of sulfation and acetylation modifications that constitute different domains. The resulting unique modification "sequences" on GAG molecules are instrumental in the binding of various biologically active proteins. Through the highly regulated affinity towards effector molecules, GAGs are important modulators of numerous biological processes. However, the sequence-specific recognition of a GAG sequence has only been shown for the serine protease inhibitor antithrombin-III, whereas less-defined preferential-binding requirements have been postulated for some growth factors, for example, FGF-1 and FGF-2. Investigating the exact role(s) of GAGs in physiological and pathological processes has historically been hampered by a lack of appropriate tools. Using phage display, we have generated a large panel (~100) of epitope-specific antibodies against CS, DS, HS, and heparin (van Kuppevelt *et al*., 1998; Jenniskens *et al*., 2000; Smetsers *et al*., 2003). Anti-GAG antibodies were used to probe changes in the topological distribution of GAG epitopes during skeletal muscle development (Jenniskens *et al*., 2002) and GAG expression aberrations in various pathologies, notably melanoma and psoriasis (Smetsers *et al*., 2004). Next to the histological staining of GAGs in healthy versus diseased tissue, antibodies were used to analyze the biological functions of GAGs: anti-heparin antibodies display differential affinities toward the antithrombin-III pentasaccharide sequence and interfere to different extents with heparin anticoagulant activity (van de Westerloo *et al*., 2002). The endogenous expression of anti-HS antibodies by myogenic cells results in a functional knockout of specific HS epitopes, which severely impairs ion housekeeping (Jenniskens *et al*., 2003). The initial characterization of the GAG epitopes recognized by our antibodies, using ELISA, immunoprecipitation, and SDS–PAGE-based techniques, has identified some of the general characteristics of the GAG sequences involved (Dennissen *et al*., 2002; ten Dam *et al*., 2004). In close collaboration with leaders in the field of GAG preparation and analysis, we are currently implementing recently acquired expertise and tools for the use of HPLC, CE, and MS to determine the exact chemical composition of these GAG epitopes. Our panel of anti-GAG antibodies provides a set of unique and highly versatile tools to study GAG structure and function. The antibodies can be used for the immunohistologic analysis of GAGs in development and pathology, probing biological activities, and for the purification and structural characterization of specific GAG epitopes.

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(11) Msulf1 and Msulf2 Differentially Modify Heparan Sulphate 6-O-Sulphation Patterning

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Heparan sulphate (HS) is a moderately sulphated type of cell surface glycosaminoglycan which has recently been shown to specifically modulate cell growth and differentiation through enzymatic modification of its specific sulphate patterning. Two novel human sulfatases, Hsulf1 and Hsulf2, have been characterized to endolytically remove sulphate groups from the 6-*O*-sulphate position on intact HS chains in the Golgi and at the cell surface. Little is known about the endogenous activity of these two mammalian sulfatases with regard to substrate specificity, regulation, and subsequent effect on embryonic development and cell signalling. To answer these questions, Msulf1, Msulf2, and Msulf1/Msulf2 knockout mice were produced. HS disaccharide analysis of the mouse embryonic fibroblast (MEF) cell lines from these mice revealed marked differences in HS composition compared with wild-type MEFs, Msulf1 knockout MEFs showed an increase in all three HS 6-*O*-sulphate carrying disaccharides UA-GlcNac(6S), UA-GlcNS(6S), and UA(2S)-GlcNS(6S). Surprisingly, Msulf2 knockout MEFs showed a decrease in UA-GlcNS(6S) and UA(2S)-GlcNS and an increase in UA-GlcNac(6S). Interestingly Msulf1/Msulf2 double knockout cells exhibited an unprecedented increase in all three 6-*O*-sulphated disaccharides. The differential effects of each sulfatase knockout on the HS patterning explains the differential FGF2 signalling observed in the various knockout cell lines and the apparent physiological defects observed in the knockout mice. The observed differences in Msulf1, Msulf2, and Msulf1/Msulf2 knockout cell HS patterning indicates either an acute difference in substrate specificity between these highly homologous enzymes or an important difference in their respective regulation and activity.

(12) Functions of Heparan Sulfate Proteoglycans and the Kallman Syndrome Protein KAL-1 in *Caenorhabditis elegans* Embryogenesis

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The nematode *Caenorhabditis elegans* provides an excellent platform for studying heparan sulfate proteoglycan (HSPG) structure and function, because of the lack of redundancy in its HS biosynthetic enzymes and the availability of deletion alleles in all of the HS biosynthesis genes. Many of these mutants also have visible phenotypes providing a further handle for genetic and biochemical analysis. We are analyzing the role of specific HS modifications, HSPGs, and the HS-binding protein KAL-1 (orthologous to human anosmin-1) during *C. elegans* embryonic development. Anosmin-1 is mutated in X-linked Kallmann syndrome, a genetic disorder manifested by a defective sense of smell and hypogonadism. Previous work identified mutations in *C. elegans* C5-epimerase and 6-*O*-sulfotransferase (*hse-5* and *hst-6*, respectively) as suppressors of *kal-1* over expression-induced axon branching. In addition, a null mutation in *kal-1* was previously reported to cause defects in embryonic epidermal morphogenesis similar to those seen in Eph-signaling mutants. Finally, the HS-copolymerase RIB-2 has been shown to be required for normal development in *C. elegans*. Using time-lapse videomicroscopy, we showed that HS biosynthesis is required during *C. elegans* embryonic development. Animals that lack HS-copolymerase (*rib-1* and *rib-2* mutants) or *N*-deacetylase-*N*-sulfotransferase activity (*hst-1* mutants) exhibit gross defects in ventral neuroblast (VNB) migration following gastrulation. However, mutants in C-5-epimerase (*hse-5*), 2-*O*-sulfotransferase (*hst-2*), 3-*O*-sulfotransferase (*hst-3*), and 6-*O*-sulfotransferase (*hst-6*), all genes required later in the HS biosynthetic pathway, are superficially healthy and viable but show subtle defects in embryonic development. Specifically, HSE-5 and HST-6 activities are required to modulate short-range migrations of VNBs before epidermal enclosure. However, 2-*O*-sulfotransferase activity (2-*O*-ST) is not required for VNB migration. *kal-1* mutants show similar VNB migration defects to those seen in *hse-5* and *hst-6* null mutants. Analysis of *kal-1; hse-5* and *kal-1; hst-6* double mutants showed no enhancement of phenotype, suggesting that HSE-5 and HST-6 modify one or more KAL-1-binding HSPGs which likely comprise a linear genetic pathway with KAL-1. In contrast, *kal-1; hst-2* double mutants showed enhanced VNB migration defects, indicating that 2-O-sulfation is not required for KAL-1-mediated developmental processes. We predicted that null mutations in a KAL-1 interacting HSPG

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should exhibit a *kal-1*-like morphogenetic profile during embryogenesis, provided there is no functional redundancy. Using a candidate gene approach, we found that VNB migrations were grossly normal in syndecan (*sdn-1*) and glypican (*gpn-1*) mutants. However, *sdn-1 gpn-1* double mutant embryos display cell migration defects similar to those of *kal-1* mutants suggesting that SDN-1 and GPN-1 might function redundantly during VNB migration. We used a KAL-1 affinity matrix to identify HSPGs that bind to KAL-1. We found that both SDN-1 and GPN-1 were selectively bound by the KAL-1 matrix in a HS-dependent manner, suggesting that KAL-1, SDN-1, and GPN-1 function redundantly to modulate VNB migration during *C. elegans* embryogenesis. We are currently making GFP-tagged KAL-1, SDN-1, and GPN-1 to elucidate how and where these molecules interact at the cellular level.

Session Topic: Evolution of Glycans and Glycan Function

(13) The Basic Principles of N-Linked Protein Glycosylation

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N-linked protein glycosylation is the most frequent protein modification in eukaryotic cells. This essential process initiates at the membrane of the endoplasmic reticulum (ER). The oligosaccharide $Man_5GlcNAc_2$ is assembled in the cytoplasm from nucleotide-activated sugars on the lipid carrier, dolichylpyrophosphate. After translocation to the lumenal side of the membrane, the oligosaccharide is extended to $Glc_3Man_9GlcNAc_2$, dolichylphosphomannose and dolichylphosphoglucose serve as donors in these reactions. Oligosaccharyltransferase, a complex enzyme consisting of eight membrane-anchored subunits in the model organism *Saccharomyces cerevisiae*, transfers the oligosaccharide to asparagines residues within the N-X-S/T consensus sequence of nascent polypeptide chains. In the ER and the Golgi compartment, the protein-bound $Glc_3Man_9GlcNAc_2$ oligosaccharide is subsequently trimmed and modified in a species and cell-type-specific manner. N-linked protein glycosylation does also take place in archaea and in bacteria. The recently discovered N-linked protein glycosylation process in the human pathogenic bacterium *Campylobacter jejuni* was functionally transferred into *Escherichia coli*, enabling a detailed genetic and biochemical analysis of the pathway. As in eukaryotic cells, an oligosaccharide, $GlcGalNAc_5Bac$ in the case of *C. jejuni*, is assembled at the cytoplasmic side of the plasma membrane on an isopreonoid carrier, bactoprenylpyrophosphate. After translocation across the membrane, the oligosaccharide is transferred to protein. A single membrane protein, PglB, with a high sequence similarity to one of the components of the eukaryotic oligosaccharyltransferases, catalyzes this transfer. D/E-X-N-X-S/T was found to be essential for glycosylation (X can be any amino acid except proline). The requirements for the protein acceptor sequence in the bacterial glycosylation system are more stringent than in eukaryotes. In contrast, a wide variety of oligosaccharides, presented on the lipid carrier bactoprenylpyrophosphate, can be transferred to protein in the bacterial system: oligosaccharides that are structurally very different from the $GlcGalNAc_5Bac$ unit and that consist of 50 or more hexose units are efficiently linked to protein. The high-sequence similarity of the bacterial oligosaccharyltransferase with one subunit of the eukaryotic enzyme, the very similar protein acceptor sequence as well as the finding that bactoprenylpyrophosphate- and dolichylpyrophasphate-linked oligosaccharides serve as substrates in the reactions suggest that the bacterial and the eukaryotic N-linked protein glycosylation are homologous processes. The differences between the two systems make it possible to raise hypotheses about the function of the additional subunits of the eukaryotic oligosaccharyltransferase. The basic principles of the prokaryotic N-linked protein glycosylation, in combination with a wide variety of tools offered by the *Escherichia coli* model system, pave the way to novel approaches in glycoprotein engineering.

(14) Egghead and Brainiac are Essential for Glycosphingolipid Biosynthesis *in vivo*

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The Drosophila genes, brainiac, and egghead encode glycosyltransferases predicted to act sequentially in early steps of glycosphingolipid biosynthesis, and both genes are required for development in Drosophila. Egghead encodes a β4-mannosyltransferase, and brainiac encodes a β3-*N*-acetylglucosaminyltransferase predicted by *in vitro* analysis to control synthesis of the glycosphingolipid core structure, GlcNAcβ1-3Manβ1-4Glcβ1-Cer, found widely in invertebrates but not vertebrates. In this report, we present direct *in vivo* evidence for this hypothesis. Egghead and brainiac mutants lack elongated glycosphingolipids and exhibit accumulation of the truncated precursor glycosphingolipids. We demonstrate that despite fundamental differences in the core structure of mammalian and Drosophila glycosphingolipids, the Drosophila egghead mutant can be rescued by introduction of the mammalian lactosylceramide glycosphingolipid biosynthetic pathway (Galβ1-4Glcβ1-Cer) using a human (β4-galactosyltransferase (β4Gal-T6) transgene. Conversely, introduction of egghead in vertebrate cells (CHO) resulted in near complete blockage of biosynthesis of glycosphingolipids and accumulation of Manβ1-4Glcβ1-Cer. This study demonstrates that glycosphingolipids are essential for development of complex organisms and suggest that the function of the Drosophila glycosphingolipids in development does not depend on the core structure.

(15) Structural and Evolutionary Aspects of Animal Lectins: Diversity in Glycan Recognition

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Cell surface glycans encode information that modulates interactions between cells or between cells and the extracellular matrix, by specifically regulating the binding to cell surface-associated or soluble carbohydrate-binding receptors, such as lectins. For example, the recognition of non-self glycans by humoral- or cell surface-associated lectins is a critical component of innate immune mechanisms. Furthermore, the rapid modifications of exposed carbohydrate moieties by glycosidases and glycosyltransferases and the equally dynamic patterns of expression of their receptors during early development suggest that both play important roles during embryogenesis. Among a variety of biological roles, galectins have been proposed to mediate developmental processes, such as embryo implantation and myogenesis. In the past few years, substantial progress has been accomplished in the elucidation of the structural diversity of the lectin repertories of invertebrates, protochordates, and ectothermic vertebrates, providing particularly valuable information about their biological roles in those groups that constitute the invertebrate/vertebrate boundary and beyond. Although representatives of the lectin families typical of mammals, such as C-type lectins, galectins and pentraxins, have been described in these taxa, the detailed study of selected model species have yielded either novel variants of the structures described for the mammalian lectin representatives or novel lectin families with unique sequence motifs, multidomain arrangements (chimaeric structures), and a new structural fold. Along with the high structural diversity of the lectin repertoires in these critical taxa, their functional diversity is only starting to be elucidated. Relationships between lectin structure and diversity in glycan recognition in the context on innate immunity will be discussed (supported by grant MCB-00-77928 from the National Science Foundation and grant R01 GM70589 from the National Institutes of Health).

(16) Regulation of Notch Signaling by Glycosylation

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Notch is a receptor protein that mediates a wide range of cell fate decisions during animal development. In humans, aberrant Notch signaling has been linked to leukemia (TAN-1), and congenital syndromes associated with stroke and dementia (CADASIL), and liver, cardiovascular, and skeletal defects (Alagille, spondylocostal dysostosis). The Notch receptor and its ligands are modified by an unusual form of glycosylation, which is initiated by the attachment of fucose to serines or threonines within epidermal growth factor-like (EGF) repeats. We have studied the influence of this posttranslational modification using a combination of Drosophila genetics, cell culture, and biochemistry. Decreasing the expression of protein *O*-fucosyltransferase 1 (OFUT1), the enzyme that initiates the synthesis of O-linked fucose demonstrated that OFUT1 is positively required for Notch signaling. We recently discovered, however, that OFUT1 actually plays two distinct roles in Notch signaling. It acts both as a fucosyltransferase to modify the Notch receptor and as a chaperone to promote Notch receptor folding. These two roles are genetically separable, because the chaperone activity of OFUT1 does not require its fucosyltransferase activity. The chaperone activity is required for all Notch functions, but the fucosyltransferase activity is principally required to allow Notch to be further glycosylated. Fringe is a glycosyltransferase that modifies the O-linked fucose on Notch by the addition of β1,3 linked *N*-acetylglucosamine. This further glycosylation of Notch both inhibits the activation of Notch by one ligand, Serrate, and potentiates the activation of Notch by another ligand, Delta. The influence of this glycosylation on Notch activation can be accounted for an effect on Notch-ligand binding. By reproducing the influence of glycosylation on ligand binding *in vitro* with purified components, we have been able to demonstrate that the simple addition of *N*-acetylglucosamine to Notch is sufficient to alter the interaction

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of Notch with its ligands and that this influence of glycosylation does not require the participation of any accessory proteins. In another line of experiments, we have used site-specific mutagenesis to assess the requirements for glycosylation of different EGF repeats of Notch. Characterization of mutant forms of Notch that cannot be fucosylated on specific EGF repeats, or that lack specific EGF repeats, has indicated that Fringe acts through multiple, independent sites to modulate ligand binding and suggest a model for how Notch interacts with its ligands.

(17) *N*-Acetylglucosaminyltransferase I-Dependent *N*-Glycans are Involved in the Response of *Caenorhabditis elegans* to Bacterial Pathogens

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UDP-GlcNAc : α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT I) controls the synthesis of hybrid, complex, and paucimannose *N*-glycans. *Caenorhabditis elegans* makes paucimannose but little or no hybrid nor complex *N*-glycans. Worms have three GnT I genes. GLY-12 and GLY-13 are widely expressed, whereas GLY-14 is expressed only in gut cells. GLY-13 is responsible for ~99% of the total GnT I activity in normal worm extracts. Adult worms with a triple knockout (TKO) of all three GnT I genes (gly-14, gly-12, gly-13) (Zhu *et al*., 2004) show a normal phenotype and lifespan under standard laboratory conditions. TKO extracts have no detectable GnT I activity (<0.04% of total wild-type GnT I activity) and cannot make 31 paucimannose, complex, and fucosylated oligomannose *N*-glycans present in wild-type worms. Survival times were determined on wild type, gly-12, gly-13, and gly-14 single null, gly-14;gly-12, gly-14;gly-13, and gly-12 gly-13 double null, and TKO worms exposed to two bacterial pathogens that kill worms by infection and exotoxin, respectively. The seven mutant worms show significant differences in their sensitivity to killing by a particular pathogen, and a particular mutant worm responds differently to the two pathogens. (i) GLY-12, GLY-13, and GLY-14 differ in their protein targets and (ii) GnT I-dependent (primarily paucimannose) *N*-glycans are not essential for worm development but play a role in the ability of *C. elegans* to survive bacterial infection and exotoxin. Support by the Canadian Institutes of Health Research (CIHR) and the Canadian Protein Engineering Network Centre of Excellence (PENCE).

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(18) *O*-GlcNAc Cycling Enzymes Modulate Life Span in *Caenorhabditis elegans*

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O-Linked beta-*N*-acetylglucosamine (*O*-GlcNAc) is a dynamic posttranslational modification of serine and threonine residues on cytosolic and nuclear proteins found in all metazoans studied to date. The cycling enzymes, *O*-GlcNAc transferase (OGT) that adds *O*-GlcNAc and *O*-GlcNAcase which removes it, have been cloned and partially characterized from a number of organisms. Elevation in global *O*-GlcNAc levels induces insulin resistance, the hallmark of Type II diabetes, and increases thermotolerance in mammalian systems. *Caenorhabditis elegans* is a genetically amenable model organism for the study of insulin-like signaling. Interestingly, mutations that inhibit insulin-like signal transduction in *C. elegans* increase lifespan and stress resistance. Given that levels of the *O*-GlcNAc modification have been shown to modulate insulin resistance and that the insulin-signaling pathway plays a central role in the lifespan of *C. elegans*, we hypothesized that perturbing *O*-GlcNAc levels would modulate lifespan. In this study, OGT and *O*-GlcNAcase *C. elegans* deletion strains ogt-1 (ok 430) and T20B5.3 (ok 1207), respectively, were used for lifespan studies. The lifespans of ogt-1, T20B5.3, and wild-type *C. elegans* strains were compared. The median lifespan of the *O*-GlcNAcase deletion strain increased ~25%, whereas the median lifespan of the OGT deletion strain decreased ~25%. Maximum lifespan was also decreased in the OGT deletion strain. We are elucidating the effect of these deletion strains on stress survival in the worm. Furthermore, OGT and *O*-GlcNAcase deletion strains are being crossed with mutants in the insulin-signaling pathway to pinpoint where elevated *O*-GlcNAc levels are impinging on the signaling cascade.

(19) O-Glucosylation of Notch1 and its Significance in Notch Signaling

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The Notch protein is a transmembrane receptor that initiates a signaling pathway crucial for many cell fate decisions. In mammals, Notch is activated upon binding to its ligands, members of the Delta and Jagged families, which are present on the surface of adjacent cells. Mutations in Notch, its ligands, or other components of Notch-signaling pathway have been found in a number of human diseases (T-cell leukemia and other types of cancer, CADASIL, multiple sclerosis). The extracellular domain of Notch1 contains 36 EGF repeats, many of which contain consensus sequences for O-fucosylation and O-glucosylation. O-Fucosylation is known to be critical for Notch function. Protein *O*-fucosyltransferase 1, an enzyme adding fucose to EGF repeats, is essential for Notch signaling, and its genetic ablation in mice results in embryonic lethality. Enzymes of the Fringe family (Lunatic, Manic, and Radical) add *N*-acetylglucosamine to *O*-fucose. Knockout of Lunatic fringe in mice causes developmental abnormalities. Here we are investigating the role of *O*-glucose in Notch signaling. We have examined the glycosylation state of each site by mass spectrometry. We have expressed His-tagged fragments of mouse Notch1 in mammalian cells, purified them using Ni-NTA agarose, digested with proteases, and analyzed by ion trap mass spectrometry. Over 13 *O*-glucose sites in mouse Notch1 have been mapped. In parallel, using a cell-based signaling assay, we have determined the importance of individual O-glucosylation sites in Notch. The same assay was previously used to study the effect of *O*-fucose sites on Notch signaling. We have generated mutants in the O-glucosylation consensus sites present in different EGF repeats in mouse Notch1. The mutant constructs were transiently transfected into the cultured cells together with the luciferase reporter gene. The transfected cells were then co-cultured with the ligand expressing cells, and the Notch activity was determined by assaying luciferase activity. By comparison with wild-type Notch, we can assess the significance of the glycosylation of each mutated site. Using this assay, we have identified several specific *O*-glucose sites that have an effect on Notch activation. This work is supported by NIH award GM61126.

Session Topic: Neuroglycobiology

(20) Polysialic Acid-Dependent Cell Migration is Essential for Mammalian Brain Development

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Polysialic acid is a unique posttranslational modification of the neural cell adhesion molecule (NCAM), and its expression is developmentally regulated (Angata and Fukuda, 2003). To determine the functions of polysialic acid in neural development, we generated mutant mice in one of two polysialyltransferases ST8SiaII or ST8SiaIV. ST8SiaII-deficient mice exhibited higher exploratory drive and reduced behavioral response to Pavlovian fear conditioning. Since cell migration from amyglada to mossy fibers was not altered, altered infrapyramidal mossy fibers in the hippocampus probably likely caused this behavior anomaly (Angata *et al*., 2004). It was reported, on the other hand, that ST8SiaIV-deficient mice exhibited an impairment of long-term depression and long-term potentiation in the hippocampus, although no overt anomaly in behavior was observed (Eckhardt *et al*., 2000). These single knockout mice still express a significant amount of remaining polysialic acid. To determine the roles of polysialic acid, we thus generated double knockout mice deficient in both ST8SiaII and ST8SiaIV. In contrast to NCAM knockout mice and ST8SiaII or ST8SiaIV single knockout mice, double mutant mice completely lack polysialic acid, indicating that ST8SiaII and ST8SiaIV are sufficient in polysialic acid synthesis. The double knockout mice display severe defects in brain development and rarely survive beyond 2 months of age. Polysialic acid deficiency impairs tangential and radial cell migration of GABAergic and pyramidal neuron precursors, respectively, and results in thin cerebral cortices, small olfactory bulbs, and abnormal cerebellar foliation. Moreover, the loss of polysialic acid enhances PDGF, but not BDNF-directed differentiation of astrocytes in neurosphere assays. These results suggest that polysialic acid attenuates the interaction between PDGF and PDGF receptor. Mutant mice deficient in both of ST8SiaII and the major polysialic acid carrier NCAM have similar but milder phenotypes than polysialic acid-deficient mice. These findings combined demonstrate that polysialic acid is essential for migration and regulating the differentiation of neural cells, thereby is required for brain development. Supported by NIH grant CA33895.

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(21) Polysialic Acid is Essential to Control NCAM Functions During Mouse Development

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Polysialic acid is a unique, dynamically regulated posttranslational modification of the neural cell adhesion molecule (NCAM) tightly associated with neural development and plasticity. The vital role attributed to polysialic acid was, however, challenged by the mild phenotype observed in mice lacking polysialic acid owing to the genetic deletion of NCAM. To dissect polysialic acid and NCAM functions, we selectively abolished the carbohydrate polymer by simultaneous ablation of the two polysialyltransferases, St8sia-II and St8sia-IV. Polysialyltransferase double-null mice were completely devoid of polysialic acid and retained normal levels of NCAM in the brain. Like Ncam-knockout mice, polysialyltransferase-negative animals showed small olfactory bulbs, a massive accumulation of cells in the proximal part of the rostral migratory stream and defasciculation and aberrant lamination of the mossy fibre tract. These shared defects must, therefore, be caused by the absence of polysialic acid and not by lack of NCAM. Beyond that, the polysialyltransferase-depleted mice exhibit a severe phenotype characterized by specific brain wiring defects, progressive hydrocephalus, postnatal growth retardation, and precocious death. Because these lethal alternations could be completely reversed by the additional inactivation of the NCAM gene in triple knockout animals, the conclusion can be drawn that defects are caused by a gain of NCAM functions. With this study, we provide the first direct evidence that polysialic acid has an essential function in controlling NCAM interactions during mouse development.

(22) Glycosphingolipids in Nervous System Development, Stability, and Disease

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Sialylated glycosphingolipids, known as gangliosides, are the major class of glycoconjugates on neurons and carry the majority of the sialic acid within the central nervous system. Their synthesis occurs through the stepwise transfer of carbohydrates to a ceramide lipid anchor. Glycosphingolipid catabolism takes place within lysosomes by the concerted action of hydrolases and activator proteins. Defects in the breakdown of glycosphingolipids cause a group of lysosomal storage diseases including Tay-Sachs, Sandhoff, and Gaucher diseases. Impaired synthesis of gangliosides has recently been found to be associated with a form of childhood epilepsy. To explore the function of glycosphingolipids and their role in disease, we have established knockout mice with defects in both the synthesis and degradation pathways. Through analysis of these mice, we have determined that glycosphingolipids are critically important for the formation of stabile central and peripheral nervous systems. Glycosphingolipids appear to function by mediating axon–glial interactions. Mice with defects in the glycosphingolipid degradation pathway model the human storage diseases and have provided insight into pathogenic mechanisms that underlie these severe neurodegenerative disorders. This concerted analysis of glycosphingolipid synthesis and degradation pathways has revealed new therapeutic strategies for treatment of the storage diseases.

(23) Human GM3 Synthase Deficiency: A Novel Form of Hereditary Childhood Epilepsy

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Although there are well-documented hereditary enzyme deficiencies for most of the steps in ganglioside catabolism which result in lysosomal storage diseases, there have been no proven defects in ganglioside biosynthesis associated with human disease. In this study, we have identified a severe epilepsy syndrome associated with loss of GM3 synthase function. This disorder is inherited as an autosomal recessive trait and results in an infantile onset symptomatic epilepsy syndrome associated with developmental stagnation and blindness. We have now studied eight affected individuals with this syndrome in four families from a large old order amish pedigree. Gene sequencing identified a nonsense mutation in the SIAT9 gene, which is predicted to result in the premature termination of the GM3 synthase enzyme (CMP-NeuAc: lactosylceramide α-2,3 sialyltransferase, EC 2.4.99.9). GM3 synthase is a sialyltransferase that catalyzes the initial step in the biosynthesis of the majority of complex ganglioside species from lactosylceramide (LacCer). Biochemical analysis of plasma glycosphingolipids (GSLs) confirmed a lack of GM3 synthase activity in all the affected individuals. There was a complete absence of GM3 ganglioside and its biosynthetic derivatives and a concomitant increase in LacCer and its alternative neutral GSL derivatives, Gb3 and Gb4. These data suggest that a lack of complex gangliosides and/or the accumulation of precursors of ganglioside synthesis result in neuronal instability in the CNS. Elucidating the mechanism(s) through which this disease phenotype develops will shed light on ganglioside functions in the brain and offer new insights for the development of therapies for this novel form of childhood epilepsy.

(24) Substrate Reduction Therapy Reduces Brain Ganglioside GM2 in Neonatal Sandhoff Disease Mice

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Sandhoff disease arises from an autosomal recessive mutation in the gene for the β-subunit of β-hexosaminidase A (*hexb* gene). This results in defective β-hexosaminidase A that, together with the GM2 activator protein, catabolyzes GM2 within lysosomes. Accumulation of GM2 and asialo-GM2 (GA2) occurs primarily in the CNS, leading to progressive neurodegeneration and brain dysfunction. Substrate reduction therapy (SRT) decreases the rate of glycosphingolipid (GSL) biosynthesis to compensate for impaired catabolism. The imino sugar, *N*-butyldeoxygalactonojirimycin (*N*B-DGJ) inhibits ceramide-specific glucosyltransferase, which catalyzes the first committed step in GSL biosynthesis. We compared the concentration and distribution of brain gangliosides between postnatal day 2 (p-2) and p-5 129/SV *Hexb–/–* mice. Neonatal mice were also injected daily intraperitonealy (ip) from p-2 to p-5 with either saline or *N*B-DGJ at 600 mg/kg body weight. *N*B-DGJ did not alter body weight, brain weight, or brain water content in the *Hexb–/–* mice. Total brain ganglioside and GM2 content increased by ~25% from p-2 to p-5 in the *Hexb–/–* mice. *N*B-DGJ treatment in the *Hexb–/–* mice from p-2 to p-5 significantly reduced total brain ganglioside content by 23% and that of GM2 by 57%. GM2 content was also less in the p-5 *N*B-DGJ treated mice than in the p-2-untreated mice. Furthermore, the distribution of GM1 increased in the *N*B-DGJ treated mice at p-5. These results suggest that SRT using *N*B-DGJ during early development may be an effective early intervention therapy for the management of GM2 ganglioside storage diseases. Supported by NIH grant (HD39722) and the NTSAD association.

(25) Protein-Specific Polysialylation of NCAM by Polysialyltransferases

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Polysialic acid is an anti-adhesive protein modification that promotes cell migration and the plasticity of cell interactions. Because so few proteins carry polysialic acid, we hypothesize that polysialylation is a protein-specific event and that a specific polysialyltransferase-substrate interaction is the basis of this specificity. The major substrate for the polysialyltransferases is the neural cell adhesion molecule, NCAM. Previous work demonstrated that the first fibronectin Type III (FNIII) repeat of NCAM (FN1) was necessary for the polysialylation of the *N*-glycans on the adjacent immunoglobulin domain (Ig5) (Close *et al.*, 2003). This suggested that FN1 may be a recognition site for the polysialyltransferases. To demonstrate a requirement for FN1, we replaced FN1 of NCAM with FN2. We found that FN2 is unable to functionally replace FN1 to allow NCAM polysialylation, suggesting that specific sequences in FN1 might play a role in NCAM recognition. FNIII repeats share a similar *fÒ* sandwich structure but are diverse in sequence. We modeled the structure of FN1 using the NMR structure of rat FN2. This revealed that FN1 possessed a negatively charged surface patch, including D511, E512, and E514, which was not present on the surface of FN2. Arg substitution of theses acidic amino acids eliminated polysialylation not only of a minimal Ig5-FN1 substrate, but also of full-length NCAM. Interestingly, Ala substitution of these residues eliminated Ig5-FN1 polysialylation, but not that of full-length NCAM, suggesting that the

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two proteins are interacting differently with the enzymes and that multiple residues are involved in the enzyme-NCAM interaction. Using another truncated protein, Ig5-FN1-FN2, we confirmed the importance of enzyme-substrate positioning for optimal recognition and polysialylation. These results suggested that the acidic residues on the surface of FN1 are part of a larger protein interaction region that is critical for NCAM recognition and polysialylation by the polysialyltransferases (Mendiratta *et al*., 2005). Recently, we have solved the crystal structure of the FN1 repeat of human NCAM. This domain has a *f*Ò sandwich structure but is unique in that it has an *f*Ñ helix between strands *f*Ò4 and *f*Ò5. This *f*Ñ helix protrudes from the surface of the molecule and is positioned near the D511/E512/E514 acidic patch. We predict that the acidic amino acids and the *f*Ñ helix serve as a recognition site for the polysialyltransferases and that this interaction is the basis for the protein specificity of polysialylation.

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(26) Role of Sialyltransferase in the Nervous System Development of *Drosophila*

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In vertebrates, sialylation is implicated in many physiological and pathobiological processes, including nervous and immune system development and functioning, pathogen–host interaction, and cancer progression. In lower animals, data on sialylation are scarce. We previously characterized *Drosophila* sialyltransferase (D.SiaT), so far the only sialyltransferase described in protostomes (Koles *et al*., 2004). D.SiaT shows significant homology to the ST6Gal family of vertebrate enzymes. Interestingly, similar to its mammalian homologue ST6Gal II, *Drosophila* sialyltransferase is expressed in the CNS during development. To elucidate the biological functions and mechanisms of sialylation in animal development, we focus our research on understanding the mechanism and role of sialylation in *Drosophila* development. We found that *Drosophila* sialyltransferase is expressed in a stage-specific manner. Expression of D.SiaT is restricted to certain types of neurons in the central nervous system during embryonic and larval stages, which suggests a role for D.SiaT in the development and functioning of the nervous system. Using gene-targeting approach (Rong and Golic, 2000), we have generated several *D.SiaT* mutants. These mutants are viable and fertile, but they exhibit notable behavioral abnormalities and have a locomotor impairment that progresses with age. Our data suggest an important role for sialylation in synaptic signal transmission. New data on the expression pattern and mutant phenotypes of *D.SiaT* will be presented and discussed in the light of the potential role of sialylation in neural development. This work was supported in part by NIH grant R01 GM069952.

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Session Topic: Glycans and Lectins in Pathogen Recognition

(27) Genetic Analysis of Pathways Required for the Assembly of the Surface Glycocalyx Coat of the Protozoan Parasite *Leishmania* and Their Roles in the Infectious Cycle

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The trypanosomatid protozoan parasite *Leishmania* expresses a diverse array of glycoconjugates on its surface throughout the infectious cycle, many of which have been implicated in critical steps essential for pathogenesis. These include lipophosphoglycan (LPG), a polymer of phosphoglycan (PG) $[Gal\text{-}Man\text{-}P]_n$ repeating units attached to the surface through a heptasaccharide glycan core and glycosylphosphatidylinositol (GPI) anchor, PG-modified and/or GPI-anchored proteins, such as proteophosphoglycan (PPG) and GP63 (leishmaniolysin), free GPIs termed glycosylinositol phospholipids (GIPLs), and inositolphosphoceramides (IPCs). LPG expression is restricted to the promastigote stage carried by the sand fly vector, whereas the remaining molecules are expressed more or less constitutively and in the amastigote stage which resides within mammalian macrophages. Since the diverse families of glycoconjugates comprising the parasite surface frequently share the same or similar structural motifs, definitive assignment of the role of any given molecule or subdomain has proven elusive. Thus forward and reverse genetic approaches (now aided by the completion of the *Leishmania major* genome sequence) have proven critical in dissecting their molecular pathway(s) of assembly and roles. In most cases, genes recognized by forward genetics have proven to be novel and founding members of new protein families. Collectively, these studies have yielded genes exemplified by those affecting nucleotide sugar biosynthesis (UDP-galactofuranose; *GLF*), glycosyltransferases such as those encoded by *LPG1* (Galf-T) and *LPG4* (Man-P-T), specific chaperones such as the GRP94 relative *LPG3*, and nucleotide-sugar transporters encoded by the *LPG2* and *LPG5A*/*B* genes, as well as steps within the ether phospholipid (*ADS1*) and sphingolipid synthesis and degradation (serine palmitoyl transferase, *SPT1*/*2*, and sphingosine 1-phosphate lyase, *SPL*) pathways. From these studies, we are developing a comprehensive picture of how the parasite surface is assembled, and how each of these molecules and/or glycoconjugate domains contributes singly or in association with others to the ability of the parasite to complete its infectious cycle.

(28) Lipophosphoglycan–Galectin Interactions Controlling Sand Fly Vector Competence for *Leishmania major*

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The phlebotomine vectors of *Leishmaniasis* are in some cases only permissive to the complete development of the species of *Leishmania* that they transmit in nature. The parasite–sand fly interactions that control this specificity are related to differences in the ability of the parasite to maintain infection in the midgut during excretion of the digested bloodmeal. The evidence that the *Leishmania* surface lipophosphoglycan (LPG) mediates promastigote attachment to the midgut epithelium so as to prevent their loss during bloodmeal excretion is especially strong based on the comparison of development in sand flies using LPG-deficient mutants. LPG displays interspecies polymorphisms in their phosphoglycan domains that in most cases can fully account for species-specific vector competence. The ability of *Phlebotomus papatasi* to transmit only *Leishmania major* sp. has been attributed to the unique, highly substituted nature of *L. major* LPG that provides for multiple terminally exposed β-linked galactose residues for binding, suggesting that *P. papatasi* midguts express lectin-like molecules with specificity for polygalactose epitopes. PpGalec, a cDNA encoding a novel tandem repeat galectin, was identified by high throughput screening of a midgut library of *P. papatasi*. Recombinant PpGalec bound specifically to *L. major* promastigotes bearing poly-gal epitopes on their LPG, and native PpGalec, was shown to be used by *L. major* as a receptor for mediating its specific binding to the *P. papatasi* midgut. This is the first description of the nature and specificity of a sand fly midgut LPG receptor; and the first indication that insect galectins, which have been mainly associated with embryonic development or innate immunity against pathogens, can be exploited by parasites to promote their survival and transmission. The feasibility of using sand fly midgut molecules as target antigens for transmission-blocking vaccines has also been demonstrated.

(29) Mannose 6-Phosphate Receptors and the Pathogenesis of Infections due to Varicella Zoster Virus

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The large cation-independent mannose 6-phosphate receptor (CI-MPR) plays roles in the transport of newly synthesized lysosomal enzymes from the trans-Golgi network (TGN) to late endosomes and in the receptor-mediated endocytosis of extracellular lysosomal enzymes. Recent evidence suggests that CI-MPR is also critically involved in the pathogenesis of varicella and zoster, the two diseases caused by varicella zoster virus (VZV). At least four VZV envelope glycoproteins contain mannose 6-phosphate (Man 6-P) and thus interact with CI-MPR. Plasma membrane CI-MPR was proposed to be essential for viral entry, because Man 6-P selectively blocks infection by cell-free VZV, and cells become resistant to VZV infection when trafficking of free Man 6-P to the cell surface is inhibited by alkalinizing endosomes. The proposal that CI-MPR are essential for viral entry was confirmed by experiments with CI-MPR-deficient lines of human cells. These lines were generated by expression of antisense cDNA or siRNA-like transcripts. CI-MPR-deficient cells resist infection by cell-free, but not cell-associated, VZV. Electron microscopic (EM) observations

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and the ability of chlorpromazine, which interferes with endocytosis, to prevent infection by cell-free VZV, suggest that endocytosis, possibly CI-MPR mediated, is essential for infection by varicella virions. During lytic infection, viral envelopment occurs in the TGN. Viral and cellular glycoproteins, including CI-MPR, become separated. Viral glycoproteins become restricted to the viral envelope, whereas the CI-MPR is concentrated in the membrane of a transport vesicle, which encloses the newly assembled virion. After envelopment, these vesicles follow the itinerary of CI-MPR and transport virions to late endosomes, where VZV is inactivated. Because of this transport, infectious virions are not released, and infection is almost exclusively cell associated (infected cells fuse with their neighbors, independently of CI-MPR). In contrast, CI-MPR-deficient cells release infectious virions when infected by cell-associated VZV. Intracellular CI-MPR thus divert newly enveloped VZV to late endosomes, whereas CI-MPR at cell surfaces are necessary for entry and may mediate VZV endocytosis. Immunocytochemical and EM observations of VZV-infected human skin reveal that the expression of CI-MPR is lost in maturing superficial epidermal cells. These cells, therefore, do not divert VZV to late endosomes and constitutively secrete infectious VZV. CI-MPR are concentrated in the plasma membranes of axon terminals, which innervate the superficial epidermis and thus are bathed in infectious VZV during varicella. *In vitro* studies with animal neurons have shown that latency is established when isolated neurons are infected by cell-free VZV, but a lytic infection results when they are infected by fusion with nonneuronal cells. The release of infectious virions in the epidermis during varicella may thus be the critical event that enables VZV to become latent selectively in sensory ganglia. Reactivation of VZV in these ganglia then returns VZV by anterograde transport to the epidermis where infection of Man 6-P-deficient cells spreads VZV to new hosts. Supported by AI127187, AI24021, and NS12969.

(30) Identification and Functional Characterization of a UDP-Glucose Pyrophosphorylase from *Leishmania major*

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Leishmania are protozoan parasites and cause diseases ranging from self-healing cutaneous lesions to lethal visceral forms. In *Leishmania major*, various glycoconjugates are essential for parasite virulence. They form a dense cell surface glycocalyx allowing the survival and proliferation of the parasite in very hostile environments. Thus, enzymes involved in the biosynthesis of the parasite glycocalyx provide interesting drug targets. Essential for the biosynthesis of glycoconjugates is the metabolic activation of the monosaccharides as nucleotide sugars by pyrophosphorylases. We have isolated a UDP-glucose pyrophosphorylase (UDPGP) from *Leishmania major* that forms UDP-glucose from glucose-1-phosphate and UTP. The activation of glucose to UDP-glucose is crucial for the entry in biosynthetic pathways and is required for the synthesis of UDP-galactose, a major component of *Leishmania* glycoconjugates. The activity of the UDPGP was proven by complementation studies of an *Escherichia coli galU* mutant and by *in vitro* activity assays. Because it was postulated that the oligomeric state of UDPGPs has a critical impact upon catalysis, the oligomerization status of the recombinant protein was determined. In contrast to UDPGPs from other organisms, the *L. major* UDPGP exists as monomer exclusively, and no higher order oligomers could be detected. These data are in agreement with the simple Michaelis–Menten kinetics observed for all substrates. Finally, the role of the UDPGP in the pathomechanism of *Leishmania* has been investigated by gene deletion. The obtained mutant demonstrated drastically reduced virulence in a mouse-infection model, indicating the importance of this gene for pathogen development in the host.

(31) Origin of the Galacturonic Acid Modifications to the Inner Core of *Rhizobium leguminosarum* Lipopolysaccharides

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The outer leaflet of the outer membrane of Gram-negative bacteria is composed of a unique glycolipid known as lipopolysaccharide (LPS). The LPS of the nitrogen-fixing plant endosymbiont, *Rhizobium leguminosarum*, is strikingly different from the LPS of common enteric Gram-negative bacteria such as *Escherichia coli*. One prominent difference is the modification of the inner core domain of *R. leguminosarum* LPS with galacturonic acid (GalA) moieties. The first seven enzymes of the LPS biosynthesis pathway in *Escherichia coli* and *R. leguminosarum* are conserved, making Kdo_2-lipid IV_A a common intermediate. Using the radiolabeled intermediate, Kdo_2-[4′-^{32}P]-lipid IV_A, as probe, we screened a cosmid *R. leguminosarum* 3841 DNA library harbored in a *Sinorhizobium meliloti* background. We isolated a clone (pSGAT) that overexpressed two new putative glycosyltransferase activities. The clone catalyzed the attachment of two hydrophilic moieties to the outer Kdo of the radiolabeled substrate, as determined by thin later chromatography and mild acid hydrolysis of products. Extracts of cells harboring a 7 kb subclone (pMKG) derived from pSGAT retains the activities of the original clone. The activities are insert dependent as they are not present in the empty vector or in the host, *S. meliloti*. The activities are membrane bound, require a detergent for optimal activity, and exhibit a preference for the 1-dephosphorylated substrate. Sequence analysis of the clone indicates the presence of four open-reading frames (ORFs) of interest. Three of these are orthologs of the *E. coli* ArnT gene, an enzyme involved in the transfer of aminoarabinose from an undecaprenyl-phosphate aminoarabinose donor onto lipid A. The fourth ORF is homologous to the dolichol phosphomannose synthase family of proteins. Based on initial data and bioinformatic considerations, we suggest the ArnT orthologs are *R. leguminosarum* core GalA transferases and have renamed them RgtA, RgtB, and RgtC (*r*hizobium *g*alA *t*ransferase). We further postulate that these GalA transferases use a lipid-linked GalA donor. The individual Rgts have been isolated and analyzed in the heterologous host strain *S. meliloti*. RgtA and RgtB are able to reconstitute the two original activities seen in pSGAT in an ordered fashion. ESI mass spectrometry of an *in-vitro* synthesized product verifies the formation of a hexuronic acid adduct and its linkage to a Kdo sugar. Thus we propose RgtA and RgtB to be the two outer-Kdo GalA transferases. Reassessment of the parent clone, pMKG, using the novel radiolabeled substrate, mannosyl-Kdo_2-1-dephospho-[4′-^{32}P]-lipid IV_A, led to the generation of three hydrophilic products. We suggest the third activity represents the attachment of a GalA moiety to the mannose residue by RgtC. The heterologous expression of RgtA in *E. coli* requires the presence of a *Rhizobiaceae* membrane lipid component for activity, suggestive of a lipid-linked donor. This component is resistant to mild-alkaline hydrolysis. To identify the donor, lipids from *R. leguminosarum* 3841 have been isolated, alkali treated, and partially purified by ion-exchange chromatography. Initial results indicate the presence of a C_{60}-polyisoprene-linked hexuronic acid (likely galacturonic acid) that we purport to be the GalA donor. Supported by NIH grant GM-51796 to C.R.H.R.

(32) Pathogen Capture in Water Using Glycoprotein Micelles

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Studies have demonstrated that fimbriated bacteria can be captured from tissue fluids by exploiting the affinity of their surface adhesins for certain sugars. Binding studies with pathogenic bacteria reveal remarkable specificity of this group of adhesins (lectins) for carbohydrate configurations present on glycoproteins of host tissue surfaces. In accordance with these observations, the possibility of using glycoprotein micelles to capture pathogens based on the lectin profiles expressed in water was proposed. Glycoprotein micelles present a novel approach to pathogen capture in aqueous systems. Micelles are formed by adding oil to glycoprotein solutions. The amphipathic nature of glycoproteins provides needed stability to produce floating oil spheres coated with proteins exposing oligosaccharides to the milieu. Buoyancy of micelles in water facilitates their recovery for analysis. A lectin agglutination assay confirms the presence of glycans on the external surfaces of manufactured micelles. Currently, these micelles are being assessed for binding capacity and specificity for certain bacteria. Our model system employs micelles made with commercial yeast-derived invertase to capture *Escherichia coli* expressing Type 1 fimbriae; an afimbriated *E. coli* strain is used as a negative control. Within a short incubation time, the invertase micelles capture fimbriated *E. coli* with high specificity, indicating that invertase high-mannose glycans are powerful ligands for Type 1 fimbriae in aqueous solution. Testing preferential micelle capture of *E. coli* in a mixture of bacteria has also begun. Future studies will aim to optimize micelle size, increase binding capacity, test specificity, and selective capture in multispecies aqueous systems. We plan to explore the application of glycoprotein micelles to capture toxins.

Session Topic: Glycan Immunology

(33) Development of a Conjugate Vaccine Against *Haemophilus influenzae* Type B Based on Synthetic Antigens

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Conjugate vaccines represent an important step forward in the fight again infectious diseases. Alternatives to existing technologies are continuously needed to increase the massive needs of vaccines throughout the world. The possibility of reproducing the structure of protein or polysaccharide antigens by chemical synthesis was demonstrated in many cases. However, the development of vaccines using these synthetic antigens was interfered by many issues. We developed a process for the chemical synthesis of *Haemophilus influenzae* Type b oligosaccharides as a base for a new conjugated vaccine prototype. After complex preclinical and technological development that includes clinical testing in the target population, we demonstrated that the vaccine containing fully synthetic oligosaccharides representing a fragment of the bacterial capsular polysaccharide is as effective as their natural counterpart (Verez-Bencomo *et al*., 2004). The vaccine was registered in Cuba in 2003 and is now part of the nation immunization program. Further large scale production is now implemented.

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(34) C-Type Lectins on Dendritic Cells: Antigen Receptors and Modulators of Immune Responses

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Dendritic cells (DC) are specialized in the recognition of pathogens and play a pivotal role in the control of immunity. Yet DC are also important for homeostatic control recognizing self-antigens and tolerizing its environment, indicating that the nature of the antigen it recognizes may steer a DC toward immunity or tolerance. C-Type lectin receptors expressed by DC are involved in the recognition and capture glycosylated self-antigens or pathogens. To date, seven different C-type lectins have been identified on DC. It is now becoming clear that these C-type lectin receptors may not only serve as antigen receptor recognizing pathogens to allow internalization and antigen presentation, but may also function in the recognition of self antigen or as adhesion molecules and signaling molecules. We have studied in great detail the function and the glycan specificity of the DC-specific C-type lectin DC-SIGN and MGL. DC-SIGN recognizes high-mannose structure and nonsialylated Lewis antigens (Lex, Ley, Leb, and Lea), and MGL recognizes GalNAc which are expressed on many pathogens and have suggested to lead to immune escape. To date, little is known on the specificity by which C-type lectins interact with self-glycoproteins. Lewis antigens are recognized on glycoproteins present on PMNs and mediate a cellular interaction between PMN and DC allowing proper antigen delivery. Also Lewis antigens on colon carcinomas are recognized by DC-SIGN on DC, and identification of the tumor antigens revealed that DC-SIGN strongly binds the tumor antigen CEA through Lex and Ley carbohydrate structures. Similarly, also MGL recognizes self-glycoproteins on a subset of PBL, but also interacts with colon carcinoma-associated MUC1 carrying GalNAc structures. Currently, we are analyzing how glycan modifications in Lewis antigen or GalNAc during oncogenesis may suppress DC function in benefit for tumor growth. The finding that especially C-type lectins recognize carbohydrate structures on tumor cells opens up a new area of research that studies the potency of C-type lectins to interact with distinct glycosylated tumor antigens. Understanding the diversity of C-type lectins being expressed on DC as well as their carbohydrate-specific recognition profile will be instrumental to understand DC pathogen recognition in many pathogenic disorders, as well as the regulation of cellular interactions of DC that are essential in the control of immunity.

(35) Structural Basis of DC-SIGN Ligand Specificity

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The dendritic cell receptor DC-SIGN functions in the initial recognition of pathogens and also mediates adhesion of T cells that scan the surface of dendritic cells for the presence of peptide antigens. Both DC-SIGN and the related endothelial cell receptor DC-SIGNR bind high-mannose N-linked carbohydrates presented on the surfaces of HIV and other enveloped viruses. Screening of a glycan array has revealed that DC-SIGN and DC-SIGNR display distinct specificities: DC-SIGN binds to certain kinds of branched fucosylated structures as well as high-mannose N-linked structures, whereas DC-SIGNR only binds to high-mannose structures. The molecular basis of these specificity differences have been investigated by determining high-resolution co-crystal structures of these receptors bound to appropriate carbohydrate ligands. The structures reveal that a few amino acids present in DC-SIGN create two distinct subsites which confer its dual ability to recognize both high-mannose and branched fucoslyated structures.

(36) Glycan Processing and Presentation: The New MHC Class II Pathway

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For many years, T cell-dependent adaptive immunity has been the sole dominion of protein antigens, whether they be conventional proteins that were processed to peptides by host proteases or superantigens binding directly to MHC molecules without processing. These antigens are recognized by αβ T-cell receptors on T cells which triggers the appropriate immune response. Then, the MHC-like protein CD1 was found to present lipids and glycolipids to γδ T-cell receptors; however, the canonical MHC class I and class II mechanisms were still limited to protein-based antigens. Despite this broadly held view, the Kasper laboratory has repeatedly demonstrated that at least one class of carbohydrates, containing a zwitterionic charge motif, also have the capability of activating $CD4^+$ T cells in a manner that requires αβ T-cell receptors. Furthermore, my work in the Kasper laboratory demonstrated that these carbohydrates not only utilize the MHC class II (MHCII) pathway within antigen presenting cells, but are also processed to a low molecular weight form through an uncharacterized nitric oxide-dependent mechanism for presentation and recognition. Despite these observations, no structural model exists that could readily explain how these carbohydrates are binding to MHCII molecules. As a result, much of my more recent effort has focused on understanding the mechanism of carbohydrate presentation. An initial binding study with a model T cell-activating polysaccharide (PSA from *Bacteroides fragilis*) has demonstrated that carbohydrate binding with recombinant MHCII proteins is saturable, 1 to 1, and shows allelic selectivity. With an average affinity of ~1 μM, the binding is very similar to many known peptide antigens. Interestingly, competition experiments show that PSA competes for MHCII binding with both peptides and superantigens, suggesting that these carbohydrates contact both the normal peptide binding cleft in addition to regions outside that cleft. These data begin to paint a picture of how T-cell activating zwitterionic polysaccharides might activate the adaptive immune system in a MHCII-dependent fashion. The allelic selectivity seen within these *in vitro*-binding studies suggest that MHC restriction may play an important role in the resulting T-cell responses, although this has not been demonstrated biologically. These results also suggest that presentation is a specific event with these antigens, as seen with conventional peptide antigens, although the specificity of the T-cell recognition and response is not well established. Collectively, these observations shift the traditional MHC paradigm to include carbohydrates, opening the door to new possibilities in vaccine research and development.

(37) Exogenous and Endogenous Glycolipid Antigens Activate NKT Cells During Microbial Infections

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CD1d-restricted natural killer T (NKT) cells are innate-like lymphocytes that express a conserved T-cell receptor and contribute to host defence against various microbial pathogens. However, their target lipid antigens have remained elusive. Here we report evidence for microbial, antigen-specific activation of NKT cells against Gram-negative, lipopolysaccharide (LPS)-negative alphaproteobacteria, such as *Ehrlichia muris* and *Sphingomonas capsulata*. We have identified glycosylceramides from the cell wall of *Sphingomonas* that serve as direct targets for mouse and human NKT cells, controlling both septic shock reaction and bacterial clearance in infected mice. In contrast, Gram-negative, LPS-positive *Salmonella typhimurium* activates NKT cells through the recognition of an endogenous lysosomal glycosphingolipid, iGb3, presented by LPS-activated dendritic cells. These findings identify two novel antigenic targets of NKT cells in antimicrobial defence and show that glycosylceramides are an alternative to LPS for innate recognition of the Gram-negative, LPS-negative bacterial cell wall.

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Session Topic: N-Linked Glycan Functions

(38) Gains of Glycosylation Comprise an Unexpectedly Large Group of Pathogenic Mutations

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Mutations involving gains-of-glycosylation have been considered rare, and the pathogenic role of the new carbohydrate chains has never been formally established. We identified three children with Mendelian susceptibility to mycobacterial disease who were homozygous with respect to a missense mutation in IFNgammaR2 creating a new N-glycosylation site in the IFNgammaR2 chain. The resulting additional carbohydrate moiety was both necessary and sufficient to abolish the cellular response to IFNgamma. We then searched the Human Gene Mutation database for potential gain-of-N-glycosylation missense mutations; of 10,047 mutations in 577 genes encoding proteins trafficked through the secretory pathway, we identified 142 candidate mutations (~1.4%) in 577 genes (~13.3%). Six mutant proteins bore new N-linked carbohydrate moieties. Thus, an unexpectedly high proportion of mutations that cause human genetic disease might lead to the creation of now N-glycosylation sites. Their pathogenic effects may be a direct consequence of the addition of N-linked carbohydrate.

(39) Hexosamine, *N*-Glycans, and Cytokine Signaling—A Regulatory Network

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N-Glycans on cytokine receptors bind galectins at the cell surface, forming a high avidity lattice that opposes receptor loss to constitutive endocytosis (Partridge *et al*., 2004). The avidity of galectin-3 binding is dependent on the number of *N*-glycan chains per receptor and their modifications on passage through the Golgi. The branching of *N*-glycans is dependent on flux through the hexosamine pathway to UDP-GlcNAc, a rate-limiting substrate in the Golgi. We observe that the number (*n*) and density of *N*-glycans chains is higher in anabolic cytokine receptors than TGF-b receptors. Moreover, TGF-b and other receptors known primarily for morphogenic functions display a selectively lower number and density of *N*-glycans. Golgi multistep ultrasensitivity and *n* determine the kinetics of receptor regulation at the cell surface by the hexosamine pathway. An important emergent property of the model is that sensitivity to anabolic cytokines occurs at low glucose flux and generates the positive feedback required to increase surface receptors and autocrine TGF-b/Smad signaling. This mimics a developmental sequence where growth and proliferation is followed by differentiation and arrest. Our results suggest a system for metabolic and developmental homeostasis that requires conditional regulation of *N*-glycan processing, with implications for cancer, immunity, aging, and stem cell maintenance.

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(40) Dietary and Genetic Control of Pancreatic Beta Cell Glucose Transporter-2 Glycosylation Promotes Insulin Secretion in Suppressing the Pathogenesis of Type 2 Diabetes

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Pancreatic beta cell surface expression of glucose transporter-2 (Glut-2) is essential for glucose-induced insulin secretion thereby controlling blood glucose homeostasis in response to dietary intake. Beta cell failure associated with loss of Glut-2 expression is the earliest pathogenic feature in the development of Type-2 diabetes, resulting in the absence of glucose-stimulated insulin secretion and chronic hyperglycemia. We show that the Mgat4a-encoded Golgi resident GnT-4a glycosyltransferase is required for the production of an *N*-glycan structure which functions as a ligand for lectin receptors, including galectin-9, that maintain Glut-2 residency on the beta cell surface. This lectin–ligand binding interaction is glycoprotein- and cell-type specific. Glycoprotein analyses reveal normal expression of other similarly misglycosylated glycoprotein including insulin receptors on the beta cell surface, and expression of Glut-2 molecules is unaltered among hepatocytes that lack Mgat4a expression and GnT-4a protein glycosylation. Competitive inhibition of lectin binding to Glut-2 using exogenous ligand mimetics leads to rapid loss of beta cell surface Glut-2 expression. Furthermore, attenuation of Mgat4a expression by genetic disruption or administration of a high-fat diet diminishes Glut-2 glycosylation, resulting in a severe reduction of cell surface half-life by provoking endocytosis with redistribution into endosomes and lysosomes. GnT-4a deficiency abolishes the first phase of glucose-stimulated insulin secretion resulting in hyperglycemia, increased circulating free fatty acids, and elevated expression of liver gluconeogenic enzymes. Hepatic steatosis and insulin resistance develop with age further enhancing the resemblance of GnT-4a deficient pathology in the mouse to human Type 2 diabetes. These findings reveal that GnT-4a glycosyltransferase expression and Glut-2 glycosylation are under genetic and dietary control mechanisms that are essential for maintaining pancreatic beta cell surface Glut-2 expression and insulin secretion in normal physiologic contexts. Disabling this receptor-binding mechanism by genetic disruption or chronic ingestion of a high-fat diet is linked with pancreatic beta cell failure and is a harbinger of further metabolic dysfunction to follow in the pathogenesis of Type 2 diabetes.

(41) N-Glycosylation-Dependent Apical Trafficking of the Sialomucin Endolyn in Polarized Epithelial Cells

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Our laboratory has been investigating the role of *N*-glycans as targeting signals using the sialomucin endolyn as a model. The lumenal portion of endolyn contains two sialomucin domains separated by a nonmucin domain; each domain is N-glycosylated at two to four sites. Glycosylation of endolyn is developmentally regulated and appears to be functionally important for endolyn's role in cell adhesion and differentiation. In polarized epithelial cells, endolyn is found at the apical cell surface and in lysosomes, and apical delivery of the protein is dependent on N-glycosylation of its nonmucin domain. Specifically, terminal processing of a subset of endolyn's *N*-glycans is required for efficient apical delivery of the newly synthesized protein. Once at the cell surface, endolyn is efficiently internalized and recycles to the plasma membrane from endosomal and lysosomal compartments. Because apical recycling may contribute significantly towards regulating the steady-state distribution of the protein, we examined the role of N-glycosylation in the postendocytic sorting of endolyn. For these experiments, we compared the initial polarity of delivery of radiolabeled endolyn or endolyn constructs in which the *N*-glycan-dependent signal was disrupted with the steady-state distribution attained after multiple rounds of recycling. Wild-type endolyn maintained its initial polarized distribution throughout a 21-h course, suggesting that internalized endolyn is recycled primarily to the apical surface. In contrast, both the initial and subsequent delivery of N-glycosylation mutants was nonpolarized, indicating that apical recycling of endolyn was disrupted. Moreover, desialylation of apical endolyn resulted in acute redistribution of the desialylated pool of protein, which is consistent with a role for terminal glycan processing in postendocytic sorting. These results suggest that similar *N*-glycan-dependent sorting determinants are required for apical delivery of endolyn along both the biosynthetic and postendocytic pathways.

(42) HIV Envelope Glycoproteins: Modification of Glycans and Glycan-Dependent Folding Pathways Provide New Targets for Vaccine Design and Anti-Viral Therapies

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Carbohydrate recognition represents a major component of both adaptive and innate immunity. However, antibodies to sugars, particularly glycans attached to cell surface glycoproteins, have rarely been exploited in vaccine design (Kelly *et al*., 2004; Zimmer and Stephens, 2004). Human immunodeficiency virus Type 1 (HIV-1) is covered by large, flexible, and poorly immunogenic N-linked carbohydrates which form an "evolving glycan shield" that generally promotes humoral immune evasion. Nevertheless, we have defined a highly unusual cluster of mannose residues on the HIV envelope glycoprotein gp120 that forms the epitope for one rare, neutralizing domain swapped antibody, 2G12 (Scanlan *et al*., 2002; Calarese *et al*., 2003). We have now shown that manipulation of the glycan shield using glycosylation processing inhibitors provides an enhanced template for immunogen design. In another approach, we have selected a yeast mutant that displays 2G12 epitopes. The imino sugar, *N*-butyldeoxynojirimycin

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(NB-DNJ), is an inhibitor of endoplasmic reticulum (ER) á-glucosidases I and II and blocks the processing of nascent glycoproteins to the monoglucosylated glycoforms required for entry to the calnexin/calreticulin quality control pathway. NB-DNJ treatment of HIV-infected cells leads to misfolding of the envelope glycoprotein gp120 and significantly reduces viral infectivity (Fischer *et al*., 1995; 1996). Although NB-DNJ has an inhibition constant of 0.57 micromolar, serum concentrations of >500 micromolar are required to deliver the drug to the ER at the levels required to achieve anti-viral effects *in vivo*, resulting in serious side effects in patients. Mammalian cells expressing soluble gp120 were incubated with liposomes containing NB-DNJ. These liposomes dramatically enhance the intracellular delivery of NB-DNJ across both the plasma membrane and the endoplasmic reticulum, significantly lowering the overall dose required to reduce viral infectivity. Concentration-dependent inhibition of the processing of glycoproteins was observed, both on secreted proteins and in the ER. Intracellular delivery of NB-DNJ led to a 104- to 105-fold enhancement of the activity of the imino sugar, reducing overall concentrations to several orders of magnitude below toxic levels. This suggests that NB-DNJ delivered in liposomes may be a generally effective anti-viral treatment.

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(43) Characterization of a Human Core-Specific Lysosomal α1-6Mannosidase Involved in *N*-Glycan Catabolism

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In humans and rodents, lysosomal catabolism of core Man3GlcNAc2 *N*-glycan structures is catalyzed by the concerted action of several exoglycosidases, including a broad specificity lysosomal alpha-mannosidase (LysMan), core-specific alpha1-6mannosidase, beta-mannosidase, and cleavage at the reducing terminus by a di-*N*-acetylchitobiase. We describe here the first cloning, expression, purification, and characterization of a novel human glycosylhydrolase family 38 alpha-mannosidase with catalytic characteristics similar to those previously established for the core-specific alpha1-6mannosidase (acidic pH optimum, inhibition by swainsonine and 1,4-dideoxy-1,4-imino-D-mannitol, high K_m for cleavage of 4-methylumbelliferyl-alpha-D-mannoside, and stimulation by Co^{+2} and Zn^{+2}). Substrate-specificity studies comparing the novel human alpha-mannosidase with human LysMan revealed that the former enzyme efficiently cleaved only the alpha1-6mannose residue from Man3GlcNAc, but not Man3GlcNAc2 or other larger high mannose oligosaccharides, indicating a requirement for chitobiase action before alpha1-6mannosidase activity. In contrast, LysMan cleaved all of the alpha-linked mannose from high mannose oligosaccharides except the core alpha1-6mannose residue. Transcripts encoding the alpha1-6mannosidase were ubiquitously expressed in human tissues and expressed sequence tag searches identified homologous sequences in mouse, pig, and dog databases. No expressed sequence tags were identified for bovine alpha1-6mannosidase, despite the identification of two sequence homologs in the bovine genome. The lack of conservation in 5′-flanking sequences for the bovine alpha1-6mannosidase genes suggests that the absence of enzyme activity in this species may result from defective transcription, similar to the mechanism that eliminates transcription of the bovine chitobiase gene. These results suggest that the chitobiase and alpha1-6mannosidase function in tandem for mammalian lysosomal *N*-glycan catabolism. (Supported by NIH grants GM47533, CA91295, and RR05351.)

Session Topic: Glycans in Immune System Regulation

(44) CD22: A Multifunctional Lectin that Regulates B Lymphocyte Survival and Signal Transduction

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B lymphocytes are the central mediators of humoral immunity. B cells depend on cues from their extracellular microenvironment for development, homeostasis, activation, proliferation, and effector function. These functions are regulated through cell-surface molecules that generate transmembrane signals, regulate intercellular communication, and direct lymphocyte localization within tissues. These events rely on signaling molecules that provide important functional links between the cell surface and intracellular signaling. In this regard, CD22 represents a specialized costimulatory or coreceptor cell surface molecule expressed exclusively by all mature B-lineage cells that also function as "response regulator" to modulate the intensity, quality, and duration of homeostatic and B-cell antigen receptor (BCR)-induced signals. Response regulators carry out broader functions than costimulatory molecules, because they establish intrinsic-signaling thresholds that provide a context for other transmembrane and cytoplasmic signals. Recent advances in the study of CD22 also indicate a complex role for ligand-binding by this transmembrane lectin-like member of the immunoglobulin superfamily in regulating BCR and CD19 signal transduction and providing essential survival signals. CD22 has been previously recognized as a lectin-like adhesion molecule that binds alpha2,6-linked sialic acid-bearing ligands and as an important regulator of BCR signaling. Until recently, most of the functional activity of CD22 has been widely attributed to the ability of CD22 to recruit potent intracellular phosphatases and limit the intensity of BCR-generated signals. However, recent genetic studies in mice reveal that some CD22 functions are regulated by ligand binding, whereas other functions are ligand independant and may only require expression of an intact CD22 cytoplasmic domain at the B-cell surface (Poe *et al*., 2004). With these findings, a more complex role for CD22 has emerged, including a central role in a novel regulatory loop controlling the CD19/CD21-Src-family protein tyrosine kinase (PTK) amplification pathway that regulates basal-signaling thresholds and intensifies Src-family kinase activation following BCR ligation. CD22 ligand binding is also central to the regulation of peripheral B-cell homeostasis and survival, the promotion of BCR-induced cell cycle progression, and is a potent regulator of CD40 signaling. This seminar will discuss our current understanding of how CD22 governs these complex and overlapping processes, how alterations in these tightly controlled regulatory activities may influence autoimmune disease and the current and future applications of CD22-directed therapies in oncology and autoimmunity (Tedder *et al*., in press).

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(45) CD22-Ligand Interactions in BCR Signaling

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CD22 is a negative regulator of B-cell receptor (BCR) signaling, an activity modulated by its interaction with glycan ligands containing α2-6 linked sialic acids. CD22 interacts with glycan ligands on the B cell, in cis, and on adjacent cells (e.g., T cells), in trans, both appear to modulate CD22 function as a negative regulator of BCR signaling. We have sought to develop tools to aid in dissecting the role of CD22-ligand interactions in B-cell biology. To identify the cis ligands of CD22, we developed a novel method for *in situ* photoaffinity crosslinking of glycan ligands comprising a 9-aryl-azide sialic acid (9-AAz-NeuAc) that is readily taken up by B-cell lines and incorporated into cell surface glycoproteins. Surprisingly, sIgM and other glycoproteins that bind to CD22 *in vitro* do not appear to be major cis ligands of CD22 *in situ*. Instead, CD22 appears to recognize glycans of neighboring CD22 molecules as cis ligands, forming homo–multimeric complexes. Localization of CD22 is largely restricted to clathrin-coated pits where it undergoes receptor-mediated endocytosis, possibly accounting for its restricted recognition of glycoproteins as cis ligands. B cells from mice deficient in the

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enzyme forming the CD22 ligand (ST6–/–) exhibit suppressed BCR signaling, whereas mice deficient in both CD22 and its ligand (CD22–/– ST6–/–) exhibit restored BCR signaling, demonstrating that the suppressed signaling of cells from ST6–/– mice is mediated through CD22. Coincident with suppressed BCR signaling, B cells of ST6–/– mice exhibit a net redistribution of sIgM to clathrin rich domains, resulting in a 2-fold increase in colocalization of CD22 with the BCR. The altered microdomain localization appears to be mediated by CD22, because B cells of CD22–/– ST6–/– mice exhibit wild-type sIgM distribution. Taken together, these data suggest that association of CD22 with the BCR does not require and may even be reduced by cis ligand interactions. (Supported by NIH grants GM60938, AI50143, and GM62116.)

(46) Siglec-8: An Inhibitory Receptor on Eosinophils and Mast Cells

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Siglecs (sialic acid-binding immunoglobulin-like lectins) are a family of innate immune receptors that are transmembrane I-type lectins characterized by the presence of an N-terminal V-set immunoglobulin domain that binds sialic acid. A unique characteristic of many Siglecs is the presence of conserved cytoplasmic sequences containing tyrosine motifs, suggesting that these molecules possess inhibitory functions (e.g., immunoreceptor tyrosine-based inhibitory motifs [ITIMs]). Ongoing work has focused on characterizing Siglec expression and function on human eosinophils and mast cells, with an emphasis on Siglec-8 biology in these cells. Flow cytometric and other analyses have shown that eosinophils predominantly express CD33 (Siglec-3) and Siglec-8, with very low levels of Siglec-10, whereas mast cells derived from CD34+ precursors *in vitro* express CD22 (Siglec-2), CD33, Siglec-5, Siglec-6, Siglec-8, and Siglec-10 protein and mRNA, although the CD22 and Siglec-10 proteins are primarily or exclusively intracellular. Kinetic analysis of surface phenotype revealed that CD34+ precursor cells from peripheral blood constitutively expressed surface CD33, Siglec-5, and Siglec-10. As they matured into mast cells, their constitutive levels of CD33 changed little, Siglec-5 and Siglec-10 declined, and Siglec-6 and Siglec-8 appeared *de novo*, all in parallel with accumulation of histamine, tryptase, and other mast cell markers, such as surface expression of high-affinity IgE receptors and CD51. Because of its relatively selective expression on eosinophils and mast cells, the biology of Siglec-8 has been explored in greater detail. For eosinophils, crosslinking of Siglec-8 results in caspase-dependent and mitochondria-dependent apoptosis that is enhanced by survival promoting cytokines like IL-5 and GM-CSF. Based on data with pharmacologic inhibitors, this enhanced apoptosis appears to be because of enhanced production of reactive oxygen species. In mast cells, unlike in eosinophils, Siglec-8 crosslinking does not induce their apoptosis. Instead, marked inhibition of IgE receptor-dependent mediator release (e.g., histamine, PGD2) is observed. Through the efforts of the Consortium for Functional Glycomics, ligand screening for Siglec-8 was performed, and among ~180 carbohydrate ligands, only one, 6′-sulfo-sialyl Lewis X, was identified, with a relative affinity of ~2 μM. Using biotinylated multivalent polyacrylamide polymers, selective binding of 6′-sulfo-sialyl Lewis x, but not structurally related molecules, was confirmed using flow cytometry. Separate studies in mice suggest that the functionally convergent paralog of Siglec-8 is Siglec-F. Its expression on mouse eosinophils and mast cells has been confirmed, but prominent expression on mouse alveolar macrophages was also seen. Preliminary studies in IL-5 transgenic mice displaying hypereosinophilia show that the administration of Siglec-F antibody to these mice results in rapid and profound reductions in eosinophil numbers. This is consistent with *in vitro* studies incubating mouse eosinophils with Siglec-F antibody where, as with human eosinophils, the mouse eosinophils undergo enhanced apoptosis. These data demonstrate that activation of innate immune receptors, like Siglec-8, can profoundly inhibit various aspects of eosinophil and mast cell biology and raises the possibility that anti-mast cell, anti-eosinophil, and allergy-related therapies could be developed using antibodies or glycomimetics that target Siglec-8 and other Siglecs.

(47) Probing the Functions of Siglecs Expressed on Myeloid Cells

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Sialoadhesin (Siglec-1, CD169) is a prototypic member of the Siglec family of sialic acid-binding Ig-like lectins expressed selectively on tissue macrophages. It has an unusually long extracellular region made up of 17 Ig domains which is highly conserved in all mammals examined so far (mouse, rat, dog, pig, cow, chimpanzee, and human) and a relatively short cytoplasmic tail which is poorly conserved and contains no obvious signalling motifs. To gain insight into the biological functions of this receptor, sialoadhesin-deficient mice have been generated by gene targeting and a range of studies performed. In contrast to sialoadhesin, the CD33-related Siglecs contain two well-conserved tyrosine-based motifs implicated in inhibitory signalling via recruitment of SHP-1 and SHP-2 protein tyrosine phosphatases. We have expressed wild-type and mutant forms of the human myeloid Siglecs-5, -7, and -9 in rat basophil leukaemia cells to examine their potential inhibitory functions in modulation of serotonin secretion and Siglec-dependent cell adhesion. Our findings show that CD33-related Siglecs have the potential to regulate cellular activation in the absence of tyrosine phosphorylation, suggesting a role in constitutive down-regulation of myeloid cell activation (Avril *et al.*, 2005). (Supported by a grant from the Wellcome Trust.)

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(48) Regulation of Intracellular Immune Signal Transduction by Protein Glycosylation: Setting Thresholds for B Lymphocyte Activation and Immunoglobulin Homeostasis

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The immune system generates and responds to endogenous posttranslational protein modifications that are topologically separated, with phosphorylation confined to intracellular compartments and glycosylation occurring predominantly in the secretory pathway. We show that the ST6Gal-I sialyltransferase generates cell surface glycan counter receptors for the B-cell transmembrane CD22 Siglec glycoprotein that reduce colocalization with the antigen receptor and lower the threshold for protein phosphotyrosine induction in immune signaling. ST6Gal-I further selectively masks ligands for asialoglycoprotein receptors on Kupffer cells that prevent rapid clearance of serum immunoglobulin M and diminished functional levels. Depressed humoral immunity owing to ST6Gal-I deficiency reflects enforced glycan changes typically restricted to postactivated B cells and which are capable of attenuating the development of autoimmune disease. The glycoprotein-binding specificities of endogenous lectins comprise mechanisms integrating protein glycosylation, phosphorylation, and homeostasis in pathways of immune regulation.

(49) P-Selectin Expression in the Thymus is Required for Importation of T-Cell Progenitors and is Modulated by Thymic T-Cell Production

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Thymic homeostasis in adult mice is maintained by gated importation of circulating T-cell precursors (CTPs). The mechanisms controlling thymic homing of CTPs are poorly understood. We have recently found that P-selectin–PSGL-1 interaction is important in the control of thymic precursor homing and importation. Indeed, circulating CTPs and the earliest thymic progenitors bind to P-selectin, which is constitutively expressed at low a level in mouse thymus. To gain a better insight into the underlying mechanisms controlling thymic P-selectin expression, we established real-time quantitative PCR (rtqPCR) procedures to measure the relative transcript levels for P-selectin in adult thymi of wild-type mice and transgenic mouse lines with a phenotype of altered T-cell development. IL7R knockout mice have an impaired thymocyte expansion and a profound reduction of thymic cellularity, owing to a requirement of thymic progenitors for IL7. rtqPCR analysis showed that relative P-selectin RNA levels are 9-fold higher in thymi of IL7R knockout mice than in thymi of wild-type mice. Reconstitution of IL7R knockout mice with wild-type prothymocytes resulted in a down-regulation of P-selectin RNA to levels comparable with wild-type controls 3 weeks after reconstitution. The down-regulation of P-selectin mRNA expression is indicative of a negative feedback mechanism for P-selectin expression that is controlled by the occupation status of the intrathymic niches. T cells bearing the male antigen (HY) T-cell receptor are positively selected in female mice and negatively selected in male mice. Thymi from female HY mice are of normal size, whereas thymi from male HY mice are small and contain few double positive and few single positive thymocytes. PCR analysis showed that male thymi had an ~2-fold higher P-selectin RNA expression than female HY thymi, suggesting that P-selectin expression is modulated by the numbers of double positive and single positive thymocytes.

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(50) Lymphocyte Trafficking in Mice Deficient in MECA-79 Antigen: Analysis of Core 1 Extension Enzyme (β1,3-*N*-Acetylglucosaminyltransferase-3) Knockout Mice

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High endothelial cells in lymph nodes express several sialomucins such as CD34 and GlyCAM-1 modified with a functional L-selectin ligand, 6-sulfo sialyl Lewis x structure. This terminal structure caps internal core *O*-glycan structures such as extended core 1 and core 2 branch. The significance of core 2 branch was shown by core 2 *N*-acetylglucosamynyltransferase-1 (core2GlcNAcT-1) knockout mice, in which the lymphocyte homing was reduced to ~50% of wild-type mice (Hiraoka *et al*., 2004). 6-Sulfo sialyl Lewis x structure in extended core 1 structure is recognized by MECA-79 antibody, a function-blocking antibody of peripheral lymph node addressin, although the minimum epitope for this antibody is 6-sulfo *N*-acetyllactosamine in extended core 1 structure (Yeh *et al*., 2001). The synthesis of extended core 1 structure requires β1,3-*N*-acetylglucosaminyltransferase-3 (β3GlcNAcT-3) which is also called core1-β1,3GlcNAcT or core 1 extension enzyme (Yeh *et al*., 2001), one of the seven β1,3-*N*-acetylglucosaminyltransferases reported so far. We have also demonstrated that β3GlcNAcT-3 is the only β1,3-*N*-acetylglucosaminyltransferase which reconstitutes MECA-79 antigen when cultured cells are transfected with L-selectin ligand sulfotransferase (Mitoma *et al*., 2003). To determine the significance of the extended core 1 *O*-glycans in lymphocyte homing, we generated β3GlcNAcT-3 knockout mice. β3GlcNAcT-3 (–/–) mice developed normally and lacked any obvious gross anomaly. Significantly, MECA-79 reactivity was completely absent in high endothelial venules of β3GlcNAcT-3 (–/–) mice, demonstrating that β3GlcNAcT-3 is the only β3GlcNAcT which can add GlcNAc to core 1 *O*-glycan in β1,3-linkage in HEV. The binding of L-selectin-IgM chimera to HEV was appreciably reduced, and in Stamper-Woodruff assay lymphocytes failed to bind *ex vivo* to PLN prepared from β3GlcNAcT-3 (–/–) mice. Moreover, lymphocyte homing into PLN was also decreased to ~60% of wild-type mice. These results indicate that the formation of extended core 1 structure participates in the synthesis of functional L-selectin ligand in PLN, and other structures including 6-sulfo sialyl Lewis x in core 2 branch may contribute to the residual lymphocyte homing. By taking advantage of intravital microscopy of PLN, lymphocyte rolling of T cells and B cells was assessed *in vivo*. B-cell rolling was more severely reduced than T-cell rolling in β3GlcNAcT-3 (–/–) mice. This result is very similar to the result obtained with core2glcnact-1 knockout mice (Gauguet *et al*., 2004) suggesting that extended core 1 and core 2 branched structure equally contribute to the expression of functional L-selectin ligand. Supported by NIH grants CA48737 and CA71932.

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Session Topic: Glycans in Disease

(51) Inactivation of the Golgi CMP-Sialic Acid Transporter Gene Reveals a New Human Typeii Congenital Disorder of Glycosylation (CDGIIf)

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The human Golgi CMP-sialic acid transporter is a Type III multi-transmembrane protein of 337 amino acids, responsible for the transport of the nucleotide sugar from the cytosol to the Golgi lumen. The Lec2 cells, derived from the Chinese hamster ovary cell line (CHO), are deficient in Golgi CMP-sialic acid transport. We have identified a homozygous G>A substitution in the intron 6 donor splice site (IVS6+1G>A) of the CMP-sialic acid transporter gene, responsible for their asialo phenotype. These cells were used for complementation studies, to test the activity of the two CMP-sialic acid transporter cDNA alleles of a 4 month-old-boy patient, with macrothrombocytopenia, neutropenia, and complete lack of sialyl-Lex antigen (NeuAc-a2,3Galb1,4(Fuc-a1,3)GlcNAc-R or CD15s), on polymorphonuclear cells (PMN). He presented initially a spontaneous massive bleeding of the posterior chamber of the right eye and cutaneous hemorrhages. The clinical status worsened over a period of 30 months with more severe hemorrhages because of severe thrombocytopenia (2–6 × 109/L), respiratory distress syndrome, and opportunistic infections. Treatment with transfusions and steroids ameliorated temporarily his condition, and bone marrow transplantation was performed at the age of 34 months. However, complications that included graft versus host disease, pulmonary viral infection, and massive pulmonary hemorrhage with refractory respiratory failure led to death at the age of 37 months. Ultrastructural studies showed hypogranular giant platelets. Bone marrow aspirates gave normocellular marrow with megakaryocytic hyperplasia. No complementation was obtained with either of the two patient alleles, whereas full restoration of the sialylated phenotype was obtained in the Lec2 cells transfected with the corresponding human wild-type transcript. Inactivation of one patient allele by a double microdeletion inducing a premature stop codon at position 327 and a splice mutation in the other allele, inducing a 130-bp deletion and a premature stop codon at position 684, are proposed to be the causal defects of this disease. An homozygous four base insertion (CACT) in the intron 6 was found in the mother and in one allele of the patient, and we propose that it is responsible for the splice mutation giving the 130-bp deletion of the transcript. This CACT insertion in the intron 6 creates a new U2 snRNA site, which is in competition with the putative normal U2 snNRA site and can induce splice alterations leading to deletions of exon 6. Therefore, this mutation must be leaky, as many congenital disorder of glycosylation (CDG) mutations allowing for the expression of enough wild-type transcripts in the homozygous mutated individuals, to avoid the disease. But, the addition of this maternal leaky splice mutation to the other completely inactivated allele of paternal origin induced the disease in the patient. We conclude that this defect is a new CDG of Type IIf, affecting the transport of CMP sialic acid into the Golgi apparatus.

(52) Functional Domains in Dystroglycan Processing and Laminin Binding

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Reduced ligand-binding activity of α-dystroglycan is associated with muscle and central nervous system pathogenesis in a growing number of muscular dystrophies. Posttranslational processing of α-dystroglycan is generally accepted to be critical for the expression of functional dystroglycan. Both the N-terminal domain and a portion of the mucin-like domain of α-dystroglycan are essential for high-affinity laminin-receptor function. Posttranslational modification of α-dystroglycan by glycosyltransferase, LARGE, occurs within the mucin-like domain, but the N-terminal domain interacts with LARGE defining an intracellular enzyme-substrate recognition motif necessary to initiate functional glycosylation. Gene replacement in dystroglycan-deficient muscle demonstrates that the dystroglycan C-terminal domain is sufficient only for dystrophin–glycoprotein complex assembly, but to prevent muscle degeneration the expression of a functional dystroglycan through LARGE recognition and glycosylation is required. Therefore, molecular recognition of dystroglycan by LARGE is a key determinant in the biosynthetic pathway to produce mature and functional dystroglycan.

(53) Galectins and the Inflammatory Response

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Galectins are a family of animal lectins with affinity for beta-galactosides. They are differentially expressed by various cell types, and their expression levels are dependent on cell differentiation and activation. They can interact with cell surface and extracellular matrix glycoconjugates, through lectin–carbohydrate interactions. Through this action, they can promote cell growth, affect cell survival, modulate cell adhesions, and induce cell migration. Galectins do not have a classical signal peptide and are often localized in the intracellular compartments, including the nucleus. Intracellularly, they can regulate cell growth and

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survival by interacting with cytoplasmic and nuclear proteins, through protein–protein interactions, thus affecting intracellular signaling pathways. This presentation focuses on various extracellular and intracellular functions of galectin-3 related to the inflammatory response. Galectin-3 has been shown to activate various cell types, including mast cells, neutrophils, monocytes, and eosinophils, when added exogenously to these cells. The action results in mediator release and superoxide production but also suppression of cytokine production in some cases. Galectin-3 has also been shown to induce migration of monocytes and macrophages *in vitro* and *in vivo*. This function is inhibitable by pertussis toxin, suggesting the involvement of a G-protein coupled receptor(s). In addition, galectin-3 has been shown to be associated with the T-cell receptor (TCR) complex, in a fashion that is dependent on glycosylation of TCR polypeptides by beta 1,6 *N*-acetylglucosaminyl-transferase V (Mgat5). Galectin-3 is implicated in restricting TCR-initiated signal transduction, by forming multivalent complexes with the glycans on TCR. Finally, galectin-3 has been shown to be able to induce apoptosis in T cells, through binding to cell surface glycoproteins. Various intracellular functions have been documented for galectin-3. A number of groups including ours have demonstrated the anti-apoptotic functions of galectin-3 through binding to intracellular proteins. Studies with bone marrow-derived macrophages from gal3–/– mice revealed the role of galectin-3 in FcR-dependent phagocytosis of erythrocytes and phagocytic clearance of apoptotic cells. Galectin-3 was found to be localized in the phagocytic cups and phagosomes and responsible for actin polymerization induced by the phagocytic stimuli. More recently, we found that migration of macrophages and dendritic cells from gal3–/– mice are significantly impaired when compared with wild-type counterparts, both *in vitro* and *in vivo*, suggesting a role for galectin-3 in regulation of cell migration. Under chemokine simulation of macrophages, galectin-3 is colocalized with F-actin and vinculin at podosomes, suggesting that this protein may regulate cell migration by interacting with intracellular apparatus involved in this process. The role of galectin-3 in the inflammatory response *in vivo* has been documented by using gal3–/– mice. Previously, we have shown that gal3–/– mice exhibited reduced pertitoneal inflammation induced by thioglycollate. Subsequently, we have shown that galectin-3 deficiency resulted in reduced airway inflammation induced by airway challenge with an antigen, in mice previously sensitized with the antigen. More recently, we found that gal3–/– mice exhibited significantly reduced response in a model of contact hypersensitivity. Therefore, endogenous galectin-3 plays an important role in promotion of the inflammatory response.

(54) Hepatic Clearance of Triglyceride-Rich Lipoproteins Depends on Heparan Sulfate

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Heparan sulfate proteoglycans bind to several proteins that play a role in lipoprotein metabolism: apolipoprotein B (apoB), apolipoprotein E (apoE), lipoprotein lipase (LPL), and hepatic lipase (HL). *In vitro* studies have indicated a role for the proteoglycans located in the space of disse in the sequestration and hepatic uptake of lipoprotein remnants and lipolytic enzymes. To examine the role of hepatic heparan sulfate *in vivo*, mice conditionally knocked out in hepatocyte heparan sulfate *N*-deacetylase/*N*-sulfotransferase 1 (Ndst1) were created by crossing a loxP-flanked allele (*Ndst1*f/f) with *Alb–Cre* mice in which the bacteriophage Cre recombinase was expressed under the control of the rat albumin promoter. Southern blotting showed that ~65–75% of hepatocytes contained a deletion of *Ndst1*. Analysis of the heparan sulfate chains showed a decrease in glucosamine N-sulfation and uronic acid 2-O-sulfation. This change in liver heparan sulfate structure caused an accumulation of apoE-bearing triglyceride-rich particles, resulting in a 2- to 3-fold higher level of circulating plasma triglycerides. Additionally, plasma hepatic lipase was increased 2-fold. Mutant mice synthesized lipoproteins normally but showed reduced plasma clearance of injected VLDL. Interestingly, selective alteration of *Ndst1*f/f in endothelial cells had no effect on lipoprotein metabolism. Analysis of the proteoglycan expression by semiquantitative PCR and a new proteomic method showed that hepatocytes produce both membrane bound and secreted heparan sulfate proteoglycans. Genetic analysis of these proteoglycans is underway to determine whether the accumulation of apoE triglyceride rich particles in *Ndst1*f/f*AlbCre*+ mice results from changes in specific proteoglycans. These findings demonstrate a crucial role for hepatic heparan sulfate in the normal metabolism of triglyceride-rich lipoproteins and suggest the possibility that alterations in hepatic heparan sulfate could cause hypertriglyceridemia in humans.

Session Topic: New Technologies for Glycobiology

(55) Effect of *N*-Acetylmannosamine Kinase Gene Deletion for the Synthesis of Cytidine 5′-Monophosphate *N*-Acetylneuraminic Acid in *Escherichia coli* K12

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Cytidine 5′-monophosphate *N*-acetylneuraminic acid (CMP-NeuAc) is an essential precursor for the synthesis of sialooligosaccharides. To produce CMP-NeuAc, we employed a recombinant *Escherichia coli* system which was engineered by gene knockout and gene transformation techniques. Genes for NeuAc synthase (*neu*B) and CMP-NeuAc synthetase (*neu*A) were transformed and coexpressed in *E. coli* K12. The *neu*B gene was for the conversion of *N*-acetylmannosamine and phosphoenolpyruvate to NeuAc, and the neuA gene was for the production of CMP-NeuAc from CTP and NeuAc. Additionally, reversible degradation reaction of accumulated NeuAc by NeuAc aldolase (*nan*A) was avoided by knockout of nanA gene from *E. coli* K12 strain. The sialic acid synthase and CMP-sialic acid synthetase were successfully expressed in *E. coli* K12 nanA(–) strain and produced both sialic acid and CMP-sialic acid in cells. On the basis of previous study, additional gene knockout was carried out as follows, a disruption cassette with chloramphenicol-resistance (Cm^R) of pKD3 plasmid was used for the deletion of *N*-acetylmannosamine kinase (*nan*K) involved in the metabolic pathway of CMP-sialic acid catalyzing the irreversible reaction of ManNAc into ManNAc-6-P. ManNAc is the significant precursor of sialic acid, therefore the deletion of *nan*K gene was expected to improve the productivity of CMP-sialic acid. A simple method to disrupt chromosomal genes in *E. coli* was employed by using template plasmids carrying antibiotic resistance gene that are flanked by FLP recognition target (FRT) sites, recombination by ?-Red recombinase and FLP recombinase on temperature-sensitive plasmids. The effect of gene knockout was compared with host recombinant *E. coli*, and the influence of *nan*K gene on the metabolic pathway of aminosugar synthesis was analyzed.

(56) Synthesis of New Nonnatural Carbohydrates and Analogs Proposed as Glycomimetics

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An impressive variety of regulatory processes including cell adhesion and migration, proliferation, and so on has been found to be mediated by specific protein–carbohydrate interactions. Natural oligosaccharides like sialyl Lewis x and like maltohexaose demonstrated that even a reduced number of sugar units could play relevant biological actions towards selectin (Fuster *et al*., 2003) and towards heparanase (Parish *et al*., 1999), respectively. Synthesis of nonnatural carbohydrates and glycomimetics is a topic strictly connected to these fundamental findings. More precisely, our results fall down in this topic, as we are able to synthesize original carbohydrate-based structures with an original electrochemical approach. This approach allowed doubling the units of a starting sugar moiety by a one-pot synthesis creating rigid anomeric C–C bonds. Glucose-based glycomimetics characterized by a direct C-interglycosidic bond were synthesized from acetobromomaltotriose, acetobromomaltose, and acetobromoglucose: hexasaccharide-like mimics from acetobromomaltotriose, tetrasaccharide-like mimics from acetobromomaltose, and C-disaccharide-like mimics from acetobromoglucose (Guerrini *et al*., 2005). Potentiostatic electroreduction of glycosyl bromide on silver cathode generates carbohydrate radicals suitable to dimerise statistically to the three diastereoisomers, thus confirming the radical mechanism. Further reduction of the intermediate anomeric radical is a side reaction leading to carbanium that induces acetate as a leaving group. Although this side reaction decreases the reaction yields, this one-pot dimerization on silver cathode is a very intriguing method to produce a new rigid C–C anomeric bond between sugars. Also iodosugars could be successfully reduced on silver. 1,2:3,4-di-*O*-isopropylidene-6-deoxy-6-(α-D-1,2:3,4-di-*O*-isopropylidene-6-deoxy-galactopyranos-6-yl)-α-D-galactopyranose characterized by a CH2–CH2 flexible connection between two galactose rings was synthesized from 1,2:3,4-di-*O*-isopropylidene-6-deoxy-6-iodo-α-D-galactopyranose (Rondinini *et al*., 1998). This time it was impossible to distinguish whether the doubling is a radical dimerization or an ionic nuclephilic substitutions by the intermediate anion on the starting material. Anyway, the reaction product is obviously unique. We present with this contribution that other halosugars are now synthesized and electrolyzed to enlarge the application field of the proposed methodology. **3,4,6-tri-*O*-acetyl-2-deoxy-2-phtalimido-α-D-glucopyranosyl bromide was chosen to check whether the methodology works on the glucosamine monomer, present in glycosaminoglycans like heparin, whose

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importance is well known by everybody. The synthesis of the aminoglucosyl dimers was successfully performed. Noteworthy, the presence of the amino group instead of an acetyl group in position two totally eliminates the by-products owing to acetyl elimination in position two. §§Both 1,2,3-tri-*O*-acetyl-6-deoxy-6-iodo-4-*O*-(2,3,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-β-D-glucopyranose and 1,2,3-tri-*O*-acetyl-6-deoxy-6-iodo-4-*O*-(hepta-*O*-acetyl-α-D-maltopyranosyl)-β-D-glucopyranose were prepared to buildup a flexible tetrasaccharide and a flexible hexasaccharide, respectively. They were successfully electrolyzed to a unique product of coupling CH2 with CH2. As closing remarks, apart the mechanism, it appears meaningful, as conformation is a crucial parameter in determining interactions, that the electrochemical approach introduces flexible bonds by reducing C–I bond at the nonreducing end of a sugar and rigid bonds by reducing C–Br bond at the reducing end.

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(57) O-Linked Glycosylation in Maize-Expressed Human IgA1

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O-Linked glycans vary between eukaryotic cell types and play an important role in determining a glycoprotein's properties, including stability, target recognition, and potentially immunogenicity. We describe O-linked glycan structures of a recombinant human IgA1 (hIgA1) expressed in transgenic maize. Up to six proline/hydroxyproline conversions and variable amounts of arabinosylation (Pro/Hyp+Ara) were found in the hinge region of maize-expressed hIgA1 heavy chain (HC) using a combination of MALDI MS, chromatography, and amino acid analysis. Approximately 90% of hIgA1 was modified in this way. An average molar ratio of six Ara units per molecule of hIgA1 was revealed. Substantial sequence similarity was identified between the HC hinge region of hIgA1 and regions of maize extensin-family of hydroxyproline-rich glycoproteins (HRGP). We propose that because of this sequence similarity, the HC hinge region of maize-expressed hIgA1 can become a substrate for posttranslational conversion of Pro to Hyp by maize prolyl-hydroxylase(s) with the subsequent arabinosylation of the Hyp residues by Hyp-glycosyltransferase(s) in the Golgi apparatus in maize endosperm tissue. The observation of up to six Pro/Hyp hydroxylations combined with extensive arabinosylation in the hIgA1 HC hinge-region is well in agreement with the Pro/Hyp hydroxylation model, and the Hyp contiguity hypothesis suggested earlier in literature for plant HRGPs. For the first time, the extensin-like Hyp/Pro conversion and O-linked arabinosylation are described for a recombinant therapeutic protein expressed in transgenic plants. Our findings are of significance to the field of plant biotechnology and biopharmaceutical industry developing transgenic plants as a platform for production of recombinant therapeutic proteins.

(58) Developing of Lectin-Enhanced Laser Adsorption/Ionization Microarray

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Traditional methods for sequencing glycans conjugated to glycoproteins, such as HPLC, affinity chromatography, ESI, MALDI-TOF, and GC MS are time consuming and require a larger number of purification and chemical modifications steps. In recent decade, microarray-based technologies for glycan profiling has been suggested to overcome these difficulties. However, these methods are incapable of providing information, such as the number of glycosylation sites and glycoforms, the presence of O-linked glycans and sequence determination. We are developing molecular arrays of lectins, portraying the different binding specificities of different carbohydrates. The lectin arrays are absorbed onto MALDI plate, thus enabling a simultaneous identification of the bounded carbohydrate sample according to its mass. For a proof of principle, three glycoproteins, ribonuclease B, transferrin, and ovalbumin, which their glycan types and profile are well known, were digested by proteases to yield glycopeptides. After binding to the lectin arrays, they were assessed by MALDI-TOF MS and compared with the sequence of their glycans obtained by reversed-phase HPLC. The glycan profiles detected by the novel lectin array/MALDI assay were similar to those obtained by HPLC analysis. Most remarkable, our novel lectin array enabled providing the glycosylation sites on these proteins. At present, we are trying to show the ability of the array to determine glycan sequences. By applying a series of exoglycosidases on the glycopeptide mixture (after protease treatment), we anticipate to reveal sugar-reduced peaks by the MALDI-TOF MS. Our future goals are to optimize the binding capabilities of the microarrays, show O-linked glycosylation sites, use highly glycosylated and unknown glycoproteins and, finally, to develop an algorithm and MALDI-sugar database.

(59) Specificity and Inhibition of Beta1,4-Galactosyltransferase

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Galactosyltransferases are important enzymes involved in the extension of the glycan chains of glycoproteins and play critical roles in cell surface functions and in the immune system. In this work, the acceptor specificity and inhibition of bovine beta4-Gal-transferase T1 (beta4GalT, EC 2.4.1.90) was studied. A series of *N*-acetylglucosamine (GlcNAc) analogs as well as GlcNAc-carrying glycopeptides were synthesized and found to be good acceptor substrates. Modifications were made at the 3-, 4-, and 6-positions of the sugar ring of the acceptor, in the anomeric linkage, in the aglycone moiety, and in the 2-acetamido group. The acceptor-specificity studies showed that the 4-hydroxyl group of the sugar ring was essential for beta4GalT activity, but the 3-hydroxyl could be substituted with an electronegative group. The anomeric beta-configuration was superior to alpha and *O*-, *S*-, and *C*-glycosides were all active as substrates. The aglycone group was a major determinant for the rate of Gal transfer. Derivatives containing a 2-naphthyl aglycone were inactive as substrates, although similar quinolinyl groups supported activity. Several compounds with bicyclic structures as the aglycone were found to bind to the enzyme and potently inhibited the transfer of Gal to control substrates. Galactosyltransferase activity from bone and intestinal cancer cell homogenates was also inhibited. These studies help to delineate beta4GalT-substrate interactions and will aid in the development of biologically applicable inhibitors of the enzyme. This work was supported by a grant from the Natural Sciences and Engineering Research Council.

(60) Development of New Chemistries for Labeling Glycans *in vivo*

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There is a great need for tools that allow the imaging and proteomic analysis of glycoproteins. One method developed by our laboratory, metabolic oligosaccharide engineering, involves the chemical tagging of glycans within their native environment. In this technique, unnatural sugars containing the azido functional group are metabolically incorporated into a host of intracellular and cell-surface glycoconjugates (Dube *et al*., 2003). To visualize these azide-labeled glycans, the azide must be chemically elaborated using appropriately functionalized secondary reagents. Existing chemistries to modify the azide in this context are limited to (1) the Staudinger ligation, using a phosphine reagent, (2) "click" chemistry, involving a terminal alkyne and a copper catalyst, and (3) the strain-promoted cycloaddition, employing a cyclooctyne reagent (Prescher and Bertozzi, 2005). The first two methods have been utilized in numerous biological applications, but each has a major drawback. First, the phosphine reagents required for the Staudinger ligation are difficult to synthesize and susceptible to oxidation. Second, the required copper catalyst for click chemistry is cytotoxic, precluding its use *in vivo*. As it is neither prone to degradation *in vivo* nor cytotoxic, the recently reported strain-promoted cycloaddition, however, shows great promise for use in bioorthogonal ligation (Agard *et al*., 2004). The initially reported cyclooctyne reagent, however, shows slower kinetics than both the Staudinger ligation and click chemistry. In addition, it has been shown to decompose slowly, even when stored at –80°C. Thus, there is a need for improved cyclooctyne reagents that address these issues. Here, the synthesis, kinetic evaluation, and preliminary *in vitro* and *in vivo* data are presented for second-generation cyclooctyne reagents with enhanced stability and reactivity.

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(61) Chemical Synthesis of Fluorinated UDPGalNAc Analogs as Probes for the Study of the Retaining Polypeptidyl GalNAc Transferases

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Mucin-type O-linked glycoproteins decorating the outer surface of the cell membrane are key mediators in many biologically relevant interactions between the cell and its environment. Even though the importance of these structures is well recognized, very little is known about their mode of assembly and most importantly, about the enzymes involved in their construction. The enzymes responsible for the initiation and the eventual existence of O-linked glycoproteins are the polypeptidyl *N*-acetylgalactosaminyltransferases (ppGalNAcTs). These enzymes catalyze the glycosyl transfer of *N*-acetylgalactosamine (GalNAc) onto a serine or threonine residue in a protein. Further elaboration off this core monosaccharide residue by other glycosyltransferases results in the eventual construction of complex oligosaccharides off the peptide backbone. The use of fluorinated carbohydrates as tools in enzyme mechanism studies, particularly with the retaining glycosyl hydrolases, is well precedented. Utilizing this same approach, we have designed a panel of fluorinated UDPGalNAc analogs to carry out mechanistic studies on the ppGalNAcTs. One of the fluorinated analogs bears the fluorine atom at the C2 position of the galactopyranosyl residue in place of the *N*-acetyl group. The remaining two compounds are truly fluorinated analogs of UDPGalNAc. The first one of these bears a fluorine atom at the C5 position of the galactopyranosyl residue (5-fluoro UDP-GalNAc), whereas the second one bears two fluorine atoms, one at C5 and the other at the C6 positions of the galactopyranosyl residue. The chemical synthesis of these compounds is described.

(62) Novel Assays for Cell-Binding Studies as well as for Identification of Compounds that Inhibit or Enhance Cell Attachment

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The ability of cells to adhere to each other or to constituents of the extracellular matrix (ECM) is important in normal cellular function in mammals. Cell-matrix interactions depend to a large extent upon the engagement of specific ECM proteins with cell surface integrins. Besides protein–protein interactions, protein–carbohydrate interactions are also utilized in cell adhesion mechanisms. Galectins are a family of carbohydrate-binding adhesion molecules (lectins) with affinity for lactose and other β-galactosides. Laminin I, a major component of basement membranes, has numerous biological activities, including promotion of cell adhesion, migration, growth, and differentiation. The basement membrane protein complex (BMC) is a solubilized basement membrane preparation extracted from Engelbreth–Holm–Swarm (EHS) mouse sarcoma. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, and entactin. It represents a physiologically relevant environment for studies of cell morphology, biochemical function, migration, and invasion. The 96-well cell adhesion assays for galectin-1/galectin-3 and Laminin I/BMC are designed for the determination of the relative attachment of adherent cell lines to galectin-1/galectin-3 involving protein–carbohydrate interaction or to Laminin I/BMC involving protein–protein interactions, for evaluation of cell adhesion receptors and for assessment of inhibitory or stimulatory substances for cell attachment. Several adherent tumor cell lines were tested for attachment. For the galectin-1/galectin-3 assay, cells were incubated in wells coated with galectin-1 or galectin-3 followed by cell staining with Calcein-AM. Cell attachment was measured, and binding was presumed to involve protein–carbohydrate interaction as no binding was seen in the presence of lactose. Out of the various cell lines tested, A431 cell line showed similar binding to both galectin-1 and galectin-3 where as SK-BR-3 cell line showed preferential binding to galectin-1. No binding to BSA-coated wells was observed.

(63) Immobilization of 2-Aminopyridine-Oligosaccharides: Bridging Structural Analysis and Glycoarrays

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Printed carbohydrate micro array reported recently (Blixt *et al*., 2004) requires nanomolar quantities of carbohydrate ligands. On the other hand, routine structure analysis of glycoprotein carbohydrate chains based on chromatographic separation of oligosaccharides (OS) is also often performed within nanomole range. This coincidence opens an attractive prospect of using OS obtained after *analytical* HPLC for immobilization on chip followed by assaying lectins and other carbohydrate-binding proteins. Such a "link" variant when OS bears a fluorescent label is especially attractive: on the one hand, this makes easier the HPLC separation and more sensitive OS detection; on the other hand, this makes real quantitative dosage of glycans during chip fabrication. The experimental technique is the following: (1) carbohydrate chains are cleaved from protein core, (2) oligosaccharide pool is labeled with fluorescent reagent, (3) HPLC is carried on, and (4) the collected peaks in the optimal concentration are that they are placed to the microfluidistic system of the microarrayer. In this work, we demonstrated the possibility of grafting onto three-dimensional gel microchip (Rubina *et al*., 2004) of the widely used and commercially available 2-aminopyridine (2AP) derivatives of oligosaccharides. The chip is developed earlier for oligonucleotide assaying.

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(64) Oligosaccharide Preferences of β1,4-Galactosyltransferase-I: Crystal Structures of Met340His Mutant of Human β1,4-Galactosyltransferase-I with a Pentasaccharide and Trisaccharides of the *N*-Glycan Moiety

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β-1,4Galactosyltransferase-I (β4Gal-T1) transfers galactose from UDP-galactose to *N*-acetylglucosamine (GlcNAc) residues of the branched N-linked oligosaccharide chains of glycoproteins. In an N-linked biantennary oligosaccharide chain, one antenna is attached to the 3-hydroxyl- (1,3-arm) and the other to the 6-hydroxyl- (1,6-arm) group of mannose, which is β-1,4-linked to an N-linked chitobiose, attached to the asparagine residue of a protein. During remodeling of the *N*-glycan chains of glycoproteins in the Golgi apparatus GlcNAc, Gal and sialic acid are added to the chains by corresponding glycosyltransferases. At least seven *N*-acetylglucosaminyltransferase (GlcNAc-T) family members (I–VII) add GlcNAc sequentially to the branched antenna of *N*-glycans, and the branch specificity of these enzymes has been well established. For a better understanding of the branch specificity of β4Gal-T family members (I-VI) towards the GlcNAc residues of *N*-glycans, we have carried out kinetic studies with one member, the wild-type human β4Gal-T1 (h-β4Gal-T1) and its mutant Met340His (h-M340H-β4Gal-T1), and their crystal structures in complex with a GlcNAc-containing pentasaccharide and several GlcNAc-containing trisaccharides present in *N*-glycans. The oligosaccharides used were pentasaccharide GlcNAcβ1,2-Manα1,6 (GlcNAcβ1,2-Man α1,3) Man; the 1,6-arm trisaccharide, GlcNAcβ1,2-Manα1,6-Manβ-OR (1,2-1,6-arm); the 1,3-arm trisaccharides, GlcNAcβ1,2-Manα1,3-Manβ-OR (1,2-1,3-arm) and GlcNAcβ1,4-Manα1,3-Manβ-OR (1,4-1,3-arm); and the trisaccharide GlcNAcβ1,4-GlcNAcβ1,4-GlcNAc (chitotriose). With the wild-type h-β4Gal-T1, the K_m of 1,2–1,6-arm is ~10-fold lower than for 1,2–1,3-arm and 1,4–1,3-arm and 22-fold lower than for chitotriose. Crystal structures of h-M340H-β4Gal-T1 in complex with the pentasaccharide and various trisaccharides at 1.9 to 2.0 Å resolution showed that β4Gal-T1 is in a closed conformation with the oligosaccharide bound to the enzyme. The dihedral angle ϕ of the GlcNAcβ1,2-Man linkage in the trisaccharides 1,2–1,6-arm and 1,2–1,3-arm takes similar values, whereas the ψ values are different. After interacting with the protein atoms, the reducing end mannose (α1,6-Man or α1,3-Man) stabilizes the conformation of the trisaccharide, dictating a particular conformation for the dihedral angle ψ of the preceding β1,2-linkage in the respective trisaccharides. As a consequence, the rms deviation between the endocyclic atoms of the middle mannose of the 1,2–1,6-arm and the 1,2–1,3-arm trisaccharides is large (1.76 Å). These structural investigations reveal that, compared with other trisaccharides, the 1,2–1,6-arm, owing to its greater conformational flexibility, makes maximum number of interactions with the protein atoms of h-β4Gal-T1, which correlates well with its lowest K_m for β4Gal-T1. Among the trisaccharides tested, the 1,2–1,6-arm trisaccharide causes substrate inhibition at low concentrations, suggesting that because of its higher affinity towards the enzyme, it induces the conformational change in the

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flexible loop of the enzyme, closing the lid that covers the donor binding site before the donor substrate can bind to it. The kinetic and crystallographic studies suggest that at a lower concentration, the 1,2–1,6-arm, rather than the 1,2–1,3-arm, of a biantennary *N*-glycan is the preferred antenna for galactosylation by β4Gal-T1, whereas at a higher concentration, the 1,2–1,3-arm is the preferred antenna. Other members of the β4Gal-T family may be preferentially transferring galactose to the 1,2–1,3-arm and acting in concert to galactosylate the various antennae of the *N*-glycans of glycoproteins.

(65) Synthesis and Analytical Evaluation of New Oligosaccharide Tags

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Carbohydrates play vital roles in numerous biological processes. Separation and structural elucidation of carbohydrate oligomers is crucial for meaningful structure-activity studies. The structural elucidation of constituent glycans by techniques similar to those used in proteomics is still at a nascent stage. The analysis of biologically important oligosaccharides is complicated by structural complexity (stereochemistry, linkage, and anomericity), poor detectability in chromatography and minute amounts (Harvey, 1996). Currently, the lack of chromophores in native carbohydrates is typically addressed by introducing fluorescent or chromophoric tags to improve detectability during chromatographic separation. The labeling is most commonly done by reductive amination of the aldehyde in the presence of sodium cyanoborohydride (Hase, 1996). Whereas low-molecular mass carbohydrates can be labeled very efficiently in this manner, high-molecular mass oligosaccharides often suffer from reduced labeling efficiency presumably because of steric reasons. In our previous studies, a biotinylated tag with a benzene core was successfully synthesized and introduced into different oligosaccharides. This tag combines bioaffinity for purification and UV activity for photo-diode array detection in HPLC. Also, the tag can be deuterium coded through reductive amination in the presence of $NaCNBD_3$, and the carbohydrate derivative can be N-quaternized for electrophoretic applications. To increase the UV detectability, we synthesized a variety of tags with substituents at the benzene core. In this poster, we present the synthesis of new multifunctional biotinylated tags with m-xylylenediamine, 4-nitroanisole, 4-methoxybenzonitrile, and 4 methoxybenzamide. Examples for reductive amination of oligosaccharides with the new tags, HPLC separation, and detection of the derivatives by MALDI-TOF and ESI mass spectrometry will be discussed. We also investigated click chemistry (1,3-dipolar Huisgen cycloaddition) as an alternative approach to reductive amination (Kolb and Sharpless, 2003; Kuijpers *et al.*, 2004). We demonstrated that the tag with a pendent alkyne group could be successfully clicked to benzylazide to give the 1,2,3-triazole in high yield under very mild conditions. We are currently perusing the introduction of an alkyne group into the tags for reaction with sugar azides. The results of these studies will be presented.

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(66) Biodegradation of Xanthan by Newly Isolated *Sphingomonas* sp. XT-11 and Biological Activity of Xantho Oligosaccharides

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Xanthan gum is an anionic heteropolysaccharide produced by a plant-pathogenic bacterium, *Xanthomonas campestris*. Xanthan is composed of cellulosic backbone with linear trisaccharide side chains consisting of a mannosyl–glucuronyl–mannose sequence linked at the C-3 position on every other glucosyl residues. The internal and termian mannosyl residues of the side chain are frequently acetylated and pyruvylated, respectively, depending on both the growth conditions and the bacterial strain. Owing to its exceptional pseudoplasticity, high viscosity at low concentration, and tolerance toward a wide range of temperatures and pHs, its numerous areas of application cover a broad range, from the food industry to oil drilling. Xanthan is not easily degraded by most microorganisms, though a few strains can decompose xanthan used for oil drilling. Two types of xanthan-degrading enzymes are known to exist in microbes. One is the xanthanase catalyzing the hydrolysis of the main chain of xanthan. A few xanthanases have been identified, some of which were categorized as cellulase family members. Cadmus *et al*. described the isolation of a bacteriumcapable of degrading xanthan. The xanthanase they obtained was a mixture of the enzymes that attacked all of the side chain linkages in the xanthan molecule, including the one involving (1¡ú3) linkage of acetylated mannose to the glucosidic backbone. They found no depolymerase activity in their cultures, because the ¦ Â-1,4-linked glucan backbone remained intact. The other type is the xanthan lyase, which eliminatively cleaves the terminal pyruvated ¦ Â-D-mannosyl- ¦ Â-D-1, 4-glucuronosyl linkage of the side chain of xanthan. A xanthan lyase was first obtained from a mixed culture and recently purified from *Bacillus* and *Paenibacillus*. However, this enzyme can be only used for modifying xanthan with novel physicochemical and physiological functions for exploiting new application fields. In this study, the biodegradation of xanthan by newly isolated *Sphingmonas* sp. XT-11 from an enrichment culture on xanthan is described. The isolate XT-11 was assigned tentatively to the genus *Sphingmonas* mainly because of the following characteristics: gram-negative, acid-fast negative, slender and irregular rod in young culture but coccoid in old culture, some of rods are arranged at an angle to each other giving V formations, facultatively anaerobic, catalasepositive, and no endospores formation. The data from 16S rRNA gene sequence also support that isolate XT-11 is the member of the genus *Sphingmonas*. 16S rRNA sequence database searches indicated that the nearest relatives of isolate XT-11 were *Sphingmonas* spp. Displaying ~95.6–99.8% 16S rRNA similarity. Degradation was inhibited by glucose addition. Xanthan-degrading enzyme activity was found in the culture supernatant when *Sphingmonas* sp. XT-11 was grown in the medium with xanthan as carbon source. The optimal pH and temperature for the xanthandegrading reaction was 7.0 and 30°C, respectively. Xantho oligosaccharides showed good antifungal activity against fungi and high radical scavenging activity towards the 1,1-diphenyl-2-picrylhydrazyl-2-radical (DPPH).

(67) The Effects of Elevated Ammonium on Gene Expression in CHO Cells Producing a Glycoprotein

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Ammonium is a toxic waste product, which has been reported to negatively inhibit cell growth and recombinant glycosylation in Chinese hamster ovary (CHO) cells; however, the effect of this toxicity on intracellular gene expression has only been limitedly investigated. In this study, two methods were used to evaluate genes in response to elevated ammonium. The first method used the exploratory method of differential display method. The second method targeted 12 glycosylation-related genes in CHO cells and used quantitative real-time reverse transcriptase PCR. Eight partial cDNA sequences were identified by differential display and confirmed by northern blots to be ammonium sensitive. Five of the putative CHO cell genes were identified to have lower expression under ammonium stress, whereas three of the putative CHO cell genes were identified to have higher expression. Sequence homology with other mammalian organisms was used to attribute function to these newly identified genes. The identified ammonium sensitive genes were grouped into three main functional groups: cellular processes, energy metabolism, and genetic information processing. Specifically, three of genes with lower expression (anaphase-promoting complex subunit 5, eukaryotic initiation factor 5A II, KIAA 1091 protein) are cellular process related. The two genes with higher expression under ammonium stress (ATP synthase subunit C and mitofusin 1) are energy metabolism related. The other two genes with lower expressions (ER-resident protein ERdj5 and structure-specific recognition protein 1) are genetic information processing related. One gene, 26S proteasome subunit ATPase 3, had higher expression and also belonged to genetic information processing group. These preliminary results indicate that ammonium stress down-regulates expression of genes controlling cell cycle, protein folding, and quality and up-regulates genes that control energy metabolism and degradation. The qRT–PCR method determined that numerous cytosol and endoplasmic reticulum localized genes associated with early glycosylation steps were insensitive to the ammonium condition. The initial expression of UDP-galactose transporter was higher for the ammonium-treated culture, whereas the initial expressions of CMP-sialic acid transporter, β(1,4)-galactosyltransferase, and UDP-glucose pyrophosphorylase were higher for the control culture. α(2,3)-Sialyltransferase was observed to have lower expression level under the elevated ammonium condition compared with the control culture; however, both expression profiles were insensitive to culture time. This study indicates that galactosylation and sialylation inhibition is mainly because of decreased gene expression of galactosyltransferase, sialyltransferase, and CMP-sialic acid transporter and not because of sialidase. These unbalanced initial glycosylation and branching steps can explain the higher molecular heterogeneity under ammonium stress. Moreover, this study indicates that elevated ammonium has limited effects on the glycosylation genes associated with the endoplasmic reticulum and cytosol compared with the Golgi.

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(68) Heteronuclear NMR Methods for Structural Studies of Intact Asparagine-Linked Glycoproteins

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We are using multidimensional NMR spectroscopy to characterize the structure and dynamics of intact asparagine-linked glycoproteins. We have previously shown that expression of recombinant Thy-1 in Lec1 CHO cells substantially reduces glycoform heterogeneity. Here, we show that the carbohydrates and some amino acids of Thy-1 are easily and economically isotopically enriched by expressing protein in sugar-free medium supplemented with uniformly labeled metabolic precursors. We also present NMR chemical shift assignment methods that narrow the resonance linewidth of the carbohydrate resonances, which improves the overall spectral quality and increases sensitivity. The development of economically efficient isotope labeling strategies and high resolution NMR methods for the characterization of N-linked glycoprotein carbohydrates will benefit glycobiologists investigating the structures of intact glycoproteins.

(69) Monitoring Tunicamycin-Induced Apoptosis by Fourier Transform Infrared Spectroscopy

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Angiogenesis is the formation of new blood vessels from pre-existing vasculature by migration and proliferation of capillary endothelial cells. It is essential for normal growth and development and is a "key step" in tumor progression and invasion. Many cytokines/chemokines accelerate angiogenesis by switching the quiescent endothelial cells to a new agiogenic phenotype that is dependent on the dolichol pathway of protein N-glycosylation. This conclusion was reached after observing the capillary endothelial cells growth arrested in G1 and entered into apoptosis following 32 h of tunicamycin (TM) treatment. TM inhibits *N*-acetylglucosaminyl-1-phosphate transferase, the enzyme responsible for transferring phospho-D-GlcNAc from UDP-GlcNAc to dolichol phosphate to initiate $Glc_3Man_9GlcNAc_2$-PP-Dol oligosaccharide biosynthesis in the rough endoplasmic reticulum (ER). This finally gets transferred to gamma-N of Asn on the respective glycoprotein(s). Earlier observations, using flow cytometry, indicated that TM induced "ER stress" and resulted in unfolded protein response-mediated growth arrest in G1 and apoptosis. To better evaluate the relationship between unfolded protein and the induction of apoptosis in capillary endothelial cells by TM, diffuse reflectance Fourier transform infrared spectroscopy (FT-IR) was utilized. Cells cultured with or without TM for varying times were analyzed. Quadruplicate spectra were collected in 32 scans at room temperature. At 3 h, the spectra of cells grown in TM displayed the amide I band characteristic of proteins [1700–1600 cm^{-1} from the C=O stretching of monosubstituted amides (MSA)] shifted from 1644 cm^{-1} (in the zero and 3 h controls) to 1648 cm^{-1}. Well-resolved bands around 1087 and 1047 cm^{-1}, which are characteristic of symmetric stretching vibrations of the P–O bond of phosphates and the C–O bond of –COH in Ser, Thr, Tyr, and carbohydrates, were detectable. At 12 h, the results were about the same as in the 3 h TM-treated cells, but the amide I band was shifted to 1659 cm^{-1}, and at 24 hr it was shifted to 1665 cm^{-1}. Also at 24 h, the amide II band (peak at 1506 cm^{-1} in the control) that arises from N–H bending and C–N stretching vibrations of MSA was fused with the band at 1450–1400 cm^{-1} (peak at 1472 cm^{-1}); the latter band is known to originate from symmetric vibrations of ionized carboxyl groups (-COO-). Besides, the bands around 1087 and 1047 cm^{-1} became undetectable in the control and in the TM-treated sample. The data show that TM induces a gradual alteration of secondary or tertiary protein structure that is detectable as early as 3 h after TM addition to the cultures. Because IR amide I band shifting to higher frequency indicates protein denaturation, these results demonstrate that TM-induced apoptosis is characterized by protein denaturation. Thus, FT-IR spectroscopy provides a valuable tool to monitor the anti-angiogenic effect of TM on capillary endothelial cells and supports protein unfolding with induction of growth arrest in G1 and apoptosis. Supported partly by Department of Defense grant DAMD 17-03-1-0754 and by National Institutes of Health grant U54-CA096197.

(70) MS^n Fragmentation of Glycosaminoglycan Oligosaccharides: Identification of Sequence- and Isomerism-Informing Fragment Ions

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Glycosaminoglycans (GAGs) are biofunctional polysaccharides known to have a regularly alternating backbone consisting of uronic acid and *N*-acetylhexosamine. Some of them usually undergo modifications, such as sulfation and uronic acid epimerization, to complete their mature structures. Difference in glycosidic linkage, position of sulfation, and kind of *N*-acetylhexosamine, that is, *N*-acetylglucosamine or *N*-acetylgalactosamine, gives rise to a lot of isomeric structures among them, which cannot be differentiated easily by using conventional analytical techniques. This study aims at offering a rapid and facile method to identify GAG oligosaccharides using a mass spectrometry (MS) technique, that is, by means of systematic MS^n fragmentation, to accelerate further biochemical studies. (1) Unsulfated GAG oligosaccharides: saturated and unsaturated hyaluronan (HA), chondroitin (CH), and *N*-acetylheparosan (NAH) oligosaccharides were prepared, and their MALDI-LIFT-TOF/TOF-MS/MS and ESI-CID-MS/MS spectra were acquired. In all of the spectra, sequence-informing fragment ions generated by glycosidic cleavages were clearly identified, reflecting their alternating regular structures. Some isomerism-informing fragment ions could also be identified, which differentiated a pair of linkage isomers, that is, HA and NAH oligosaccharides. However, the isomerism between HA and CH oligosaccharides, differing only in the configuration of C_4–OH group in *N*-acetylhexosamine, that is, diastereomer, was not determined easily. (2) Sulfated GAG oligosaccharides: So far, a few research groups have reported MS/MS fragmentation data of some species of chondroitin sulfate (CS) oligosaccharides. However, CS oligosaccharide samples prepared by a simple fractionation procedure can be heterogeneous by sequence, because natural CS, even if it is a reagent grade, contains various disaccharide units. To investigate contribution of a particular structure to its MS/MS fragmentation pattern, structurally defined homogeneous CS oligosaccharides, that is, those consisting of a simply alternating sequence, were prepared and analyzed. Sequence- and isomerism- (as regards sulfation position) informing fragment ions were successfully identified. The information obtained herein should be useful to determine sequences of structurally heterogeneous CS oligosaccharides. This work is partly supported by the New Energy and Industrial Technology Development Organization (NEDO) in Japan.

(71) Construction of Advanced □gLectin Map□h by Comprehensive Interaction Analysis Between Lectins and PA Oligosaccharides Using Frontal Affinity Chromatography

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Rapid progress in glycobiology field proved that glycans play important role in physiological phenomena. However, structural complexity and poor availability of functionally important glycans still make it difficult to profile them. To overcome these difficulties, the use of lectins, which have diverse specificities with a much wider range of binding affinity than antibodies, is promising. Under this concept, we proposed □ghect-by-hect project□h, which intends to analyze interaction between 100 lectins and 100 glycans by means of frontal affinity chromatography (FAC). The project has been performed using a prototype automated-FAC machine (FAC-1), and obtained data were stored in □glectin database□h to utilize them efficiently. In the previous meeting, we reported construction of the first step □gLectin Map□h, which concisely shows affinity strength between selected 49 oligosaccharides and 42 lectins from various sources. It also implied that various glycan structures can be discriminated from one another by comparing the patterns of multiple lectin affinities. To develop a more comprehensive glycan profiler, we further analyzed interactions between oligosaccharides (>100) and other lectins (>50). As a result, advanced □gLectin Map□h was obtained, which enables us to profile glycans more precisely. We will also report □glectin database□h and improved an automated FAC machine (FAC-2, tentative name) in this meeting. This work is supported, in part, by the New Energy and Industrial Technology Development Organization (NEDO) in Japan.

(72) Alignment of Low-Complexity Glycoprotein Sequences: Composition-Modified Scoring Matrices Allow Alignment of Yeast Cell Wall Proteins

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Although low-complexity sequences are extremely common in glycoproteins and comprise >10% of the proteome, reliable and accurate homology detection

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and alignment have not been achieved. The alignment of such sequences is essential for structural and evolutionary studies. BLAST and FASTA use the default scoring matrix BLOSUM62, which is optimized for sequences with diverse amino acid composition, that is, high Shannon entropy. With these tools, low-complexity sequence alignments place identical residues in nonhomologous positions and thereby generate anomalously high scores with small *e*-values. These low *e*-values are a consequence of deviations from the extreme value distribution of alignment scores, a phenomenon called low-complexity corruption. This corruption prevents BLOSUM62-based BLAST and FASTA from identifying correct homologs for a test query set of low entropy: wall proteins in *Saccharomyces cerevisiae*. We have devised strategies to restore the extreme value distribution of alignment scores by altering matrix score elements to compensate for amino acid frequencies in any query sequence. That is, matrix score elements were reduced for alignment pairs with high probability of occurrence, and in some cases scores were increased for low-probability events. Empirical tests of these modified matrices used cell-wall proteins as queries in a data set consisting of the yeast proteome plus randomized pseudo-protein sequences of conserved entropy. BLAST or FASTA using the best-performing matrix modification, called gtQ, reliably identified 95 homologs of a set of cell wall query proteins, where BLOSUM62-based searches identified 0–15 even if low-complexity regions were masked out. The gtQ modifications decreased sensitivity by a modest 15% for searches with high-complexity sequences. These composition-modified matrices generated alignment scores that complied with the expected extreme value distribution and generated *e*-values more accurate that did BLOSUM62 for queries with cell wall proteins. In both BLAST and FASTA searches, five types of modified matrix identified a consistent set of yeast proteins as homologous to the cell-wall test set and consistently rejected nonwall proteins and randomized pseudo-protein sequences. The modified matrices were computationally efficient and generated alignment scores that conformed to the extreme value distribution. Therefore, selective reduction of scores for high-frequency residue pairs yielded searches with high sensitivity and discrimination for low-complexity sequences. The PERL and shell scripts, customized databases, and supplemental sensitivity curves presented in this article can be obtained at http://wallace.hunter.cuny.edu/~jc/docs/.

(73) Further Developments in a Lectin Microarray: Complex Systems and Quality Control

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The complexity of glycosylation at both the molecular and biosynthetic levels argues for a system-based approach to the study of cellular glycans. One of the principle requirements of such an approach is the ability to obtain large amounts of data. The development of microarray technology has created new avenues for bioinformatic analysis of complex systems. We have recently developed a lectin microarray, a new tool for the emerging field of glycomics. This array allows us to rapidly analyze glycoproteins. To extend this technology to the analysis of more intricate systems, such as whole cells, refinements in quality control, sample preparation, and standardization must be made. Herein, we describe our work towards enabling fast analysis of cellular glycans using the microarray technology. Quality control issues, fluorescence standards, and sample analysis are discussed. The development of this system will enable us to utilize bioinformatics to approach crucial questions in glycobiology.

(74) Conformational Aspects of GalNAc Transferase Glycopeptide Substrates

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Structural characteristics of a series of glycopeptides based on a MUC2-like primary peptide sequence PTTTPLK are being investigated using solution NMR methods. The glycopeptides have various degrees and permutations of GalNAc glycosylation on the T residues and have been prepared by solid-phase synthesis methods (Liu *et al*., in press). Some of these glycopeptides have previously been examined by others as substrates for several GalNAc transferases (Takeuchi *et al*., 2002), thus our studies can provide a context for understanding their structure–function relationships and factors contributing to the ultimate pattern of O-linked glycosylation. Further, these studies can provide insights into the onset of conformational change as a function of the density of glycosylation, helping to elucidate the impact of incomplete glycosylation on the overall mucin glycoprotein organization. The NMR data show that conformational order is found to increase with the degree of glycosylation. Interestingly, the spectral fingerprint found for the peptide backbone when all three threonine residues are glycosylated is quite similar to that found for the case of the S*T*T* sequence and an S*S*S* sequence we have also studied (Coltart *et al*., 2002), indicating that the glycosylation rather than the specific choice of S or T residues dominates the molecular organization and the orientation of the sugar residues. In reported studies of antibodies arising from vaccination with clustered Tn-glycopeptide anti-tumor constructs (Kagan *et al*., 2005), antibody cross-reactivity among Tn-glycosylated triplet constructs with various T and S substitutions have been observed. This also supports our contention of a common structural motif. In this motif, the GalNAc residues are directed in the C-terminal direction, which may explain why enzymatic addition to open adjacent sites is favored by N-terminal to an existing glycosylated residue rather than C-terminal. NMR studies of constructs using amino acid analogs based on glycosylated hydroxynorleucine with a longer side chain do not indicate peptide backbone ordering. This is also consistent with the hypothesis that proximity of the GalNAc to the peptide backbone is crucial for the structural interactions.

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(75) Application of 2Dical, a New Platform for Large-Scale Proteomics, to Glycoprotein Analysis

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We developed a new proteome platform, two-dimensional image converted analysis of liquid chromatography and mass spectrometry (2DICAL), to enable reproducible, large-scale quantitative analysis, and semi-automated identification of proteins. Protein samples were completely digested with modified trypsin, separated with the constant-flow splitless nano-HPLC system (200 nL/min), and detected by high-resolution hybrid quadrupole time-of-flight mass spectrometry. The intensity of the peptides was converted to the maximum value every 1 mass-to-charge ratio (*m/z*) and displayed in a two-dimensional plane with the *m/z* value along the X axis and retention time along the Y axis. More than 100,000 peptide peaks above the predetermined threshold value were detected by 2DICAL from 60 μg of cellular protein samples or 1 μL of serum samples. 2DICAL cannot only quantify naked peptides, but peptides by modified glycosylation, and so on. In this conference, we report the detection by 2DICAL of carcinoembryonic antigen (CEA) molecules altered by glycosylation in cancer cell lines. Lysates of pancreatic cancer cell lines BxPC3, PK9, and HPAF-II were adjusted to a protein concentration of 3 mg/mL. The cell lysates were immunoprecipitated with anti-CEA antibody, eluted under the alkaline condition, and were analyzed with western blotting and 2DICAL. Western blotting revealed CEAs of different molecular weight in the BxPC3, PK9, and HPAF-II cell lines. After the deletion of N-linked glycosylation with *N*-glycanase F, the molecular weight of CEA was 87 Kd, suggesting different N-glycosylations of CEA. The 159 peaks were detected as candidates for differently glycosilated peptides derived from CEA among BxPC3, PK9, and HPAF-II cells by 2DICAL system. 2DICAL can array peptide fragments with different glycosylation. 2DICAL was concluded to be applicable to analyses of the glycosylation status of *N*-glycoproteins.

(76) High-Efficiency Production and Enzyme Properties of a Soluble Recombinant α2,6-Sialyltransferase

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Beta-galactoside α2,6-sialyltransferase from *Photobacterium damselae* JT0160 shows unique acceptor specificity. For example, this enzyme catalyzes the transfer of NeuAc from CMP-NeuAc to 2□f-fucosyllactose and 3□f-sialyllactose, which are not good acceptor substrates for mammalian sialyltransferase. The deduced amino acid sequence of the enzyme appears to have a multidomain structure; the N-terminal hydrophobic domain with a signal function, the putative catalytic domain, and the C-terminal domain with a putative membrane-binding function. Thus this enzyme seems to be quite different in structure from those of mammalian sialyltransferases. We constructed many expression vectors,

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which contained a series of truncated sequences of the sialyltransferase, and proteins were produced in *Escherichia coli* to identify portions of the protein essential for the activity. It was confirmed that the central domain had the catalytic function. Interestingly, active gene products were quite different in the amount of proteins produced in *Escherichia coli*. One of the truncated proteins designated as N2C1, which consisted of 393 residues corresponding to 58% of the full-length protein N0C0, was expressed >30 times higher compare with that of N0C0 enzyme. N2C1 protein was found in the soluble fraction from the lysate of the *E. coli*, whereas N0C0 protein was primarily found in fractions insoluble without detergents. Because N2C1 lacked the C-terminal domain, the hypothesized membrane-binding role of the domain was supported by this observation. The property of the N2C1 enzyme is under investigation.

(77) Molecular Cloning and Production of a β-Galactoside α2,3-Sialyltransferase of *Vibrio* sp. JT-FAJ-16

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Sialyl oligosaccharides are of great importance in industrial applications as well as research purposes. The advantages of enzymatic sialylation over chemical sialylation include high reaction yields and stereoselectivity. Furthermore, bacterial sialyltransferases may be prepared easily in large quantities and very much stable compared with the mammalian enzymes. Therefore, we have been screening a large number of bacteria for novel sialyltransferase activities. During the course of the study, we isolated a marine bacterium which expressed an α2,3-sialyltransferase activity. This bacterium appeared to be closely related to *Vibrio rumoiensis* and was designated as *Vibrio* sp. JT-FAJ-16. Using the α2,3-sialyltransferase gene from *Photobacterium phosphoreum* JT-ISH-467 as a probe, the genomic library prepared from JT-FAJ-16 was screened, and a gene encoded for a protein of 402 amino acids was identified. This protein showed homology of 64.7, 30.5, and 27.3% to the α2,3-sialyltransferase from JT-ISH-467, the α2,6-sialyltransferase from *P. damselae* JT-0160, and the α2,3/2,8-sialyltransferase from *Pasteurella multocida* subsp. multocida strain Pm70, respectively. The DNA fragments that encoded for the full-length protein and its putative mature form were amplified by PCR and cloned into expression vector pTrc99A. Both of the genes were expressed well in *Escherichia coli*. It was revealed that total soluble proteins from both of the strains of *E. coli* showed a sialyltransferase activity, which transferred NeuAc from CMP-14C-NeuAc to lactose. The α2,3-sialylation of the PA-labeled lactose by the proteins was confirmed by HPLC analysis. The productivity of the putative mature form of the recombinant enzyme was much higher than that of the full-length form. Therefore, the former was produced in *E. coli* in a 10-L scale culture. The putative mature form of the enzyme was purified from 10.8-L culture by two steps of anion-exchange column and a hydroxyapatite column chromatography with a purification factor of 127.3. The enzyme migrated as a single band on SDS–PAGE. The specific activity of the purified enzyme reached 57.5 U/mg. The substrate specificity and kinetic parameters is under investigation.

(78) Purification, Cloning, and Production of an α2,3-Sialyltransferase from *Photobacterium phosphoreum* JT-ISH-467

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The oligosaccharide chains in glycoconjugates, such as glycoproteins and glycolipids, are playing important roles in interaction between cells, cell differentiation, cell multiplication, and so on, in diverse organisms. Bacterial glycosyltransferases have been shown to be convenient tools for syntheses of various oligosaccharides, which are key materials for the functional studies, because, in general, bacterial enzymes are easier to prepare in large quantities and stabler than eukaryotic counterparts. Thus, we have been searching for bacteria with novel glycosyltransferase activities. A novel α2,3-sialyltransferase was isolated in the process from the cell lysate of a luminous squid habitant, *Photobacterium phosphoreum* JT-ISH-467. The enzyme catalyzed transfer of NeuAc from CMP-NeuAc to galactosides of lactose and lactosamine. The purified enzyme had a molecular mass of 39 kDa in SDS–PAGE analysis. The gene encoding for the α2,3-sialyltransferase was cloned from the genomic library of the bacteria using probes derived from the N-terminal and internal amino acid sequences. The nucleotide sequence was then determined, and an open-reading frame (1230 bp) for a 409 residue protein with a predicted molecular mass of 46.7 kDa was identified. The amino acid sequence of the protein shows 32% homology to the α2,6-sialyltransferase in *P. damselae*. Because the purified enzyme lacked the 21 amino acids in the N-terminus, the molecular mass of the mature protein was calculated to be 44.3 kDa, which was close to the measured value. The DNA fragments that encoded for the full-length protein and its putative mature form lacking the N-terminus were amplified by polymerase chain reaction and cloned into an expression vector pTrc99A. It was demonstrated that both of the genes were expressed in *Escherichia coli*, and the lysate from both of the strains of *E. coli* retained the activity.

(79) Prediction of Glycan Structures by Combining DNA Expression and Mass Data

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To understand the biological functions of glycans, it is important to determine glycan structures (sequences) like DNA and proteins. In spite of the improvements in purification and analytical methods for glycans such as high-performance liquid chromatography, capillary electrophoresis, nuclear magnetic resonance, and mass spectrometry, the determination of glycan structures are still difficult. There are some prediction methods of glycan structures using mass data (Goldberg *et al*., 2005) and DNA microarray data (Kawano *et al*., in press). Mass data reveal a combination of monosaccharides and a fundamental framework structure, but stereoisomeric monosaccharides, covalently linked position of nonreducing ends and anomeric configurations, are indistinctive. Although DNA microarray data reveal expressed glycosyltransferases and reaction information (bond information) catalyzed by them, the information from expression data is qualitative rather than quantitative. These two methods have both advantages and disadvantages. A combination of these methods to supplement the disadvantages of each other makes it possible to predict glycan structures with high accuracy. To integrate both types of data, we constructed an exhaustive glycan structure map, called composite structure map (CSM) (Hashimoto *et al*., 2005, in press). CSM is constructed by combining glycan structures in the KEGG GLYCAN database. Mapping of both types of data onto CSM enables prediction of glycan structures with correct linkage information.

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(80) Tissue-Specific Expression and Function of a Novel Human pp-GalNAc-T, O-21

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Mucin type O-glycosylation, one of the major posttranslational modifications, starts with the attachment of a GalNAc to a Ser/Thr in a peptide backbone, and the enzymes that catalyze this attachment are UDP-GalNAc : polypeptide *N*-acetylgalactosaminyltransferases (pp-GalNAc-Ts). There have been 16 human pp-GalNAc-Ts/pp-GalNAc-T like molecules published. Here we show the cloning and characterization of another pp-GalNAc-T, O-21. The O-21 gene encodes a 601 amino acid protein with conserved motifs of pp-GalNAc-T family proteins and is most homologous to pp-GalNAc-T10 (T10). Real-time PCR experiments revealed that the O-21 transcript was expressed in testis and neuronal tissues. *In situ* hybridization revealed that mouse O-21 gene was transcribed in pachytene stage primary spermatocytes. Recombinant O-21 protein showed marginal pp-GalNAc-T activity toward unglycosylated peptide substrates but attached GalNAcs to mono-GalNAc peptides. This specific characteristic in the O-21 catalytic activity resembles that of T10. However, there appeared to be differences in acceptor preference between O-21 and T10. The tissue expression of T10 is more ubiquitous than that of O-21. Thus, O-21 appears to play specific roles in O-glycosylation in testis and neuronal tissues. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO).

(81) Binding Specificity of Jacalin Towards O-Glycosylated Peptides; Exclusion of the Binding to 6-Glycosylated GalNAc

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In the course of search for lectins to capture O-glycopeptides, such as those derived from mucin, jacalin, a lectin from *Artocarpus integrifolia*, was found to

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be most relevant. Jacalin is known as a T/Tn-antigen-specific plant lectin. However, its detailed sugar-binding specificity has not been elucidated, especially with respect to glycopeptides. Thus, we performed sugar-specificity experiments by the following two methods; that is, semi-quantitative HPLC and quantitative frontal affinity chromatography (FAC). For this purpose, a series of mucin-type glycopeptides was synthesized using glycosyltransferases, ST3GalNAc1, Core1Gal-T1 and -T2, Î²3Gn-T6, and Core2GnT1n. As a result, jacalin showed significant affinity for Tn-antigen (GalNAc-a) and Core1 (Galb1-3GalNAca)-attached peptides, consistent with the previous observation. In addition, however, jacalin also showed significant affinity for Core3 (GlcNAcb1-3GalNAc-a) and ST (NeuAca2-3Galb1-3GalNAca)-attached peptides. On the other hand, it could bind Core2, Core6, nor STn-antigen. The results were also confirmed by FAC using *p*-nitrophenyl-derivatized saccharides. Thus, we conclude that jacalin binds to GalNAcÎ±1-peptides, in which C6-OH of Î±GalNAc is free (i.e., Core1, Tn, Core3, ST), whereas it cannot bind to those having substitution at the C6 position (Core2, Core6, STn). These findings provide useful information when applying jacalin for glycoproteomics targeting O-glycopeptides. This work was supported by Mitsubishi Chemical Corporation and the New Energy and Industrial Technology Development Organization (NEDO).

(82) Rapid Mass Spectrometric Screening Methodology for the Glycome of Glycolipids

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Glycosphingolipids (GSLs) are found in the membranes of nearly all living cells and are implicated in a wide range of cellular functions. GSLs consist of a ceramide lipid component attached to a glycan through either a galactose or a glucose residue. Mutations in genes that encode enzymes involved in the catabolic pathway of glycolipids have been associated with human disorders such as Fabry's disease, Gaucher disease, and Tay Sachs disease. In these instances, a block in the breakdown of the glycan portion of the glycolipid causes accumulation of substrate in the lysosome and leads to a disease state and finally clinical symptoms. In this study, we describe the development of a highly sensitive and rapid mass spectrometric screening strategy for defining the glycosylation repertoire of glycolipids from a wide range of tissues from mice, including brain, liver, kidney, and testes. Glycolipids partially purified from tissue were digested with ceramidase, and the glycan component further purified by Sep-Pak and Hypercarb chromatography. Glycans were deuteroreduced, permethylated, and analyzed by matrix-assisted laser desorption ionisation mass spectrometry (MALDI) to gain information on the composition of the glycans. Further structural information was also achieved by collisionally activated tandem mass spectrometry (CAD-ESI-MS/MS) and gas chromatography mass spectrometry. The glycosylation profiles documented here will facilitate future studies of diseased tissues by enabling the glycolipid glycome to be easily screened. Furthermore, this methodology can be employed on knockout mice to test the effect of mutated genes on the glycan profile of the glycolipids.

(83) Structural Analysis of UDP-GlcNAc 2-Epimerase/ManNAc Kinase, the Key Enzyme of Sialic Acid Biosynthesis, by Biophysical Methods

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Sialic acids are essential molecules in biological recognition systems. They are synthesized in the cytosol of mammalian cells by five consecutive steps. This pathway is initiated and regulated by the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE/MNK). The enzyme consists of two functional domains, an N-terminal GNE-domain and a C-terminal MNK domain. Furthermore, previous reports revealed assembly of the protein as a fully active hexamer and as a partially active dimer. To get more detailed information about structural features of GNE/MNK, we applied several biophysical methods to the wild-type enzyme and a set of active site mutants and mutants which occur in the GNE/MNK gene of hereditary inclusion body myopathy (HIBM) patients. First, we applied CD spectroscopy to recombinant wild-type GNE, which revealed a secondary structure composition of ~33% alpha-helices, 17% beta-folds, and 21% turns. For point mutants of GNE active site amino acids, which display drastic loss of enzyme activity, no significant changes in secondary structure were observed. In contrast, a few HIBM mutants show major structural alterations in combination with less-affected enzyme activities. This may indicate diverse effects of active site and HIBM mutations. Dynamic light scattering of GNE/MNK, which was expressed in and purified from insect cells, demonstrated existence of several subpopulations, which correspond to different oligomeric forms of the protein. Therefore, we used analytical ultracentrifugation for calculation of the native sizes of these subpopulations. Sedimentation equilibrium experiments revealed a dynamic equilibrium of GNE/MNK in three different oligomeric states. These three states were determined as monomers, dimers, and tetramers. Furthermore, aggregates of molecular masses in the range of MDa were formed, which tend to precipitate in solution. In summary, GNE/MNK seems to have a complex and dynamic quaternary structure, which may contribute to regulation of this key enzyme of sialic acid biosynthesis.

(84) Defining the Binding Specificity of Commercially Available Plant Lectins Using a Printed Glycan Array

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Purified plant lectins supplied by commercial vendors are important tools in exploring the carbohydrates elaborated on numerous cells and organisms. The binding specificities of these lectins are often defined by hapten inhibition studies with mono-, di-, and trisaccharides, agglutination assays, and FACS analysis. The Consortium for Functional Glycomics (CFG) has developed a glycan array for high throughput screening of protein–carbohydrate interactions (Blixt *et al*., 2004). As part of an ongoing effort to further develop the tools and reagents available to glycobiologists, the CFG has undertaken a large-scale effort to screen all commercially available plant lectins to characterize their binding specificity. The CFG-printed array allows simultaneous screening of ~300 glycans including most major terminal carbohydrate epitopes, blood group determinants, oligomannosides, Lewis structures, gangliosides, polylactosamines, sialosides, and biantennary *N*-glycans from glycoproteins. To date, the array has been successful in defining the specificity of numerous human and murine lectins, carbohydrate-specific antibodies, and bacterial and viral glycan binding proteins (GBP) (Blixt *et al*., 2004). This project represents an unprecedented opportunity to refine our understanding of the binding specificity of plant lectins and expand the utility of these important glycobiology reagents. The binding specificity results from this project will be available to the public, along with all other GBPs screened by the Consortium, on the CFG Website at http://www.functionalglycomics.org/static/consortium/main.shtml. The glycan-array analyses were conducted by the protein–carbohydrate interaction and carbohydrate synthesis and protein expression cores of the consortium for functional glycomics funded by the National Institute of General Medical Sciences grant GM62116.

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(85) Prominent Role of Tryptophan in Carbohydrate–Protein Interactions

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Carbohydrate–protein interactions are implicated in a variety of cell–cell and cell–matrix recognition events, ranging from fertilization, cellular differentiation, and development to pathological situations like inflammation, viral and bacterial infections, immune response, metastasis, and apoptosis. These events require a specific recognition of different carbohydrate structures by carbohydrate-binding proteins, the lectins (Lis and Sharon, 1998). To be able to understand these processes in detail, knowledge of the three-dimensional structures of the protein–carbohydrate complexes is often indispensable. The largest publicly available source of such three-dimensional structures is the Protein Data Bank (PDB) (http://www.pdb.org). The PDB was searched for carbohydrate using the *pdb2linucs* software (Lutteke *et al*., 2004). For the detected carbohydrate residues, information about amino acids within a 4Å radius was stored in an XML file, which is analyzed by the *GlyVicinity* software. Both *pdb2linucs* and *GlyVicinity* are available at the glycosciences.de Web portal (http://www.glycosciences.de). Polar amino acids are overrepresented in the spatial vicinity of carbohydrates, whereas nonpolar amino acids are underrepresented compared with their natural abundance. The aromatic residues Tyr and Trp form a remarkable exception. Especially the latter one is highly overrepresented around most types of carbohydrate residues, which indicates a special role of

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Trp in carbohydrate binding in general. Analysis of the atoms that are involved in the interactions reveals that Trp is also important to distinguish between different carbohydrates. Galactoses mainly form stacking (CH-p) interactions with Trp via the nonpolar B-face of the galactose rings. These interactions are quite evenly distributed over the indole ring of Trp. Between glucoses and Trp also stacking interactions are observed, but because glucoses do not have one side that is much more preferred to interact with Trp than the other one, like the galactoses B-face, Trp is found to interact with both sides of glucose rings. Neuraminic acids, in contrast, do not form stacking interactions to Trp at all. The pattern of interactions between neuraminic acids and Trp exhibits a decrease from the Ch2 atom to the backbone atoms. Such a decrease of interactions from the outer tip of the side chain to the backbone is mainly observed with polar or charged amino acids, which form hydrogen bonds or other electrostatic interactions to carbohydrate residues. Sulfated residues exhibit an interaction pattern with Trp that is similar to that of galactoses. Indeed, the majority of sulfated carbohydrates interacting with Trp are sulfated b-D-Galp.

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(86) Development of a High-Throughput Transcript Analysis of Glycan-Related Genes and a Cross-Platform Comparison with Microarray Expression Analysis and Correlation with Relative Quantitation of Glycan Mass Spectral Analysis

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Quantitative real-time RT–PCR (qRT–PCR) is an emerging technique for determining transcript abundance at the cellular level. We are using qRT–PCR to determine the abundance of transcripts encoding glycan-related genes in mouse tissues and embryonic stem cells at various stages of differentiation. Our approach employs a uniform strategy for gene-specific primer design and the intercalating fluorescent dye SYBR green to detect the cDNA amplification products without the need for more expensive fluorescent probes or beacons. Each primer set is first validated using mouse genomic DNA as a template before use in transcript quantitation. Using previously optimized parameters for RNA isolation, cDNA synthesis and robotic assembly of reaction mixtures, we present transcript analysis of mouse glycan-related genes found in the CAZY database (CAZY, http://afmb.cnrs-mre.fr/CAZY/), including glycosyltransferases, glycosylhydrolases, carbohydrate esterases, and carbohydrate-binding module genes in mouse embryonic stem cells and several mouse tissues. Because the glycan-related genes are generally expressed at low levels, we have compared our qRT–PCR methodology to established microarray approaches for transcript analysis. Our poster will present our qRT–PCR-derived transcript abundance data with comparison to parallel analyses employing the Glyco-gene chip microarray v2 from the Consortium for Functional Glycomics. We will also compare our relative transcript abundance measurements in various mouse tissues with parallel glycan profiling data generated by the Consortium Analytical Glycotechnology Core. Our goal is to determine whether correlations exist between transcript abundance and glycan structures in an effort to test the hypothesis that transcript abundance can act as an effective surrogate measurement for enzyme activity levels in animal tissues. (Supported by NIH grant RR018502.)

(87) A Metabolic-Labeling Approach to Proteomic Analysis

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Posttranslational modification of proteins is a major point of diversification distinguishing the proteomes of higher organisms from their more simple evolutionary ancestors. Glycosylation, the most complex posttranslational modification, is known to regulate many aspects of protein function. Therefore, increased attention to a system-wide analyses of glycan structure and function is necessary. We have recently validated a new method to detect and characterize glycoproteins on a global scale. This detection methodology is based on the incorporation of monosaccharide analogs containing a bio-orthogonal tag into cellular glycoproteins and subsequent chemical detection. Because detection is dependent on the monosaccharide chosen, profiling of individual classes of glycoproteins is possible. Also, unlike other methods of glycoproteomics, detection of both N-linked and O-linked glycoproteins is possible using the same techniques. Currently, we are attempting to apply this novel method in various settings, such as cancer biology as well as T-cell activation.

(88) *N*- and *O*-Glycans of CHO Glycosylation Mutants Determined by High-Throughput Glycomic Screening Using MALDI-TOF Mass Spectrometry

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Individual sugar residues on complex glycans expressed at the cell surface have a wide variety of functions including the mediation of cell–cell interactions important in cell adhesion and cell migration. Identifying the specific sugars and the glycan structures which carry them is key to defining the roles of sugars and the basis of their recognition by carbohydrate-binding proteins (CBP). It is also important to establish the range of structures that can be synthesized by given cell types and organisms and that defines the glycome of mammals. To determine a wide range of glycan structures on small amounts of biological samples, highly sensitive, high-throughput glycomic screening techniques have been developed and are being applied to cell and tissue samples. A demonstration of the power of these techniques will be presented by comparing the structures of *N*- and *O*-glycans determined from matrix-assisted, laser desorption ionization-time-of-flight (MALDI-TOF) spectra obtained from permethylated glycans released from total glycoproteins of Chinese hamster ovary (CHO) cells and glycosylation mutants derived from them. The glycosylation mutants have well-characterized molecular defects in specific glycosylation reactions that give rise to either a more simplified range of structures (loss-of-function mutants) or a more complex range of structures (gain-of-function mutants) compared with parent CHO. The structural characterization of the major *N*- and *O*-glycans expressed by these CHO glycosylation mutants makes them valuable tools with which to define the sugar-binding specificities of CBPs. This work was supported by the Consortium for Functional Glycomics funded by the National Institutes of Health, NIGMS, and grant RO1 36434 to P.S.

(89) Tools for Glycoproteomic Analysis: Size Exclusion Chromatography Facilitates Identification of Tryptic Glycopeptides with N-Linked Glycosylation Sites

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The development of proteomic techniques, such as HPLC coupled to tandem mass spectrometry (LC-MS/MS), has also proved useful for the identification of specific glycosylation sites on glycoproteins (glycoproteomics). Glycosylation sites on glycopeptides produced by trypsinization of complex glycoprotein mixtures, however, are particularly difficult to identify both because a repertoire of glycans may be expressed at a particular glycosylation site and because glycopeptides are usually present in relatively low abundance (2 to 5%) in peptide mixtures compared with nonglycosylated peptides. Previously reported methods to facilitate glycopeptide identification require several preenrichment steps or involve complex derivatization procedures. Because the N-linked glycans expressed on tryptic glycopeptides contribute substantially to their mass, we demonstrate that size exclusion chromatography (SEC) provides a significant enrichment of N-linked glycopeptides relative to nonglycosylated peptides. The glycosylated peptides are then identified by LC-MS/MS after treatment with PNGase-F by the monoisotopic mass increase of 0.984 Da caused by the deglycosylation of the peptide. Preprocessing of the protein database used as input for peptide identification by ion searching further facilitates this procedure. Analyses performed on human serum showed that this SEC glycopeptide isolation procedure results in at least a 3-fold increase in the total number of glycopeptides identified by LC-MS/MS, demonstrating that this simple, rapid method is an effective tool to facilitate the identification of peptides with N-linked glycosylation sites.

(90) Tandem Mass Spectrometry Approaches for the Mapping of O-Glycosylation Sites

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Several methodologies exist for the identification and characterization of glycoproteins. At least three important steps are necessary, identification of the

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glycosylated protein, characterization of the glycans, and mapping the sites of modification. Although various methods have been perfected and applied for the first two steps, mapping of sites, especially for complex O-glycans, has been difficult. Previously, a strategy for mapping O-GlcNAc sites was developed termed BEMAD (beta-elimination followed by Michael addition with dithiothreitol). This method has several advantages for site mapping. Using BEMAD, the labile glycan is replaced with a covalently bound, stable DTT tag, that can be used to enrich the peptides of interest using thiol-chromatography. Furthermore, this method is amenable to relative quantification by the use of isotope light and heavy DTT. Here, we demonstrate that this methodology can be adapted to the mapping of other types of *O*-glycan structures. We also demonstrate that we can couple this methodology with neutral loss scanning LC-MS3 analysis to gain information about the *O*-glycan structures present at the site of modification. This combination of methods is used to map multiple sites of *O*-Man modification on proteins from *Aspergillus niger*. Further, we map sites of complex O-glycosylation on fetuin, alpha-dystroglycan, and proteins from complex mixtures. Also, we demonstrate the usefulness of the BEMAD method for quantifying relative site occupancy. Finally, we illustrate the power of combining neutral loss MS3, BEMAD, PNGaseF with O-18 water, and electron capture dissociation fragmentation to gain more complete information about the sites of both N-glycosylation and O-glycosylation on proteins.

(91) Glycomics Using Negative Ion Tandem Mass Spectrometry of Native Glycans

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In the field of glycomics, it is necessary to produce as much structural information as possible on limiting sample quantities. Mass spectrometry (MS) is particularly useful for generating information on samples available in submicrogram quantities. One approach for analysis of glycans using tandem MS is to permethylate the structures. This step serves to improve the ionization responses, minimize ion suppression, and maximize the informational value of the tandem mass spectra. Despite these advantages, permethylation is not suitable for many glycomics experiments. The recovery of glycans with sulfate and phosphate substituents is quite poor, and these molecules must be analyzed in native forms. In addition, classical permethylation is suitable only for samples available in quantities ~>1 μg. Thus, the mass spectrometric analysis of native glycans is an important analytical option in glycomics. Most classes of animal glycans are acidic, and the use of negative ionization is a natural choice. Sialylated glycans and those modified with sulfate or phosphate groups produce abundant ions in the negative mode. Recently, however, the use of negative ionization tandem mass spectrometry for analysis of neutral glycans has received renewed attention in the literature; the tandem mass spectral patterns have been shown to be particularly useful for determining branching and fucosylation patterns and for distinguishing Type 1 versus Type 2 structures in the antennae. The chemistries of neutral and acidic glycans are rather different, and it is the aim of this work to describe fragmentation processes for both classes. Particular attention will be paid to the influence of acidic functional groups on the product ion patterns in the negative mode. The glycan classes examined include asialo and sialylated N-linked, asialo and sialylated milk, asialo and sialylated Lewis structures, chondroitin sulfate, chondroitin, heparan sulfate, and heparosan oligosaccharides. All glycans were analyzed in the negative nano-electrospray mode using an Applied Biosystems/MDS Sciex Qstar pulsar-i mass spectrometer. The asialo glycans were analyzed as both deprotonated and nitrated ions. The sialylated, sulfated, and uronosyl glycans were analyzed as deprotonated ions. The results demonstrated that the deprotonated asialo N-linked, milk and Lewis glycans require significantly less input of energy to cause fragmentation than do the nitrated or the sialylated forms. In addition, the abundances of structurally useful cross-ring cleavages and D-type ions are highest for the deprotonated ions. The product ion patterns of glycosaminoglycan oligosaccharides are complex and reflect the interplay of acidic effects caused by uronic acid residues and sulfate groups. Fragmentation mechanisms may be rationalized based on proton transfer reactions between the acidic functional groups and the ring hydroxyl groups. The results are significant because they form the basis for analytical MS-based analytical platforms for glycomics of native structures.

(92) Discrimination of Isomeric/Isobaric Glycosphingolipid Glycan Structures Using Ion Trap MS^n in Combination with Glycan Fragment Library and Decomposition Pathway Constraint Strategies

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Multistep disassembly of sodium adducted permethylated glycans by ion trap MS^n has already shown considerable potential for deriving information about linkage and branching. In more recent comparative studies with a variety of di- and trisaccharides of known structure, reproducible differences in fragmentation have been observed not only as a function of linkage position but of differences in monosaccharide identity and anomeric configuration as well (Ashline *et al*., in press). Moreover, MS^n of permethylated glycans has been combined with computerized glycan fragment library searching and high-throughput tree analysis of decomposition pathways (Lapadula *et al*., in press; Zhang *et al*., in press). In work to be described in this presentation, the newly developed fragment search and decomposition pathway constraint algorithms were applied to ion trap MS^n spectra of permethylated GSLs from insect (*Drosophila melanogaster*, cultured High Five cells) and mammalian sources, as well as to permethylated glycosylinositols derived from glycosylinositol phosphorylceramides (GIPCs), a structurally diverse subclass of sphingolipids found in plants and fungi, but not in mammals or other vertebrates. We focused particular attention on the prospects for discriminating between pairs of GSLs whose glycans differ with respect to a single feature, for example, glycosidic linkage position or identity of nonreducing terminal monosaccharide. Such pairs included the core diglycosylceramides LacCer (Galβ4Glcβ1Cer) and MacCer (Manβ4Glcβ1Cer), and the triglycosylceramides Gb3Cer (Galα4Galβ4Glcβ1Cer) and iGb3Cer (Galα3Galβ4Glcβ1Cer). In this study, we both used and added to a growing glycan Fragment Library (FragLib) and applied the spectral search and comparison algorithms developed to interface with this database (Zhang *et al*., in press). In addition, GSL MS^n data were submitted to the algorithm, oligosaccharide subtree constraint algorithm (OSCAR), that accepts multiple MS^n ion fragmentation pathways and proposes topologies that are consistent with all input ions (Lapadula *et al*., in press). The effect of the adducting cation (lithium versus sodium) on the sensitivity and quality of discrimination between terminal monosaccharide residues was also examined. The performance of OSCAR and FragLib search algorithms with the data sets generated from isomeric GSL glycans will be reported.

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(93) A Universal High Throughput Screening Assay for Glycosyltransferases

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Glycosyltransferases (GTs) are increasingly being targeted for therapeutic intervention, both in humans and microbes; however, the diversity of donors and acceptor substrates complicates development of screening assays that can be used across family members. Many GTs use UDP-activated sugars, so detection of UDP provides a generic GT assay method. We have previously shown that a competitive fluorescence polarization immunoassay for UDP can be used with high sensitivity to detect the activity of hepatic UDP-glucuronosyltransferases involved in xenobiotic conjugation. In this study, we extend these results to a mammalian UDP-galactosyltransferase that uses lactose as acceptor. Because it relies on detection of the product of donor substrate cleavage, the assay can be used in two modes: (1) with an acceptor present, to screen for inhibitors or (2) to screen directly for acceptors. A small library of bioactive compounds was screened in the presence and absence of lactose, enabling identification of both inhibitors and acceptors. These results establish proof of concept for a broadly applicable HTS assay that can be used to detect and profile modulators across diverse members of the GT superfamily.

(94) MonoSaccharideDB: A Reference Resource to Unify the Notation of Carbohydrate Residues

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Carbohydrates are much more complex than other biopolymers like proteins or nucleic acids. The most often stated reason for this complexity is the fact that their single residues, the monosaccharides, can be linked in several ways, which allows the formation of branched structures. Another reason for the complexity of carbohydrates is the large number of different monosaccharides, which by far exceeds the number of amino acids (20) or nucleotides (8 [4 DNA + 4 RNA]). For an efficient handling of carbohydrate structures in databases and

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computer software, a unique nomenclature for the monosaccharide units is indispensable. For example, *a-D-6-deoxy-Glcp* and *6-deoxy-a-D-Glcp* or *b-D-Glcp2NAc* and *b-D-GlcpNAc* are different residues for a computer. The same applies, for example, to *b-D-4-deoxy-Galp* and *b-D-4-deoxy-Glcp*. Without further knowledge about carbohydrates, the latter ones appear to be different residues for humans as well. Lacking a normalization of monosaccharide residue notation, it is not only difficult to crosslink carbohydrate databases but even possible that one structure is represented by two or more entries within one single database. MonoSaccharideDB is an attempt to provide a reference for the nomenclature of carbohydrate residues. It can be accessed in two ways: The fuzzy search yields, for example, all residues based on D-glucose. In the exact search, the user can directly enter a residue name. This name is parsed by a check routine, which tries to generate a unified residue name. Rules to provide a unique monosaccharide name are based on CarbBank/IUPAC notation. If such a name could be created, it is used to query the database and, in case no match is found, a new entry is entered. By this means, MonoSaccharideDB is growing on demand. This proceeding is necessary because it is virtually impossible to prefill the database with all thinkable combinations of monosaccharide base types and modifications. Besides, such a prefilled database would contain a large amount of residues that do not exist in nature. Each single database entry contains the unified residue name, properties like basetype, anomeric, absolute configuration, or modifications, a Haworth projection and a three-dimensional structure view of the residue. An XML format summarizing the monosaccharide properties is also offered. MonoSaccharideDB is available online at http://www.dkfz.de/spec/monosaccharide-db/. The Website also contains a set of rules for unique monosaccharide nomenclature and a notation check program based on the check routine mentioned above. A simple object access protocol (SOAP) interface to the check routine and the XML format is also available, so that it can be easily accessed by external programs or databases.

(95) A New Tandem LC-MS-Based Method for Glycolipids

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A new simple LC-MS methodology we originally developed for a broad range of lipids is here applied and optimized for separation and analysis of gangliosides, glycerophosphoglycoinositols, lipid A components, and permethylated glycans. A PVA-Sil normal-phase column coupled to a triple quadrupole mass spectrometer is used for the detection and initial characterization of different kinds of glycolipids, with further characterization of the fractions using nanospray MS or chromatography on a reversed phase capillary column followed by MS and/or MS/MS. For the smaller structures, sharp peaks in a reproducible chromatogram are easily achieved on the normal phase column, while on the same system, especially for lipid A, peak broadening is more difficult to avoid. The main advantages of this methodology are its robustness, the dual information from retention times and mass spectra, and the easier characterization of minor components in a complex mixture.

(96) Automated Analysis and High-Throughput Mass Spectrometric Glycomics Profiling of Mammalian Cells and Tissues

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The Consortium for Functional Glycomics (CFG) is a large research initiative funded by NIGMS to understand the role of carbohydrate–protein interactions at the cell surface in cell–cell communication. The analytical core of the CFG utilizes high throughput mass spectrometric glycomics strategies, complemented by metabolic labelling/chromatographic profiling, to structurally define the glycans of glycoconjugates from murine and human cells and tissues. In the course of our studies, we have thus far analyzed a total of 108 murine tissues (brain, colon, kidney, liver, lung, lymph nodes, ovaries, small intestine, spleen, testes, and thymus), 63 human tissues (colon, heart, kidney, large intestine, liver, lung, lymph nodes, pancreas, skin, small bowel [ileum and jejunum], and spleen), and 22 cell lines. The data are all freely available in both raw and annotated formats from http://www.functionalglycomics.org/static/consortium/main.shtml/. We describe here further developments in *Cartoonist*, a novel algorithm/viewer for high throughput interpretation and annotation of *N*-glycan MALDI-MS profiles. *Cartoonist* is now capable of generating its own archetypal cartoons, vastly increasing its flexibility. Improvements to the peak detection process have extended the reliable range of the algorithm to beyond 5000 Da and has enhanced the sensitivity with regard to low intensity signals with incomplete isotope envelopes. It is also now possible to load a specific analysis for a given sample, allowing the user the freedom to decide between a preset expert analysis and the flexibility of a more tolerant annotation. The viewer aspect of *Cartoonist* has been improved with respect to user friendliness and functionality, with a Web-based version currently being developed. Data from the murine and human analyses will be presented, along with a fully functional iteration of the program itself.

(97) Modulation of Cell Adhesion or Attachment Using Sialic Acid Engineering Methods

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"Metabolic substrate-based sialic acid engineering" is an established technique that exploits the remarkable ability of the sialic acid pathway to biosynthetically convert nonnatural *N*-acetylmannosamine (ManNAc) analogs into the corresponding nonnatural forms of sialic acid and then install these novel epitopes onto the cell surface. This presentation explores the ability of this methodology to modify the adhesive properties of a cell and, by demonstrating that it can be used to both enhance and diminish the "stickiness" of the cell surface, establishes new research directions in cancer therapy, tissue engineering, and nanobiotechnology. In the first part of this project, based on the rationale that sialic acid plays a role in many steps of the metastatic process by altering cell surface adhesive molecules, we investigated the extensively studied set of analogs with *N*-acyl hydrocarbon chain extensions, along with the novel analog, 1,3,4,6-tetra-*O*-acetyl-2-acetylthioacetamido-2-deoxy-á-D-mannopyranose (Ac5ManNTGc). These analogs were shown to change the behavior of cells in *in vitro* assays commonly used to predict the metastatic potential of cancer cells; the biological basis of these changes is now under investigation. Cancer cells incubated with Ac5ManNTGc, and therefore displaying thiol-bearing sialic acids on their surfaces, showed dramatic cell–cell condensation. In the long term, this provocative result may benefit the treatment of metastatic cancer if the increased display of thiols on the cell surface have the ability to crosslink cancer cells before metastasis and slow their progression through sulfide-rich extracellular matrix. In the short term, this result suggested that sialic acid-displayed thiols could be exploited in tissue engineering applications. Specifically, we confirmed that surface thiols were useful cell surface "handles" for new modes of directed cell attachment by demonstrating that analog-treated, naturally nonadhesive Jurkat (human t-lymphoma) cells selectively bound to gold-plated, or maleimide-derivatized, surfaces. As a first step toward developing tissue engineering applications for sialic acid engineering technology, we demonstrated that ManNAc analogs were successfully metabolized by embryoid body-derived (EBD) stem cells. Flow cytometric quantitation revealed that levels of cell surface thiol expression in these cells increased 15-fold upon incubation with Ac5ManNTGc, which was supported by quantum-dot labeled confocal imaging. In addition, the analog appears to favor neuronal-like differentiation of EBD cells, especially when grown on gold-coated cover slips. Efforts are currently underway to exploit the sugar-expressed thiols for tissue engineering by developing biocompatible, polymeric scaffolds with maleimide groups and for nanobiotechnology applications by attaching cells to micro- and nano-patterned surfaces as well as developing technologies for the electromagnetic field directed positioning of analog-treated cells on gold-patterned microelectrode arrays. The panel of analogs included a diverse array of alkyls, ketones, and thiols. Thiols, in particular, were introduced to create additional disulfide bridges between cells in an effort to mimic cell condensation, a hallmark of differentiation. Additional disulfide bridges were also intended to crosslink cancer cells before metastasis and slow their progression through sulfide-rich extracellular matrix.

(98) *N*-Glycan Chemoenzymatic Synthesis Using Oligosaccharide Oxazolines as Donor Substrates

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Natural and modified *N*-glycopeptides were chemoenzymatic synthesized in a regio- and stereospecific manner. Endo-A can transglycosylate the synthetic oxazoline donors, the mimics of the presumed oxazolinium ion intermediate to GlcNAc-peptide acceptors in very high yield (60–75%). This method enhanced the synthetic efficiency, expanded the substrate availability. It might be useful to synthesis the natural *N*-glycan and modified nonnatural *N*-glycopeptides.

(99) A Simple Method for Separation of N- and O-Linked Oligosaccharides from Glycoproteins and Analysis by HPLC and Electrospray Mass Spectrometry

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The need for methods of analysis of oligosaccharides is growing because of the increased demand for analytical methods to study carbohydrate posttranslational modifications. Although there are methods for the analysis of N- and

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O-linked oligosaccharides but none, to our knowledge, that simply separate the two sets of glycoforms from the same sample. Hydrazine has been used in the past to release oligosaccharides; however, this is a tedious method, requiring anhydrous conditions, a toxic substance, different reaction temperatures for optimum N- and O-linked cleavages, and *N*-acetyl groups must be reacetylated with acetic anhydride and it gives both oligosaccharides in one fraction. Our method requires no toxic reagents, does not require anhydrous conditions that will produce two separate fractions and will eliminate the need to remove protein from the sample. In addition, it gives the added capability of isolating *O*-glycopeptides which can be further analyzed by MALDI. Separation of the N- and O-linked glycoforms aids in identification and structural analysis of glycoprotein structure. N-linked oligosaccharides are obtained by digestion with PNGase F. We place them through a Dionex H column (ammonium form). We wash off the N-linked oligosaccharides with water and use ammonium hydroxide to elute O-linked glycopeptides. We use ammonium hydroxide/borohydride to remove O-linked oligosaccharides from serine/threonine residues via β elimination. We place this mixture over Dionex H column again (ammonium form) and elute the reduced O-linked alditols with water leaving behind the peptides. Nonvolatile cations are exchanged to aid in ESI/MS analysis. Starting from as little as 10 µg fetuin, the N-linked alditols can be analyzed by HPLC, using a Dionex Carbo Pac PA 200 column. We have used this approach to analyze mg quantities of fetuin. Characterization of samples is in progress.

(100) Synthesis of Oligo-β-(1-6)-*N*-Acetylglucosamines, Fragments of the Polysaccharide Intercellular Adhesin of Staphylococci

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It has been shown recently that antibodies against partially de-*N*-acetylated β(1-6)-linked poly-*N*-acetyl glucosamine (PNAG) surface polysaccharide antigen-mediated effectively killing of a variety of strains of *S. aureus* and *S. epidermidis*. However, the exact chemical nature of the protective epitopes is not known. To define the exact chemical structure of the most important fragments giving rise to protective immunity, chemical synthesis of oligoglucosamines with glucosamine units bearing N-acetylated and free amino groups in defined places was necessary. As a first step toward this goal, preparation of oligosaccharides either with all N-acetylated or all N-unprotected glucosamine units was studied. First, oligomerization of mono- or oligosaccharide glucosamine derivatives that contained both glycosyl donor and glycosyl acceptor sites was explored. However, this approach afforded mainly cyclic products of intramolecular glycosylation and only low yield of linear β(1-6)-glucosamines. Another approach consisted in step-by-step elongation of the oligosaccharide chain with the use of di- or tetrasaccharide glycosyl donors. It provided a series of protected higher β(1-6)-oligoglucosamines. Subsequent removal of protecting group with or without N-acetylation resulted in the formation of fully N-acetylated or N-unprotected oligoglucosamines, respectively. Protecting groups pattern allowed preparation of oligosaccharides containing thiol functionality in a spacer group for further conjugation with a protein carrier. A set of orthogonal N-protecting groups for preparation of β(1-6)-glucosamines having *N*-acetyl groups in defined glucosamine residues and thiol in a spacer arm has been elaborated. The work is supported by CRDF (grant RUB1-2639-MO-05) and the Russian Foundation for Basic Research (grant 05-03-08107).

Session Topic: Proteoglycan Functions

(101) Global Assessment of Iduronic Acid and 2-O-Sulfated Iduronic Acid in Heparan Sulfate with Mass Spectrometry

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Heparan sulfate (HS) is a linear polysaccharide that contains iduronic acid (IdoA) and 2-O-sulfated iduronic acid (IdoA2S) residues. IdoA has significant contribution to the overall flexibility of HS chain. IdoA2S is important for various protein binding. Here, we describe a new approach to determine overall distribution of IdoA and IdoA2S residues on bovine kidney HS with mass spectrometry. An HS sample was first digested with low pH nitrous acid to expose these residues and then digested with iduronate-2-sulfatase and/or á-L-iduronidase. The digests were monitored with HPLC coupled mass spectrometry. Our results showed that IdoA2S residue exclusively located between two consecutive GlcNS residues, and the degree of epimerization of a UA was positively related to the sulfation content of its immediate context. In particular, higher degree of epimerization of a UA was observed when it was bordered by two *N*-sulfates. Although oligosaccharides from NA domains showed significant amount of zero-, mono-, and disulfated species, oligosaccharides from NS and NS/NA domains only showed significant amount of mono- and disulfated species. All those oligosaccharides could begin with either IdoA or GlcA. A trisulfated disaccharide was found to contain only IdoA2S as its UA moiety. Our data suggest that 2-*O*-sulfates are preferentially coupled to consecutive *N*-sulfates, and 3-*O*- and 6-*O*-sulfates may be coupled to both consecutive and alternating *N*-sulfates during HS biosynthesis. The advantages for using LC/MS to study functional groups on HS are summarized.

(102) Non-Reducing End Structures of Heparan Sulfate Polysaccharide

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The reducing end of heparan sulfate has been known for a long time, but information on the nonreducing end has been lacking. Recent studies indicate that the nonreducing end of heparan sulfate might be the place where FGF-signaling complex forms. The nonreducing end also changes with heparanase digestion and thus might serve as a marker for tumor pathology. Using HPLC-coupled mass spectrometry, we have identified and characterized the nonreducing end of bovine kidney heparan sulfate. We find that the nonreducing end region is highly sulfated and starts with a GlcA residue. The likely sequences of the nonreducing end hexasaccharides are GlcA-GlcNS6S-UA ± 2S-GlcNS ± 6S-Ido2S-GlcNS ± 6S. Our data suggest that the nonreducing end of bovine kidney heparan sulfate is not trimmed by heparanase and is capable of supporting FGF-signaling complex formation.

(103) Analysis of Sulfated Disaccharides from Keratan Sulfate and Chondroitin/Dermatan Sulfate During Chick Corneal Development by Electrospray Ionization Tandem Mass Spectrometry

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In cornea, keratan sulfate (KS) and chondroitin/dermatan sulfate (CS/DS) are the two major glycosaminoglycans (GAGs). KS does not contain uronic acids, and its repeating disaccharide unit is composed of alternating residues of D-galactose (Gal) and *N*-acetyl-D-glucosamine (GlcNAc) linked ¦ Â-1,4 and ¦ Â-1,3, respectively. Chains of CS/DS consist of *N*-acetylgalactosamine (GalNAc) residues alternating in glycosidic linkages with glucuronic acid or iduronic acid residues. The GalNAc residues are predominantly sulfated in the C-4- or C-6-hydroxyl position, interspersed with a few nonsulfated residues. In this study, electrospray ionization tandem mass spectrometry (ESI-MS/MS) was employed to quantify changes in KS and CS/DS-sulfated disaccharides in the developing chick cornea. The concentration of KS monosulfated disaccharide (MSD) Gal-¦ Â-1,4-GlcNAc(6S) in embryonic day 8 (E8) cornea equals that at E20, falls to its lowest level by E10, rises to a second peak by E14, falls to a second low by E18, peaks again by E20, and remains high in adult corneas. A similar concentration profile is observed for KS disulfated disaccharide (DSD) Gal(6S)-¦ Â-1,4-GlcNAc(6S) and thus also for total sulfated KS disaccharides. The molar percent of DSD is higher than that of MSD from E8-E18, equivalent at E20, and less than that of MSD in adult corneas. In contrast, total concentration of CS/DS ¦ ¤di-4S plus ¦ ¤di-6S decreases as development progresses and is lowest in adult corneas. Concentration and molar percent of ¦ ¤di-6S is highest at E8, then decreases through development as the concentration and molar percent of ¦ ¤di-4S increases from E8 and exceeds that of ¦ ¤di-6S after E14. New rapid, direct chemical analysis of extracellular matrix components obtained from sections from embryonic and adult chick corneas reveals heretofore undetected changes in sulfation characteristics of KS and CS/DS disaccharides during corneal development.

(104) Unraveling the Molecular Basis of the Role of Pectins in Human Health

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Glycosaminoglycans (GAGs) are naturally occurring, heterogeneous, negatively charged, and N-acetylated unbranched matrix polysaccharides. They form a major part of the extracellular matrix of connective tissues and are implicated in a wide number of biological activities including the development and function of cartilage and bone, neural development and repair, thrombosis and haemostasis, fertilization, inflammation, and cell adhesion. Pectins are heterogeneous acidic polysaccharides that are primary structural elements of the matrix of the plant cell wall and have been implicated in morphogenesis, pH regulation, ion balance, wall permeability, and plant defense. Thus, GAGs and pectins serve similar functions within their respective organisms. There is substantial evidence as to the roles of pectins in medicine including potentiation of human colonic adenocarcinoma cells, immunostimulating activity, anti-ulcer

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activity, anti-metastasis activity, anti-mutagenic activity, anti-nephrosis, and cholesterol decreasing activity. Our laboratory has been actively looking at the relationships between pectins and glycosaminoglycans, as well as between the microbial pectin degrading enzymes (PDEs) and microbial GAG degrading enzymes. We noted that the enzymes that cleave GAGs and those that degrade pectin often share structural similarities. The similarity in function of GAGs and pectins, and the structural similarities of the enzymes that degrade them, led us to wonder whether these were clues that could shed light on the mechanism of the role of pectins in human health. As a first step, we investigated the effects of GAGs on selected pectin degrading enzymes and of pectin on GAG degrading enzymes. Our investigations demonstrate effects on both enzyme structure and activity, indicating that pectins are able to affect certain GAG degrading enzyme activities, and GAGs are able to affect pectin degrading enzymes (PDEs). It is a reasonable assumption that the receptors for GAGs involved in such activities as neural development and angiogenesis could act as potential targets for pectins and that the role of the GAGs themselves in neural development or angiogenesis may be altered by pectin degrading enzymes. Based on this hypothesis and our *in vitro* data, we are currently investigating the *in vivo* role of pectins and PDEs in modulating GAG function.

(105) Proteolytic Processing of the 315 kDa Human HARE/Stab2 Hyaluronan Receptor Generates the Smaller Functional 190 kDa HARE Isoform

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The predominant endocytic clearance receptor for circulating hyaluronan (HA) and other glycosaminoglycans that originate from tissue ECMs throughout the body is the hyaluronic acid receptor for endocytosis (HARE) (also called Stabilin-2). Full-length human HARE is a glycoprotein of 2551 amino acids (~315 kDa in SDS–PAGE) that is highly expressed in the sinusoidal endothelial cells of lymph node, spleen, and liver. Immunopurified HARE from human or rat spleen and liver exists as two receptor isoforms (a ~315 kDa form and a 190 kDa form whose N-terminus corresponds to Ser-1136 in full-length hHARE). There is no detectable mRNA for the 190 kDa isoform, which appears to be generated *in vivo* by specific proteolytic cleavage of the full-length protein. The cleavage region does not contain consensus sequences for any known proteases. Flp-In 293 cell lines expressing the full-length human HARE cDNA were created to study this recombinant receptor for the first time. In support of the above cleavage mechanism, we observed the same proteolytic processing to create the 190-kDa isoform in 293 cell lines stably expressing only the full-length HARE cDNA. Based on pulse-chase experiments using 35S-Cys/Met, the full-length 315-kDa HARE receptor is synthesized, glycosylated, and then presented on the cell surface before the appearance of the 190-kDa HARE. An N-terminal GFP-190 HARE fusion protein expressed in 293 cell lines was also cleaved to produce free GFP and free 190 kDa HARE. Conversely, full-length hHARE lacking the cytoplasmic and transmembrane domains (a secreted ectodomain) was not proteolytically cleaved, indicating that membrane anchorage is vital for receptor processing to create the 190-kDa isoform. We conclude that the proteolytic cleavage of full-length HARE to create the second smaller isoform is a natural, nonartifactual process and that both isoforms are actively engaged in glycosaminoglycan clearance. (This research was supported by NIH grants R01 GM69961 and F32 GM070262.)

(106) Cells Expressing Full-Length Recombinant Human HARE/Stab2 Receptor Mediate the Binding, Endoctyosis, and Degradation of Multiple Glycosaminoglycans

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The systemic clearance of hyaluronan (HA) and chondroitin sulfates (CS) from the circulatory and lymphatic systems is mediated by the HA receptor for endocytosis (HARE) (also designated Stabilin-2). HARE is present as two major membrane-bound isoreceptors, a full-length ~315 kDa form and a truncated ~190 kDa form, which is derived from the larger protein by specific proteolysis. Both HARE species are highly expressed in sinusoidal endothelial cells of liver, lymph node, and spleen. Flp-In 293 cell lines expressing the full-length human HARE cDNA were created to study this recombinant receptor for the first time. Cells expressing the 315-kDa HARE were able to endocytose and degrade HA, chondroitin, and a variety of CS types, but not heparin, heparan sulfate, or keratan sulfate. All 315HARE 293 cell lines stably expressed both receptor isoforms (315 kDa and 190 kDa) in culture, although the clones differed in total receptor expression levels and their HA binding, endocytosis, and degradation activities. Three monoclonal antibodies, raised against rat HARE, crossreacted with both human receptor isoforms. These antibodies also partially block specific endocytosis of HA by HARE. We also developed cell lines that secrete full-length hHARE without the transmembrane and cytoplasmic domains. The secreted 315 kDa HARE ectodomain was purified from media via metal-chelate chromatography. In an ELISA format, the ectodomain binds to HA with a higher affinity than to chondroitin sulfates A–E. Unlabeled chondroitin sulfates A–E also blocked HA endocytosis by 315-kDa HARE stable cell lines, to different degrees. We are currently biotinylating intact or permeabilized cells to determine the cellular distribution of the two receptor isoforms; the total or biotin-labeled 315 kDa and 190 kDa HARE proteins are then examined by western analysis using HARE-specific antibodies and streptavidin-HRP. Preliminary results indicate that only a small fraction of total cellular receptors reside on the cell surface. We conclude that both the 315 kDa and 190 kDa HARE receptor isoforms can mediate the clearance of multiple types of glycosaminoglycans via endocytosis and contribute to their normal homeostasis. (This research was supported by NIH grants R01 GM69961 and F32 GM070262.)

(107) Proteoglycan Profiles in Chicken Gastrocnemius Tendons Change with Age and Exercise

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Tendon function depends on the proper organization of Type I collagen fibrils in the tendon. Collagen fibrillogenesis is an essential process during embryonal development as well as during remodeling. The biochemical structure of the tendon adjusts to facilitate the required function. Fibrillogenesis, including the rate of formation and final sizes of the fibrils is regulated by proteoglycans (PGs). PGs consist of core proteins to which glycosaminoglycan (GAG) chains are attached. Though many PGs, for example, biglycan, aggrecan, fibromodulin, and versican are present in the tendon, the extent of their involvement in tendon organization, and in collagen fibrillogenesis in particular is not well understood. Only the role of decorin, a small leucine-rich proteoglycan, has been described to some detail. Decorin, a major proteoglycan in the tendon, limits collagen fibril growth and thus directs tendon remodeling owing to tensile forces. Mechanical tension induces the synthesis of decorin, whereas the production of the large PG aggrecan is stimulated in a tendon subjected to compression. Such data underscore the importance of PGs in normal function of tendons and other connective tissues. We hypothesized that rapid growth and moderate exercise induce changes in PG synthesis in the gastrocnemius tendon of young chickens. To test our hypothesis, we compared the PG content in gastrocnemius tendons from growing chickens between 1 day and 6.5 weeks old and from control 6.5-week-old chickens with that in tendons from 6.5-week-old chickens that underwent exercise. Using guanidine HCl and CsCl fractionation, we have extracted PGs from gastrocnemius tendons of young chickens, some of which underwent moderate exercise. The presence of specific PGs and GAGs was analyzed with Sepharose CL-2B chromatography, PAGE, HPLC, and immunoblotting for GAGs and core proteins. An increase in the size of tendons, and the content of GAGs, specifically of keratan sulfate, chondroitin sulfate (indicative of increase in decorin), and hyaluronan was observed in rapidly growing avian gastrocnemius tendons. Our results show high levels and a wide variety of GAGs in 6.5-week-old tendons. Chondroitin-4-sulfate disaccharide was the major GAG disaccharide in control and exercised 6.5-week-old gastrocnemius tendons. Exercise led to an increase in the size of the tendons, the content of hyaluronic acid, and the level of decorin. High levels of keratan sulfate (KS) were found in the lower halves of gastrocnemius tendons; although the amount of KS decreased with exercise. This corresponded well with lower content of aggrecan in lower halves of exercised tendons. In conclusion, our data support the hypothesis that exercise alters the content of PGs in chicken tendons. It remains to be seen whether such changes affect collagen fibril formation.

(108) An LC/MS/MS Platform for Glycoform Quantification of Chondroitin Sulfate

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Glycomics, known as the study of the structure and function of glycans, is a rapidly growing field. Glycosaminoglycan (GAG) chains are made of repeating disaccharide units that are attached to proteoglycan core proteins on adherent animal cell surfaces and in extracellular matrices. Chondroitin sulfate (CS) is a glycosaminoglycan that consists of repeating disaccharide units of [(GlcAβ(1-3)GalNAcβ(1-4)]. Three types of CS exist as CS-A, CS-B, and CS-C. Presently, the field of glycomics lacks an effective analytical method for the isomeric differentiation and relative quantification of GAGs in small (1–10 μg) biological samples. This work describes the development of a method for quantification of glycoforms using a stable isotopic labeling technique and its application to sulfated GAGs. CS samples, both standard and unknown, were partially depolymerized by chondroitinase ABC. The standard CS was then derivatized via a reductive amination reaction with 2-anthranilic acid, whereas the unknown CS

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was derivatized with 2-anthranilic-3,4,5,6-d4 acid. The derivatized CS samples were cleaned, and the excess reagent removed via a cellulose microspin column. Equimolar mixtures of the standard and unknown CS samples were made. The isotopically labeled CS mixture was subjected to size exclusion liquid chromatography in a 10% acetonitrile, 50 mM ammonium formate buffer with online electrospray ionization mass spectrometrometric detection in the negative mode. Automated tandem mass spectrometry was acquired, and quantification of unknown samples was found using relative ion abundances of the diagnostic ions. A sample of lyase-digested CSA was used as a reference against which unknown CS samples were compared. The reference was reductively aminated with d0-anthranilic acid and the unknown CS with d4-anthranilic acid. The samples were mixed and separated using SEC with online negative ESI MS/MS detection. The HPLC flow was split before the inlet, allowing 10 uL/min of flow into the mass spectrometer. Tandem MS was performed using the automated MS^n feature of the ion trap. The isolation and fragmentation windows were set to 12.0 u so that CID spectra of heavy and light forms were acquired simultaneously. Tandem MS resulted in Y ions and $[M - H - SO_3]$ ions containing the reducing end and differing by four mass units and B ions that are isobaric for heavy and light forms of AA-labeled CS oligosaccharides. The abundances of Y and $[M - H - SO_3]$ heavy and light ions were used as glycoform distribution predictors for unknown CS samples. The method is validated by acquiring automated tandem mass spectra on several isotopically labeled CS mixtures in triplicate. The mixtures were analyzed, and the percent total ion abundances of light and heavy predictive ions containing the reducing end were calculated. Light and heavy predictive ion contributions from the unknown were then put into a set of three equations. The three equations were solved for three unknowns that represent the percentage of CSA, CSB, and CSC in a mixture. The results demonstrate that tandem mass spectrometry can be used for the isotopic quantification of glycoforms of CS. NIH grants P41 RR10888 and R01 HL74197.

(109) Oxidation of Proximal Cysteine Residues Reversibly Inactivates the *Streptococcus equisimilis* Hyaluronan Synthase by Formation of Disulfide Bonds

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The hyaluronan synthase (HAS) from *Streptococcus equisimilis* (seHAS) contains four free Cys residues (at positions 226, 262, 281, and 367) that are generally conserved among mammalian HASs. HASs are normally assayed and purified in the presence of reducing agents. The activity of recombinant seHAS, assayed immediately or after weeks of frozen storage, was very low or not detectable, when the enzyme was purified in the absence of reducing agent. HAS activity, however, was rescued after treatment with a reducing agent such as b-mercaptoethanol or dithiothreitol (DTT), indicating the involvement of Cys residues in enzyme inactivation. Freshly purified inactivated enzyme was predominantly monomeric, with a small fraction of dimer, indicating that oxidative conditions during solubilization and purification did not enhance intermolecular disulfide bond formation. The ability to reduce and reactivate seHAS indicates that some Cys residue pairs may be close enough to form disulfide bonds, which inactivates the enzyme. Consistent with this hypothesis, Cys-null seHAS (which contains four Cys-to-Ala changes and retains ~20% of wild-type activity) was not inactivated in the absence of DTT. To identify the Cys residues involved in the activity changes associated with oxidation–reduction, we examined a panel of seHAS mutants in which various combinations of the four Cys residues were changed to Ala. All four single Cys mutants of seHAS were capable of being inactivated and then reactivated in the presence of DTT; they showed essentially the same behavior as wild-type enzyme. Thus, the ability of enzyme to be inactivated and then reactivated is not dependent on a particular Cys residue. The triple Cys mutants are still being examined. Four of the six possible seHAS double Cys mutants showed substantial activity after solubilization and purification and behaved essentially like wild-type seHAS: C(226,281)A, C(281,367)A, C(226,367)A, and C(262,281)A. The results indicate that multiple pairs of Cys residues can account for the inactivation of enzyme under oxidative conditions and subsequent reactivation in the presence of DTT. We conclude that a recently identified (Kumari and Weigel, 2005) spatially close cluster of Cys residues in seHAS is important for efficient catalysis and that oxidative conditions reversibly inactivate the enzyme by the formation of disulfide bonds. (This research was supported by NIH grant GM35978.)

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(110) Hyaluronan Product Size is Altered by Modification of an Intramembrane Polar Pair that is Well Conserved Within the Hyaluronan Synthase Family

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Hyaluronan synthases (HAS) have two well-conserved polar amino acids within different predicted membrane domains (MD). These residues in the *Streptococcus equisimilis* HAS (seHAS) are K48 located in MD2 and E327 located in MD4. The former Lys residue is replaced by a conserved polar Gln residue, and the latter Glu residue is conserved positionally, in eukaryotic HAS family members. To assess whether K48 and E327 might interact within the membrane region of seHAS or be required for activity, we investigated the effects of site directed mutation to change K48 to Arg, Leu, or Glu, and to change E327 to Lys, Asp, or Gln. All mutants, as well as a double-switch mutant, in which K48 and E327 were exchanged, were expressed in *Escherichia coli* SURE cell membranes. The seHAS mutants E327Q and particularly E327K were expressed at very low levels, whereas the other mutants were expressed well, or even at wild-type levels, for example, mutant E327D. The specific enzyme activities of seHAS mutants K48L, K48R, and K48E were 85, 17, and 7% of wild-type, respectively. The E327Q and E327D mutants had 26 and 38% of wild-type activity, respectively. In contrast, the activity of seHAS(E327K) was only ~0.16% relative to wild type. The very low activity of seHAS(E327K) was rescued over 46-fold by changing K48 to Glu. The expression of the double-switch seHAS(E327K,K48E) protein was also rescued to near wild-type levels. The size of HA made by these seHAS mutants also varied greatly. For example, based on SEC-MALLS analysis of membrane preparations, seHAS(E327K,K48E) did not make large weight-average molar mass HA; its HA products were only ~10% of the size made by wild-type seHAS. The results indicate that E327 within MD4 is a critical residue for the stability of seHAS, that it may interact with K48 within MD2, and that this interaction is involved in the ability of HAS to synthesize large HA. Conservation of identical or similar polar residues within the large class I HAS family suggests that interactions between MD2 and MD4 are an important general feature for HAS activity and for the synthesis of large molar mass HA. (This research was supported by NIH grant GM35978.)

(111) Molecular Mechanisms of *Drosophila glypicans* Dally and Dally-Like in Controlling Wingless Morphogen Gradient Formation

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During animal development, tissue patterning is controlled by a small group of secreted signaling molecules called morphogen. Morphogens are produced by a subset of cells in a tissue and form concentration gradients which provide positional information for cell fate specifications. *Drosophila wingless* (Wg), a founding member of the Wnt family of secreted proteins, functions as a morphogen during wing development. The molecular mechanism(s) of Wg gradient formation is not fully understood. Our laboratory has been studying the function of heparin sulfate proteoglycan (HSPG) in morphogen gradient formation. In this report, we provide essential evidence that Dally and Dally-like (Dlp), two *Drosophila* glypican members of HSPG play critical roles in regulating the Wg morphogen gradient formation. Using powerful genetic and cell biology approaches, we systematically analyzed the roles of glypicans Dally and Dlp, the Wg receptors Frizzled (Fz) and Fz2, and the Wg co-receptor Arrow (Arr) in Wg gradient formation in the wing disc. We found that both Dally and Dlp are essential and have different roles in Wg gradient formation. The specificities of Dally and Dlp in Wg gradient formation are at least partially achieved by their distinct expression patterns. To our surprise, although Fz2 was suggested to play an essential role in Wg gradient formation by ectopic expression studies, removal of Fz2 activity does not alter the extracellular Wg gradient. Interestingly, removal of both Fz and Fz2 or Arr causes enhanced extracellular Wg levels, which is mainly resulted from upregulated Dlp levels. We further show that Notum, a negative regulator of Wg signaling, down-regulates Wg signaling mainly by modifying Dally. Lastly, we demonstrate that Wg movement is impeded by cells mutant for both dally and dlp. Together, these new findings suggest that the Wg morphogen gradient in the wing disc is mainly controlled by combined actions of Dally and Dlp. We propose that Wg establishes its concentration gradient by a restricted diffusion mechanism involving Dally and Dlp in the wing disc.

(112) Experimental Evidence for All-Or-None Cooperative Interactions Between the G1-Domain of Versican and Multivalent Hyaluronan Oligosaccharides

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Cooperative interactions between extracellular matrix molecules are central to the processes of tissue assembly, morphogenesis, and repair. Here, we show how a simple polyacrylamide gel based assay, when interpreted quantitatively, can be used to investigate interactions between the G1-domain of human recombinant versican (VG1) and multivalent hyaluronan (HA) oligosaccharides. Results indicate that the oligosaccharides make a direct transition from the free state to the completely bound state (containing multiple proteins) with increasing VG1 concentration. Therefore, we hypothesize that VG1 interacts with polymeric HA in an all-or-none cooperative manner, where each VG1 molecule occupies 10 sugar residues of the HA chain. This mode of binding may contribute to spontaneous HA-protein self-assembly necessary for the formation of massive HA-proteoglycan complexes that provide mechanical stability in cartilage and other tissues.

(113) HSulf-2, an Extracellular Endoglucosamine-6-Sulfatase, is Secreted by MCF-7 Breast Carcinoma Cells and Selectively Mobilizes Heparin-Bound VEGF, FGF-1, and SDF-1

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Heparin/heparan sulfate (HS) proteoglycans are found in the extracellular matrix (ECM) and on the cell surface. A large body of evidence has established that heparin and heparan sulfate proteoglycans (HSPGs) interact with numerous protein ligands including fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), cytokines, and chemokines. These interactions are highly dependent upon the pattern of sulfation modifications within the glycosaminoglycan chains. We previously cloned a novel human endosulfatase, HSulf-2, which removes 6-O-sulfate groups on glucosamine from subregions of intact heparin. Previously, a serial analysis gene expression (SAGE) analysis from public databases showed that the level of HSulf-2 transcripts in human breast cancer tissue was significantly up-regulated as compared with that in normal breast tissue. We have found that HSulf-2 mRNA was highly expressed in MCF-7, a human breast cancer cell line, and that the protein was secreted by the cell line in an enzymatically active form into the conditioned medium. In this study, we have employed both recombinant HSulf-2 and the native enzyme from conditioned medium of the MCF-7 cell line. To determine whether HSulf-2 modulates the interactions between heparin-binding factors and heparin, we developed ELISAs, in which soluble factors were allowed to bind to immobilized heparin. Our results show that the binding of VEGF, FGF-1, and SDF-1/CXCL12 to immobilized heparin was abolished or greatly diminished by pretreating the heparin with HSulf-2. Furthermore, HSulf-2 released these soluble proteins from preassociation with heparin. Native Sulf-2 from MCF-7 cells reproduced all of these activities. Our results validate Sulf-2 as a new tool for deciphering the sulfation requirements in the interaction of protein ligands with heparin/HSPGs and expand the range of potential biological activities of this enzyme. The ability of Sulf-2 to mobilize ECM-bound factors could be an important mechanism by which tumor cells modify their microenvironment to facilitate tumor angiogenesis or their own growth.

(114) Functional Involvement of Keratan Sulfate Carbohydrate for Corneal ECM Organization

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Keratan sulfate (KS) proteoglycans are largely found in cornea and cartilage and are suggested to have biological function for the maintenance of corneal transparency. Previously, we identified *CHST6* as a gene encoding corneal GlcNAc 6-*O*-sulfotransferase (human CGn6ST, also called as GlcNAcT-5 and GST-4beta) for KS production and found that mutations on *CHST6* result in macular corneal dystrophy (MCD), a hereditary disease in which the patients develop clouding of the cornea due to abnormal carbohydrate deposits. In mouse, *Chst5* is the authologue of *CHST6* in human and encodes a GlcNAc 6-*O*-sulfotransferase (mouse IGn6ST, also called as GlcNAcT-3 and GST4) that also has activity for KS sulfation. Here, we generated a *Chst5*-null mouse strain that lacks mIGn6ST activity and determined whether the mutant mice exhibit morphological and biological defects comparable with MCD in human. Highly sulfated KS, which is detected by 5D4 antibody, is not detected in the cornea of *Chst5*-null mice, indicating that mIGn6ST is one of the responsible enzymes for corneal KS production. Unlike the typical phenotype found in MCD patients, *Chst5*-null mice did not show obvious opacity in their corneas even at 1-year-old. By X-ray fibre diffraction analysis, we found altered collagen fibril organization in the corneal extracellular matrix of *Chst5*-null mice. Collagen fibrils in *Chst5*-null corneas are more widely spaced and more disorganized than those in wild type. Some of these features are also reported in MCD corneas suggesting that *Chst5*-null mouse can serve an animal model for this disease. These results obtained by *Chst5*-null mice demonstrate the important role of KS carbohydrate in the maintenance of corneal extracellular matrix structure. Supported by grant NIH EY014620.

Session Topic: Evolution of Glycans and Glycan Function

(115) *N*-Acetylglucosaminyltransferase I-Null *Drosophila melanogaster* is Unable to Compete for Survival in the Presence of Wild-Type Flies

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UDP-GlcNAc : α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GnT I) controls the synthesis of hybrid, complex, and paucimannose *N*-glycans. *Drosophila melanogaster* makes paucimannose but little or no hybrid nor complex *N*-glycans. The single GnT I gene in flies has been cloned and expressed (Sarkar and Schachter, 2001). GnT I-null *D. melanogaster* lines were obtained by imprecise excision of a P-element located 546-bp upstream of the start codon; a 1301-bp deletion was produced downstream of the P-element. No flanking genes were disrupted. GnT I –/– adults were recovered only when animals were removed from the vial at the larval stage and allowed to develop together with a limited number of mutant larvae. Mutant embryos enclosed normally but had a significantly reduced life span (98% dead within 15 days; 80 days for heterozygotes). GnT I –/– adults are viable with a normal external morphology. Locomotor activity (open grid method) showed that –/– flies are significantly more sluggish than wild-type flies. No eggs were obtained on attempts to mate mutant males and females. Extracts of GnT I –/– flies showed no GnT I activity. Mass spectrometric analysis of these extracts showed dramatic changes in *N*-glycans compatible with GnT I lack. The data indicate that GnT I-dependent *N*-glycans are required for normal development of the nervous system of the fly. Support by the Canadian Institutes of Health Research (CIHR) and the Canadian Protein Engineering Network Centre of Excellence (PENCE).

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(116) Purification and Characterization of *Helicobacter pylori* α1,3/4Fucosyltransferases

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The minimal catalytic domain of fucosyltransferases (FucT) from *Helicobacter pylori* strain NCTC11639 and UA948 was mapped by N-terminal and C-terminal truncations. The C-terminus but not the N-terminus can be truncated without significant activity loss. Deletion of the heptad repeats, which connect the catalytic domain with the C-terminal putative amphipathic α-helices, almost completely abolished enzyme activity. Strikingly, addition of only one heptad repeat fully reactivates 11639FucT, whereas UA948FucT regains partial activity. Removal of the two putative amphipathic α-helices dramatically increased protein expression so these constructs with a C-terminal His_6-tag were purified at milligrams per liter yield. Steady-state kinetic analysis of the purified FucTs showed that 11639FucT possessed slightly tighter binding affinity to both Type II acceptor and GDP-fucose donor than UA948FucT, and its K_{cat} of 2.3 s^{-1} was double that of UA948FucT, which had a K_{cat} value of 1.1 s^{-1} for both Type II and Type I acceptors. UA948FucT strongly favors Type II over Type I

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acceptor with a 20-fold difference in acceptor K_m. Sixteen modified Type I- and Type II-series acceptors were employed to map the molecular determinants of acceptors required for recognition by *H. pylori* α1,3/4 FucTs. Deoxygenation at 6-C of the galactose in Type II acceptor caused 5000-fold decrease in α1,3 activity, whereas in Type I acceptor it completely abolished α1,4 activity, indicating that this hydroxyl group is a key polar group. Less dramatic but significant effects were observed by modifying the polar groups at C-4, C-6 of galactose, and the *N*-acetamido group of GlcNAc. Our results support a shared structural mechanistic basis for mammalian and *H. pylori* α1,3/4 FucTs.

(117) Acceptor Protein Requirements for Recombinant N-Glycosylation in *Escherichia coli*

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The N-glycosylation system of *Campylobacter jejuni* is encoded by the so-called *pgl* cluster. Its expression in *Escherichia coli* reconstitutes the N-glycosylation machinery *in vivo*. In this system, we analyze the structural elements of the *C. jejuni* N-glycoprotein AcrA required for attachment of an *N*-glycan. By deleting the polypeptide sequences flanking the native glycosylation site asparagine N123 in AcrA, we find that, compared with eukaryotic primary consensus sequence for N-glycosylation, asparagine-X-serine/threonine (where X cannot be proline), the bacterial consensus sequence is N-terminally extended to form aspartate/glutamate-X-asparagine-Y-serine/threonine (where Y and X cannot be proline). *N*-Glycan addition was prevented when the Y residue was mutated to proline indicating a conformational requirement for recognition by the bacterial oligosaccharyltransferase PglB. The polypeptide sequences next to the consensus site were shown not to be significant for glycosylation of asparagine N123 in AcrA. Interestingly, not all consensus sequons were used when they were either artificially introduced or present in non-*C. jejuni* proteins. This indicates an additional unknown requirement for glycosylation as it has been observed in eukaryotes. However, introduction of bacterial consensus into AcrA and cholera toxin B subunit produced recombinant glycoproteins with one or more engineered N-glycosylation sites. Our data show that bacterial N-glycosylation is a homologous process to eukaryotic N-glycosylation with a more stringent protein acceptor sequence requirement. The presented methodology permits the production of tailor-made, recombinant N-glycoproteins in *E. coli*.

(118) Galectins Bind to the Multivalent Glycoprotein Asialofetuin with Enhanced Affinities and a Gradient of Decreasing Binding Constants

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Our previous isothermal titration microcalorimetry (ITC) studies of the binding of synthetic multivalent carbohydrates to the Man/Glc-specific lectins concanavalin A (ConA) and Dioclea grandiflora lectin (DGL) showed negative binding cooperativity that was due to the carbohydrate ligands and not the proteins (Dam *et al*., 2002). The negative cooperativity was associated with the decreasing functional valence of the carbohydrates upon progressive binding of their epitopes. This study also shows negative cooperativity in the ITC binding data of asialofetuin (ASF), a glycoprotein that possesses nine LacNAc epitopes, to galectins-1, -2, -3, -4, -5, and -7, and truncated, monomer versions of galectins-3 and -5, which are members of a family of animal lectins. Although the observed K_a values for ASF binding to the galectins and two truncated forms are only 50- to 80-fold greater than that of LacNAc, analysis of the data in terms of the relationship between the observed macroscopic free energy of binding and the decreasing microscopic free energies of binding of the epitopes shows that the first LacNAc epitope of ASF binds with ~6000-fold higher affinity than the last epitope. Thus, the microscopic binding constants of the galectins to the first epitope(s) of ASF are in the nM range, with a gradient of decreasing binding constants of the remaining epitopes. The results indicate that the above galectins bind with fractional, high affinities to multivalent glycoproteins, such as ASF, independent of the quaternary structures of the galectins. These findings have important implications for the binding of galectins to multivalent carbohydrate receptors.

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(119) Role of the Low-Affinity Mannose 6-Phosphate Binding Site in the Cation-Independent Mannose 6-Phosphate Receptor

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The 300-kDa cation-independent mannose 6-phosphate receptor (CI-MPR) directs soluble acid hydrolases to lysosomes. These newly synthesized lysosomal enzymes are marked with mannose 6-phosphate (M6P) residues to enable binding to the CI-MPR. The extracytoplasmic region of the CI-MPR consists of 15 homologous domains. Domains 1–3 and 9 contain high-affinity M6P binding sites with residues critical for binding located in domains 3 and 9. A structure-based sequence alignment revealed similarities between domains 3, 9, and 5 which prompted investigation of domain 5 as a possible third M6P-binding site. In our recent studies, (Reddy *et al*., 2004) a construct encoding domain 5 alone (Dom5His) was expressed in Pichia pastoris and was shown to bind the lysosomal enzyme, β-glucuronidase with a K_d of 54 μM. In contrast, constructs encoding domains 1–3 or 9 bind β-glucuronidase with an affinity of ~1 nM. The CI-MPR's ability to bind a diverse population of M6P-containing proteins is likely facilitated by having three M6P binding sites (domains 1–3, 5, and 9), each with different binding characteristics and in different proximity to each other. The role of domain 5s low affinity, M6P binding site with respect to lysosomal enzyme trafficking will be further evaluated by expressing the full-length CI-MPR containing a nonfunctional domain 5. Amino acid residues known to be crucial for M6P binding in domains 3 and 9 were identified in analogous locations in domain 5. These conserved residues were mutated, and the resulting domain 5 constructs containing single amino acid substitutions were analyzed with surface plasmon resonance. M6P binding was diminished to a K_d of 480 μM for E709Q, 300 μM for E709D, and >500 μM for the Q644E mutant. No detectable M6P binding was observed by either the R687K or the Y714F mutant domain 5 constructs. Full-length CI-MPR constructs containing point mutations in domain 5 (R687K or Y714F) will be expressed in mouse fibroblast cells lacking endogenous CI-MPR. Characterization of lysosomal enzyme trafficking in these cell lines will be presented. (Supported by NIH grant DK 42667.)

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(120) Characterization of Siglec-13, a Cell Surface Molecule Specifically Deleted in Humans

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Siglecs are sialic acid recognizing lectins belonging to the Ig superfamily. These proteins have an extracellular domain that includes the sialic acid-binding region, a transmembrane domain and an intracellular domain that can include signaling motifs. The CD33-related Siglecs are a rapidly evolving subfamily of Siglecs whose genes are found mostly clustered together in a single chromosomal region. Our recent multispecies comparative study of this region (Angata *et al*., 2004) showed multiple differences between humans and great apes, of which the most striking was the complete absence in the human genome of a putative new primate Siglec named Siglec-13. Chimpanzees and other great apes are the most evolutionarily close species to humans. Despite this, they are phenotypically and biomedically different from us in many aspects. Consequently, a study of such genetic differences could lead to a better understanding of the human condition. Comparison of the genomic sequences surrounding Siglec-13 in the chimpanzee genome and the corresponding region of the human genome indicates that an *Alu:Alu* recombination event apparently lead to the deletion of this sequence in humans. The extracellular domain of Siglec-13

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was expressed as soluble fusion protein with the Fc region of human IgG. This molecule also showed binding to sialic acids in ELISA-type assays. The Siglec-13 extracellular domain was cleaved from this fusion protein and used to raise rabbit polyclonal antibodies. Serial adsorption of the antiserum by human IgG and human Siglec-9 was needed to ensure that it was specific for Siglec-13, showing no crossreactivity to any other Siglecs tested. Using this antibody in flow cytometry analyses, Siglec-13 was found to be present on chimpanzee peripheral blood leukocytes. Tissue immunohistochemistry showed the prominent expression of Siglec-13 along the apical border of colonic mucosal epithelial cells from multiple chimpanzees, whereas the same region did not show staining in human samples. Given the ability of CD33rSiglecs to induce apoptosis and/or inhibition of cell proliferation, this observation could be relevant to the fact that colon cancer is very common in humans but has never so far been reported in great apes. Further studies of the binding preferences and tissue distribution of Siglec-13 are underway.

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(121) Human Salivary Mucins: MG2 (MUC7) Glycosylation is Consistent, Whereas MG1 (MUC5B) Glycosylation Varies Extensively Between Healthy Individuals

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The study aimed to characterize the natural glycosylation variation of the high molecular-weight glycoprotein fraction MG1 which mainly consists of MUC5B (Thornton *et al*., 1999) and the low molecular-weight glycoprotein fraction MG2 (MUC7) (Bobek *et al*., 1993). Mucins from 30 individuals were isolated by composite gel electrophoresis of 50 uL reduced and alkylated saliva, blotted to PVDF membranes, and visualized with Alcian Blue. O-Linked oligosaccharides were released from mucin bands by reductive O-elimination and analyzed with liquid chromatography ion trap mass spectrometry. MUC7 appeared to carry a consistent glycosylation with predominantly sialylated oligosaccharides and with a mass spectrometric profile similar to previously published results (Schulz *et al*., 2002). In contrast, MUC5B glycosylation varied extensively between individuals, with regard to terminal epitopes and the relative distribution of neutral and charged glycoforms. Slow electrophoretically migrating MUC5B components were found to be dominated by neutral oligosaccharides and fast migrating components by sulfated oligosaccharides. ABO histoblood group specific sequences were frequently expressed on 22 individuals. These individuals were assigned as "secretors." Eight remaining individuals lacked blood group sequences and were assigned as "nonsecretors." The nonsecretors were characterized by a high degree of sialylation. Western blot assays with antibodies confirmed increased expression of Si-Lea on MUC5B from the nonsecretors. One nonsecretor lacked fucosylated epitopes on both MUC7 and MUC5B. Genotyping confirmed this individual as a nonsecretor and Lewis negative. Our results highlight that the two salivary mucin glycoprotein fractions are very different with regard to the natural variation among healthy individuals.

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(122) N-Linked Glycan Diversity in the *Drosophila* Embryo

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Developmental phenotypes arise in the embryos of many animal species as a result of mutations in genes that normally contribute to N-linked glycan synthesis or processing. We have undertaken a systematic and complete characterization of the diversity of N-linked glycans expressed in wild-type *Drosophila* embryos to provide an essential baseline for interpreting mutational effects. Our optimized method routinely achieves high glycan recovery (>95%) and is applicable to small amounts of starting material (200–300 mg wet weight equivalent of embryos). Total protein extracts are delipidated and subjected to digestion with trypsin/chymotrypsin before enzymatic glycan release (PNGaseF or PNGaseA). Released glycans are fluorescently tagged with 2-aminopyridine and fractionated into acidic and neutral pools by UNO Q-1 anion exchange chromatography. Further fractionation by TSK-amide chromatography provides preliminary structural information based on retention relative to standards. Subsequent analyses of the glycans within each TSK-amide fraction by MALDI-TOF/MS and by ESI/MS^n (linear ion trap) demonstrate that the *Drosophila* embryo is capable of synthesizing a broad range of glycan structures. Although total glycan profiles are dominated by high-mannose structures, minor species are detected that indicate the capacity of the embryo to synthesize both hybrid and complex glycans. Furthermore, core fucosylation is detected at the 3-position alone, or at the 6-position alone, or simultaneously at both positions of reducing terminal GlcNAc residues. The predominance of high-mannose structures relative to the low abundance of hybrid/complex oligosaccharides indicates that glycosylation in the *Drosophila* embryo can be regulated largely by limiting flux through terminal processing pathways. Characterization of glycan profiles in various mutant backgrounds will be essential to delineate the control points that determine specific glycan expression. Supported by funding from NIH/NIGMS 1R01GM07283901.

(123) Purification, Characterization, and Cloning of a New *Spodoptera frugiperda* Sf9 β-*N*-Acetylhexosaminidase that Hydrolyzes Terminal *N*-Acetylglucosamine on *N*-Glycan Core

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N-Glycans produced in insects or insect cells mostly are of the oligomannosidic or the paucimannosidic type, in contrast to the more complex *N*-glycans in mammals (Tomiya *et al*., 2004). The generation of paucimannosidic *N*-glycans in insects has been attributed to the presence of a β-*N*-acetylhexosaminidase activity that removes β(1,2)-linked terminal *N*-acetylglucosamine residues on the *N*-glycan core. We report the purification of a β-*N*-acetylhexosaminidase (Sfhex) from the culture medium of *Spodoptera frugiperda* Sf9 cells. The purified Sfhex showed 10 times higher activity toward a terminal *N*-acetylglucosamine on *N*-glycan core than tri-*N*-acetylchitotriose. Sfhex appeared to be a homodimer of 110 kDa, with a pH optimum of 5.5. With a biantennary *N*-glycan substrate terminated with *N*-acetylglucosamine residues, it exhibited a 5-fold preference for removal of the β(1,2)-linked *N*-acetylglucosamine from the Manf_i(1,3) branch than the Manf_i(1,6)-branch. In contrast to the β-*N*-acetylhexosaminidase activity in homogenate of insect cells (Altmann *et al*., 1995), Sfhex showed activity toward β(1,2)-linked *N*-acetylglucosamine on the Manf_i(1,6)-branch on the *N*-glycan core and on the Manf_i(1,3)-branch in $GlcNAcMan_5GlcNAc_2$. We isolated two corresponding cDNA clones for Sfhex that encode proteins with >99% amino acid identity. A recombinant Sfhex expressed in Sf9 cells exhibited the same substrate specificity and pH optimum as those with the purified enzyme. Phylogenetic analysis suggested that Sfhex is similar to mammalian β-*N*-acetylhexosaminidase, but unlike previously cloned enzymes from other lepidopteran insects.

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(124) Endothelial *O*-Glycans are Essential for Vascular Development

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Serine- or threonine-linked mucin-type *O*-glycans are commonly found on membrane and secreted proteins, yet their functions are not well understood.

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O-Glyans have four main core structures. Among them, core 1 and 2 *O*-glycans are expressed in many tissues. Core 1 is a precursor for core 2 and for many extended *O*-glycans and is formed by the enzyme core 1 beta 1,3-galactosyltransferase (T-synthase). We engineered mice that are globally deficient for T-synthase (T-syn–/–). The T-syn–/– mice developed brain hemorrhage that was uniformly fatal by embryonic day 14. The T-syn–/– brains formed a disorganized microvascular network with distended endothelial cells and defective association of endothelial cells with pericytes, extracellular matrix, and neural tissues. These data revealed a novel requirement for core 1-derived *O*-glycans (*O*-glycans) during vascular development. To identify cell types requiring *O*-glycans for vascular development, we developed mice with the T-syn gene flanked by loxP sites (T-synflox/flox). To examine the contribution of endothelial *O*-glycans to vascular development, we made mice lacking T-synthase specifically in endothelial cells by breeding T-synflox/flox mice with Tie2Cre transgenic mice that mediate endothelial-specific deletion (EC T-syn–/–). In addition, we generated transgenic mice that express T-synthase specifically in endothelial cells under control of the Tie2 promoter (T-syn Tg) to test whether the breeding of T-syn Tg with T-syn–/– mice would rescue its defective vasculature. To examine the roles of *O*-glycans in extracellular matrix and neural tissues, we generated mice lacking T-synthase specifically in these tissues (Neu T-syn–/–) by breeding T-synflox/flox mice with NestinCre transgenic mice in which the expression of Cre is controlled by Nestin, a neural specific promoter. The EC T-syn–/– mice exhibited no embryonic lethality but had growth retardation and a high neonatal mortality rate (over 90%). Almost all the mice that died had signs of gastrointestinal (GI) bleeding. Gross morphology and imaging analyses revealed that the mice developed chaotic macro and microvasculatures in different organs, especially in the GI tissues, which resembled the abnormality in the T-syn–/– brain. Breeding of T-syn Tg mice with T-syn–/– mice completely rescued the embryonic lethality of T-syn–/– mice. Furthermore, the rescued mice exhibited normal vascular development. Analyses of the Neu T-syn–/– mice revealed no bleeding phenotype, and the Neu T-syn–/– brains developed normally but exhibited a modest abnormal microvascular network in comparison with wild-type littermates. Collectively, these data indicate that endothelial *O*-glycans are critical in vascular development, whereas the *O*-glycans of neural cells and extracellular matrix have a modest contribution. To characterize how lack of *O*-glycans in endothelial cells causes vascular abnormality, we have developed endothelial cell lines from the wild-type and T-syn–/– mice. Preliminary analysis showed that the T-syn–/– endothelial cells formed abnormal tubular structures on Matrigel, which is consistent with the *in vivo* data.

(125) CMP-Sialic Acid Transporter Trafficking and Golgi Localization

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CMP-sialic acid reaches the lumen of the Golgi apparatus through the CMP-sialic acid Transporter (CST) and is used for the sialylation of proteins and lipids. Although the membrane topology and substrate recognition regions have been determined for CST, the localization of CST in the Golgi and the signals and mechanisms mediating its localization remain elusive. We predict that CST is localized all across the Golgi stack because the sialyltransferases involved in glycoprotein sialylation are found in the late Golgi, whereas some involved in glycolipid sialylation are found in the early Golgi. To test this prediction, we used immunofluorescence microscopy and compared the localization of the CST with other Golgi subcompartment markers. The CST partially overlapped with Erv46 (IC/cis Golgi), GM130 (cis, medial Golgi), and TGN46 (trans Golgi network), suggesting its presence throughout the Golgi stack. To evaluate the role of the N- and C-terminal cytosolic tails of the CST in its trafficking and localization, we deleted the N- and C-terminal tails and evaluated the localization of the mutant proteins (ΔN-CST, ΔC-CST). We found that ΔC-CST was retained in the endoplasmic reticulum (ER), whereas ΔN-CST was found in the Golgi. This suggested that the C-terminal tail sequences of CST are involved in its ER export. Further studies revealed a di-Ile motif and a Val motif found at the very C-terminus were required for CST ER export. Either motif alone was sufficient to mediate CST ER export. In summary, this work has demonstrated that the CST is localized throughout the Golgi to provide CMP-sialic acid to sialyltransferases localized throughout this compartment and that specific motifs in the C-terminal tail of the CST mediate its ER export.

(126) Distinct Carbohydrate-Binding Properties of *Cymbosema roseum* Lectins

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Two lectins, designated as CRL I and CRL II, have been isolated from the legume species of *Cymbosema roseum* of *Diocleinae* subtribe. CRL I, like other Diocleinae lectins, is a Man/Glc-specific lectin that binds with high affinity to the "core" trimannoside of N-linked oligosaccharides. Thermodynamic data obtained with a complete set of monodeoxy analogs of the core trimannoside indicate that CRL I recognizes the 3-, 4-, and 6-hydroxyl groups of the α(1,6) Man residue, the 3- and 4-hydroxyl group of the α(1,3) Man residue, and the 2- and 4-hydroxyl groups of the central Man residue of the trimannoside. Binding thermodynamics of the tetradeoxy analog (lacking the 3-and 4-hydroxyl group of the α(1,3) Man residue and the 2- and 4-hydroxyl groups of the central Man residue of the trimannoside) are consistent with the involvement of these hydroxyl groups in binding. Other Diocleinae lectins also recognize the same set of hydroxyl groups of trimannoside; however, subtle differences exist in the thermodynamics of binding of hydroxyl groups. CRL I possesses enhanced affinities for the Man5 oligomannose carbohydrate and a biantennary complex carbohydrate. CRL II, on the other hand, is a blood group H antigen-specific lectin. Thermodynamic binding studies reveal that the binding site of CRL II is complementary for blood group H Type II trisaccharide. 2′fucosyllactose is a slightly weaker ligand compared with H Type II trisaccharide; however, addition of a second fucose residue as in lactodifucotetraose further impairs the binding affinity. Blood group (O)H Type I determinant shows considerably lower affinity compared with H Type II antigen. A second fucosylation on the Type I determinant drastically reduces its association constant. CRL II binds very weakly to D-galactose, D-fucose, LacNAc, and blood group H disaccharide. This thermodynamic binding study shows that lectins synthesized in the same tissue can have discrete carbohydrate specificities. The results also indicate that other species of Diocleinae subtribe, beside the Man/Glc binding lectin, may possess a second lectin that is specific for blood group H Type II antigen.

(127) N-Glycosylation of Proteins by the *pgl* System of *Campylobacter jejuni* Requires an Extended Sequon, Asp/Glu-Xaa-*Asn*-Xaa-Ser/Thr

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The Gram-negative bacterium *Campylobacter jejuni* was the first bacterium shown to *N*-glycosylate proteins in the manner of eukaryotes. The enzymes of its *pgl* locus synthesize a heptasaccharide, GalNAc-(1,4-GalNAc-(1,4-(Glc-(1,3)-GalNAc-(1,4-GalNAc-(1,4-GalNAc-(1,3-Bac, where Bac is 2,4-diacetamido-2,4,6-trideoxy-D-Glc (Young *et al*., 2002). It is then attached by the oligosaccharyltransferase PglB to Asn residues that have Ser or Thr residues at the +2 position, as occurs in the sequon utilized in eukaryotic glycoprotein biosynthesis, Asn-Xaa-Ser/Thr. The *pgl* locus was successfully introduced into *Escherichia coli* (Wacker *et al*., 2002) and shown to be functionally active, N-glycosylating two coexpressed Campylobacter proteins, AcrA and Peb3. These findings raised the possibility of synthesizing glycoproteins in recombinant *E. coli* (Feldman *et al*., 2005). However, the intrinsic *E. coli* proteins were apparently not being modified by the *pgl* system, demonstrating that there were additional factors required beyond the presence of the Asn sequon (Nita-Lazar *et al*., 2005). Previously, we reported the sequences of six glycopeptides from periplasmic glycoproteins of *C. jejuni*, whereas ~20 more glycoproteins were identified from two-dimensional gels of the products from lectin affinity chromatography of soluble protein extracts (Young *et al*., 2002). The majority of these proteins were annotated as periplasmic ones. Mass spectrometry of additional glycoproteins from membrane fractions led to sequence data for several more glycopeptides, and the N-glycosylation sites in two other proteins have been reported, from site-directed mutagenesis experiments. Alignment of all these glycopeptide sequences around the modified Asn residues disclosed that, in addition to the requirement for Ser or Thr at the +2 position, the oligosaccharyltransferase PglB also requires an Asp or Glu at the –2 position. In other respects, the residue preferences in the local sequences were similar to those found around sequons in eukaryotic glycoproteins. The extended sequon occurs in many of the other putative glycoproteins identified in the previous two-dimensional gel experiment. The requirement for a longer sequon with an acidic residue at the –2 position, D/E-X-N-X-S/T, is clearly one factor that strongly influences the behavior of the *pgl* system in *C. jejuni* and in recombinant *E. coli*.

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(128) Aberrant Protein N-Glycosylation in *Caenorhabditis elegans* GnT-I Triple Knockout Worms

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Postgenomics research on the biology of *Caenorhabditis elegans* is focused on dynamic aspects of the worms' life cycle including proteomics and posttranslational modifications such as glycosylation. Here we report significant differences among *N*-glycan structures obtained from wild type (WT) and GnT-I triple knockout (TKO) animals. Protein *N*-glycans obtained from WT *C. elegans* include unusual structures with a branched reducing end GlcNAc substituted with one or two fucoses as well as one or two galactoses. Multiple structural isomers are observed within all MS peaks corresponding to $GlcNAc_2Hex_{4\text{-}8}Fuc_{2\text{-}4}$ that dominate the WT glycan mass spectrum (MS). In contrast to WT animals, high-mannose glycans dominate the *N*-glycan MS profiles from TKO worms. Abundances of the most highly core-substituted glycans are significantly reduced in TKO worms and are observed in conjunction with differential overall fucosylation patterns. The presence of distinct sets of fucosylated *N*-glycan isomers among glycans in WT and TKO worms is made apparent following facile cleavage between the core GlcNAcs in MS^2. Further molecular disassembly of nonreducing end B-type ions and reducing end Y-ions by sequential mass spectrometry (MS^n) leads to structural insights and an ability to define the topologies of multiple isomeric structures. Using this approach, we have developed new bioinformatics tools, including two algorithms (OSCAR, Isosolve) for reconstructing *N*-glycan topologies using MS^n fragmentation pathway data. Results from these analyses provide considerable insight into *N*-glycan biosynthesis in *C. elegans*. It is apparent that *N*-glycan biosynthesis in *C. elegans* is highly complex, and all the structures, enzymes, and pathways are not yet not well defined. However, this simple model organism is an excellent system to use for achieving an improved understanding of *N*-glycan biosynthesis and the effects of gene mutations and perturbations. New tools and approaches for glycan structural analysis may also emerge through such studies.

(129) Analysis of the Oligomeric State(s) of Mouse Lunatic Fringe

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The Notch receptor is anchored to the membrane with a single transmembrane pass, as are its ligands Delta and Serrate/Jagged. Thus, neighboring cells utilize Notch signaling in various capacities, such as boundary formation in development, or lateral inhibition in neuron formation or cell specification. Notch knockouts are embryonic lethal, and Notch signaling has been implicated in numerous human disease states. The Notch receptor (four exist in mammals) is modified by O-linked carbohydrates in the extracellular domain, namely O-fucose and, in some contexts, subsequently with *N*-acetylglucosamine (GlcNAc). These sugar modifications can modulate Notch signaling, presumably by affecting the interaction of Notch with its ligands. Knockouts of GDP-fucose protein *O*-fucosyltransferase 1 (*O*-FucT-1), the enzyme responsible for the addition of *O*-fucose to Notch, resemble the embryonic lethal Notch knockouts. The *O*-FucT-1 knockout exhibits a stronger phenotype than any single Notch gene knockout, strongly suggesting that the *O*-fucose modification is absolutely necessary for proper Notch function. The addition of GlcNAc to *O*-fucose on Notch is mediated by Fringe enzymes. There are three such enzymes in mammals termed Lunatic (Lfng), Radical (Rfng), and Manic (Mfng). Knockouts of Lfng show dramatic segmentation defects. Rfng knockouts have no known phenotype, and Mfng knockouts have not yet been reported. Thus, all four of these glycosyltransferases are potentially important players in human disease. We have initiated structural studies on these enzymes with a long-term goal of developing inhibitors that may be useful for modulation of Notch activity. During these studies, we have observed that Lfng forms oligomers. Lfng has seven cysteines, and we have recently observed that in the absence of reducing agents, Lfng runs as a ladder of oligomers on SDS–PAGE, presumably due to one or more unpaired cysteine residues. Lfng is able to form disulfide linkages both with itself and with other proteins, as has recently been reported in the case of Notch3 mutations that cause cerebral autosomal dominant arteriopathy with sub-cortical infarcts and leukoencepholopathy (CADASIL) (Arboleda-Velasquez *et al.*, 2005). CADASIL is caused by mutations in the EGF-like repeats of Notch3 which result in the gain or loss of a cysteine residue, resulting in an unpaired cysteine. Lfng can form a disulfide-linked dimer with fragments of Notch3 bearing such mutations. Thus, we are characterizing the oligomeric state of Lfng and its propensity to form disulfide linkages using reducing agents, iodo-acetamide, dynamic light scattering, gel filtration chromatography, and an *in vitro* enzyme activity assay. This work was supported by NIH grant GM61126.

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(130) Expression of the UDP-GalNAc : Polypeptide *N*-Acetylgalactosaminyltransferase Family is Spatially and Temporally Regulated During *Drosophila* Development

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The UDP-GalNAc : polypeptide *N*-acetylgalactosaminyltransferase enzyme family is responsible for the first committed step in the synthesis of mucin-type *O*-glycans on protein substrates. Previous work from our group has demonstrated both sequence and functional conservation between members of this family in mammals and the fruit fly, *Drosophila melanogaster*. One member (pgant35A) of this family in *Drosophila* has been shown to be essential for viability and development. In an effort to understand the developmental stages and processes in which this enzyme family is involved, we have determined the expression pattern of nine functional family members and three putative isoforms during *Drosophila* embryonic and larval development. Our studies indicate that each isoform is expressed in discrete spatial and temporal fashions during development, with some isoforms being found uniquely in restricted areas of the developing embryo (brain, trachea, pharynx, esophagus, proventriculus), whereas others are found in multiple embryonic regions and overlap with the expression of other isoforms (salivary glands, posterior midgut, anterior midgut, and the fore-/hindgut). As development proceeds, most isoforms are expressed in the third instar larval imaginal discs, implicating this enzyme family in the development of the adult structures as well. Thus, these results provide insight into the specific regions in the developing embryo and larvae that may require O-linked glycosylation *in vivo* as well as which isoforms may act cooperatively in certain tissues and which may be uniquely responsible for glycosylation in others. This data will aid us in deciphering the phenotypes unique to the pgant35A mutants, which die during embryogenesis in the absence of wild-type pgant35A RNA.

(131) Laminin 5, Netrin-4, and Lumican Have Potential to Serve as Counterreceptors of Galectin-3

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We have previously shown that (1) a carbohydrate-binding protein, galectin-3 is expressed in corneal epithelium, (2) re-epithelialization of corneal wounds is significantly slower in galectin-3 deficient mice compared with the wild-type mice, and (3) the exogenous addition of galectin-3 stimulates re-epithelialization of corneal wounds in a mouse animal model (Cao *et al.*, 2002). Cell–matrix interactions play a key role in re-epithelialization of corneal wounds, and it is well established that galectin-3 contains binding sites for some ECM molecules such as laminin 1. Laminin 5 (3β3 2), netrin, and lumican are among ECM molecules known to be present in corneal epithelial basement membrane and play a role in re-epithelialization of corneal wounds or epithelial cell migration. Because these ECM molecules are glycosylated, it is logical to hypothesize that they may serve as counterreceptors of galectin-3 and that galectin-3 may influence

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re-epithelialization of corneal wounds by binding to and modulating the function of one or more of these counterreceptors. Therefore, the goal of this study was to determine whether galectin-3 binds to laminin 5, netrin-4, or lumican. Dot blots and/or electrophoresis blots of purified laminin 5, heterologously expressed netrin-4, a fragment of the laminin 2 chain, and affinity-purified lumican from amniotic membranes were probed with peroxidase-conjugated galectin-3 in the presence and absence of a competing disaccharide, β-lactose. The blots were developed by a chemiluminescence detection system (PerkinElmer, Life Sciences). Galectin-3 bound to laminin 5, 2 chain of laminin 5, netrin-4, and lumican. The binding was abolished by a competing disaccharide, β-lactose, but not by a noncompeting disaccharide, sucrose. Also, as expected, galectin-3 did not bind to bovine serum albumin, which was used as a negative control. Galectin-3 may influence re-epithelialization of corneal wounds by modulating the function of key ECM molecules (laminin 5, netrin-4, and/or lumican) known to play a role in cell migration.

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(132) O-Fucosylation of Thrombospondin Type I Repeats: Analysis of Mouse *O*-FucT-2

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The thrombospondin Type I repeats (TSRs) of human thrombospondin 1 (TSP 1), human properdin and rat F-spondin have been shown to bear the disaccharide Glc-Fuc-*O*-S/T. This modification is distinct from and occurs through a different pathway than the GlcNAc-Fuc-*O*-S/T modification of epidermal growth factor-like (EGF) repeats mediated by protein *O*-fucosyltransferase 1 (*O*-FucT-1) and fringe. We have recently demonstrated that *O*-FucT-1 does not add *O*-fucose to TSRs, but a homologue, *O*-FucT-2, does. Members of the TSR superfamily, all containing one or more potential O-fucosylation sites, are secreted or transmembrane proteins playing roles in such diverse biological processes as guidance of neuronal growth, regulation of wound healing and angiogenesis, and processing other ECM proteins into their mature form. Many of those functions, most notably the anti-angiogenic/anti-cancer activity of human TSP 1/2, map to the TSR domains. To begin studies on the mouse enzyme, we cloned the mouse *O*-FucT-2 and showed that it can fucosylate TSR repeats when overexpressed in NIH3T3 or COS-1 cells. Mutation of predicted catalytic residues gave inactive enzyme with a slight dominant negative character. Preliminary data suggest that the enzyme localizes to the ER. By analogy to OFT1, with which OFT2 shares 58% sequence similarity and the requirement for properly folded substrates, we predict this enzyme may play a role in quality control of TSR folding. We are currently testing several members of the TSR superfamily as substrates of *O*-FucT-2 activity, as well as the effect of the modification on their rate of secretion. In addition, we have shown that mouse embryonic stem (ES) cells in which one copy of the *O*-FucT-2 gene has been disrupted show 50% reduction in enzyme activity. We have used these ES cells to generate chimeric mice and achieved successful germline transmission of the mutation. Intercrosses of heterozygotes are underway. This work was supported by NIH grant GM61126.

(133) Glycosyltransferases Involved in Type 1 Chain and Lewis Antigen Biosynthesis Exhibit Glycan and Core Chain Specificity

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Lewis A (Le^a) and Lewis B (Le^b) are two important epitopes, which have been studied in many different biological contexts, such as in transfusion medicine, cellular and microbial adhesion, lectin binding, and cancer. The biosynthesis of Le^a and Le^b is complex with several enzyme families involved. They are based on the Type 1 chain, which are synthesized by β1,3-galactosyltransferases (β1,3Gal-Ts). Based on data from enzymatic studies *in vitro*, it has been suggested that Type 1 chain biosynthesis by β3Gal-T1 is restricted to glycolipids, by β3Gal-T2 to *N*-glycans and that Type 1 chain biosynthesis by β3Gal-T5 occurs almost exclusively on *O*-glycans. Other studies have shown that β3Gal-T1 and T5 both can act on *N*-glycans. *O*-Glycans with different core structures have been identified, and the ability of the β3Gal-Ts to use these as substrates has not been resolved even though β3Gal-T5 has been claimed to be responsible for Type 1 chain elongation of core 3 *O*-glycans. From the Type 1 chain precursor, the biosynthesis of Le^b and Le^a is considered to proceed via the products of the FUT2 (Fuc-T2) and/or the FUT3 gene (Fuc-T3), respectively. Fuc-T5 has also shown α1,4-fucosylation activity *in vitro* and *in vivo*. However, conflicting results exist and, most importantly, it is not clear on which glycans (N-, O-linked, or glycolipid) the synthesis takes place. Furthermore, Fuc-T1 has been seen as an additional candidate for α1,2 fucosylation of Type 1 chains *in vitro* and *in vivo*. To complicate matters even more, it has been demonstrated *in vitro* that the specificity of the fucosyltransferases differs; Fuc-T1 and Fuc-T5 prefer Type 2 to Type 1 and Fuc-T2 and Fuc-T3 prefer Type 1 to Type 2. Many studies have been carried out *in vitro* on some of these enzymes, but very few have addressed this issue in a physiological context, such as in cultured cells. To examine in more detail the *in vivo* specificity of enzymes involved in Type 1, H Type 1, Le^a, and Le^b synthesis, we have transfected CHO-K1 cells with relevant human glycosyltransferases and detected the resulting Lewis antigens on secreted reporter proteins carrying N- or O-linked glycans, respectively. All three studied β3Gal-Ts could synthesize Type 1 chains on N-linked glycans, but only β3Gal-T5 worked on O-linked glycans. Interestingly, the latter enzyme could use both core 2 and core 3 precursor structures. Furthermore, the specificity of Fuc-T5 and Fuc-T3 in Le^a and Le^b synthesis was different, with Fuc-T5 fucosylating H Type 1 on core 2, but Fuc-T3 fucosylating H Type 1 almost only on core 3. Finally, Fuc-T1 and Fuc-T2 were both found to direct α2-fucosylation on Type 1 chains on both N- and O-linked structures. A detailed characterization of the glycan- and core chain-specificity of the glycosyltransferases involved in Lewis antigen biosynthesis will allow us to engineer recombinant glycoproteins with defined substitution. These tools will be important for investigations on the fine carbohydrate specificity of lectins, such as *Helicobacter pylori* adhesins and DC-SIGN, and may also prove useful as therapeutics.

(134) A Novel Approach to Study *in vivo* Sialyltransferase Protein Expression in *Drosophila melanogaster*

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With the complete genome sequence and emergence of novel reverse genetic approaches in *Drosophila melanogaster*, one can study any gene of interest. Most commonly *in situ* hybridization and immunohistochemistry techniques are employed to follow the expression levels of genes of interest in different tissues and at different developmental stages. However, *in situ* hybridization patterns do not necessarily reflect the distribution of the corresponding proteins, whereas antibodies are not always easy to produce. We have applied a novel method to overcome these limitations. Gene targeting was used to "knockin" a small heamoagglutinin (HA) epitope to our gene of interest, *Drosophila* sialyltransferase. A targeting construct was designed where the N-terminal part of the sialyltransferase contained two tandem HA tags ~70 amino acids away from the catalytic domain. The construct was then injected into embryos where it has been incorporated into the genome via P-element-mediated transformation. This "targeting construct" was then mobilized to replace the endogenous sialyltransferase in a two-step manner. The first targeting step led to the duplication of sialyltransferase locus, with one copy containing the HA-tagged sialyltransferase gene. In the final "copy-reduction" step, one of these duplicated genes was removed, and flies were selected for the copy that contained the HA epitope. Our immunostaining results confirm the validity of this approach. Even if somewhat time consuming, it offers a new way to study endogenous protein expression during different stages of *Drosophila* development. This work was supported in part by NIH grant R01-GM54594 to V.M.P.

(135) Three-Dimensional Structure of Human *N*-Acetylglucosamine Kinase

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N-Acetylglucosamine kinase (GlcNAc kinase, EC 2.7.1.59) converts endogenous GlcNAc from lysosomal degradation or nutritional sources into GlcNAc 6-phosphate. GlcNAc 6-phosphate then enters the pathway leading to the formation of UDP-GlcNAc, which serves as a substrate of the GlcNAc transferases in oligosaccharide biosynthesis. Furthermore, UDP-GlcNAc is used for the formation of intracellular *O*-GlcNAc modification of proteins and for the biosynthesis of sialic acid. Here, we present the crystal structure of human GlcNAc kinase with a resolution of 2.6 A. The complex of GlcNAc kinase with bound ADP and GlcNAc is a dimer which shares common features of other sugar kinases. The catalytic centre exhibits an ATP-binding motive, and our data support an induced fit mechanism during substrate binding which is already described for hexokinase. Determination of the kinetic properties of cysteine mutants C131S and C143S of GlcNAc kinase showed that the decreased enzyme activities were because of a strongly decreased affinity of GlcNAc and ATP, respectively. These cysteine residues are located in the active

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site of GlcNAc kinase with a potential role in the binding of the transferred gamma-phosphate group of ATP within the catalytic mechanism.

(136) Mucin Splice Variants in Ocular Surface Tissues

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Mucins are highly glycosylated proteins that are vital to the maintenance of healthy epithelial surfaces including the ocular surface. Mucins act as lubricants, protectants, and mediators of signal transduction. The best characterized transmembrane mucin, variously termed episialin, polymorphic epithelial mucin, epithelial membrane antigen, MUC1/REP, or MUC1/B, is encoded by the MUC1 gene which features a core region containing 30–100 tandem repeats. Although at least 12 splice variants of MUC1 have been found in other tissues, no splice variants have been reported in human ocular surface tissues. We have analyzed those tissues by RT–PCR to identify MUC1 splice variants that were then confirmed by sequencing. Human cornea, conjunctiva, and lacrimal gland express a variant transcript that retains 27 bp from the 3′ end of intron 1,2. This splice event was previously described in the variants MUC1/A and MUC1/Yalt and is predicted to add nine amino acids to the MUC1 sequence before the tandem repeat region. Cornea and conjunctiva both contain another MUC1 variant, previously identified as MUC1/SEC, that lacks the transmembrane domain and, therefore, results in a soluble, secreted form of MUC1. Cornea, conjunctiva, and lacrimal gland also express a new MUC1 variant transcript that retains 99 bp from the 5′ end of intron 1,2 and 27 bp from the 3′ end of intron 1,2, resulting in a frame shift and premature stop codon. This transcript is predicted to produce a novel 27 amino acid peptide after signal peptidase cleavage. The functional consequences of mucin variants at the ocular surface remain to be determined but include possible effects on surface wetting and resistance to inflammation and infection.

(137) The PM1138 Gene Product of *Pasteurella multocida* Pm70 is an α-1,3-*N*-Acetylgalactosaminyltransferase Belonging to the GT-4 Family of Glycosyltransferases

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The lipopolysaccharide of *Pasteurella multocida* Pm70 has 13 sugar residues and contains GalNAca-1,3-GalNAcb- at its nonreducing end. The availability of the genome sequence of *P. multocida* Pm70 has allowed investigators to identify LPS biosynthesis genes and to suggest enzymatic function based on sequence similarities. Thus, the terminal GalNAc residue α-1,3-linked to GalNAcb- was proposed to be added by the PM1138 gene product during LPS biosynthesis. Consecutive GalNAc residues are a rare occurrence in bacterial polysaccharides. The N-linked heptasaccharide of *Campylobacter jejuni* NCTC 11168 contains five consecutive GalNAc residues. It consists in GalNAca-1,4-GalNAca-1,4-[Glcb-1,3]GalNAca-1,4-GalNAca-1,4-GalNAca-1,3-Bacb-, where Bac is bacillosamine, 2,4-diacetamido-2,4,6-trideoxy-b-D-glucopyranose, which synthesized from GlcNAc. The PM1138 gene product shares aa sequence similarities with the three GalNAc transferases (PglA, PglH, and PglJ) involved in N-linked heptasaccharide synthesis. In addition, PM1138, PglA, PglH, and PglJ have all been assigned to GT-4 family of glycosyltransferases in the carbohydrate active enzymes (CAZy) database, suggesting that they share structural similarities. The GalNAc transferase activity of PglH and PglJ has previously been demonstrated using GalNAca-FCHASE as an acceptor, but the reaction yields have always been low. This acceptor might not be optimal for the PglH and PglJ GalNAc transferase assays. The availability of a functional PM1138 enzyme would allow the synthesis of a GalNAca-1,3-GalNAcb-FCHASE acceptor that could be a better acceptor for the study of the PglH and PglJ GalNAc transferases. Despite our best efforts, the production of either PglH or PglJ has never been achieved in amounts sufficient to support crystallization trials. If an active PM1138 enzyme could be produced and purified in high amounts, it would become an alternative structural target for the GT-4 family of glycosyltransferases, for which no structure is currently available. The PM1138 gene has been cloned in an expression vector and expressed as a fusion with the malE gene of *Escherichia coli*. The PM1138 enzyme was shown in cell lysates to be a GalNAc transferase using the synthetic acceptor GalNAcb-FCHASE. The reaction product was purified and analyzed by CE-MS and NMR. The mass spectrogram of the product showed the expected *m/z* for GalNAc-GalNAc-FCHASE, and standard carbon-correlated proton spectra of the FCHASE compound confirmed that the distal GalNAc is α-1-3 linked to the proximal GalNAc residue. These data support the assignment of PM1138 as an α-1,3-GalNAc transferase. Additional investigations of PM1138 activity in cell lysates showed that it does not transfer GalNAc to GalNAca-FCHASE nor to GlcNAc-based acceptors.

(138) A Chinese Hamster Ovary Cell Line Deficient in UDP-Xylose Synthase

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Xylose is not only the first sugar residue within the core of all proteoglycans but is also found in other O-linked glycans. These proteins are very diverse in structure and function, and the individual role of Xylose in these complexes is still unknown. UDP-Xylose, the substrate for all xylosyltransferases, is produced from UDP-glucuronic acid within the ER/Golgi lumen by UDP-Xylose Synthase (UXS), formerly also named UDP-glucuronic acid decarboxylase. Many mutant CHO cell lines that are deficient in proteoglycan biosynthesis have been isolated. Here, we show that one of these cell lines not only lacks proteoglycans but is also not able to incorporate Xylose in other glycans. We showed this by transient expression of an *Arabidopsis thaliana* xylosyltransferase. Although wild-type cells become positive for an antibody that recognizes xylosylated *N*-glycans upon expression of this plant enzyme, the mutant cell line remains negative. The phenotype is corrected by expression of UXS, which shows that these cells are deficient in UDP-Xylose synthesis. Indeed, sequencing of the UXS messenger RNA produced in the mutant revealed that no active enzyme can be produced, as a point mutation results in the appearance of a premature stop codon within the open-reading frame. Interestingly, the mutant phenotype is also corrected by expression of UXS in the cytoplasm, which shows that there is active transport of UDP-Xylose over the Golgi membrane and confirms earlier observations that UDP-Xylose can actively be imported into Golgi vesicles. This is further strengthened by the fact that one of the human members of the nucleotide sugar transporter gene family (SLC35B4) is able to transport UDP-Xylose. The physiological role of UDP-Xylose transport, however, remains obscure. Although present in plants, the existence of a cytoplasmic pool of UDP-Xylose has not been reported in mammals.

(139) >Mice with Notch1 Lacking *O*-Fucose in the Ligand-Binding Domain are Viable and Fertile

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Notch receptors play key roles in regulating cell fate determinations in the metazoa. The Notch1 receptor has 36 EGF repeats in its extracellular domain, 23 of which contain a consensus site for O-linked fucose. Fucose is transferred by protein *O*-fucosyltransferase 1 encoded by the *Pofut1* gene. Inactivation of the *Pofut1* gene in mice or flies is lethal because of severe defects in Notch signaling. Mutation of *O*-fucose sites on *Drosophila* Notch has suggested that *O*-fucose residues in different regions of the Notch receptor are important for Notch ligand binding, and deletion analyses have shown that EGF repeats 11 and 12 are required for ligand binding (Xu *et al*., 2005). There is a single *O*-fucose site in EGF12 conserved in all Notch receptors, but there is no *O*-fucose site in EGF11. When the EGF12 site was mutated from Ser to Ala in *Drosophila* such that *O*-fucose could not be transferred, the ectopically expressed mutant Notch receptor was more active in Notch signaling, leading to the conclusion that *O*-fucose at Notch EGF12 represses Notch signaling (Lei *et al*., 2003). However, when a similar mutation was made in mouse Notch1, the mutant receptor was markedly reduced in signaling activity in a coculture reporter assay, suggesting that *O*-fucose in EGF12 is required for Notch1 signaling to occur (Rampal *et al*., 2005). We now report the consequences of generating mice with the same mutation. A Cre/LoxP targeting strategy was used in which the *O*-fucose site in Notch1 EGF12 was eliminated by replacement of Thr with Ala (T12A). WW6 embryonic stem cells targeted at the *Notch1* locus were microinjected into C57Bl/6 blastocysts to obtain chimeric mice. Heterozygotes obtained from chimeras were crossed with MeuCre40 mice to remove the selection cassette and to obtain knockin T12A mice. Homozygous Notch1 knockin mice carrying the T12A mutation ($N1^{T12A/T12A}$) were obtained by crossing heterozygous mice. The T12A mutation was confirmed by Southern blot analysis and sequencing of RT–PCR products. The ratio of pups obtained from heterozygous crosses was $N1^{+/+}$: $N1^{T12A/+}$: $N1^{T12A/T12A}$ = 1:2:1, a normal Mendelian frequency. $N1^{T12A/T12A}$ mice have survived >6 months. They are viable and fertile but have a slightly reduced body weight. This is very surprising given that the same mutation caused activation of Notch signaling in the fly and inhibited mouse Notch1 signaling in a reporter assay. The T12A mutation in the mouse was predicted to give the embryonic lethality typical of mouse mutants with an inactive Notch1. $N1^{T12A/T12A}$ mice are being investigated for more subtle Notch signaling defects that might affect cellular differentiation in different organs and in immunity. This work was supported by National Institutes of Health grant RO1 95022 to P.S.

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(140) Subtle Somitogenic and Skeletal Defects in Mouse Embryos Lacking β4Galactosyltransferase-1

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The formation of somites and the development of the skeleton in vertebrates require that Notch signaling is intact. Notch signaling is mediated by Notch receptors, a family of single transmembrane glycoproteins containing 29–36 EGF repeats in their extracellular domain. Many of these EGF repeats may be modified with *O*-fucose by the action of protein *O*-fucosyltransferase-1 (Pofut1). *O*-Fucose residues on Notch may be extended with *N*-acetylglucosamine (GlcNAc), galactose, and sialic acid. Fringe is the β3N-acetylglucosaminyltransferase that is responsible for the transfer of GlcNAc to *O*-fucose. Fringe action results in the modulation of Notch signaling. It was shown previously that the addition of Gal to the *O*-fucose-GlcNAc disaccharide on Notch EGF repeats by β4GalT-1 is required for Fringe inhibition of Jagged1-induced Notch signaling in a Chinese hamster ovary (CHO) coculture assay (Chen *et al*., 2001). This led to the suggestion that, in some instances, the action of Fringe may be necessary but not sufficient for the modulation of Notch signaling. In an attempt to identify an *in vivo* circumstance that might reflect the coculture results, the expression of Notch pathway and somitogenic genes was examined in E9.5 mouse embryos lacking β4GalT-1. Four of the Notch pathway genes were altered in expression pattern or expression level. Expression of Notch signaling target genes *Hes5* and *Mesp2* was reduced in all mutant embryos. The Notch ligand genes *Dll1* and *Dll3* were reduced or altered in expression in a significant proportion of mutant embryos. Although there were no differences in the number or morphology of somites in E9.5 β4GalT-1 null embryos, the number of lumbar vertebrae in mutant embryos differed from control littermates ($p = 0.01$). This finding is consistent with the effects of Notch signaling on Hox gene functions during mouse skeletal development. Thus, a transient effect on somitogenesis consistent with a transient disruption of Notch signaling was observed in embryos lacking β4GalT-1. The subtlety of the *in vivo* phenotype may be owing to redundancy, because several of the five β4GalT genes related to β4GalT-1 are expressed during embryogenesis. This work was supported by National Institutes of Health grant RO1 95022 to P.S.

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(141) Cloning and Characterization of the Phosphoglucomutase of *Trypanosoma cruzi* and Functional Complementation of a *Saccharomyces cerevisiae* PGM Null Mutant

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Trypanosoma cruzi is the etiological agent of Chagas' disease, a chronic illness characterized by progressive cardiomyopathy and/or denervation of the digestive tract. The parasite surface is covered with glycoconjugates such as mucin-type glycoproteins and glycoinositolphospholipids (GIPL) whose glycans are rich in galactopyranose (Galp) and/or galactofuranose (Galf) residues. These molecules have been implicated in attachment of the parasite to and invasion of mammalian cells and in modulation of the host immune responses during infection. In *T. cruzi*, galactose (Gal) biosynthesis depends on the conversion of UDP-glucose (Glc) into UDP-Gal by an NAD-dependent reduction catalyzed by UDP-Gal 4-epimerase. Phosphoglucomutase (PGM) is a key enzyme in this metabolic pathway catalyzing the interconversion of Glc-6-phosphate (P) and Glc-1-P which is then converted into UDP-Glc. We here report the cloning of *T. cruzi* PGM, encoding *T. cruzi* PGM, and the heterologous expression of a functional enzyme in *Saccharomyces cerevisiae*. *T. cruzi* PGM is a single copy gene encoding a predicted protein sharing 61% amino acid identity with *Leishmania major* PGM and 43% with the yeast enzyme. The 5′ trans-splicing site of PGM RNA was mapped to a region located 18 base pairs upstream of the start codon. Expression of *T. cruzi* PGM in a *S. cerevisiae* null mutant lacking genes encoding both isoforms of PGM (pgm1Δ/pgm2Δ) rescued the lethal phenotype induced upon cell growth on Gal as sole carbon source.

(142) Molecular Characterization of a Novel Cytoplasmic UDP-Gal : Fucoside α3-Galactosyltransferase that Modifies Skp1 in *Dictyostelium*

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Skp1 is a cytoplasmic and nuclear protein which is posttranslationally modified by a pentasaccharide, Galα1,Galα1,3Fucα1,2Galβ1,3GlcNAcα1O-, at a 4-hydroxylated derivative of Pro143 in Skp1 from the amoebazoan *Dictyostelium discoideum*. An enzymatic activity capable of transferring Gal from UDP-Gal to Fucα1,2Galβ1,3GlcNAcα1O-Bn and to the corresponding glycoform of Skp1 was described previously in cytosolic extracts of *Dictyostelium*. A protein GT72 associated with this enzyme activity was purified to apparent homogeneity by a combination of conventional and affinity column chromatography. In-gel tryptic digestion followed by Q-TOF-MS analysis yielded eight peptides which mapped onto a predicted gene with three exons on chromosome 4 (nt 144665-146830) with unknown function. The candidate DNA and predicted cDNA sequence for the protein obtained from DictyBase were confirmed by PCR and RT–PCR using genomic DNA and total RNA as templates, resulting in a predicted protein of 648 amino acids consisting of a potential N-terminal glycosyltransferase domain and a predicted C-terminal β-propeller domain. Overexpression of the gene with an N-terminal His6-tag resulted in detection of the His6-tagged protein and a 120-fold increase in α3GalT-activity in cytosolic extracts compared with the parental strain, and expression of the truncated N-terminal region suggested that this domain mediates the catalytic activity. In contrast, disruption of the gene resulted in absence of detectable enzyme activity in the extracts. GT72 represents a novel α3GalT whose specificity suggests that it is the Skp1 α3GalT and whose mechanism is consistent with the sequential model of glycosylation of Skp1 proposed based on studies of the earlier modification enzymes in the pathway. The occurrence of the C-terminal predicted β-propeller domain suggests an interesting mechanism of enzyme regulation. Informatics studies suggest that related catalytic domains are expressed in the Golgi of plants and other protozoans.

(143) Mucin *O*-Glycans Mediate Cell–Cell Adhesion in Corneal Epithelial Cells Under Dynamic Flow Conditions

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We have hypothesized that *O*-glycans on membrane-associated mucins confer disadhesive properties to the apical surfaces of human corneal epithelial cells, preventing the tarsal conjunctival epithelial surfaces of the eye from adhering to the cornea while blinking or sleeping. The purpose of this study was to evaluate the role of membrane-associated mucins and their *O*-glycans on cell–cell adhesion, using a transient adhesion assay on telomerase-immortalized human corneal-limbal epithelial (HCLE) cells. The production of mucins and *O*-glycans in HCLE cells was evaluated by immunofluorescence microscopy and western blot analysis using monoclonal antibodies to MUC1 (HMFG-2), MUC16 (OC125), and a terminal O-acetylated sialic acid epitope on MUC16 (H185). HCLE cultures were grown on cell culture slides and fitted onto a parallel plate laminar flow chamber. Trypsinized HCLE cells grown without serum and labeled with a fluorescent dye (6-CFDA) were perfused over the HCLE culture through the flow chamber at a shear stress of 0.8 dyn/cm^2. Cell–cell interactions were monitored by fluorescence video microscopy. The number of transient adherent (rolling) cells was quantified in eight different areas of each slide ($n = 6$). HCLE cultures grown without serum on cell culture slides produced MUC1 on their apical surfaces but no MUC16. HCLE cells grown in serum for 7 days produced MUC1, MUC16, and its carbohydrate epitope, H185. Addition of 2 mM benzyl-alpha-GalNAc, an inhibitor of mucin O-glycosylation, to HCLE cells cultured in serum resulted in lack of binding of H185, without affecting either MUC1 or MUC16 apomucin production. The number of transient adherent cells on HCLE cells producing both MUC1 and MUC16 was significantly lower (3.8 + 1.9) than in the more adhesive, no-MUC16 condition (71.2 + 23.6, $p < 0.005$). The number of transient adherent cells on benzyl-alpha-GalNAc-treated cultures (46.0 + 12.9) was higher than in control cultures grown with serum ($p < 0.05$). These data indicate, that under dynamic flow conditions, mucin *O*-glycans mediate cell–cell adhesion in HCLE cells and suggest that

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glycosylated membrane-associated mucins may contribute to the disadhesive properties of the apical epithelial surfaces on the eye. Supported by NIH/NEI R01EY012847 to P.A.

(144) Identification of a Novel Class of Chondroitin Proteoglycans in *Caenorhabditis elegans*

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Chondroitin sulfate proteoglycans (CSPGs) are a large and diverse protein family, with over 20 different varieties expressed in mammalian systems. CSPGs function in many biological processes ranging from structural support in cartilage to integrity of the dermis, to nervous system axon guidance and inhibition of mature neurite outgrowth. Some of these functions are specific to organisms located higher in the evolutionary tree. Previous work has shown chondroitin also plays a fundamental biological role in lower organisms such as *Caenorhabditis elegans*. Studies of the *sq*uashed *v*ulva (*sqv*) mutants demonstrated that chondroitin is required for proper cell division of the single-celled embryo, as well as larval vulval morphogenesis. Interestingly, *in silico* analysis suggests *C. elegans* does not express obvious orthologs of any mammalian CSPG core proteins. We have developed a biochemical purification and mass spectrometry approach that identifies proteoglycan core proteins and at the same time tags the site of glycosylation. Nine novel chondroitin proteoglycans (CPGs) were identified, none of which are present in the mammalian genome. The proteins were shown to carry chondroitin chains by recombinant protein expression in mammalian COS-7 cells. Two genes, *cpg-1* and *cpg-2*, were selected for further functional characterization. Although RNAi depletion of either gene alone showed no phenotype, simultaneous depletion of both genes resulted in gonadal defects and multinucleated embryos that die at the single-cell stage. The embryonic lethal phenotype resembles that seen in the *sqv* mutants, suggesting that CPG-1 and CPG-2 are two novel, functionally redundant chondroitin proteoglycans. These findings have interesting evolutionary implications. The biosynthetic machinery that generates the chondroitin chain is conserved between worms and humans, but the core proteins that carry these chains are distinctly different.

(145) Identification of Three Pseudogenes for Human Core 1 (beta)3Gal-T (T-synthase)

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Core 1 (beta)3galactosyltransferase (C1GalT-1, T-synthase) is a key enzyme in mucin type *O*-glycan biosynthesis. Human C1GalT-1 is composed of three exons localized on 7p13-14. Unlike most other glycosyltransferases, which have more than one gene, there is only a single functional gene for T-synthase found in human, mouse, and *Caenorhabditis elegans*. Blastn search of the human genome using human T-synthase cDNA coding sequence revealed three highly conserved DNA sequences on chromosomes 5, 8, and 12 related to C1GalT-1, which we termed human C1GalT-1 pseudogenes-1, -2, and -3 (pC1GalT-1, pC1GalT-2, and pC1GalT-3). In contrast to the functional gene, all three pC1GalTs are composed of a single "exon" with several mutations. The sequence of pC1GalT-1 on chromosome 5 is the most conserved exhibiting 93% identity to the cDNA of C1GalT-1, although pC1GalT-1 contains many single base changes, a single base insertion, and two single base deletions. pC1GalT-3 on chromosome 12, which is 91% identical to the cDNA of C1GalT-1, has many single base changes in different positions, five 2–5 base deletions, and one 2 base insertion, and one 15 base insertion containing 14 thymidines. pC1GalT-2 on chromosome 8 is only 80% identical to the cDNA of C1GalT-1. The first 150 base pairs of pC1GalT-2 do not match Exon I (220 bp) of C1GalT-1, thus accounting for the major overall differences between pC1GalT-2 and C1GalT-1. pC1GalT-2 also contains many consecutive bases changes, deletions, and insertions compared with the cDNA of C1GalT-1. All these changes in the pseudogenes result in many stop codons and ORF shifts leading to non-ORF DNA sequences. Furthermore, neither reversed nor reversed and complementary sequences of the pseudogenes contain any ORF, indicating that they are not other genes or parts of other genes, further indicating they are pseudogenes. In addition, searches of human ESTs in the database show that there are two other sequences that match perfectly to pC1GalT-2, thus indicating that pC1GalT-2 on chromosome 12 probably arose from a transcript. Based on the identity of the three pseudogenes to human C1GalT-1, it is likely that the pC1GalT-2 on chromosome 8 was probably the earliest evolutionarily, whereas pC1GalT-1 on chromosome 5 is the most recent. Furthermore, the single "exon" nature of these pseudogenes suggests that they resulted from reverse transcription of the mRNA of human C1GalT-1 and subsequent integration into the human genome. The existence of three nonfunctional genes or pseudogenes of human C1GalT-1 suggests an interesting evolution of human C1GalT-1 and also suggests that the pseudogenes may play a role in gene expression or regulation of human C1GalT-1.

(146) Terminal Differentiation and Morphogenesis in *Dictyostelium* Depends on its Skp1 Prolyl 4-Hydroxylase

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In the free-living social soil amoeba *Dictyostelium*, the small nucleocytoplasmic protein Skp1 is modified by a hydroxyproline-linked pentasaccharide consisting of a substituted Type 1 blood group H antigen. The Skp1 prolyl hydroxylase (P4H1) is, like HIF?-type animal prolyl 4-hydroxylases, rate limited by physiological levels of O_2. Disruption of the P4H1 gene (phyA) was previously shown to block Skp1 glycosylation. When starved, *Dictyostelium* executes a developmental cycle which involves the determination and differentiation of prestalk and prespore cells and then differentiation into stalk and spore cells as they culminate in an O_2-dependent fashion to form a fruiting body. P4H1-null cells grow and aggregate normally under laboratory conditions. However, P4H1-null slugs fail to induce the early prestalk marker EcmA and late prespore marker SpiA and do not culminate. The effect may be mediated via Skp1, the only biochemically identifiable acceptor substrate in lysates. A mutant lacking the PgtA glycosyltransferase, required for addition of the second sugar, culminates with only a slight delay and modest reduction of spore number. This indicates that the P4H1 defect is not simply because the Skp1 glycan chain is not extended but leaves open the role of the HyPro-linked ?GlcNAc whose transferase gene resists disruption. Skp1 glycosylation and culmination are rescued by specific expression of P4H1 in either prestalk or prespore cells. Culmination and spore differentiation can be rescued by the presence of a small number of wild-type cells which, however, do not themselves form spores. These and other studies using GFP-tagged strains reveal that mutant cells exert a "cheater" phenotype in which they successfully compete with normal cells to become surviving spores. Differentiation and culmination are also rescued by hyperoxic conditions (40% O_2). These phenotypes are similar to though distinct from those of strains whose genes for cullinA and FbxA, two proteins that interact with Skp1 in E3(SCF)Ubiquitin ligases, are disrupted. The results suggest that in concert with the Skp1 ?GlcNAc-transferase, P4H1 regulates the activity of a noncell autonomous protein modification signaling pathway that couples a decision point in terminal differentiation to the environment of the slug.

Session Topic: Neuroglycobiology

(147) Targeted Disruption of *N*-Acetylglucosaminyltransferase GnT-VB in Human Neuroblastoma Cells Elevates the Expression of Beta 1 Integrin Leading to Impaired Neurite Outgrowth, Increased Adhesion, and Reduced Rates of Migration on Laminin

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Neuroblastoma is a childhood malignancy that is often associated with a high rate of metastasis and a poor clinical prognosis. The addition of complex branched oligosaccharides to glycans destined for the cell surface has been associated with increased metastatic potential. We are investigating the possible role of the *N*-acetylglucosaminyltransferase known as GnT-VB (GnT-IX) in neuroblastoma metastasis. Using RNA interference, we have established neuroblastoma cell lines with reduced GnT-VB expression. Neuroblastoma cells with reduced GnT-VB expression display increased adhesion, reduced migration, and impaired neuritogenesis when plated on the extracellular matrix protein laminin. Expressing a recombinant form of GnT-VB that is not targeted for RNA interference can reverse these changes indicating the direct involvement of GnT-VB-mediated glycosylation. To better understand the mechanisms regulating these changes in adhesion and migration, we examined the expression levels of various laminin receptors and found that GnT-VB-deficient cells express increased levels of integrin subunits compared with control cells. GnT-VB is capable of performing both N- and O-linked glycosylation. To confirm that changes in cell adhesion, neurite extension, and cell migration observed for GnT-VB-deficient neuroblastoma cells were because of O-linked glycosylation, we suppressed the expression of the enzyme, POMGnT1, that is required before GnT-VB can add β(1,6) GlcNAc to O-linked substrates. Reduced expression of POMGnT1 leads to a phenotype similar to that seen for cells with reduced GnT-VB and confirms that these phenotype changes are because of O-linked mannose glycosylation. Taken together, our results suggest that O-linked glycosylation of glycoprotein acceptors by GnT-VB may contribute to the decreased

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cell adhesion and increased rates of metastasis that occur in human neuroblastoma. Therapies targeted at the inhibition of GnT-VB expression may be useful for the prevention of neuroblastoma metastasis.

(148) Defects in Radial Neuronal Migration in the Cerebellum of the Largemyd Mouse are Associated with Disruptions in Bergmann Glia Organization and Delayed Migration of Granule Neurons

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The LARGE gene encodes a putative glycosyltransferase that is required for normal glycosylation of dystroglycan, and defects in either LARGE or dystroglycan cause abnormal neuronal migration. The mechanism(s) for this effect is(are) not fully understood. This study analyzes the Largemyd mouse cerebellum during postnatal cerebellar development as a model for radial neuronal migration. LARGE is shown to be expressed most strongly in the Bergmann glial cells and Purkinje cells throughout cerebellar development, which is similar to what is known for dystroglycan expression. Discontinuities of the pial surface of the developing Largemyd mouse cerebellum correlate with disruption of the normal organization of the external granule cell layer and Bergmann glial fibers. At early time points, granule neurons express differentiation markers normally, both temporally and spatially, and show no defects in neurite outgrowth in *in vitro* assays. However, granule neuron migration is delayed within the external granule and molecular layers, resulting in granule neurons undergoing their intrinsically programmed differentiation in inappropriate locations. Consequently, cells expressing mature granule neuron markers become stranded within these layers. The cause of the less efficient migration is likely because of both physical disruption of the glial-guide scaffolding, as well as to suboptimal neuronal–glial guide interactions during migration.

(149) Defects in Tangential Neuronal Migration of Pontine Nuclei Neurons in the Largemyd Mouse are Associated with Stalled Neuronal Migration at a Migrational Choice Point in the Ventro-Lateral Hindbrain

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The LARGE gene encodes a putative glycosyltransferase that is required for normal glycosylation of dystroglycan, and defects in LARGE can cause abnormal neuronal migration in congenital muscular dystrophy (CMD). Both radial and tangential migration appear to be disrupted in CMD, although the mechanisms for these effects are not fully understood. This study analyzes tangential neuronal migration in the development of the precerebellar nuclei in the Largemyd mouse hindbrain. Large and dystroglycan are expressed widely throughout the embryonic hindbrain, including the pontine nuclei neuron tangential migratory stream (the anterior extramural stream, AES). Neurons comprising the precerebellar nuclei [pontine reticular tegmental nuclei (PRTN), the pontine grey nuclei (PN), inferior olivary nuclei (IO), external cuneate nuclei (ECN), and the lateral reticular nuclei (LRN)] derive from the neuroepithelium of the dorsal hindbrain known as the rhombic lip. They all undergo relatively long-range tangential neuronal migration to reach their final destinations, although they all show unique characteristics. We show that their development is differentially affected by the presence of the LARGE mutation. Analyses of cell stains in the adult Largemyd mouse indicate that the PN and the PRTN are severely disrupted, whereas the IO, ECN, and LRN appear relatively unaffected. Thus, not all tangential migratory pathways are disrupted. Immunohistochemical studies in both adult and embryonic mouse suggest that the LARGE mutation does not interfere with the early stages of PN neuronal development, in that normal numbers of neurons begin their journey toward the ventral midline in the AES. However, migration stalls and PN neurons fail to reach the midline, surviving as ectopic clusters of cells located under the pial surface dorsally and laterally to where they normally would finish their migration near the ventral midline. The location at which the PN neurons stall is the same at which these neurons fail to finish tangential migration in mutations affecting responses to the axon guidance molecules netrin-1 and slit-2 (the Netrin-1 and Rig-1 knockout mutants, respectively). These observations suggest that glycan-dependent dystroglycan interactions are also required for PN neurons to correctly respond to signals at this important migrational choice point.

(150) Caloric Restriction Extends Longevity Without Altering Brain Glycolipids in Sandhoff Disease Mice

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Caloric restriction (CR), which improves health and increases longevity, was studied as a therapy in a mouse model of Sandhoff disease (SD), an incurable neurodegenerative disease involving accumulation of ganglioside GM2 and asialo-GM2 (GA2). Adult mice were fed a rodent chow diet either ad libitum (AL) or restricted to reduce body weight by 15–18%. Although GM2 and GA2 were elevated, no significant differences were seen between the Hexb –/– and the Hexb +/– mice for most phospholipids and cholesterol. Cerebrosides and sulfatides were reduced in the Hexb –/– mice. In addition, rotorod performance was significantly worse in the Hexb –/– mice than in the Hexb +/– mice. CR, which decreased circulating glucose and elevated ketone bodies, had no significant effect on brain lipid composition or on cytoplasmic neuronal vacuoles, but significantly improved rotorod performance and extended longevity in the Hexb –/– mice. The number of CNS apoptotic cells and expression of CD68 and F4/80 was also significantly less in the CR-fed Hexb –/– mice than in the AL-fed Hexb –/– mice. We suggest that CR delays disease progression in SD and possibly in other ganglioside storage diseases through anti-inflammatory mechanisms.

(151) Silencing the Expression of RAGE by RNA-Interference Inhibits Neurite Outgrowth in Neurons

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Previously, we have shown that embryonal carcinoma, P19 cells on treatment with retinoic acid (RA) differentiated into neuron-like cells and concomitantly induced expression of sulfoglucuronyl carbohydrate (SGC), its binding protein Amphoterin, and the latter's interacting signaling molecule receptor for advanced glycation end product (RAGE) on their cell membranes, coordinated with extensive neurite outgrowth (Chou *et al*., 2003). Direct interactions between SGC-proteins and Amphoterin and between Amphoterin and RAGE were shown by co-immunoprecipitation of the proteins from RA-treated P19 cells and also with proteins isolated from granule neurons of neonatal cerebellum. Furthermore, anti-RAGE antibodies inhibited neurite out growth and cell migration in explant and slice cultures, similar to anti-Amphoterin and anti-SGC antibodies (Chou *et al*., 2004). These results suggested that RAGE could act as a signaling molecule for neurite outgrowth by its interaction with Amphoterin and that of Amphoterin with SGC *in vivo*. RAGE signaling is mediated by GTPases, Rac, and Cdc42, which regulate the cytoskeletal protein actin. Actin polymerization is necessary both for the extension of neurites and neuronal migration. We have initiated studies to target the mouse RAGE gene expression by RNAi. We generated three different siRNA expression constructs, R1, R2, and R3, in the plasmid pFIV-H1/U6-coGFP as targeting sequences for the mouse RAGE gene. In transient cotransfection assays with RAGE-myc in HEK293T cells, all three siRNA constructs were found to knockdown the expression level of the ectopic RAGE expression as determined by western blotting. Among them, construct R1 knocked down RAGE expression most efficiently. The knockdown efficiency of R1 was ~94%; R2, 57%; and R3, 77%. The R1 siRNA target sequence was cloned into pBabe-H1/U6-Puro vector based on Moloney murine leukemia virus, and the virus was propagated in Bosc23 cells (a derivative of 293T cells). The virus-infected P19 cells were grown as aggregates for 4 days with RA, and the dissociated cells were allowed to culture for 6 days. Mock-infected P19 cells after treatment with RA expressed RAGE, Amphoterin, SGC, and MAP2 and produced neurite outgrowth. However, the R1-construct-infected cells did not express RAGE and did not elaborate neurite outgrowth. Thus, knockdown of RAGE in P19 cells does not allow the formation of neurites after treatment with RA. Although the R1-construct abolished RAGE expression along with the loss of neurite formation, the P19 cells continued to express SGC, Amphoterin, and neuronal markers MAP2 and TUJ1 in the cell membranes, showing that the effect of R1 construct was specific for abolishing the RAGE expression and other antigens were not affected. Also, these neuronal marker expressions would indicate that the P19 cell differentiated into neuron-like cells with RA, except the neurite formation was inhibited because of the absence of RAGE. Preliminary studies showed that the knockdown of RAGE in primary granule neurons of the cerebellum also inhibited neurite formation.

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(152) GnT-III Potentiates Dendritic Neuritogenesis by Introducing the Bisecting GlcNAc into *N*-Glycans on Beta1 Integrin

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Although many glycoproteins are involved in neuritogenesis, the biological significance of their sugar chains remains unknown. *N*-Glycans containing bisecting GlcNAc, which are catalyzed by beta1,4-*N*-acetylglucosaminyltransferase III (GnT-III), are major sugar chains in brain tissue. To investigate roles of *N*-glycans containing bisecting GlcNAc on neural differentiation, we transfected GnT-III gene to the Neuro2A, neuroblastoma cells. Overexpression of GnT-III resulted in increasing in dendrites with neurite swellings (also called transport packets) containing N-CAM and Golgin97 (one of Trans Golgi marker) under serum-starvation induced neuronal differentiation. By contrast, overexpression of dominant-negative GnT-III (Ihara *et al*., 2002) significantly suppressed dendrite formation. The enhancement of dendritic neuritogenesis by GnT-III was abrogated by adding E4-PHA lectin which preferentially recognizes bisecting GlcNAc, but not by L4-PHA lectin as a control. We found that beta1 integrin partially colocalized with N-CAM at neurite tips and swellings in differentiated Neuro2A. Interestingly, E4-PHA treatment induced abnormal axonal expansion and disrupted the localization of beta1 integrin, whereas N-CAM remains at neurite tips, implying that beta1 integrin is one of major targets of GnT-III. In fact, beta1 integrin was identified as a major component in E4-PHA associated complexes. Furthermore, treatment with anti-beta1 integrin antibody blocked GnT-III-enhanced dendrite formation. Collectively, our data indicate that GnT-III is involved in the regulation of dendritic neuritogenesis through a beta1 integrin-dependent pathway.

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(153) Transcriptional Regulation of a Glycosyltransferase Gene B3galt2 by Creb in Rat Cortical Neurons

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Regulation of gene transcription by neuronal activity is thought to be key to the translation of sensory experience into long-term changes in synaptic structure and function. Glycosylation status of synaptic proteins and lipids determines functional cell surface presentation of neurotransmitter receptors, regulates ligand–receptor interactions, and contributes to cell–cell communication between presynaptic and postsynaptic neurons as well as between neurons and astrocytes. Transcriptional regulation of glycosyltransferase genes in response to synaptic activities is likely involved in the modification of glycosylation statues of synaptic components. However, mechanisms that govern the expression of various glycosyltransferase genes are still poorly understood. Transcription factor CREB plays a fundamental role in synaptic plasticity, neurogenesis, neuroprotection, and long-term memory (LTM) formation. We have identified a conserved CREB binding site (CRE) in the promoter of the B3GALT2 (UDP-Gal : betaGlcNAc beta1,3-galactosyltransferase, polypeptide 2) gene through a genome-wide survey of putative CREB targets using a bioinformatics approach. Here, we show that B3GALT2 gene expression is up-regulated in rat cortical neurons in response to membrane depolarization and consequential CREB activation. The increased expression of B3GALT2 is owing to Ca^{2+} influx through L-type voltage sensitive calcium channels (L-VSCC) because application of nimodipine, a potent L-VSCC antagonist, blocked CREB activation as well as the induced B3GALT2 gene expression. Our results suggest that B3GALT2 is a putative target of CREB and could play a role in CREB-regulated neuronal functions.

(154) GnT-IX and GnT-V are Expressed in a Different Manner in Mouse Tissues

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N-Acetylglucosaminyltransferase V, GnT-V, is the Golgi enzyme that catalyzes the transfer of GlcNAc from UDP-GlcNAc to the core α1,6-mannose arm via a β1,6-linkage, forming the tri- and tetraantennary complex-type *N*-glycans. We recently reported on a brain-specific β1,6-*N*-acetylglucosaminyltransferase, GnT-IX, the GnT-V homologue, which acts on both α-linked mannose of *N*-glycan and *O*-mannosyl glycan. But little is known about its expression in normal mouse tissues and functions. We have compared the expression of GnT-IX and GnT-V in various mouse tissues by northern blot analysis and found that the two enzymes differentially expressed in mouse tissues. GnT-IX transcripts were restricted to cerebrum, cerebellum, thymus, and testis, whereas GnT-V transcripts were expressed ubiquitously in mouse tissues. To investigate the localization of these enzymes in detail in mouse tissues, we made a polyclonal antibody against GnT-IX. First, we examined the specificity of the antibody in GnT-IX and GnT-V transfectants. The antibody specifically recognized GnT-IX, but not GnT-V, in the Golgi apparatus, which was confirmed by the costaining with a cis-Golgi marker GM130. To compare the distributions of GnT-IX and GnT-V in mouse tissues, we performed the immunochemical staining using the antibody. The staining patterns of GnT-IX and GnT-V were apparently different in mouse brain. Thus, our data suggest that these two enzymes may play distinct roles in brain.

(155) Distribution of Major Gangliosides in Brains of Wild-Type Mice and Mice Deficient for Enzymes in Ganglioside Biosynthesis

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Gangliosides, sialylated glycosphingolipids, are major glycans in the brain and the predominant glycans of nerve cells. All mammals express the same four major brain gangliosides, GM1, GD1a, GD1b, and GT1b, which vary in the number and linkage positions of sialic acids and together represent ~90% of the total brain ganglioside content. A key branch point in ganglioside biosynthesis is the conversion of GM3 to GD3 by the Siat8a gene product (CMP-NeuAc : GM3 2,8-sialyltransferase), leading subsequently to GD1b and GT1b. Alternatively, GM3 is converted to GM2 by the Galgt1 gene product (UDP-GalNAc : GM3/GD3 *N*-acetylgalactosaminyltransferase), leading subsequently to GM1 and GD1a. Wild-type, Siat8a-null and Galgt1-null mice have the same level of total brain gangliosides. Siat8a-null mice lack GD1b and GT1b and express correspondingly higher concentrations of GM1 and GD1a. Galgt1-null mice lack all of the major brain gangliosides but express the corresponding concentrations of GM3 and GD3. Using highly specific monoclonal IgG antibodies, we studied the distribution of GD3, GM1, GD1a, GD1b, and GT1b gangliosides in brains of wild-type mice, Siat8a-null mice, and Galgt1-null mice. In wild-type mice, GM1 was expressed preferentially in myelinated pathways (white matter), GD1a in gray matter, and GD1b and GT1b in both, but at higher concentrations in gray matter. GD3 staining was essentially absent in adult wild-type mice. In Galgt1-null mice, GM1, GD1a, GD1b, and GT1b were absent. GD3, which is the major ganglioside in Galgt1-null brain, was found on neuronal and glial cells, but was lacking in myelinated pathways. In Siat8a-null mice, GD3, GD1b, and GT1b were absent. As in wild-type mice, GM1 staining in Siat8a-null mice was still predominantly in the white matter, whereas GD1a staining was predominantly in the gray matter. However, the white/gray separation of GM1 and GD1a (respectively) was less abrupt in Siat8a-null mice than in wild-type mice. We conclude that the total level of brain gangliosides is controlled early in the biosynthetic pathway, that different brain structures (gray matter/white matter) express different major brain gangliosides, and that the distribution of remaining ganglioside species in genetically altered mice generally reflects interruption of the underlying biosynthetic pathways specific for the brain structures in which they are expressed. Supported by Croatian Ministry of Science, grant number 0219021 and NIH NS37096.

(156) Sialidase Enhances Spinal Axon Outgrowth

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The adult central nervous system, including the spinal cord, is a profoundly inhibitory environment for axon outgrowth, severely limiting recovery after traumatic nervous system injury. Axons have the capacity to regenerate but are inhibited from doing so by molecules that accumulate or persist at injury sites, including chondroitin sulfate proteoglycan (CSPG), Nogo, oligodendrocyte–myelin glycoprotein (OMgp), and myelin-associated glycoprotein (MAG) (Sandvig *et al*., 2004). These inhibitors, found on residual myelin or astrocytes, bind to receptors on nerve cell axons to initiate signals that halt axon outgrowth.

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Glycans are involved in each inhibitory cascade. CSPG requires glycosaminoglycan chains for inhibition, Omgp and Nogo receptor are GPI-anchored proteins, and MAG is a sialic acid binding lectin (Siglec-4). *In vitro*, treatment with chondroitinase, sialidase, or phosphatidylinositol-specific phospholipase C (PI-PLC) enhances axon outgrowth on inhibitory substrata, and chondroitinase ABC delivery to the site of experimental spinal cord injury enhances recovery in rats (Bradbury *et al*., 2002). We used an animal model of brachial plexus (nerve root) avulsion injury in the rat to extend these studies. Our model mimicked avulsion of nerve roots upon traumatic neck and shoulder displacement. Such injuries are not uncommon in difficult childbirths and motorcycle accidents, resulting in profound loss of limb use. Recent therapy includes implantation of peripheral nerve grafts near the avulsion site. Treatments that improve axon outgrowth into the graft are expected to enhance recovery. To test the efficacy of glycobiology tools in this model, chondroitinase ABC, PI-PLC, and sialidase were delivered to the graft site and spinal axon outgrowth into the graft was quantified. Ventral spinal roots (C8) in rats were cut, then a peripheral nerve graft was inserted at the injury site. Using an osmotic pump, saline (control), chondroitinase ABC (0.5 U/mL), PI-PLC (2 or 20 U/mL), or sialidase (0.1 or 0.4 U/mL) were delivered to the graft site for 2 weeks. Spinal axons extending well into the peripheral nerve graft were then retrogradely labeled with a fluorescent dye. Tissues were fixed, the spinal cord was dissected, and axon outgrowth into the graft was quantified by measuring the number of fluorescently labeled spinal neurons. Marked enhancement (>2.5-fold, $p < 0.01$) of axon outgrowth into peripheral nerve grafts was observed in animals treated with chondroitinase ABC or with 0.4 U/mL sialidase, whereas PI-PLC or 0.1 U/mL sialidase were without significant effect. These results indicate that sialidase and chondroitinase ABC enhance axon regeneration and that treatments that modify sialoglycoconjugates and CSPG may aid recovery after nervous system injury. Supported by NIH grants NS046669, HL16315, and University of Michigan Department of Neurosurgery. I.L. is a Hopkins PREP Scholar (GM064124). K.V. supported by the Croatian Ministry of Science.

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(157) Mass Spectrometry Structural Investigation to Address Stability of Glycoprotein P0 Dimer

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Glycoprotein myelin protein zero (P0) is the major protein of the peripheral nervous system myelin in higher vertebrates. It is a critical requirement for the formation and maintenance of myelin structure in the internode, through homophilic interactions at both the extracellular and intracellular domains. Mutations and deletions in the P0 gene correlate with hereditary peripheral neuropathies of varying severity. P0 contains a single N-glycosylation site and has a heterogeneous glycosylation pattern. The glycan moiety of P0 plays an important role in cell-to-cell adhesion via homophilic interactions, because non-glycosylated P0 does not show homophilic adhesion. Crystallographic studies on the recombinant extracellular domain of rat P0 and small-angle solution scattering on full-length P0 isolated from bovine myelin suggest that P0 exists as tetramers in the membrane, and SDS–PAGE of mammalian myelin shows that the predominant form of P0 is the monomer. By contrast, in Xenopus P0, which has 65% sequence identity with rat P0, the predominant form of P0 is a dimer. The dimer appears to be totally resistant to disruption by treatments used to reduce disulfides, to deacylate, and to break hydrophobic or ionic interactions. Therefore, it has been proposed that Xenopus P0 monomers are covalently bonded to form the dimer, and the presence of the glycans may be one of the important mediators during the formation. Xenopus P0 dimer and monomer were purified by SDS–PAGE. Bands of dimer (60 Kda) and monomer (30 Kda) were excised and deglycosylated in-gel with PNGase F, then protease digestion of the proteins was performed after release of the *N*-glycans. Using a combination of protease and glycosidase in-gel digestion, MALDI MS, and ESI MS/MS, we verified the amino acid sequences of P0 glycoprotein, and the identities of dimer and monomer as P0 were confirmed. The digest of the Xenopus P0 dimer was found to contain peaks that had not been present in the digest of the monomer. This finding of unique peptide fragments only in dimer but not in monomer could support the hypothesis, and LC-MS/MS analyses have been undertaken to elucidate the covalent bond in dimer. Furthermore, the preliminary data in the characterization of glycans in dimer and monomer at the single glycosylation site, Asn92, show the difference between dimer and monomer in terms of glycosylation pattern. Our result will contribute to the understanding of the phylogenetic development of P0s adhesive role in myelin and demonstrate an atypical adhesion in peripheral myelin. Characterization of the glycans in both the dimer and monomer will contribute to our understanding of the phylogenetic development of P0s adhesive role in myelin.

(158) Polysialic Acid is Required for Coordinated Migration of Neural Precursor Cells During Brain Development

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The neural cell adhesion molecule (NCAM) is posttranslationally modified with polysialic acid, a homopolymer of α2,8-linked sialic acid, which modulates the function of NCAM. Polysialylated NCAM is highly expressed in developing central nervous system but declines after birth except in areas where active neurogenesis and synaptic plasticity persist in the adult brain. Polysialic acid is synthesized by two polysialyltransferases, ST8SiaII (STX) and ST8SiaIV (PST), of which expression is developmentally regulated in spatiotemporal manner. The studies on NCAM null mice suggested that defects found in NCAM null mice, such as deficient synaptic plasticity and slower chain migration of olfactory interneuron precursors, are in part because of loss of polysialic acid. To determine the role of polysialic acid, distinguished from the role of NCAM, in neural development, we generated double mutant mice lacking the two NCAM-modifying polysialyltransferases, ST8SiaII and ST8SiaIV. In contrast to NCAM knockout mice and ST8SiaII or ST8SiaIV single knockout mice, double mutant mice, completely lacking polysialic acid, display severe defects in brain development and rarely survive by 2 months of age. Migration of the olfactory interneuron precursors through the rostral migratory stream is disturbed in polysialic acid-deficient mice, resulting in small olfactory bulb as observed in NCAM-deficient mice. Furthermore, loss of polysialic acid resulted in thin cerebral cortex, thin corpus callosum, and enlarged lateral ventricle. BrdU-labeling experiments showed that migration of cortical neurons generated in ventricular zone of polysialic acid-deficient mice was slower than that of wild-type mice. Our data demonstrated that tangential cell migration of GABAergic neurons and radial cell migration of pyramidal neurons as well as glial cells distribution are impaired in the double mutant mice. In cerebellum, lack of polysialic acid reduced the number of cerebellar folia because of the loss of preculminate fissure. Thus, polysialic acid deficiency under the presence of NCAM *in vivo* resulted in deficient cell migration of neurons and glial cells widely required to form cerebral cortex and cerebellum not found in NCAM null mice, indicating important roles of polysialic acid itself. Mutant mice with double deficient of ST8SiaII and NCAM have similar but milder phenotypes, compared with ST8SiaII and ST8SiaIV double knockout mice, including immature development of cortices. These results as a whole indicate that decrease of polysialic acid critically affects brain development. These findings combined demonstrate that polysialic acid is required for migration and fate of neural cells essential for brain development. Supported by NIH grant CA33895.

(159) Tandem Mass Spectrometric Analysis of Heparan Sulfate Structure in the Trigeminal Ganglion and in a Genetic Cell Model Demonstrating the Importance of *N*-Deacetylase/*N*-Sulfotransferase Isoforms on the Generation of Biologically Active Heparan Sulfate

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Heparan sulfate proteoglycans influence developmental, physiologic, and pathogenic processes; yet the molecular mechanisms responsible are largely unknown. Many of these activities stem from the structurally diversity of the heparan sulfate (HS) moieties. HS is a linear copolymer assembled from *N*-acetylglucosamine and glucuronic acid units. Structural heterogeneity arises from the remodeling of these polysaccharide chains by a relatively ordered series of reactions involving an epimerase and four families of sulfotransferases which differentially place *N*- and *O*-sulfate groups within HS. The arrangement

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of these critical groups along the HS chain creates distinct binding motifs that can activate an array of important effector proteins. Recent adaptation of liquid chromatography tandem mass spectrometry (LC/MS) to quickly and with a high degree of sensitivity detect specific modifications expressed in HS extracted from a wide variety of sources has greatly improved the ability to analyze HS structure. Here, we show that the use of LC/MS confirms the *in situ* expression profile within sensory neurons of the trigeminal ganglion for two 3-*O*-sulfotransferases (3-OSTs) that can generate entry receptors for herpes simplex virus-1 (HSV-1) in a cell culture system. In addition, we have adopted the use of collision-induced dissociation (CID) analysis to enhance our ability to detect specific HS modifications and to determine the structure of oligosaccharides comprising biologically active motifs. Using LC/MS and CID analysis, we were able to monitor the structural changes that correspond to effects on biological activity by the expression of distinct *N*-deacetylase/*N*-sulfotransferase (NDST) isoforms in a mutant cell line which is null for the entire NDST family. This allowed us to make conclusions on how structural changes effected by specific NDST isoforms can affect biological activity in a cell model system.

Session Topic: Glycans and Lectins in Pathogen Recognition

(160) Recognition Factors of an Insecticidal Lectin Isolated from the Leaves of *Glechoma hederacea*

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An agglutinin exerting a potent insecticidal activity towards the Colorado potato beetle (*Leptinotarsa decemlineata*) was isolated from the leaves of *Glechoma hederacea* (Gleheda) and showed a specificity towards human erythrocytes carrying the Tn antigen. However, no details have been reported on its binding function. To corroborate the molecular basis of its biological activity and physiological function, it is necessary to understand the recognition factors involved in the Gleheda–glycotope interaction. In this study, the requirement of high-density polyvalent carbohydrate structural units for Gleheda binding and a fine affinity profile were evaluated by enzyme-linked lectinosorbent inhibition assay (ELLSA) with our extended glycan/ligand collections, by glycan array and molecular modeling. From the results, it is concluded that a high-density of exposed polyvalent Tn-containing glycoproteins (gps) (natural armadillo salivary Tn gp and asialo ovine salivary gp) are the most potent factors for Gleheda binding. They were on a nanogram basis 6.5×10^5, 1.5×10^4, and 3.1×10^3 times more active than monovalent Gal, GalNAc, and Tn epitope, respectively. It is assumed that the combining site of Gleheda may be of a cavity type with GalNAcα 1- as the major combining site. With respect to the carbohydrate structural units studied, expressed as nanomole inhibition, the hierarchy of potencies are Tn glycopeptides (M.W.$<3.0 \times 10^3$) > Tn monomer > GalNAcα1-3Galβ1-4Glc (AL) > GalNAcα1-3Gal (A) > GalNAc > GalNAcα1-3(Fucα1-2) Gal (Ah) > Galα1-3Gal (B) > GalNAcα1-3GalNAc (F) > GalNAcβ1-4Gal (S) > GalNAcβ1-3Gal (P) > Galα1-4Gal (E) > Gal >> Galβ1-4Glc (L), whereas Galβ1-3GalNAc (T), Galβ1-3GlcNAc (I), and Galβ1-4GlcNAc (II) were inactive. The results of glycan array and docking experiments support the conclusions drawn with respect to the specificity of Gleheda based on the ELLSA assays. These distinct binding features of Gleheda for polyvalent Tn clearly illustrate the importance of polyvalency in the carbohydrate-receptor interactions in biological processes and enable optimizing the application of this novel lectin in glycobiological and clinical research.

(161) Polysaccharide Microarrays for the Diagnosis of Bacterial Infections

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The presence of structurally unique polysaccharides (lipopolysaccharide and capsular) associated with gram-negative bacterial pathogens offer a great opportunity to be fabricated into microarrays. As a "proof of concept," we developed polysaccharide microarrays for diagnosing *Burkholderia pseudomallei* and *Burkholderia mallei* infections. *B. pseudomallei* and *B. mallei* are the causative agents of melioidosis and glanders, respectively. Melioidosis is an infectious disease of humans and animals and is endemic primarily in southeast Asia and northern Australia. Glanders is naturally found in equines, which occasionally transmit the infection to humans. Both *B. pseudomallei* and *B. mallei* are biothreat agents and classified as category B pathogens by the Centers for Disease Control and Prevention. Both *B. pseudomallei* and *B. mallei* are encapsulated with a polysaccharide of the same structure. The capsular polysaccharide is a homopolymer of -3)-2-*O*-acetyl-6-deoxy-β-D-manno-heptopyranose-(1-. LPS (lipopolysaccharide) O-antigen of *B. mallei* is similar to the *B. pseudomallei* LPS O-antigen and is composed of a heteropolymer of repeating D-glucose and L-talose. However, changes are apparent in the O-acetylation pattern of *B. mallei* L-talose residue compared with the pattern in *B. pseudomallei*. We isolated polysaccharides from *B. pseudomallei* and *B. mallei* strains. These polysaccharides are converted to glycosylamines and then coated onto Super Epoxy glass slides in 16-well NUNC plates. These polysaccharide microarrays were probed with polyclonal antibody against the capsular polysaccharide and with serum of a human exposed to a *B. mallei* infection. Immunoreactivity was examined with Cy3- and Cy5-labeled secondary antibody by assaying the fluorescence with a Gene Pix Axon scanner. Results indicated the presence of antibodies against the capsular polysaccharide and LPS in postinfected human serum, whereas they were absent in the control, noninfected serum. Therefore, it is apparent that polysaccharide microarray technology with surface-immobilized bacterial polysaccharides has a significant potential for diagnosing bacterial infections.

(162) A Novel Galectin from *Coprinopsis cinerea* with an Altered Sugar Binding Specificity

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The isogalectins CGL1 and CGL2 from the model mushroom *Coprinopsis cinerea* were the first fungal galectins identified. Analysis of the recently released *C. cinerea* genome sequence revealed a third putative galectin termed CGL3. This protein which is expressed in the fungus contains all conserved residues known to be involved in β-galactoside binding except for the essential tryptophan residue which is responsible for the coordination of the galactose. Having instead an arginine residue located at the position of the essential tryptophan residue, recombinant CGL3 is no longer able to bind lactose. However, lactose binding is restored when the arginine residue is replaced by a tryptophan residue. This result suggested that the rest of the lactose-binding pocket is present and functional and that the original protein may display an altered sugar binding specificity. To determine the sugar binding specificity of CGL3, a glycan array of ~200 different glycans (resources provided by the Consortium for Functional Glycomics, Scripps Research Institute, La Jolla) was probed with recombinant CGL3. As the result of this screen, chitooligosaccharides as well as Lac-di-NAc were identified as potential CGL3 ligands. We confirmed these results by demonstrating specific binding of endogenous as well as recombinant CGL3 to chitin beads. As expected, the *C. cinerea* galectins, CGL1 and CGL2, failed to bind chitin under these conditions. Our results suggest that the sugar binding specificity of galectins can be fundamentally altered by change of a single amino acid residue.

(163) Structural Determination of Xantho Oligosaccharides and Their Biological Activities

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Xanthan gum is an anionic heteropolysaccharide produced by a plant-pathogenic bacterium, *Xanthomonas campestris*. Xanthan is composed of cellulosic backbone with linear trisaccharide side chains consisting of a mannosyl-glucuronyl-mannose sequence linked at the C-3 position on every other glucosyl residues. The internal and termian mannosyl residues of the side chain are frequently acetylated and pyruvylated, respectively, depending on both the growth conditions and the bacterial strain. Owing to its exceptional pseudoplasticity, high viscosity at low concentration, and tolerance toward a wide range of temperatures and pHs, its numerous areas of application cover a broad range, from the food industry to oil drilling. On the same time, xanthan's inertia can bring out some problems. For example, xanthan is an effective brine thicker for use in drilling mud compositions and also in the secondary and tertiary recovery of petroleum. However, the increased viscosity can often make subsequent processing more difficult. Moreover, with the potential for use of large quantities in this and other fields, there has been some concern advertisement to the effect on the environment. So the biodegradation of xanthan has become important research areas. People have found that some oligosaccharides have extraordinary biological properties, such as antimicrobial activity, antiviral activity, plant elicitor activity, and antioxidant activity. Hence, the question that whether xantho oligosaccharides have some biological activities becomes another impetus to the research on xanthan's biodegradation. In a

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continuation of our research work on searching for a xanthan-degrading strain, we obtained the partially purified xanthanase, and then xanthan's enzymic degradation products, xantho oligosaccharides. To elucidate the mixture composition, and the enzymic degradation mechanism, we used modern separation techniques and tools for structural determination, particularly HPAEC-PAD and MALDI-TOF mass spectrometry. According to xanthan gum's molecular structure, these six kinds of xantho oligosaccharides' structural formulas were deduced. Furthermore, because no oligosaccharide larger than pentasaccharide could be observed, the cleavage sites of xanthanase and the enzymic degradation process could be deduced. The xanthanase was neither a typical endoxanthanase nor a typical exoxanthanase. In fact, it could be called "a quasi exoxanthanase," which cleaved pentasaccharide units from two ends to the interior of xanthan molecular chains. Another purpose of this study was to investigate xantho oligosaccharides' biological activities. Xantho oligosaccharides showed good fungicidal activity against four fungi and high radical scavenging activity toward the 1,1-diphenyl-2-picrylhydrazyl-2-radical (DPPH).

(164) Investigating Host Glycan Influence on Adhesion of *Candida albicans* to Epithelial Cells

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When a microbe enters the milieu of the vagina or alimentary tract, it soon interacts with host glycoconjugates such as glycosylated soluble mucins or the glycocalyx of an epithelial cell. Microbes express adhesins, many of which have demonstrated specificity for different terminal carbohydrate modifications. These lectin-like interactions are specific for carbohydrate composition and linkage. Higher organisms have developed an apparent decoy mechanism whereby carbohydrates are presented to pathogens for binding but prevent deeper tissue invasion. Mucins, the predominant component of mucus, are high molecular weight glycoproteins that serve this function. Blood group antigens are major components of secretions and are primary terminal decorations on mucins. In wild-type individuals, called "secretors," FUT2 catalyzes the addition of α(1,2)fucose (E.C. 2.4.1.69) to cell surface and secreted ABO histoblood group antigens. In a subset of 15–20% of people, called "nonsecretors," null mutations of *FUT2* cause complete loss of ABO histoblood group epitopes in mucosal secretions. Nonsecretor status is associated with an increased relative risk for recurrent vaginitis by *Candida albicans*. Current antifungal treatments effectively target yeast metabolism for acute treatment, yet 5–10% of women of reproductive age are susceptible to recurrent yeast infections once they stop antifungal medication despite optimal current management. We have developed a preclinical mouse model system of experimental candidiasis whereby *Fut2*-null knockout mice that lack cervicovaginal α(1,2)fucosylated glycans are intravaginally inoculated with a clinical isolate of *C. albicans*. Similarly to human nonsecretors, *Fut2*-null mice display an increased susceptibility to *C. albicans* vaginitis compared with wild-type controls. Because mucins in secretors versus nonsecretors display different potential decoy antigens, our central hypothesis is that cervical mucins in wild-type animals displaying α(1,2)fucosylated epitopes sequester *C. albicans* and inhibit adhesion and subsequent penetration to deeper layers of vaginal epithelial cells better than nonfucosylated mucins in nonsecretors. The long-term goal of our laboratory is to develop mucin-based antiadhesive therapeutics to combat recurrent vulvo-vaginal candidiasis caused by *C. albicans*. To achieve this goal, we must determine what epitopes would provide the best decoys. A better understanding of the molecular basis for host–*Candida* interactions is needed. Hence, we have tested adhesion *in vitro* to determine the extent to which α(1,2)fucosylated epitopes mediate binding between *C. albicans* and vaginal and buccal epithelia. We discovered differences in yeast-epithelial binding between experimental groups. In addition, we have begun to pioneer methods for assaying whole yeast binding to glycan arrays available through the Consortium for Functional Glycomics to study yeast–glycan interactions. Because the yeast–hyphal morphogenic switch is important to virulence, we are testing an alternative hypothesis that fucosylated antigens inhibit this transition by performing germ tube inhibition assays. Taken together, these experiments will provide further insight into the yeast–host interaction involved in candidal pathogenesis.

(165) Cervical Mucus Plays an Essential Role in Susceptibility of *Fut2*-Null Mice to Experimental Vaginal Candidiasis

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The *Secretor* gene, *FUT2*, encodes an estrogen-responsive α(1,2)fucosyltransferase that elaborates α(1,2)fucose residues on mucosal epithelium and secreted mucins within the female reproductive tract. Approximately 20% of the human population possess a mutation within the coding region of this gene which is associated with a higher relative risk of recurrent vulvovaginal candidiasis. Using *Fut2-LacZ* null mice as an animal model for human nonsecretors, we have previously shown these mice display an increased susceptibility to *Candida albicans* infection in an estrogen-dependent model of yeast vaginitis. *Fut2* is expressed within the glandular epithelium of the uterus and endocervix, but absent in the vaginal squamous epithelium. *Ulex europaeus* agglutinin I (UEA-I) lectin staining, however, demonstrates the presence of α(1,2) fucosylated glycans at the apical surface and lumen of the vagina and on epithelial cells isolated from vaginal lavage of C57BL/6J wild-type mice. Given this discrepancy, we propose that α(1,2)fucosylated endocervical mucins descend into the vagina, coat exposed epithelial cells providing protection against fungal colonization. To test this hypothesis, vaginal epithelial cells isolated by lavage from wild-type mice which had undergone hysterectomy (including removal of ovaries and cervix) revealed the absence of UEA-I staining which correlated with a total loss of Alcian blue pH 2.5 mucin staining. To analyze the role of cervical mucus in susceptibility to vaginal candidiasis, wild-type and *Fut2*-null mice either received a total hysterectomy with oophorectomy or ovariectomy alone before inoculation with *C. albicans*. Wild-type mice that received hysterectomies displayed no differences in yeast fungal burden compared with ovariectomized control wild-type mice. In contrast, removal of uteri and cervixes of *Fut2*-null mice resulted in a 3-fold reduction in susceptibility to experimental vaginal candidiasis compared with ovariectomized control *Fut2*-null mice. These data suggest that the presence of the cervix/uterus is overall provirulent, whereas expression of Fut2 reduces susceptibility by masking epitope(s) that would otherwise promote susceptibility to *C. albicans*.

(166) A New Method for Bacterial Glycomics

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The biological role of glycosylation in eukaryotes has been studied in many important processes, such as development, differentiation, and malignancy. The concept of prokaryotic glycosylation has only recently been accepted. There is increasing evidence of bacterial glycoproteins and their role in pathogenesis. What is even less understood is the role of glycosylation in microbial cell–cell and cell–matrix interactions. The microheterogeneity of glycosylation demands more rapid and high-throughput methods of characterizing the surface glycans of bacteria. Conventional methods such as agglutination assays, lectin type ELISA, and surface plasmon resonance are not practical for high-throughput analysis of complex bacterial glycans. Microarray technology is the ideal format for the systematic analysis of surface glycosylation in bacteria. In previous work, our laboratory demonstrated that glycosylation patterns of individual proteins could be determined using a lectin array. Using a simple fluorescence protocol, we extend this technology to probing bacterial surface glycans. In our initial experiments, 21 commercially available lectins were used to discriminate between *Escherichia coli* strains. In fact, closely related K12-derived strains could be distinguished based on distinct glycan patterns. The ability to establish reproducible glycan patterns for bacterial surfaces sets the stage for studying the role of glycans in pathogenicity and host–cell interactions.

(167) Implications of Pilin Glycosylation in Pathogenesis of *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae, the gonococcus (GC), causes the sexually transmitted disease gonorrhoea (uncomplicated gonorrhoea, UG), as well as pelvic inflammatory disease (PID) and disseminated gonococcal infection (DGI); the latter two conditions are actually considered extensions/complications of the former. Pili of GC, which are outer membrane surface filaments, are made up of multimers of pilin glycoprotein subunits—the pilin polypeptides are O-glycosylated at the Ser-63 residue. Formation of pili is essential for virulence of GC as GC pili initially bind to host cell surface and play important role in early cellular adherence process on mucosal surfaces. After this initial interaction, GC forms a tighter association with the target cells that in many cases leads to subsequent internalization into the host cell. This step is followed by transcellular migration of the bacteria from the apical side of the invaded cell toward its basal end. This study was focused on understanding the pathogenic roles of pilin glycosylation (pgl) activities. We are presently studying the effect of all of the possible pgl enzymes encoded within GC genome. Genetic analysis of different pgl mutants were made by using the current molecular tools/techniques including PCR, restriction analysis, cloning, DNA sequencing, and RT–PCR. Wild-type (WT) and mutant pilin proteins were studied by SDS–PAGE, silver staining, western blotting, high pH anion exchange chromatography in combination with pulsed amperometric

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detection (HPAE-PAD), mass spectrometry (MS), GC-MS, and MS-MS linkage analysis. Adherence and invasion assays were performed using infection models employing HEC-1-B endometrial cells and ME-180 cervical cells. Monolayers of T84 human colonic epidermoidal cells which form tight junctions were mainly used for transmigration assays. Through this study, we evaluated adhesion, invasion, and transmigration abilities of isogenic mutants of several GC strains. These mutants contain shorter glycoforms, which would match the pilin glycan structures of some of the natural phase variants that naturally carry such shorter glycans. Overall, GC pilin glycan mutants showed substantially increased invasion compared with their corresponding WT. This observation correlates well with the inherent properties (i.e., phase variability) of those pilin glycosylation genes that can be switched on/off. Such switching likely creates shorter glycan structures than those of the WT mentioned above, and thus such phase variations are expected to help the bacteria to spread deeper into the body. Conversely, it seems that the longer glycoforms are more important for initial attachment of the bacteria to the host mucosal cells. However, for further invasion and transcellular migration, the shorter glycoforms appear to be advantageous. Once the bacteria are tightly adhered to the host cell, they seem to get rid of the pilin glycan by phase variations (longer pilin glycoforms to shorter glycoforms) which help them to invade faster and deeper inside the host causing complications like PID and DGI. Thus, GC pilin glycan may have a minor role in establishment of infection, but it apparently has a major role in the systemic spread of GC, as well as in the overall pathogenic mechanism of this bacterium. Both A.W. and S.K.G. contributed equally towards this project.

(168) A Beta-1,2-Xylosyltransferase from *Cryptococcus neoformans*

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Cryptococcus neoformans is a pathogenic fungus responsible for serious disease in immunocompromised individuals. The main distinguishing feature of this budding yeast is an elaborate polysaccharide capsule that surrounds the cell wall and is absolutely required for its virulence. The capsule is primarily composed of two extensive polysaccharides. One is based on an alpha-1,3-mannose backbone that is heavily 6-O-acetylated and bears monosaccharide side chains of glucuronic acid and xylose in a repeating pattern. The second is based on a linear alpha-1,6-galactose chain with slightly longer side chains of galactose, mannose, and xylose. Previous studies have shown that both the presence and arrangement of the xylose residues within these capsule polysaccharides are important determinants of virulence. The enzymes responsible for transferring xylose to these polysaccharides are therefore of great interest, but none of these xylosyltransferases (XTs) have been identified. Based on the known capsule polysaccharide structures, we hypothesize that there are multiple XTs in *C. neoformans*, each of which may be relevant to the virulence of this pathogen. Because no sequences within the cryptococcal genome align with the few known XTs from other organisms, we took a biochemical approach to identifying these enzymes. We have now purified one of the cryptococcal XTs, using an assay that monitors the transfer of ^{14}C-xylose from a UDP-^{14}C-xylose donor to an alpha-1,3-linked dimannoside acceptor. We enriched the XT activity ~3000-fold from a detergent-solubilized membrane preparation, using conventional and novel chromatography resins. Mass spectrometry data from a candidate species that migrated at ~90 kDa on SDS–PAGE matched sequence of a cryptococcal protein encoded in the newly released *C. neoformans* genome sequence. The corresponding gene was identified and named *CXT1*. Expression of this gene in *Saccharomyces cerevisiae* resulted in appearance of the XT activity. NMR analysis of the enzymatic product isolated from assays of cryptococcal material confirmed a beta-1,2 linkage between the xylose and the reducing mannose of the dimannoside acceptor, consistent with structures present in the native capsule polysaccharides. Interestingly, neither the purified nor expressed Cxt1p activities were cation dependant. We have now generated a strain of *C. neoformans* that is deleted for *CXT1*. We are analyzing this deletion strain, including NMR and electron microscopic examination of capsular polysaccharides and assessment of virulence in a mouse model. We have also identified a group of sequences in the *C. neoformans* database with homology to *CXT1*. We are expressing these proteins in *S. cerevisiae* for activity determination and disrupting the genes in *C. neoformans* to assess phenotype. Studies of cryptococcal XTs are supported by NIGMS R01 71007 to T.L.D. and NIGM F32 72341 to J.S.K.

(169) Binding of *Pseudomonas aeruginosa* Lectin LecB to Cystic Fibrosis Airway Cells is Inhibited by Fucosylated Compounds: Implications for Therapy

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Cystic fibrosis (CF) is the most common, lethal-inherited disease in the United States. The genetic defect, which underlies CF, has been identified (Riordan *et al*., 1989) and research is currently underway to define the precise mechanisms of the molecular pathogenesis of the disease. Airways from CF patients are particularly susceptible to chronic *Pseudomonas aeruginosa* colonization. This pathogen is the major cause of morbidity and mortality in patients with CF. *P. aeruginosa* synthesizes two lectins, LecA and LecB (PA-IL and PA-IIL, respectively). The crystal structure of the PA-binding protein, LecB, has been solved and shown to contain an anionic "dock" which allows optimal binding of fucosylated structures (Loris *et al*., 2003; Mitchell *et al*., 2002, 2005). Hence, the CF glycosylation phenotype of increased fucose and decreased sialic acid on airway surface membranes could predispose CF airways to *P. aeruginosa* colonization via LecB binding (Scanlin and Glick, 1999). On the basis of LecB concentration curves for both CF and non-CF cells, we used 0.16 μM lectin (prepared by Kinnakeet Biotechnology, Richmond, VA). We show that CF/T43 and CF primary airway cells bound significantly more biotinylated LecB protein than BEAS-2B (non-CF airway cells) or non-CF cells in primary culture. Incubation with lectin-binding buffer containing LecB and a specific carbohydrate (1:30) resulted in significant inhibition of binding to the cells in the following order: lacto-*N*-fucopentaose II (LNFP II) > Lewis a > L-fucose > Lewis x. Thus, we show that LecB protein binds to carbohydrate ligands containing Fuc a1,3/4GlcNAc which are in greater amounts on the surface of CF airways cells compared with non-CF airway cells. In microtiter assay, the inhibition curve for each glycomimetic oligosaccharide necessary to inhibit the binding of LecB to CF airway epithelial cells was determined. The binding of LecB to the surface of CF cells was 100% inhibited with 10 μM Lewis a or LNFP II. Recently, Tielker *et al*. (2005) demonstrated that LecB is abundantly present in the bacterial outer membrane fraction, a finding that could explain the lectin-mediated cytotoxic and adhesive properties. These results are in support of the proposal for glycomimetic blockade to prevent the colonization of *P. aeruginosa* in CF airways. Supported in part by Department of Pediatrics, RWJMS-UMDNJ, CF Foundation (JE) and the Nurmi Foundation.

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(170) Inactive *Trypanosoma cruzi Trans*-Sialidase Recognizes a 36-KDa Protein on Endothelial Cell Surface

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Communication between *Trypanosoma cruzi* (the causative agent of Chagas' disease) and mammalian cells is initiated by contact of parasite surface and cognate host cell molecules. Previously, we have demonstrated that an enzymatically inactive form of *T. cruzi trans*-sialidase (iTS) behaves as a lectin which binds and triggers contact-dependent activation of NF-êB pathway on endothelial cells. iTS increases expression of adhesion molecules, up-regulates parasite invasion of host cells, and rescues endothelial cells from apoptosis by increasing

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expression of Bcl-2. In this work, we aimed at identifying the receptor(s) involved in the interaction between iTS and human bone marrow endothelial cells (HBMEC). Using affinity purification, we demonstrate that iTS specifically binds to a 36-KDa protein from HBMEC lysate. Amino acid sequencing of tryptic peptides, followed by analysis by mass spectrometry (electron spray ionization/ion TRAP), showed an iTS coreceptor which was identified as annexin II with a confidence level approaching 100%. Flow cytometry studies demonstrated that anti-annexin II antibodies prominently stained the external surface of HBMEC cells and preincubation of these cells with iTS, abrogated the binding. Furthermore, antibodies against annexin II decreased iTS-PE binding to HBMEC. Together, these results strongly suggest that iTS interacts with annexin II on the endothelial cell surface. The ability of iTS to recognize annexin II, a molecule abundantly expressed on endothelial cells, may mediate vascular injury observed during *T. cruzi* infection.

(171) *Pseudomonas aeruginosa* Mucoid Strain 8830 Binds Glycans Containing Sialyl Lewis X Epitope

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Pseudomonas aeruginosa infection of patients with cystic fibrosis (CF) is a leading cause of their morbidity and mortality. Pathogenesis is initiated in part by molecular interactions of *P. aeruginosa* with carbohydrate residues in airway mucins that accumulate in the lungs of patients with this disease. To explore the nature of the glycans recognized by a stable, mucoid, alginate-producing strain *P. aeruginosa* 8830, we generated a genetically modified Pa8830 expressing green fluorescent protein (Pa3380-GFP) and tested its binding to a panel of glycolipids and neoglycolipids in which selected glycans were covalently attached to dipalmitoyl phosphatidylethanolamine and analyzed on silica gel surfaces. Among all glycans tested, Pa8830-GFP bound best to sialyl Lex-containing glycan NeuAca2-3Galb1-4(Fuca1-3)GlcNAc-R and bound weakly to H-type blood group Fuca1-2Galb1-4GlcNAc-R, sialyl-lactose, and nonsialylated (Lex), with only poor binding detected toward nonfucosylated derivatives. Interestingly, although the Pa8830-GFP bound to the glycosphingolipid asialoGM1, but did not appear to bind to a wide variety of other glycosphingolipids including GM1, GM2, asialoGM2, and sulfatide. These results indicate that *P. aeruginosa* 8830 has preferential binding to sialyl Lex-containing glycans and has weak recognition of related fucose- and sialic acid-containing glycans. The finding that Pa8830 binds sialyl Lex-containing glycans, which occur at increased levels in mucins from CF patients, is consistent with studies of other strains of *P. aeruginosa* and further suggest that such glycans on CF mucins contribute to disease pathogenesis.

(172) Remodeling of High-Phosphate *Penicillium mycelia* in Citrate Buffer: A Comparison of Freeze-Dried Mycelia with Control Mycelia

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Fungal cell walls contribute to the resiliency of fungi to the environment and are key components in fungal resistance to modern antifungal therapies. This study monitored the change in cell wall surfaces of two groups of *Penicillium fellutanum* mycelia, live and freeze-dried, while suspended in sodium citrate buffer for a period of 0–24 h. The mycelia were obtained from liquid-shake cultures grown in high phosphate (20 mM) standard growth media (HPSG) for 5 days. The mycelia were harvested and split into two groups, with one group serving as controls, whereas the remaining mycelia were freeze-dried at –68°C. Both groups were placed in a 0.1 M sodium citrate, pH 4.5 buffer. Samples were taken hourly for 10 h with a single sample taken at the 24-h mark. The samples were filtered, and the amount of carbohydrate, phosphate, and protein released from the mycelia was determined in the filtrates. Carbohydrate analyses were performed on filtrates and remaining mycelia samples. Carbohydrate, phosphate, and protein analyses of filtrates indicate that extensive remodeling of cell surfaces primarily involves loss of protein with concomitant smaller losses of carbohydrate and phosphate residues. In parallel to the chemical analyses of filtrates, we probed surface characteristics of the resulting insoluble fractions by atomic force microscopy (AFM). A total of 180 sample images were obtained per group of mycelia. Mycelial surfaces were mapped using AFM by imaging surface areas 800 nm × 800 nm in size. Root mean squared (RMS) surface roughness and phase shift data were collected to compare and contrast any similarities or differences in the surfaces of the two study groups. This research was supported by NIH/NIGMS MBRS SCORE grant 2S06 GM08197.

(173) Functional Analysis of a UDP-GlcNAc : Thr Polypeptide *N*-Acetyl-D-Glucosaminyltransferase-Like Gene in *Trypanosoma cruzi*

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Mucin-like glycoproteins are the major surface components in different life cycle stages of *Trypanosoma cruzi*, the causative agent of Chagas disease, and likely participate in both host– and vector–parasite interactions. Although these heterogenous and highly O-glycosylated glycoproteins are known to be encoded by a multicopy family of genes, all attached *O*-glycans have at their reducing end an α-*O*-*N*-acetylglucosamine residue linked to Thr. Therefore, inhibition of mucin-type O-glycosylation biosynthesis at the point of GlcNAc addition to the protein seems to be a good drug target to be explored against the parasite. The enzyme responsible for the addition of this first sugar residue, a pp-αGlcNAcT, has been characterized biochemically (Previato *et al*., 1998) and shown to be a Golgi resident protein (Morgado-Diaz *et al*., 2001). To characterize this enzyme at the molecular level, we initially used phylogenetic and bioinformatics approaches. An early BLAST search of the recently released *T. cruzi* genome database (El-Sayed *et al*., 2005) yielded three Type-2 membrane protein sequences showing similarity to cytoplasmic (Van Der Wel *et al*., 2002) and Golgi (Wang *et al*., 2003) pp-αGlcNAcTs of the social amoeba *Dictyostelium discoideum* in their catalytic domains. Interestingly, although candidate orthologs of all three sequences were also found in the other Tritryp organisms *Trypanosoma brucei* and *Leishmania major*, no natural acceptors containing Thr- or Ser-linked αGlcNAc have been described so far. The coding sequences of the three putative *T. cruzi* pp-αGlcNAcT proteins, except for their N-terminal signal sequences, were amplified by PCR, cloned into an integrating expression vector for secretory proteins (pVS4) and expressed in modB-mutant *D. discoideum* cells that lack the Golgi pp-αGlcNAcT. One of the putative *T. cruzi* pp-αGlcNAcT-like sequences (TcE5) partially complemented the absence of endogenous pp-αGlcNAcT, as determined by a western blotting study using mAb 54.2 that detected an α-linked GlcNAc-dependent epitope on a 110 kDa protein. Biochemical studies to establish TcE5 as a functional pp-αGlcNAcT are currently being pursued. Supported by CNPq, FAPERJ, IFS, OCAST, and NIH.

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(174) *Microbacterium nematophilum* Infection of the *Caenorhabditis elegans* Cuticle Requires Galactosyl Oligosaccharides

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The srf mutants display ectopic lectin binding at the cuticle surface. The bus mutants have altered susceptibility to *Microbacterium nematophilum* infection. The srf-3 mutants are also resistant to *M. nematophilum*. Srf-3 encodes a UDP-Gal and UDP-GlcNAc nucleotide sugar transporter. Bus-4 encodes a core-I type galactosyltransfersase homologue. In a previous study, we found that srf-3 mutants are deficient in *N*- and *O*-glycans that contain galactose. As bus-4 encodes a core-I type galactosyltransferase homologue, the increased resistance

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of both srf-3 and bus-4 suggests that galactosyl O-glycoproteins of the cuticle surface are required for *M. nematophilum* infection. Aganicus bisporus agglutinin (ABA) has high affinity to Gal*f*Ö1,3GalNAc, the core-I disaccharide. ABA blot analysis shows that cuticle associated glycoproteins of bus-4 are shifted to lower molecular weight compared with wild-type nematodes and are similar to those of srf-3 mutants. These data are consistent with a decreased amount of core-I like oligosaccharide in bus-4. Preliminary structural comparisons of the *N*- and *O*-glycans of bus-4 and wild-type stains are presented. This research is supported by NIH grant numbers P41RR10888 (C.E.C.) and R01HL074197 (J.Z.).

(175) Mass Spectrometry Strategy for the Determination of N-Glycosylation Patterns in Uroplakins

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Two structurally related glycoproteins, the uroplakins (UP) Ia and Ib, interact with UP II and III, to form 16 nm particles hexagonally packed to form two-dimensional crystals that cover almost the entire apical surface of mammalian bladder epithelium. It has been proposed that glycosylation patterns of the UPs determine the binding efficiency of bacteria that cause urinary tract infections. A rapid and sensitive MS strategy has been utilized in this study for the structural determination of the glycans and the identification of occupied glycosylation sites. The results should contribute to a better understanding of the mechanism of urinary tract infection and to improvements in its diagnosis and treatment. Murine and bovine UPs Ia and Ib were purified by SDS–PAGE. Bands of interest were excised and deglycosylated in-gel with PNGase F, and the extracted glycans were subjected to permethylation. Tryptic digestion of the proteins was performed in-gel, after release of the *N*-glycans. The peptides and the permethylated oligosaccharides were characterized using a Bruker Reflex IV matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer and further analyzed using a QSTAR Pulsar *i* quadrupole-orthogonal TOF mass spectrometer (QoTOF MS). The carbohydrates and peptides of interest were sequenced by MS/MS. By these means, we verified the amino acid sequences of the proteins and determined the pattern of glycoform heterogeneity at the single glycosylation site in UPs Ia and Ib from bovine and murine samples. Bovine UP Ia/Ib were found to contain a series of high-mannose type N-linked glycans at Asn131 of UP Ib and Asn170 of UP Ia. The N-linked glycan population at Asn169 in murine UP Ia was determined to be a series of high-mannose glycans, whereas murine UP Ib was found to contain a series of multiple-antennary complex, high mannose, and hybrid N-linked glycans at Asn131. The permethylated glycan pool generated in this study allowed relative quantification of glycan constituents. The survey on the distribution of glycoforms in UP Ia and Ib was carried out using MALDI-TOF MS. The main glycoforms of murine UP Ia were found to be the high mannose glycans containing 7, 8, and 9 mannose residues, whereas those of murine UP Ib were found to be mainly complex glycans (>85% of the glycoforms), along with small amounts of high mannose and hybrid glycans. MALDI MS profiles of the native and permethylated glycans from bovine UP Ia/Ib suggested that the observed profile of native glycans is in good agreement with the results obtained after permethylation, by both the identities and distributions of glycoforms. Our results provide a biochemical explanation for the observation that the Type 1-fimbriated, uropathogenic *Escherichia coli* bacteria bind to murine uroplakin 1a, but not to the closely related murine uroplakin Ib. This work was supported by NIH grants P41 RR10888, S10 RR15942 (to C.E.C.), and P01-DK52206 (to T.T.S.).

(176) Formation of a New O-Polysaccharide in *Escherichia coli* O86 via Disruption of Glycosyltransferase Gene Involved in O-Unit Assembly—An Example of Relaxed Substrate Specificity of O-Antigen Polymerization

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The majority of heteropolysaccharide biosynthesis in gram-negative bacteria utilizes the wzy-dependent pathway, in which repeating O-units are first synthesized in the cytosol, and the subsequent translocation into the periplasm initiates the polymerization process. Because of the vast variety of O-polysaccharide structures and the lack of common sequence motifs in Wzy polymerase, it is hypothesized that O-polysaccharide polymerization process has strict O-unit substrate specificity. In this work, we tested the abovementioned idea in *Escherichia coli* O86 strain. Glycosyltransferase gene wbnI in the O-antigen gene cluster was biochemically characterized responsible for the synthesis of α-1,3-linked galactose residue as the side chain of the O-polysaccharide. By disruption of the wbnI gene, we demonstrated that the mutant strain produced a different O-polysaccharide. Structural analysis by NMR, MS and methylation revealed that the new polysaccharide contains the backbone of the polysaccharide produced in wild-type strain without the galactose side chain. Therefore, the biochemical data presented in this work represent an example that polymerization process in O-antigen biosynthesis has relaxed substrate specificity towards O-unit side chain truncations. This work sets a new opportunity to a better understanding of O-antigen polymerization mechanism and to further bioengineer novel O-polysaccharide structures.

(177) Structural Characterization of Toxin-Binding Gangliosides by TLC/VC-FTMS

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Gangliosides which bind cholera-related AB5 toxins are desorbed directly from TLC plates, and their structures are explored by MALDI-FTMS and MS/MS. GM1 and GD1a gangliosides serve as trafficking receptors for the cholera toxin and related LTIIb toxin, respectively. LTIIb is not active in human intestinal cells because the LTIIb-GD1a complex does not move retrograde from the plasma membrane into the endoplasmic reticulum (Fujinaga *et al*., 2003). Here, we test the idea that structural variation in the GD1a lipid anchor explains the failure of this ganglioside to act as a trafficking receptor. To address this problem, we are using our previously developed method of direct coupling of TLC plates with vibrationally cooled (VC) MALDI-FTMS. This allows direct TLC-MALDI-FTMS without adversely affecting the FT high resolution by the irregular surface of the TLC plate. Collisional cooling is necessary for stabilization and detection of intact gangliosides. We are using polarized intestinal epithelial cell line T-84 and monkey kidney Vero cells for ganglioside purification and functional studies on the mechanism of toxin biology (Wolf *et al*., 1998). We have described ganglioside separations and instrumental parameters for VC MALDI-FTMS (Ivleva *et al*., 2004). In this study, the samples are MALDI-desorbed directly off TLC plate surfaces and thermalized by a pulse of the cooling gas. Subsequently, fragmentation is performed by SORI-CAD and IRMPD techniques. For GC-MS studies, the gangliosides were subject to methanolysis, followed by N-acetylation and addition of TMS reagent. Preliminary results showed that, in addition to a variety of oligosaccharide headgroup compositions, the ceramide structure in gangliosides from both cell lines exhibit substantial heterogeneity. The high separation efficiency of the HP-TLC plate allowed for observation of numerous homologs following each scanning step. This was demonstrated by analysis of the "pure" synthetic gangliosides and whole brain extract. A high level of ganglioside fucosylation was observed in both cell lines. Vibrational cooling resulted in stabilization of the labile sialic acid and fucose glycosidic linkages, and this feature was highly advantageous for the analysis of the heterogeneous mixtures. Mass accuracy and resolution were not affected by desorption from the uneven TLC plate surface. Compared with SORI-CAD, IRMPD demonstrated more efficient fragmentation of both parent and product ions. GC-MS analysis of the fatty acid methyl esters showed a predominant amount of 18:0 among all fatty acids. The two cell lines also expressed different distributions of 18:2, 20:2, and 20:0 fatty acids. Further analysis will focus on structural elucidation of the ceramide moiety by length, degree of saturation, hydroxylation, and branching.

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(178) Lactadherin: O-Linked and N-Linked Glycan Analysis

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The principal cause of gastroenteritis in infants and young children is attributed to rotavirus infection. Breast-feeding offers protection against enteric diseases

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through the milk mucin complex, which inhibits rotavirus replication. Lactadherin is an acidic component of the milk fat globule membrane, which shows the highest viral-binding activity. This glycoprotein competes with the rotavirus host cell receptor and inhibits viral propagation. Lactadherin looses its antiviral properties after deglycosylation or after desialylation. To identify the specific structures that generate viral affinity and other type of interactions, we used sequential analysis of O-linked and N-linked glycans followed by peptide mass fingerprinting. MALDI mass spectrometry profiles reveal various and abundant oligosaccharides, containing sialic acid and fucose residues. Tandem mass spectrometry was employed for detailed structural characterization of permethylated glycosides and glycosylation site occupancy. Unveiling lactadherin structure allows for understanding of its various biological interactions and for generating synthetic viral inhibitors.

Session Topic: Glycan Immunology

(179) DC-SIGN Mediates Binding of Dendritic Cells to Authentic Pseudo-Lewis Y Glycolipids of *Schistosoma mansoni* Cercariae—The First Parasite-Specific Ligand of DC-SIGN

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During schistosomiasis, parasite-derived glycoconjugates play a key role in manipulation of the host's immune response, associated with persistence of the parasite. Among the candidate host receptors that are triggered by glycoconjugates are C-type lectins on dendritic cells (DCs), which in concerted action with toll-like receptors determine the balance in DCs between induction of immunity versus tolerance. Recently, we showed that the C-type lectin DC-specific ICAM-3 grabbing non-integrin (DC-SIGN, CD209) binds to *Schistosoma mansoni* egg glycoproteins (SEA) via Galβ1-4(Fucα1-3)GlcNAc (Le^X) and GalNAcβ1-4(Fucα1-3)GlcNAc (LDNF) (van Die *et al*., 2003; Van Liempt *et al*., 2004). To investigate the role of schistosome glycoconjugates in modulation of the host immune response, we set out to characterize the natural ligands of schistosomes that interact with DC lectins. Our data show that DC-SIGN mediates adhesion of DCs to authentic glycolipids from *S. mansoni* cercariae and their excretory/secretory products. Structural characterization of the glycolipids, in combination with solid-phase and cellular binding studies, revealed that DC-SIGN binds to the carbohydrate moieties of both glycosphingolipid species with Le^X and Fucα1-3Galβ1-4(Fucα1-3)GlcNAc (pseudo-Le^Y) determinants. In contrast to Le^X that is found in mammals and several pathogens, the pseudo-Le^Y determinant has only been found within schistosomes (Wuhrer *et al*., 2004). Importantly, these data indicate that surveying DCs in the skin may encounter schistosome-derived glycolipids very early in infection, when the cercariae invade their host. Recent analysis of crystals of the carbohydrate-binding domain of DC-SIGN bound to Le^X, in combination with adhesion studies, provided insight into the ability of DC-SIGN to bind fucosylated ligands (Guo *et al*., 2004; Van Liempt *et al*., 2004). Using molecular modeling, we show here that the observed binding of the schistosome-specific pseudo-Lewis Y to DC-SIGN is not directly compatible with the model described. To fit pseudo-Lewis Y into the model, reorientation of the side chain of Phe313 in the secondary binding site of DC-SIGN was essential and resulted in a perfect stacking of Phe313 with the hydrophobic side of the galactose-linked fucose of pseudo-Lewis Y. We propose that pathogens such as *S. mansoni* may use the observed flexibility in the secondary binding site of DC-SIGN to target DCs, which may contribute to immune escape.

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(180) Intracellular Mannan-Binding Protein and Its Physiological Significance

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Mannan-binding protein (MBP) is a C-type mammalian lectin specific for mannose and *N*-acetylglucosamine. MBP synthesizes mainly in the liver and occur naturally in two forms, secretory serum MBP (S-MBP) and intracellular MBP (I-MBP), both of them are coded by a single form of MBP cDNA in human. S-MBP activates complement mediated by the MBP-associated serine proteases (MASPs) via lectin pathway. On the other hand, little is known about the function of I-MBP. In the previous studies, we reported the identification and characterization of the several I-MBP intracellular ligands isolated from rat liver and primary cultured hepatocytes (Mori *et al*., 1988). Here as an extension of the studies, the expression of human MBP cDNA reproduced the native MBP maturation and differentiation of S-MBP and I-MBP in human hepatoma cell lines. I-MBP showed distinct accumulations in cytoplasmic granules, accumulated in the ER exit site, and predominantly localized in ER and COPII vesicle mediated ER-to-Golgi transport and partially in Golgi. Interestingly, the mutant (C236/244S) MBP, which lacked the carbohydrate-binding activity, just dispersed in ER neither accumulated nor localized in COP II vesicle and Golgi. Furthermore, the binding of I-MBP with intermediate of glycoprotein occurred in the ER was carbohydrate- and calcium-ion-dependent and was affected both by untrimmed and trimmed glucose residues. The association and dissociation of I-MBP with oligomannose saccharides in organelles are regulated by pH. Our findings suggest that I-MBP may function as a cargo transport lectin in selective glycoprotein quality control.

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(181) Carboxylated *N*-Glycans on RAGE are Critical Determinants of S100A12 Binding

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RAGE is a pattern-recognition and signaling receptor protein of the immunoglobulin superfamily. Structurally diverse ligands bind RAGE through its extracellular V-type domain where two N-linked glycosylation sites are located. We earlier showed that a subpopulation of RAGE molecules are modified by carboxylated glycans. RAGE–ligand interactions activate NF-kB, increase expression of cytokines and adhesion molecules, and promote inflammation, tumor growth, and metastasis. Indeed, mAbGB3.1, a monoclonal antibody that recognizes the carboxylated glycans, blocks onset of colitis in an adaptive transfer model, and it reverses colitis in the emerging phase of disease by blocking NF-kB activation. To study the role of glycans in RAGE–ligand interactions, we first purified RAGE to homogeneity from bovine lung using an anti-RAGE immunoaffinity column. Both total RAGE and the mAbGB3.1-enriched subpopulation of RAGE bind to S100A12 (K_d = ~50 nM). However, the mAbGB3.1-binding subfraction has a 3-fold higher Bmax for S100A12, strongly suggesting that mAbGB3-reactive oligosaccharides actually select or promote assembly of multimeric S100A12 complexes. S100A12 is known to exist as a hexamer. Deglycosylation of mAbGB3.1 enriched RAGE leads to >90% reduction in S100A12 binding, suggesting that ligand binding is almost completely dependent on these glycans on RAGE. To study the nature of the oligosaccharides on RAGE and to generate soluble RAGE inhibitors of S100A12 binding, we generated a His-tagged construct of the extracellular domain of RAGE (sRAGE) and expressed it in HeLa cells. About 1–2% of purified sRAGEHela protein and 1–2% of labeled, PNGase-released *N*-glycans contain the mAbGB3.1 epitope. QAE and Con A analysis of [2–3H] mannose-labeled sRAGE oligosaccharide showed that unfractionated sRAGE carries both complex and hybrid/high mannose chains, whereas the mAbGB3.1 purified material carries predominantly multiantennary, highly charged species. As with bovine lung-derived RAGE, the Bmax for S100A12 binding/per RAGE

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molecule is 3-fold higher in the mAbGB3.1-purified fractions of sRAGEHela, reinforcing our hypothesis that mAbGB3.1 reactive glycans bind multimers or promote multimeric assembly. Again, deglycosylation leads to loss of S100A12 binding. When we compared four different sources of RAGE, we found a direct correlation between mAbGB3.1 reactivity of the individual RAGEs and their Bmax for S100A12. We also mutated the two glycosylation sites individually (N25Q and N81Q), generated recombinant retroviral constructs, and expressed them in HeLa cells. The first glycosylation site (N25IT) is modified by a fully processed *N*-glycan chain that retains S100A12 binding and mAbGB3 reactivity. Glycosylation at the second site (N81GS) is variable (complex, hybrid, or high mannose), shows reduced S100A12 binding, but is important for secretion of sRAGE. These results demonstrate that *N*-glycans on RAGE are critical for S100A12 binding. Efforts are now underway to test the effects of mAbGB3.1 and different sRAGEs on intracellular signaling induced by S100A12 in stable transfectants of HeLa cells expressing either full-length or signaling-deficient, cytoplasmic tail-deleted RAGE. (Supported by NIH grant R01-CA92608.)

(182) The Role of Neck Region Polymorphism in DC-SIGN and DC-SIGNR Tetramer Formation

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DC-SIGN plays two roles in dendritic cells. It recognizes pathogens, leading to their internalization, and it binds to ICAM-3 on T-cells to facilitate T-cell receptor interaction with MHC–peptide complexes. It also interacts with ICAM-2 to mediate rolling of dendritic cells on endothelium. Both DC-SIGN and the closely related endothelial cell receptor DC-SIGNR bind to human immunodeficiency virus (HIV), hepatitis C virus, and Ebola virus through high-mannose oligosaccharides on the virus surfaces. The interaction with HIV increases the efficiency of T-cell infection. DC-SIGN also binds to fucose-containing glycans including those found on schistosomes and *Helicobacter pylori*. DC-SIGN and DC-SIGNR are tetrameric Type II transmembrane proteins, each consisting of an N-terminal intracellular domain, a transmembrane segment, an extracellular neck region, and a C-terminal C-type carbohydrate recognition domain (CRD). Formation of the tetramer facilitates high affinity binding to clusters of high mannose oligosaccharides, such as those found on HIV. The neck region of DC-SIGN is comprised of 7.5 highly conserved repeats containing 23 amino acids. The neck region of DC-SIGNR is polymorphic. The number of repeats varies from 4.5 to 8.5, with 7.5 repeats being the most common form. Extracellular segments lacking the first and second N-terminal repeats exist as a mixture of dimers and tetramers, demonstrating that these two repeats play a major role in stabilizing the tetramer. To investigate further the mechanism of oligomer formation and the overall tetramer structure on the cell surface, we cloned DC-SIGNR polymorphic forms with 6.5, 5.5, and 4.5 repeats. These forms lack repeat 5, repeats 5 and 6, and repeats 5, 6, and 2. The recombinantly expressed extracellular segments of DC-SIGNR with 6.5 and 5.5 repeats form stable tetramers in solution. The 4.5-repeat form, lacking the second repeat, produced only monomers. This result is consistent with the earlier observation that the first two repeats are important to tetramer stabilization. Because the length polymorphisms in DC-SIGNR are common, many individuals are heterozygotes and have genes encoding two different forms of DC-SIGNR. Therefore, it was interesting to determine whether these polypeptides can form hetero oligomers. The 7.5-repeat form was tagged by modifying its CRD so that it binds galactose, and the ability of this protein to associate with smaller forms was tested. Heterotetramers were detected with the extracellular segments consisting of 7.5 + 6.5 repeat forms and with 7.5 + 5.5 repeat forms, but 7.5- and 4.5-repeat forms associated only weakly. Full-length 7.5- and 6.5-repeat forms coexpressed in fibroblasts form hetero oligomers on the cell surface. The capacity for heterotetramer formation between 7.5-repeat and shorter polymorphic forms suggests that the tetramers of DC-SIGN and DC-SIGNR might be formed by two dimers associated at the N-termini of their neck regions. The biological effects of the polymorphism are being investigated.

(183) Characterization of Carbohydrate Ligands Recognized by Mannan-Binding Protein on SW1116 Cells

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Mannan-binding protein (MBP) is a C-type mammalian lectin specific for Man, GlcNAc, and Fuc. The serum MBP activates complement through the lectin pathway and is an important component associated with innate immunity. MBP has a potent growth inhibitory activity to a human colorectal carcinoma cell line *in vivo* via a complement-independent mechanism (Ma *et al*., 1999). In this study, we isolated and characterized the MBP ligands on the surface of SW1116 cells. The MBP ligands on the surface of SW1116 cells were characterized with flow cytometry using plant lectins and anti-Lewis antibodies as inhibitors of FITC–MBP binding to the cells. Pronase glycopeptides were prepared from whole cell lysates, and oligosaccharides were liberated by hydrazinolysis followed by being tagged by pyridylamination. PA–MBP ligand oligosaccharides were isolated with an MBP-affinity column, and then their sequences were determined by MS and MS/MS analyses after permethylation, in combination with various biochemical analyses. The glycoproteins, which carry MBP ligands, were isolated by AAL- and MBP-affinity columns from SW1116 cell lysates. The major protein bands on SDS–PAGE under the reducing conditions were analyzed by MS analysis. Flow cytometry analysis of FITC–MBP binding to SW1116 cells showed that MBP recognizes and binds to the sugar chains containing Lewis a/b epitopes on the surface of the cells and that fucose plays an important role in the interaction. The MBP ligand oligosaccharides isolated from SW1116 cell lysates consisted of high molecular size polylactosamine-type *N*-glycans with high galactose, *N*-acetylglucosamine, and fucose contents. Endo-beta-galactosidase digestion of the ligand oligosaccharides resulted in a marked reduction of the binding activity to an MBP column together with the reduction of their molecular sizes. MS analysis of the MBP-ligand oligosaccharides after permethylation, in combination with endo-beta-galactosidase digestion, showed that the nonreducing terminal unit of the MBP-ligand oligosaccharides is mostly Lewis a/b, a substantial portion of which was carried on extended Type 1 chains as multimeric Lewis a units. The inner units were most likely to be dominated by Type 2 chain and not fully fucosylated. The reducing terminal structures of the MBP ligands were analyzed by MS analysis and lectin affinity HPLC. The results indicated that the core portion of the MBP ligands were tetraantennary *N*-glycans with the fucosylated trimannosyl core. These structures were unique and distinct from other previously reported tumor-specific carbohydrate antigens. It is concluded that MBP requires clusters of tandem repeats of the Lewis a epitope for recognition (Terada *et al*., 2005). The MBP-ligand glycoproteins were isolated by AAL- and MBP-affinity columns from SW1116 cell lysates. Two major bands, 120 kDa and 82 kDa on SDS–PAGE under the reducing conditions, were identified as CD26 and CD98 heavy chain, respectively, by MS analysis.

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(184) Metabolic Pathways of Natural Glycolipid Ligands for NKT Cells in Tumor Immunity

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NKT cells function as a paradoxical cell type in tumor immune surveillance through acting on antigen presenting cells, natural killer cells, and CD8 T cells. Alpha-galactosylceramide, a marine sponge glycolipid, induces Th1 cytokine production by NKT cells and prevents metastasis in some mouse experimental systems. In a methylcholanthrene (MCA) induced mouse primary fibrosarcoma system, endogenous lipids activate NKT cells to prevent tumor progression. However, in transplanted tumor systems, NKT cell activated by endogenous lipids promote tumor growth by producing Th2 cytokines specifically IL13. To identify the mechanism explaining the above paradoxical functions of NKT cells, we have tried to characterize the natural lipid ligands of NKT cells. We have used genetic approaches including gene-targeted mice and RNAi-silenced

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cell lines to dissect the pathways involved in the trafficking, processing, and loading of glycolipids onto CD1, an MHC-like glycoprotein family specialized in microbial, and self-lipid-antigen presentation. We found that the development and the function of CD1/lipid specific T cells are critically regulated by enzymes (β-hexosaminidases) and activator proteins (saposins) of glycolipid metabolism. This led us to narrow down the natural glycolipid antigens to substrates of these enzymes. By studying those glycolipids candidates, both chemically synthesized and purified from natural sources, we first identified the isoglobotrihexosylceramide (iGb3) as a lysosomal endogenous ligand for T cells. Genetic evidence suggests that natural ligands must be products of β-hexosaminidases. Thus, iGb3 might be the only natural ligand for NKT cells based on our current knowledge on structures of mammalian glycolipids. Ongoing studies are focused on the identity of the natural NKT cell ligands in tumor models and the metabolic pathways in generating these glycolipid ligands. We intend to identify the natural lipids responsible for the beneficial or detrimental activation of NKT cells, and the subtle changes in either lipid part or carbohydrate part that might cause switching of cytokine production profiles. With regard to the detrimental activation, we intend to find approaches to reverse the Th2 (IL13) cytokine production profile, by introducing exogenous glycolipid ligands that induce Th1 cytokine production. In collaboration with synthetic chemists, we will use the α-galactosylceramide and iGb3 glycolipids as the templates to design better structures that might possess therapeutic function.

(185) Crystallographic Analysis of the NNA7 Fab and Model for the Recognition of a Human Glycopeptide Blood Group Antigen

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The NNA7 Fab fragment recognizes the human N blood group antigen comprised of the N-terminal glycopeptide of glycophorin A (GPA). A single-site mutant form of this Fab fragment, NNA7-G91S, has dramatically decreased affinity for N antigen. To provide insight into how these Fab fragments recognize this glycopeptide antigen, the wild-type and mutant Fabs were crystallized, and the crystal structures were solved and refined to 1.83 Å and 1.97 Å resolution, respectively. In each case, the antigen-combining site forms a large crescent-shaped cleft consistent with the need to accommodate a glycopeptide. Due to the absence of antigenic ligand during crystallization, a morpholino-ethane sulfonic acid (MES) buffer molecule was trapped within the antigen-combining site and interacted with residues derived from the H chain complementarity determining regions (CDRs). The six-membered heterocyclic ring of the MES molecule adopted a chair conformation, thereby resembling a hexose subunit. Comparing the structure of the NNA7-MES complex to the binding modes of natural protein–carbohydrate recognition suggested that the buffer molecule may mimic the natural mode of glycan binding by the Fab fragment. The G91S mutation of the NNA7 L chain falls within the crescent-shaped antigen-combining cleft and correlates well with the location of antigen binding. In addition, the G91S substitution has structural consequences resulting in a deflection of the adjacent peptide backbone of the H chain CDR3. This steric clash changes the variable region structure at the confluence of the H and L chain CDRs, which is manifested by changes in crystal packing. Although the crystal packing of both structures is very similar, NNA7-G91S displayed a more compact structure in which two symmetry-related objects are closer by ~3 Å as compared with NNA7. Such differences suggest how the G91S mutation causes subtle changes in the molecular shape of the Fab fragment and its interaction with the relevant ligand. In addition, there are crystal contacts between the antigen-binding site and a Ser'-Ser'-Thr'-Lys'-Val' sequence in the constant region of an adjacent Fab, which is similar to the N-antigen peptide sequence. Finally, nearby glycerol molecules occupy the antigen-combining pocket, which may mimic the *O*-glycans of the N-antigen.

(186) Suppression of Tumor Formation in Lymph Nodes by L-Selectin-Mediated Natural Killer Cell Recruitment

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Natural killer (NK) cells largely reside in the spleen and in the peripheral blood. We have recently detected NK cells in lymph nodes in mouse. We further showed that the NK cell subsets in lymph nodes are different from those in the blood or the spleen. There are less Ly49C/I+, Ly49D+, or Ly49H+ NK cells in the lymph nodes. On the other hand, more lymph node resident NK cells expressed sialyl Lewis x oligosaccharides that are mainly carried on core 2 branched *O*-glycans. Lymph node-derived NK cells had cytolytic activity toward YAC-1 and RMA/S tumors equivalent to splenic NK cells, but splenic NK cells produced more INF-gamma in mice stimulated with endotoxin. When NK cells from wild-type and L-selectin-deficient mice were simultaneously introduced via tail vein into a wild-type mouse, we found that L-selectin-deficient NK cells were defective in migration to resting lymph nodes as well as complete Freund's adjuvant (CFA)-stimulated lymph nodes. CFA stimulation recruited less NK cells to regional lymph nodes in L-selectin ligand deficient mice (fucosyltransferase-IV and -VII double deficient mice) than wild-type animals. These results indicate that L-selectin on NK cells and L-selectin ligands in endothelial cells are essential for NK cell recruitment to lymph nodes. NK cells are known to reject certain tumors *in vivo*; however, the ability of NK cells to prevent metastasis of tumors into secondary lymphoid organs has not been addressed. We demonstrated that metastasis of B16 melanoma cells to draining lymph nodes was suppressed in wild-type or RAG-1-deficient mice, but not when NK cells were depleted by anti-NK1.1 antibody treatment. Although L-selectin-deficient NK cells lysed tumor cells *in vitro* as efficient as wild-type NK cells, NK cell-dependent suppression of tumor metastasis was diminished in mice deficient for L-selectin or fucosyltransferase-IV and -VII, as a result of insufficient NK cell recruitment to the lymph nodes. These findings indicate that L-selectin-mediated NK cell recruitment plays a crucial role in the control of tumor metastasis into secondary lymphoid organs.

Session Topic: N-Linked Glycan Functions

(187) High-Mannose Type N-Linked Oligosaccharide does not Affect the Biological Function of a Monoclonal Antibody

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MAb x is a fully human IgG1 monoclonal antibody that suppresses the growth of human tumor xenografts grown in mice. MAb X is produced in CHO cells and contains one N-linked glycosylation site in the CH2 domain, which is occupied mostly by complex type biantennary glycans, and much less by high mannose type glycans. The amount of high mannose type glycans varies from lot to lot, which may pose product quality issue for the molecule. In this study, we investigated whether MAb X containing 0, 11, or 50% high-mannose type glycans would exhibit different bioactivities. MAb X molecules containing high-mannose type glycans were isolated by lectin affinity chromatography. To assess anti-tumor activity *in vivo*, the three MAb X samples and buffer controls were injected into CB.17 SCID mice bearing COLO 205 human colon carcinoma tumor xenografts. All three MAb X samples exhibited a dose-dependent suppression of tumor growth when compared with controls. However, no significant differences in tumor growth suppression were found between the three MAb X samples. The result suggests that the activity of MAb X is not affected by whether the N-linked glycan is complex type or high-mannose type.

(188) Dietary and Genetic Control of Pancreatic Beta Cell Glucose Transporter-2 Glycosylation Promotes Insulin Secretion in Suppressing the Pathogenesis of Type 2 Diabetes

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Pancreatic beta (β) cell surface expression of Glucose transporter-2 (Glut-2) is essential for glucose-induced insulin secretion thereby controlling blood glucose homeostasis in response to dietary intake. Beta cell failure associated with loss of Glut-2 expression is the earliest feature in the development of Type 2 diabetes, resulting in the absence of glucose-stimulated insulin secretion and hyperglycemia. We show that the *Mgat4a*-encoded Golgi resident GnT-4a glycosyltransferase is required for the production of an *N*-glycan structure which functions as a ligand for lectin receptors, including galectin-9, that maintain Glut-2 residency on the β cell surface. This novel lectin–ligand binding interaction is glycoprotein- and cell-type specific. Glycoprotein analyses reveal normal expression of other similarly misglycosylated glycoprotein including insulin receptors on the β cell surface, and expression of Glut-2 molecules is unaltered among hepatocytes that lack *Mgat4a* expression and GnT-4a-dependent protein glycosylation. Competitive inhibition of lectin binding to Glut-2 using exogenous ligand mimetics leads to rapid loss of β cell surface Glut-2

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expression. Furthermore, attenuation of *Mgat4a* expression by genetic disruption or administration of a high-fat diet diminishes Glut-2 glycosylation, resulting in a severe reduction of cell surface half-life by provoking endocytosis with redistribution into endosomes and lysosomes. GnT-4a deficiency abolishes the first phase of glucose-stimulated insulin secretion resulting in hyperglycemia, increased circulating free fatty acids, and elevated expression of liver gluconeogenic enzymes. Hepatic steatosis and insulin resistance develop with age further enhancing the resemblance of GnT-4a deficient pathology in the mouse to human Type 2 diabetes. These findings reveal that GnT-4a glycosyltransferase expression and Glut-2 glycosylation are under genetic and dietary control mechanisms that are essential for maintaining pancreatic β cell surface Glut-2 expression and insulin secretion in normal physiologic contexts. Disabling this lectin receptor binding mechanism by genetic disruption or chronic ingestion of a high-fat diet is correlated with the earliest disease markers in the pathogenesis of Type 2 diabetes.

(189) Sialylation of N-Linked Glycans Influenced PK of a Glycoprotein in Rats

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Protein Y is a CHO cell produced receptor-Fc fusion protein. Twelve N-linked glycosylation sites in the molecule are fully occupied by a mixture of complex type glycans. Most of the glycans contain terminal sialic acid. It was reported that asialoglycoprotein receptors, a carbohydrate-binding protein in liver, captures unsialylated glycoproteins as a means for protein elimination. Therefore, the level of sialylation might be an important factor to determine the rate of protein Y clearance. To investigate whether differences in sialylation would influence protein Y pharmacokinetics (PK), a rat PK study was conducted with samples at 8, 12, and 19 moles SA per mole protein. Significant differences in PK parameters (t1/2, area under curve and clearance) were found between the three samples. When the values of area under curve (AUC) are plotted against the levels of sialylation, the correlation clearly indicated that a small change in protein Y sialylation would significantly influence its drug exposure (AUC).

(190) Evidence for Nuclear Factor-KappaB-Mediated Transcriptional Regulation of the β1,6-*N*-Acetylglucosaminyltransferase GnT-VB

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Alterations of cell surface glycan structures, particularly branched N-linked oligosaccharides have been linked with oncogenic transformation. The *N*-acetylglucosaminyltransferase known as GnT-V is expressed ubiquitously in most tissues, whereas its recently identified homologue, known as GnT-VB, is expressed primarily in the brain and testis of both mice and humans. The selective expression pattern of GnT-VB indicates that complex control mechanisms exist to regulate GnT-VB transcription. In our attempt to better understand GnT-VB transcriptional regulation, we have characterized the nucleotide sequences of the region flanking the 5′ end of the GnT-VB gene. We have identified distal and proximal putative promoter regions upstream of a CpG island that contain several NF-kappaB and Oct-1 elements. Functional analysis of these promoter regions reveal that GnT-VB promoter activity is negatively regulated by NF-kappaB and Oct-1 in human glioblastoma cells (U373), human embryonic kidney cells (HEK293), and human neuroblastoma (SH-SY5Y and NBFL). Expression of a dominant negative IKBalpha mutant that inhibits nuclear localization of all NK-kappaB subunits results in increased GnT-VB mRNA levels as well as increased reactivity of the lectin leukophytohemagglutinin (L-PHA) which binds beta (1,6) branched N-linked oligosaccharides. These results suggest that NF-kappaB activity in neuronal cells may be required to control the expression of highly branched *N*-glycans by GnT-VB.

(191) Apical Golgi Localization of *N*,*N*-Diacetyllactosediamine Synthase, β4GalNAc-T3, is Responsible for LacdiNAc Expression on Gastric Mucosa

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β1,4-*N*-Acetylglucosaminyltransferase III (β4GalNAc-T3), which was recently cloned and identified exhibits GalNAc transferase activity toward a GlcNAcβ residue with β1,4-linkage, forming the *N*,*N*′-diacetyllactosediamine, GalNAcβ1,4GlcNAc/LacdiNAc (Sato *et al*., 2003). Though LacdiNAc has not been found in the gastric mucosa, a high amount of transcript was detected in our previous study. To increase our knowledge of β4GalNAc-T3 expression and its product LacdiNAc, we examined the exact localization of β4GalNAc-T3 in human gastric mucosa using a newly developed antibody, mAb K1356. This antibody specifically detected the enzyme that transfected the *β4GalNAc-T3* gene into MKN45 cells, and the terminal βGalNAc epitope yielded on the cell surface was recognized by a lectin, *Wisteria floribunda* agglutinin (WFA). β4GalNAc-T3 was localized in the supra-nuclear region of surface mucous cells in gastric mucosa, and WFA stained positively the mucins secreted by these cells. In contrast, in the cells of the glandular compartment in the fundic gland and a few cells in the pyloric glands, β4GalNAc-T3 was observed in the basolateral position of nucleus, where no WFA reactivity was detected. The anti-Tn (GalNAcα-*O*-Ser/Thr) antibody staining did not overlap with the WFA staining. WFA binding to LDN was best among the sugar chains probably expressed in the gastric mucosa, according to automated frontal chromatography. Intestinal metaplastic cells presented neither β4GalNAc-T3 nor WFA reactivity. These results suggest that the supranuclear expression of β4GalNAc-T3 is critical to form LacdiNAc on the surface mucous cells and that LacdiNAc and β4GalNAc-T3 are novel differentiation markers of surface mucous cells in the gastric mucosa.

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(192) Core Fucosylation of Low Density Lipoprotein Receptor-Related Protein is Required for the Function as a Internalization for IGFBP3

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The α1,6 fucosyltransferase (FUT8) involved in core fucosylation of *N*-glycans in mammals. To know the biological roles of FUT8, we established FUT8-deficient mice. Although ~80% of the mice die within 3 days after birth, the survived mice showed severe growth retardation. Interestingly, levels of insulin-like growth factor binding protein 3 (IGFBP3), which has a strong growth inhibitory effect, were dramatically increased in FUT8-deficient mice serum. Expression of IGFBP3 mRNA in kidney, lung, and liver of FUT8-deficient mice was not changed as compared with the wild type of mice, suggesting that FUT8-deficient mice have an abnormality in the catabolism of IGFBP3. Because low density lipoprotein receptor-related protein (LRP) is a multiligand scavenger receptor that was identified as a fucosylated molecule in this study, internalization of IGFBP3 via LRP was investigated using FUT8-deficient kidney epithelial cells. The internalization of ^{125}I-GFBP3 was decreased in FUT8 KO epithelial cell (KK1 cell), compared with stable transfectant of human FUT8 gene to KK1 cell (KK1F cell). These results suggested that loss of core fucosylation on LRP increased IGFBP3 in the serum of FUT8-deficient mice, leading to the growth retardation.

(193) Dysregulation of TGF-β1 Receptor Activation Leads to Abnormal Lung Development and Emphysema-Like Phenotype in Core Fucose-Deficient Mice

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The core fucosylation (α1,6-fucosylation) of glycoproteins is widely distributed in mammalian tissues and is altered under pathological conditions. To investigate physiological functions of the core fucose, we generated α1,6-fucosyltransferase (*Fut8*)-null mice and found that disruption of *Fut8* induces severe growth retardation and death during postnatal development. Histopathological analysis revealed that *Fut8*–/– mice showed emphysema-like changes in the lung, which were verified by a physiological compliance analysis. Biochemical studies indicated that lungs from *Fut8*–/– mice exhibit a marked overexpression of matrix metalloproteinases (MMPs), such as MMP-12 and MMP-13, highly associated with lung destructive phenotypes, and a down-regulation of extracellular matrix (ECM) proteins such as elastin, as well as retarded alveolar epithelia cell differentiation. These changes should be consistent with a deficiency in transforming growth factor-β1 (TGF-β1) signaling, a pleiotropic factor which controls ECM homeostasis by down-regulating MMP expression and inducing

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ECM protein components. In fact, *Fut8–/–* mice have a marked dysregulation of TGF-β1 receptor activation and signaling, as assessed by TGF-β1 binding assays and Smad2 phosphorylation analysis. We also show that these TGF-β1 receptor defects found in *Fut8–/–* cells can be rescued by reintroducing *Fut8* into *Fut8–/–* cells. Furthermore, exogenous TGF-β1 potentially rescued emphysema-like phenotype and concomitantly reduced MMP expression in *Fut8–/–* lung. We propose that the lack of core fucosylation of TGF-β1 receptors is crucial for a developmental and progressive/destructive emphysema, suggesting perturbation of this function could underlie certain cases of human emphysema.

(194) Overexpression of *N*-Acetylglucosaminyltransferase III Results in an Increasing Activity of Adenylyl Cyclase III

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The enzyme β1,4-*N*-acetlylglucosaminyltransferase III (GnT-III) catalyzes the addition of a bisecting *N*-acetylglucosamine (GlcNAc) residue to glycoproteins and resulting in a modulation in biological function. Our previous studies have shown that modification of bisecting GlcNAc on cell surface receptors affects receptor-mediated intracellular signals. In this study, we report that the effects of overexpression of GnT-III on expression levels of cyclic AMP (cAMP), a second messenger in cells, produced by adenylyl cyclase (AC). ACIII, which contains two potential N-glycosylation sites in predicated extracellular domains, is highly expressed in Neuro-2a and B16 mouse melanoma cells, compared with other isoforms. As expected, ACIII is a target protein of GnT-III, confirmed by E_4-PHA, which preferentially recognizes bisecting GlcNAc. The overexpression of GnT-III, but not that of an enzymatic inactive GnT-III (D323A), resulted in an increase in the forskolin-induced AC activities, subsequently enhanced its product, cAMP and phosphorylation of downstream transcriptional factor CREB in those cells. The enhancement of forskolin-induced ACIII activities by GnT-III could be attributed to alteration in conformation of ACIII, because overexpression of GnT-III did not affect its expression levels on cell surfaces, confirmed by biotinylation. This is the first time to describe that GnT-III may participate in regulation of cAMP-mediated signal pathway.

(195) A New Method for the Detection of GlcNAcβ1-6Manα1- Branches in N-Linked Glycoproteins Based on the Specificity of *N*-Acetylglucosaminyltransferase VI

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Malignant transformation is often accompanied with the aberrant glycosylation profile of cell surface glycoproteins, in particular, the presence of β1-6GlcNAc branching *N*-glycans with poly-*N*-acetyllactosamine structures. The enzyme responsible for generating β1-6GlcNAc-branching *N*-glycans is UDP-*N*-acetylglucosamine : α-6-D-mannoside β1-6*N*-acetylglucosaminyltransferase (GnT V). It has been reported that the activity of this enzyme elevates depending on the malignancy of hepatocarcinoma or the expression of the oncogenes (v-src, T24-H-ras), infection with Polyoma virus, Rous sarcoma virus. So far the plant lectin leucoagglutinin (L_4-PHA) is the sole reagent to detect β1,6 branch which preferentially binds with complex type tri- and tetraantennary oligosaccharides containing β1,6 branches. However, identification of glycoproteins which are modified with GnT V still remains largely unknown with this lectin precipitation assay. Our group succeeded in purifying and cloning hen UDP-GlcNAc : GlcNAcβ 1-6(GlcNAcβ1-2)Manα1-R [GlcNAc to Man]-β1,4-*N*-acetylglucosaminyltransferase VI (GnT VI) (Sakamoto *et al*., 2000; Taguchi *et al*., 2000). GnT-VI activity is defined as that catalyzing the transfer of GlcNAc to the Manα1-6 arm and forms GlcNAc β1-4Manα1-6 linkage of *N*-glycan. This enzyme does not act on biantennary oligosaccharides and stringently requires GlcNAcβ1-6Manα1- structure on acceptor substrate for its activity. The activity of this enzyme was not detected in human yet. In this study, we took advantage of this strict substrate specificity of GnT-VI to establish a new assay method to detect glycoproteins with GlcNAcβ1-6Manα1- branch in *N*-glycan. With the use of recombinant hen GnT VI purified with a Ni chelating sepharose column as secreted protein from a insect cell line. Radiolabeled UDP-GlcNAc, human α1-acid glycoprotein(AGP), transferrin, and bovine fetuin digested with sialidase and β-galatosidase were used for control acceptor substrates. AGP is known to harbor GlcNAcβ1-6Manα1- branch on its *N*-glycans, whereas transferrin and fetuin do not possess GlcNAcβ1-6Manα1- structure. Only asialo-agalacto AGP was radiolabeled, whereas asialo-agalacto transferrin and asialo-agalacto fetuin were not. To confirm whether it is possible to develop this method for clinical field, human serum proteins which had been treated with sialidase and β-galactosidase were used as acceptor substrates for GnT-VI reaction as well. Even with crude materials such as human serum, this assay detected several proteins, one of which was identified AGP, as judged by its mobility on SDS–PAGE. This assay method may provide a useful complement to the current method relying on the specificity of L_4-PHA lectin.

(196) All-Atom Molecular Dynamics Simulations of the Diglycosylated and Membrane-Bound Prion Protein

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The prion protein is a cell-surface glycoprotein that has been implicated in various transmissible spongiform encephalopathies including Creutzfeldt-Jakob disease, fatal familial insomnia and Kuru in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle. The central hypothesis in prion disease is that it is a protein-only disease, whereby the prion protein is the only agent necessary for propagation and transmission of the disease. However, nonprotein moieties, such as the prion protein's N-linked glycans and its glycosylphosphatidylinositol anchor, are involved in the disease process *in vivo*. The glycans and the anchor can be found on both the innocuous prion isoform PrP^C and the disease-related aggregate PrP^{Sc}. The N-linked glycans have been found to influence PrP expression, distribution (within regions of the brain and among different types of neuronal cells), and deposition of PrP^{Sc} plaques *in vivo*. The glycans have also been hypothesized to modify the conformation of PrP^C and/or affect the affinity of PrP^C for a particular strain of PrP^{Sc}. We have previously used molecular dynamics simulations to observe the early misfolding of PrP^C to PrP^{Sc} at atomic resolution. Here, we describe all-atom, explicit solvent, molecular dynamics simulations of the soluble, and membrane-bound forms of diglycosylated PrP^C. Simulations were performed under amyloidogenic and nonamyloidogenic conditions to assess the effects these nonprotein moieties on the structure and dynamics of the prion protein. In agreement with experimental findings, neither the glycans nor the glycosylphosphatidylinositol anchor significantly impacts the structure of the globular region of PrP^C. However, the glycans do alter the dynamics of the unstructured N-terminus. The glycans, and to some extent the presence of the membrane, increase the kinetic stability of the disordered region, allowing the formation of short, sometimes transient, segments of secondary structure. Along with the analysis of the prion protein, analysis of the interactions amongst the different constituents of the system has revealed ways in which the nonprotein moieties participate in a "protein-only" disease.

(197) Multiple Modes of Interaction of the Deglycosylation Enzyme mPNGase with the Proteasome

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Peptide *N*-glycanase (PNGase) is involved in the cleavage of oligosaccharide chains from misfolded glycoproteins that are destined for degradation by the proteasome. Earlier a number of potential binding partners of mouse PNGase (mPNGase) were detected using the yeast two-hybrid system. In this study, an *in vitro* system was set up to directly investigate direct interactions between mPNGase and these candidate proteins. Although the yeast two-hybrid system suggested an interaction of six different proteins with mPNGase, only mHR23B and the proteasome subunit mS4 were found to interact with mPNGase. In fact, mS4 competes with mHR23B for binding to mPNGase. These results suggested two possible pathways for the interaction between mPNGase and the proteasome: in one pathway, mHR23B mediates the interaction between mPNGase and the proteasome. In an alternative pathway, mPNGase directly binds to the proteasome subunit, mS4. In either case, it is clear that PNGase is located in proximity to the proteasome and is available for deglycosylation of glycoproteins destined for degradation. Surprisingly, mPNGase was also found to mediate binding of the cytoplasmic protein, p97, to the proteasome through the formation of a ternary complex made up of mHR23B, mPNGase, and p97. Because p97 is known to bind to the ER membrane protein AMFR (gp78), an E3 ligase, we propose a model in which p97, mPNGase, and mHR23B mediates interaction of the ER with the proteasome.

(198) Computational Model of Cytokine Receptor Regulation by Hexosamine and Golgi N-Glycosylation Pathways

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Glucose is the preferred carbon and energy source in single cell eukaryotes, as well as the ligand for a family of glucose-sensing receptors that regulate cell

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migration, replication, and metabolism. Homologous signaling pathways in metazoans regulate tissue morphogenesis as well as growth, but the role of glucose as a ligand appears to be largely superseded by cell–cell contacts and cytokine signaling in animals. Although intracellular nutrient supply is sensed through the ATP/ADP energy charge, glucose supplies additional feedback to cytokine signaling pathways in a poorly understood manner. The *N*-acetyllactosamine branched *N*-glycans on cytokine receptors bind galectin-3 at the cell surface, forming a molecular lattice that opposes receptor loss to constitutive endocytosis. We show experimentally that surface cytokine receptors in Mgat5–/– tumor cells are rescued by supplementing the hexosamine pathway, which increases triantennary *N*-glycans and receptor association with galectin-3. We observe that the number and density of N-X-S/T sites in mammalian receptor kinases is higher for those that mediate anabolic signaling compared with receptors that mediate primarily morphogenesis, suggesting an unappreciated role of *N*-glycan multiplicity in receptor regulation. To better understand the relationships between the hexosamine pathway, *N*-glycan processing and *N*-glycan multiplicity, we developed a mathematical model. Both computational and experimental data reveal that the branching *N*-glycans pathway is ultrasensitive to stimulation through the hexosamine pathway. Flux through the hexosamine pathway creates positive feedback by increasing sensitivities to anabolic cytokines, followed at higher flux by increased autocrine TGF-b signaling, which drives epithelial-mesenchymal transition (EMT), and finally, growth suppression at high ratios of nuclear Smad2/3 to Erk1/2. Our results also support the idea that coevolution of the Golgi *N*-glycan processing pathway and low *N*-glycan multiplicity in morphogenic receptors in vertebrates enhances conditional control by creating distinct response kinetics to hexosamine (D50 and nH). Although the hexosamine pathway and a minimal level of *N*-acetyllactosamine branching (Mgat1, Mgat2) are required for embryogenesis in mammals, Mgat5 and possibly Mgat4 appear to be required for metabolic and developmental homeostasis in postnatal tissues.

(199) Topological Studies of Rft1 Protein, the Putative Man5GlcNAc2-PP-Dol Flippase

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Translocation of the Man5GlcNAc2-PP-Dol glycolipid intermediate is a crucial step in the biosynthesis of the lipid-linked oligosaccharide precursor for N-linked glycosylation, Glc3Man9GlcNAc2-PP-Dol, at the membrane of the endoplasmic reticulum (ER). Recently, genetic evidence has been obtained in *Saccharomyces cerevisiae* that the RFT1 locus is involved in this process (Helenius *et al*., 2002). RFT1 encodes a highly hydrophobic protein which is predicted to span the membrane multiple times. As the membrane topology of Rft1 protein could reveal important clues about possible mechanisms of the still poorly understood process of glycolipid flipping, we have initiated topological studies of yeast Rft1p. Here, we present a compilation of *in silica* data, *in vivo* data gained with SUC2-HIS4 topology probe fusions, and data from *in vitro* protease protection assays.

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(200) The Honeybee Mouse—A Biochemical Follow-Up

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We have recently reported that ablation of the murine Mpi gene, encoding for phosphomannose isomerase (PMI), causes embryonic lethality. PMI interconverts fructose-6-phosphate (Fru-6-P) to mannose-6-phosphate (Man-6-P) connecting glycolysis with glycosylation. In humans, MPI mutations, leading to 85–95% decreased PMI activity, cause congenital disorder of glycosylation Ib (CDG-Ib). Patients with CDG-Ib can be treated with orally administered Man, which is phosphorylated by hexokinase (HK) to Man-6-P, therefore, bypassing PMI. Complete loss of PMI activity in MPI–/– mice cannot be corrected with exogenous Man. Surprisingly, Man supplements even accelerate lethality and increase resorption of Mpi–/– embryos. We hypothesize that Man-6-P, which is usually converted to Fru-6-P, and eliminated through glycolysis, accumulates in Mpi–/– embryos and creates a futile cycle of Man-6-P-dephosphorylation and -rephosphorylation through HK. This causes ATP depletion and energy starvation in Mpi–/– mice similar to honeybees which have high HK and low PMI activities and die when given Man. To test our hypothesis, we generated primary murine embryonic fibroblasts from E11.5 Mpi+/+ and Mpi–/– embryos and treated them with Man. Man-6-P accumulated in a concentration- and time-dependent manner in Mpi–/– but not in Mpi+/+ cells. After 8-h incubation with 500 uM Man, Man-6-P levels reached 28.3 mM in Mpi–/– compared with 2.8 mM in Mpi+/+ cells. In parallel, ATP levels in Mpi–/– began to decrease by >20% after 8 h and by >70% after 24 h. To understand the mechanisms linking Man-6-P accumulation and ATP depletion, we investigated the ability of Man-6-P to perturb glycolytic flux and measured the effect of Man-6-P on key metabolic enzymes from cell lysates. About 28 mM Man-6-P inhibited HK activity by 70%. Man-6-P also inhibited phosphoglucose isomerase and glucose-6-phosphate dehydrogenase but not phosphofructokinase. To verify that Man-6-P inhibits HK in living Mpi–/– cells, we incubated them with 3H-2-deoxyglucose (2DG) and measured its intracellular phosphorylation through HK. Incubation with 500 uM Man reduced the amount of phosphorylated 2DG by >60% in Mpi–/– cells but not in Mpi+/+ cells. Our results using murine embryonic fibroblasts suggest that Man toxicity in Mpi–/– embryos is caused by Man-6-P accumulation, which inhibits glycolysis and depletes intracellular ATP levels. Indeed, as in fibroblasts, Man-6-P levels in E10.5 Mpi–/– embryos were increased >10 times and ATP levels were decreased by 50% compared with Mpi+/+ littermates, but protein glycosylation remained normal. We speculate that Mpi–/– embryos survive until E11.5 because metabolic studies have shown that the mouse embryo slowly switches from anaerobic glycolysis to oxidative phosphorylation between E8.5 and E10.5. At this stage of development, the contribution of oxidative phosphorylation is still low and anaerobic glycolysis is slowly decreased. An accumulation of Man-6-P further impairs glycolysis and appears to exacerbate this looming energy crisis.

(201) Glycoprotein Specificity of a Novel Group of Ubiquitin Ligases

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A significant fraction of all glycoproteins are misfolded and must be degraded to preserve cellular homeostasis. The glycoprotein endoplasmic reticulum-associated degradation (GERAD) pathway provides a quality control mechanism to eliminate these misfolded proteins from the ER. Although the precise steps involved in this system are not fully understood, one marker used by the ER to identify misfolded glycoproteins is the retention of a high-mannose (Man5-9GlcNAc2) glycan. This high-mannose tag serves as the signal for retrotranslocation into the cytosol and subsequent targeted destruction by the ubiquitin proteasome pathway. Recently, two members—FBX02 and FBXO6—of a five-protein ubiquitin ligase family (the FBA family) were shown to bind high-mannose-containing glycoproteins and participate in GERAD. This FBA family is the only group of ubiquitin ligases known to specifically target glycosylated substrates. We have cloned all five human FBA family members, generated a panel of FBA-specific antibodies, and demonstrated a marked divergence in their tissue and subcellular distribution. FBXO2 and FBXO17 are largely brain specific, FBXO6 and FBXO44 are found predominantly in abdominal organs, whereas FBXO27 is found in muscle. FBXO44 localizes to both the cytoplasm and nucleus, in contrast to the mainly cytoplasmic localization of FBXO2 and 6. We also found differences in high-mannose glycan affinity. Lectin blots revealed that only FBXO2, 6, and 27 displayed significant binding to high-mannose glycans. Additional glycan-binding studies will be described for a subset of FBA proteins using glycan array chips. To begin to understand the importance of these proteins in the liver, an important glycoprotein processing organ, we focused on two liver-enriched family members, FBXO6 and FBXO44. We tested the hypothesis that these ubiquitin ligases would degrade a known liver-specific ERAD substrate, alpha-1 antitrypsin (A1AT). Consistent with our lectin binding studies, FBXO6 alone bound A1AT with high affinity and reduced steady-state levels of A1AT. One variant of A1AT, the Z-variant, is known to misfold and accumulate in the ER of liver cells, causing neonatal hepatitis in some patients. Ongoing studies are investigating the effect of FBXO6 on the degradation of mutant A1AT (Z). To date, our findings suggest that the FBA family of ubiquitin ligases regulate the accumulation of misfolded glycoproteins and that FBX06, in particular, functions in this capacity in the liver.

Session Topic: Glycans in Immune System Regulation

(202) Modification of Cell Surface Glycosylation Could Affect Macrophage Function

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The macrophages (M) is considered the first line of defense in immune response to tumor growth. M recognize and eliminate tumor cells independently of

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MHC or specific antigens that make them an attractive tool in immunotherapy. M carry various molecules to recognize tumor cells including mannose-binding receptor, Siglecs, and galectins. Recently we have shown that phagocytosis of melanoma MELJUSO apoptotic bodies by THP-1 cells (macrophage origin) strongly inhibited by Galβ1-3GalNAcβ ($T_{\beta\beta}$?-containing probes, all the other probes were inactive (Rapoport *et al*., 2003). Elimination of apoptotic bodies generated from MCF-7 cells (human breast carcinoma) by M obtained from mononuclear cells of breast cancer patients was inhibited if Ms were pretreated with 3′SiaLac-PAA, LacNAc-PAA, asialoGM1-PAA, or $T_{\beta\beta}$-PAA (Rapoport *et al*., 2005). The aim of this study was to stimulate macrophage activity via lectins interaction with complementary carbohydrates incorporated as glycolipids in membrane of tumor cells or apoptotic bodies. We synthesized neoglycolipids by condensation of 3-aminopropyl glycoside with activated phosphatidylethanolamine (PE). Synthetic glycolipids 3′SiaLac-sp-PE, Lac-sp-PE, $T_{\beta\beta}$-sp-PE, asialoGM1-sp-PE, potent ligands of macrophage Siglecs, and galectins were incorporated into apoptotic bodies generated from tumor human cells MELJUSO (melanoma), Jurkat (T-lymphocytes), Raji (B-lymphocytes), and HT-29 (colon carcinoma); phagocytosis of apoptotic bodies by THP-1 cells was studied. Inserted glycolipids did not affect the elimination of apoptotic bodies; apoptotic bodies possibly have enough sites for lectin binding. To create a glycosylation pattern similar to that of apoptotic bodies, the tumor cells were loaded with glycolipids, phagocytosis degree of tumor MELJUSO, HT-29, and Jurkat cells loaded with $T_{\beta\beta}$ or asialoGM1 was higher than of intact tumor cells or tumor cells loaded with 3′SiaLac or Lac. Incorporation of glycolipids into Raji cells (B-lymphocytes origin) did not affect their phagocytosis by THP-1 cells. Furthermore, BALB/c mice bearing mammary adenocarcinoma were treated with apoptotic bodies loaded with 3′SiaLac-sp-PE or $T_{\beta\beta}$-sp-PE or intact apoptotic bodies. $T_{\beta\beta}$ significantly improved mice survival, whereas 3′SiaLac lead to increase of tumor growth and decrease of mice survival. *In vitro* experiments demonstrated that incorporation of $T_{\beta\beta}$-sp-PE provides increase of M cytotoxity and IFN-γ expression. Thus, change of glycosylation pattern modulates macrophage activity. (The work is supported by the grant of Russian Foundation for Basic Research N 04-04-49689.)

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(203) Proinflammatory Activities of Galectin-1, -3, and -9 *in vitro* and *in vivo*

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The interaction between oligosaccharide chains of the glycocalix and host lectins plays a role in innate immune responses, such as pathogen recognition and leukocyte recruitment. The most of those lectins, as represented by selectins and DC-SIGNs, are membrane proteins. In contrast, the roles of soluble lectins that recognize host glycans in innate immunity remain relatively unknown. One of those lectin families is soluble beta-galactoside-binding protein family, galectin, which expression and secretion are up-regulated during infection and inflammation. We investigated the proinflammatory properties of galectin-1, -3, and -9 *in vitro* by using neutrophils which migrate as the first line of defense against infection to the affected site and *in vivo* by employing a murine subcutaneous air pouch model. Galectin-3 induced L-selectin shedding and IL-8 production in both unprimed (naive) and primed neutrophils. These activities were shown to be dependent on the presence of both the C-terminal lectin domain and the N-terminal nonlectin domain of galectin-3, which is involved in oligomerization of this lectin. We also found that after galectin-3 binds to neutrophils, primed but not naive neutrophils can cleave galectin-3, mainly through elastase, resulting in the formation of truncated galectin-3 lacking the N-terminal domain. Together, these results suggest that galectin-3 activates both naive and primed neutrophils, whereas galectin-3-activated primed neutrophils have an ability to inactivate galectin-3. *In vivo*, galectin-1 (at low dose), -3, and -9 induced a rapid, transient recruitment of leukocytes, more specifically neutrophil, which was preceded by the accumulation of TNF-alpha and chemokines (MIP-2, KC, and MIP-1alpha) in the air pouches. *In vitro*, air pouch lining cells synthesized those cytokines when incubated with galectins, suggesting that cytokine production by those resident cells is involved in this galectin-induced leukocyte recruitment. In the case of galectin-1, biphasic effects on neutrophil recruitment were observed with different concentrations of galectin-1. Neutrophil recruitment was observed only in the pouches injected with a low dose of galectin-1, whereas galectin-1 induced TNF-alpha accumulation regardless of the concentration. Moreover, LPS-induced neutrophil recruitment was nearly abolished when a high dose of galectin-1 was coinjected with LPS, suggesting that galectin-1 could act as an anti-inflammatory factor. Thus, based on our results, we propose that galectin-1, -3, and -9 play a role as a new kind of proinflammatory cytokine, or "lectinocytokine," in the innate immunity.

(204) Role of Macrophage Galactose-Type C-Type Lectin 1 in Antigen-Induced Granulation Issue

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C-Type lectins are diverse, and some of them are involved in cellular immune responses. The interactions of macrophages and related cells with extracellular milieu are mediated by a variety of cell surface recognition molecules including C-type lectins. However, the role of a macrophage galactose-type calcium-type lectin 1 (MGL1, CD301) in antigen-specific chronic inflammatory tissue formation was not previously investigated. Mice preimmunized with a specific immunogen, azobenzene arsonate-conjugated acetylated BSA, were repeatedly challenged in dorsal air pouches. The effects of MGL1-deficient status (MGL1-deficient mice) and the effects of MGL1-specific blocking mAb, LOM-8.7, were investigated. MGL1-positive cells present in the granulation tissue were collected and investigated for their cell surface markers and cytokine production. The persistent presence of granulation tissue induced by a protein antigen was observed in wild-type mice but not in mice lacking MGL1 in an air pouch model. The anti-MGL1 antibody suppressed the granulation tissue formation in wild-type mice. A large number of cells, present only in the pouch of MGL1-deficient mice, were not myeloid or lymphoid lineage cells and the number significantly declined after administration of IL-1α into the pouch of MGL1-deficient mice. Furthermore, granulation tissue was restored by this treatment, and the cells obtained from the pouch of MGL1-deficient mice were incorporated into the granulation tissue when injected with IL-1α. The cells from granulation tissue injected with IL-1α are likely to be fibroblasts, because they were found to express the mAb ER-TR7 epitope. MGL1 was shown to play an essential role in antigen-induced granulation tissue formation, the final stage of cellular immune responses. MGL1 expressed on a specific subpopulation of macrophages that secrete IL-1α was proposed to regulate specific cellular interactions crucial to this process.

(205) Effects of Fucoidan on the Systemic and Mucosal Immune System

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Fucoidan, a complex sulfated polysaccharide purified from the brown seaweed, jelly coat of the sea urchin eggs, and body wall of the sea cucumber, shows various biological activities and high affinity to the CD62L (L-selectin). We have already reported that sulfated polysaccharide such as chondroitin sulfate up-regulates the antigen-specific Th1 dominant immune response of murine splenocytes sensitized with ovalbumin *in vitro* (Sakai *et al*., 2002; Akiyama *et al*., 2004). In this study, the structural characterization using spectrochemical analyses have been carried out on a fucoidan sample isolated from the brown seaweed, and the effects of fucoidan on the immune response have also been investigated. Detailed structural analysis has been previously reported of different fucoidan samples showing various features of branched chain structures and sugar compositional diversity. The fucoidan sample from the brown seaweed shows a typical one-dimensional 1H NMR spectrum that is fucoidan; [-3Fuc-4(}OSO_3^-)α1-]n, and also suggests that the brown seaweed might slightly be containing other neutral saccharides (mostly mannose, but also glucose, xylose, rhamnose, and galactose) obtained by hydrolysis with trifluoroacetic acid. To examine the effects of fucoidan on the systemic and mucosal immune system, splenocytes and intraepithelial lymphocytes were obtained from antigen-sensitized mice and were challenged with same antigen in the presence of fucoidan, and the cytokine levels in the medium of the cultured cells were measured. Fucoidan from the brown seaweed showed a significantly higher secretion of interferon gamma by antigen-sensitized splenocytes than that of a control group. Additionally, to evaluate the effect of fucoidan on the differentiation of lymphocytes, the cell surface markers on splenocytes and intraepithelial lymphocytes were analyzed using flow cytometry. This is the first demonstration that fucoidan structure impacts immunological activities on murine lymphocytes sensitized with antigen, and this finding may contribute a potential use of fucoidan in nutraceutical and/or supplements.

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(206) Cells Expressing Macrophage Galactose-Type C-Type Lectin1 and MGL2 with Unique Localization Correspond to a Distinct Subset of Dendritic Cells in Mouse Lymph Nodes

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C-Type lectins expressed on innate immune cells play important roles in the immune regulation. These molecules potentially take up antigens, mediate cell–cell interactions, and intracellular signals. Murine MGL/CD301 belongs to a family of Type II transmembrane C-type lectins and includes highly homologous members, MGL1 and MGL2. In skins, distribution of cells expressing MGL1 and/or MGL2 is highly restricted to the dermis. These dermal cells were shown to migrate to the draining lymph nodes (LNs) after irritation with a mixture of acetone and dibutylphthalate, an adjuvant used in the experimental contact hypersensitivity and may contribute to induction or modulation of this process. However, distribution of these cells within LNs were not described. In our previous studies, the cells expressing MGL1/2 in the dermis were tentatively described as macrophages, because they expressed F4/80 antigen. However, some of these cells were localized within the T-cell cortex and had dendritic morphology in the draining LNs. Therefore, these cells likely contain heterogeneous population of DCs and macrophages. The aim of this study is to identify the cell populations expressing MGL1 and MGL2 and determine their localization in the skin and LNs. By the use of anti-MGL1 and anti-MGL2 mAbs, we found that MGL2 was expressed in a restricted population of MGL1+ cells in LNs. FITC dissolved in a mixture of acetone and dibutylphthalate was painted onto the mouse forelimbs. Frozen sections of draining LNs were stained with anti-MGL1 or anti-MGL2 mAbs and with macrophage/DC markers. In addition, MGL1+ and MGL2+ LN cells were analyzed by flow cytometry using the same set of mAbs. In naïve LNs, MGL1+ cells were mainly observed in the subcapsular and medullary sinuses. MGL2 expression was restricted to cells with dendritic morphology which reside at the outer T-cell cortex. In confocal microscopy, most of the MGL2+ cells seemed to express MGL1, indicating that MGL2+ cells consisted of a restricted subset in a MGL1+ population. The area corresponds to where antigen presentation in LNs occurs. Judging from the DC marker expression, MGL1+ cells included interstitial DC-like (MHC class II^{hi}, $CD11c^{hi}$, $CD86^{hi}$), plasmacytoid DC-like (MHC class II^{int}, $CD11c^{int}$, $B220^{+}$, $Ly6G^{+}$), and probably macrophage-like (MHC class II^{lo}, $CD86^{-}$) subsets. The MGL2+ subset seemed to be more homogenous corresponding to interstitial DCs (a part of the first subset of MGL1+ cells). Among these putative subsets in MGL1+ cells, the interstitial DC-like population was associated with a higher level of FITC. These results indicate that expression of MGL1 and MGL2 is differentially regulated in distinct macrophage/DC subsets in LNs and that some of the MGL1+ cells including MGL2+ subset may present antigens to T cells after immunization.

(207) Altered Granulopoietic Profile and Exaggerated Acute Neutrophilic Inflammation in Mice with Targeted Deficiency in the Sialyltransferase ST6Gal I

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It has long been known that the systemic inflammatory response is accompanied by up-regulation of the sialyltransferase, ST6Gal I. However, whether or not ST6Gal I contributes directly to the inflammatory process has remained elusive. Here, we report that mice with a systemic ST6Gal I-deficiency (Siat1-null) exhibit significantly greater inflammatory cell recruitment in the thioglycollate model of experimental peritonitis. This observation was recapitulated in another ST6Gal I-deficient mouse, the Siat1dP1, which was created by disruption of P1, one of six known promoters driving tissue and developmental expression of Siat1, the ST6Gal I gene. Peritoneal accumulation of inflammatory cells was 2-fold greater in Siat1dP1 mice when compared with C57BL/6 wild-type cohorts at 5 h after thioglycollate challenge i.p. Neutrophils recovered from the peritoneum of elicited Siat1dP1 mice exhibited only a subtle increase in viability, suggesting that delayed apoptosis is not a significant mechanism contributing to the increased peritonitis. The exaggerated neutrophilic peritonitis in response to thioglycollate was preceded by a 3-fold greater peripheral blood neutrophil leukocytosis in Siat1dP1 mice. A significantly larger pool of marginated neutrophils resides in the Siat1dP1 mice, and these marginated cells can be released into circulation by epinephrine i.v. Within 30 min of G-CSF infusion, a close to 2-fold greater mobilization of granulocytes into peripheral circulation also occurred in Siat1dP1 mice. Siat1dP1 mice also demonstrated greater granulopoietic capacity, as reflected by greater *in vitro* marrow-derived myeloid colony forming units, by greater numbers of myeloid precursors visualized in marrow differentials, and by significantly enhanced recovery from cyclophosphoramide-induced myelosuppression. Together, these biologic phenotypes associated with ST6Gal I deficiency suggest a role for α2,6-sialylation in acute neutrophilic inflammation, granulopoiesis, and in the maintenance of hematologic stasis.

(208) Mouse Siglec-F and Human Siglec-8 are Functionally Convergent Paralogs that are Selectively Expressed on Eosinophils, Recognize 6□f-Sulfo-sLeX as a Preferred Glycan Ligand, and Recruit SHPs

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Sialic acid-binding immunoglobulin-like lectins (Siglecs) can be subdivided into two categories: the CD33-related Siglecs whose composition varies amongst mammals and a second group that includes CD22 (Siglec-2), sialoadhesin (Siglec-1), and MAG (Siglec-4). There are currently 11 human and eight mouse Siglecs identified so far. Siglec-F is a mouse eosinophil surface receptor, which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain, implicating it as a negative regulator in eosinophil signaling. We have found that the sialoside sequence 6 f-sulfo-sLeX (Neu5Ac*f*¿2-3[6-SO_4]Gal*f*À1-4[Fuc*f*¿1-3]GlcNAc) is a preferred glycan ligand for Siglec-F. In glycan array, screening of 172 glycans, recombinant Siglec-F-Fc chimeras bound with the highest avidity to 6 f-sulfo-sLeX. Secondary analysis showed that related structures, sialyl-Lewis x (sLeX) and 6-sulfo sLeX containing 6-GlcNAc-SO_4, showed much lower binding avidity, indicating significant contribution of 6-Gal-SO_4 on Siglec-F binding to 6 f-sulfo-sLeX. The lectin activity of Siglec-F is constitutively masked on mouse eosinophils and is unmasked by removing cis sialic acids. Unmasked eosinophils reveal Siglec-F-dependent binding and adhesion to 6 f-sulfo-sLeX structure, suggesting a role for Siglec-F as an eosinophil adhesion receptor. Although there is no clear-cut human ortholog of Siglec-F, Siglec-8 is gene paralog that is expressed selectively by human eosinophils and has recently been found to recognize 6 f-sulfo-sLeX (Bochner *et al.*, 2005). Furthermore, we have found that Siglec-F and Siglec-8 were phosphorylated and associated with SHP-1 and SHP-2 protein tyrosine phosphatases after pervanadate treatment. These observations suggest that mouse Siglec-F and human Siglec-8 have undergone functionally convergent evolution and implicate them as negative regulators of eosinophils and in interactions of these cells with the preferred 6 f-sulfo-sLeX ligand (Tateno *et al.*, 2005). (Supported by NIH grants GM60938, AI50143, and GM62116.)

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(209) Threshold Contribution to Selectin Ligand Formation by Polypeptide GalNAcT-1 Directs Tissue-Specific Lymphocyte Retention in Sustaining Humoral Immunity

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Cell surface expression of L-selectin is an essential component in cell adhesion mechanisms that populate lymph nodes with T and B lymphocytes. Glycan ligands for L-selectin are produced on high endothelial cells (HECs) and form L-selectin counter receptors on various glycoproteins. Normal B lymphocytes produce 50% of the level of L-selectin molecules of T lymphocytes thereby impairing the rate of B-cell homing to lymph nodes in comparison. These findings

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suggest the possible presence of a cell type-specific mechanism comprised of one or more glycosyltransferase enzymes that establishes thresholds for humoral immune responses by modulating L-selectin ligand formation on HECs. Several glycosyltransferases have been shown to contribute to L-selectin ligand formation and lymphocyte homing, yet thus far none have been found to selectively and significantly influence B lymphocyte colonization of lymph nodes and humoral immune responses. We show that the polypeptide GalNAcT-1 glycosyltransferase contributes to HEC L-selectin ligand formation among peripheral and mesenteric lymph nodes to the extent of supporting residency among the majority of peripheral B lymphocytes. Deficiency of ppGalNAcT-1 in the mouse severely reduces lymph node follicle development and depresses immunoglobulin-G production in pre- and postimmunization. These findings reveal that the initiation of *O*-glycan formation by ppGalNAcT-1 is a key determinant in the development of humoral immunity by increasing L-selectin ligand expression to threshold levels that support B lymphocyte retention among peripheral and mesenteric lymph nodes.

(210) Control of Postimmune CD8+ T-Cell Apoptosis by *O*-Glycan-Dependent Sialylation

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Apoptosis plays an essential role in peripheral CD8+ T-cell homeostasis; however, the molecular interactions initiating this process remain undefined. A mechanistic component has recently emerged from the study of the ST3Gal-I sialyltransferase and its role in altering cell surface *O*-glycan structures in response to immune activation. Induction of ST3Gal-I expression among mature thymic CD8+ T cells catalyzes the addition of terminal sialic acid to the Core 1 *O*-glycan structure Gal(1-3GalNAc-Ser/Thr before peripheral emigration. Within 48 h of immune stimulation through the T-cell receptor (TCR) complex, a significant reduction in this sialic acid modification is detected at the T-cell surface by an increase in peanut agglutinin lectin (PNA) binding, accompanied by a simultaneous increase in the expression of Core 2 *O*-glycans detected by 1B11 antibody binding. Post-activated CD8+ T cells that retain this *O*-glycan phenotype die by apoptosis, whereas viable memory T cells have increased Core 1 *O*-glycan sialylation and diminished Core 2 *O*-glycan expression. Absence of Core 1 *O*-glycan sialylation by ST3Gal-I induces peripheral CD8+ T cell apoptosis in both naïve and memory T cells, which can be blocked by TCR activation. We now show that loss of Core 1 *O*-glycan sialylation cannot be dissociated from CD8+ T-cell apoptosis and requires TCR complex activation. Stimulation of naïve CD8+ T cells with ionomycin and PMA fails to induce both Core 1 *O*-glycan desialylation and cellular apoptosis. We have also analyzed ST3Gal-I-deficient mice lacking potential genetic modifiers in this process and find that peripheral CD8+ T cell apoptosis does not involve CD43 and continues in the absence of Core 2 *O*-glycans produced by C2GlcNAcT-1. Furthermore, *in vivo* and *in vitro* studies with transgenic mice bearing constitutive T-cell expression of ST3Gal-I demonstrate that Core 1 *O*-glycan desialylation is dominant and invariably linked with CD8+ T-cell apoptosis, as opposed to Core 2 *O*-glycan branch participation, thereby resolving the *O*-glycan structural component essential in apoptotic signal generation. Additionally, we find that loss of ST3Gal-I and resulting CD8+ T-cell apoptosis cannot be rescued by overexpression of Bcl-2, implying that ST3Gal-I does not operate by modulating cytokine survival factor-mediated signals but instead by regulating a dominant death receptor signal transduction pathway emanating from an *O*-glycoprotein expressed on the mature CD8+ T-cell surface.

(211) Galectin-3 Stimulates Phagocytosis in Normal and Glaucomatous Trabecular Meshwork Cells *in vitro*

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The trabecular meshwork (TM) cells in culture are known to be actively phagocytic, and a reduction in the phagocytic response of TM cells is thought to play a role in the pathogenesis of glaucoma. The goal of this study was to investigate the effect of galectin-3 on the phagocytic activity of the human TM cells *in vitro*. Cell cultures derived from three normal and three glaucomatous TM were incubated in Opti-MEM I with rhodamine-conjugated polystyrene latex beads for 18 h in the presence or absence of recombinant human galectin-3. At the end of the incubation period, the cells with phagocytosed beads were quantified by flow cytometry. TM cells derived from all three glaucomatous specimens exhibited significantly reduced phagocytic capacity ($27.5 \pm 2.53\%$) compared with the normal TM cells ($38.12 \pm 1.54\%$). Exogenous galectin-3 stimulated photocytic capacity in both normal and TM cells. In every case, the stimulating effect of galectin-3 was inhibited by β-lactose, suggesting that the carbohydrate recognition domain of the lectin is directly involved in the stimulating effect of exogenous galectin-3 on phagocytic capacity of TM cells. Our *in vitro* data support the notion that glaucomatous TM cells have reduction in phagocytic capability, which may be important in the pathogenesis of glaucoma. Our findings that galectin-3 stimulates phagocytic activity of TM cells lead us to speculate that the lectins (1) as likely to be among the factors that modulate phagocytic property of TM cells and (2) may serve as a physiological agent to enhance the phagocytic capability of TM cells to remedy the progression of glaucoma.

(212) Characterization of *N*-Glycans on Murine CD25+ and CD25– CD4+ T Lymphocytes

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Glycosylation of the cell surface plays a pivotal role in the immune system. Decorating all mammalian cells, surface glycans expressed by T lymphocytes serve many diverse functions, ranging from facilitating the orientation of plasma membrane proteins to preventing nonspecific protein–protein interactions. We hypothesized that glycan–lectin interactions may participate in the molecular crosstalk between murine regulatory and nonregulatory CD4+ T lymphocytes. As a first step to exploring possible glycan-mediated interactions, we immunomagnetically selected peripheral lymphoid CD4+ CD25+ regulatory T cells (Tregs) and CD4+ CD25– lymphocytes from BALB/c and C57BL/6 mice, before undertaking a comparative study of their *N*-glycan expression. Owing to the difficulty of selecting large numbers of CD4+ CD25+ Tregs, we developed effective strategies to probe the major *N*-glycan components of small populations of murine lymphocytes, typically 0.5–3 million cells. By means of ultrahigh sensitivity MALDI-TOF, we screened the major *N*-glycans expressed by murine CD4+ CD25+ Tregs and CD4+ CD25– lymphocytes. Predicted structures from the mass mapping experiments were confirmed by GC-MS linkage analysis and tandem MS/MS CID MALDI TOF/TOF experiments. Certain preparations of both CD25+ and CD25– CD4+ T cells were richly N-glycosylated, with hypersialylated *N*-glycans, but distinct glycomic differences between these two populations were not observed. The most abundant *N*-glycans in these preparations were nonfucosylated triantennary structures carrying up to five *N*-acetylneuraminic acid residues. These glycans have not previously been observed in murine leucocytes. Moreover, the unusually high level of sialylation could have implications for Siglec-associated interactions. This study demonstrates that this powerful technology is ideally suited for probing the *N*-glycomes of small populations of murine lymphocytes, which may share surprisingly uniform glycan repertoires despite very different functions *in vitro* and *in vivo*.

(213) Regulation of FucT-VII Expression in Leukocytes

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The alpha(1,3)-fucosyltransferase FucT-VII is essential for the biosynthesis of E-, P-, and L-selectin ligands. Expression of FucT-VII is constitutive in myeloid cells but inducible and strictly regulated in T cells. However, little is known about the molecular mechanisms controlling FucT-VII expression in myeloid or T cells. We have previously shown that enforced expression of constitutively active H-Ras, but not K-Ras or N-Ras, induces FucT-VII expression in Jurkat T cells. This H-Ras-mediated FucT-VII induction requires both activation of the Raf-MEK-ERK cascade and the constitutive phosphoinositide-3 kinase (PI3K) activity characteristic of Jurkat T cells. However, mutational and complementation analysis of FucT-VII induction show that PI3K and Raf are required but not sufficient for FucT-VII expression and suggest the existence of a third, H-Ras-specific pathway which is also involved in FucT-VII induction. Gene reporter assays indicated that only the H-Ras isoform induces AP-1 activity in Jurkat T cells, and comparative analysis of the mouse and human FucT-VII loci revealed multiple conserved binding sites for the transcription factor AP-1, thereby implicating AP-1 in FucT-VII regulation. Moreover, preliminary analysis of H-Ras–/– mice revealed a defect in E- and P-selectin ligand formation, suggesting that H-Ras may be involved in FucT-VII regulation in primary T cells. To independently gain further insight into the molecular events regulating transcription of FucT-VII in myeloid cells compared with T cells, we utilized the adenovirus E1A protein, which sequesters and thereby inhibits specific coactivators of transcription. Although E1A is widely used as an inhibitor of

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p300/CBP, E1A can inhibit the activity of several other transcriptional regulators as well. Data gathered with E1A and specific E1A mutants strongly implicate p300/CBP in constitutive expression of FucT-VII in myeloid cells, suggest some role for p300/CBP and other coactivators in H-Ras-inducible FucT-VII in Jurkat cells, and indicate that the modes of transcriptional regulation are fundamentally distinct in these two cell types.

(214) Human Galectins-1, -2, and -4 Induce Surface Exposure of Phosphatidylserine in Activated Human Neutrophils but not Activated Lymphocytes

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Cellular turnover is associated with exposure of surface phosphatidylserine (PS) in apoptotic cells, leading to their phagocytic recognition and removal. But recent studies indicate that surface PS exposure is not always associated with apoptosis. Here, we show that several members of the human galectin family of glycan-binding proteins (galectins-1, -2, and -4) induce PS exposure in a carbohydrate-dependent fashion in activated, but not resting, human neutrophils and in several leukocyte cell lines. PS exposure was not associated with apoptosis in activated neutrophils. The exposure of PS in cell lines treated with galectins was sustained and did not affect cell viability. Interestingly, galectins bound well to activated T-lymphocytes, but did not induce either PS exposure or apoptosis, indicating that galectin effects are cell specific. These results suggest a novel immunoregulatory contribution of galectins in potentially regulating leukocyte turnover independently of apoptosis.

(215) Analysis of the *O*-Glycan Structures in PSGL-1 from the Wehi Murine Leukocyte Cell Line

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Human P-selectin glycoprotein ligand-1 (PSGL-1) has been shown to contain a single core 2-based sialyl Lewis x *O*-glycan at its extreme N-terminus that is essential for binding to P-selectin. To date, no studies have been performed on the glycan structures of murine PSGL-1. Interestingly, studies by the Consortium for Functional Glycomics employing MALDI-TOF-MS analysis of *O*-glycans from mouse Wehi cells, which express PSGL-1 and bind P-selectin, did not detect any Lewis-related, fucose-containing glycans. However, previous studies by several groups using mouse genetic approaches demonstrated that expression of both FucT-VII and core 2 GlcNAc-T is required for murine leukocyte adhesion to selectins. To address this apparent anomaly, we used metabolic radiolabeling with ^{3}H-monosaccharide precursors ($GlcNH_2$ and Fuc) to detect low abundance *O*-glycan structures in Wehi cells and on PSGL-1 purified from these cells. These studies were carried out using HPAEC-PAD (Dionex) chromatography with an online flow scintillation counter and involved the synthesis of several radiolabeled *O*-glycan structures for use as comparative standards. The results of our analyses show that PSGL-1 from Wehi cells contains the same core 2 sialyl Lewis x *O*-glycan structure as found on the human PSGL-1. However, this *O*-glycan structure is present in very low abundance in Wehi cells and is not detectable in total glycoproteins. Thus, the results also suggest that Wehi cells selectively fucosylate the *O*-glycans on PSGL-1 to generate the functionally important core 2-based sialyl Lewis x *O*-glycans. (This work was supported by grant GM62116 from the NIGMS/NIH.)

(216) Homo-Multimeric Complexes of CD22 in B Cells Revealed by Protein–Carbohydrate Crosslinking

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CD22 is a negative regulator of B-cell receptor signaling, an activity mediated by recruitment of SH2 domain containing phosphatase 1 through a phosphorylated immunoreceptor tyrosine inhibitory motif in its cytoplasmic domain. As in other members of the sialic acid-binding immunoglobulin-like family (Siglecs), the extracellular N-terminal immunoglobulin domain of CD22 binds to NeuAc?2-6Gal of glycoproteins on the same cell (in cis) and on adjacent cells (in trans). B-cell glycoproteins bind to CD22 in cis and "mask" the ligand-binding domain, modulating its activity as a regulator of B-cell signaling. To investigate the identity of the B-cell glycoproteins that serve as cis ligands, a method of photo-crosslinking glycans to CD22 was developed, employing the endogenous biosynthetic pathway to incorporate sialic acid bearing a photoactive aryl-azide moiety at C-9 (9-AAz-NeuAc). 9-AAz-NeuAc was incubated with BJAB (K20) cells, and formation of the cell surface 9-AAz-NeuAc?2-6Gal was verified by staining with *Sambuccus nigra* agglutinin (SNA) and CD22-Fc that are specific for NeuAc?2-6Gal linkage as monitored by flow cytometry. The presence of azide functionality on the cell surface was verified by Staudinger–Bertozzi ligation, which resulted in covalent attachment of a biotin group to the cell surface. Irradiation of BJAB cells cultured with 9-AAz-NeuAc with UV resulted in extensive crosslinking of CD22, with negligible crosslinking to previously implicated cis ligands glycoproteins, including CD45 and CD19. Thus, despite the fact that these glycoproteins carry glycan ligands on the same B-cell surface, and their glycans can be recognized by CD22 *in vitro*, none of them appear to represent significant cis ligands of CD22 in resting B cells *in situ*. Instead, CD22 recognizes glycans of neighboring CD22 molecules as cis ligands, forming homomultimeric complexes. Supported by NIH grants GM 60938 and AI50143.

(217) Sialidase Activity in Human Monocyte-Derived Cells Influences the Response to Bacterial Lipopolysaccharide

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Monocytes and monocyte-derived cells play key roles in potentiating diverse immune activities in response to microbial pathogens. The functional capacity of these cells is influenced by changes in the sialic acid content of cell surface glycoproteins and glycolipids. Desialylation of glycoconjugates on the surface of purified monocytes using exogenous neuraminidase-activated extracellular signal-regulated kinase 1/2 (ERK 1/2) and led to production of IL-6, MIP-1a, and MIP-1b. Exposure of monocytes to gram-negative bacterial lipopolysaccharide (LPS) also led to enhanced expression of IL-6, MIP-1a, and MIP-1b. The amount of each of these cytokines that was produced, though, was markedly increased when monocytes were desialylated before exposure to LPS. Monocytes differentiate into immature dendritic cells by growth *in vitro* in defined medium-containing IL-4 and GM-CSF. These cells differentiate further into mature dendritic cells after exposure to LPS. The cellular response to LPS includes a change in cell function and the induction of specific cytokines. Endogenous sialidase activity of monocyte-derived cells is up-regulated during the differentiation of monocytes, with up to a 20-fold increase in lysosomal Neu1 activity and a 2-fold increase in the activity of plasma membrane associated Neu3. Although localized predominantly in lysosomes in freshly isolated monocytes, Neu1 was detected on the surface of dendritic cells by flow cytometry and confocal microscopy using Neu1-specific antibodies. Differentiation of monocytes in the presence of sialidase inhibitors resulted in an increase in the amount of total cellular sialic acid and a reduction in binding of galactose-recognizing lectins to the cell surface. Inhibition of sialidase activity in differentiating monocytes also led to reduced expression of IL-6, TNFa, MIP-1a, MIP-1b, and IL-12p40 after immature dendritic cells were exposed to LPS. These results suggest that endogenous sialidase activity of monocyte-derived dendritic cells may play a role in the interaction of cells with microbial pathogens, and their products during inflammation and infection.

(218) Sialidase Activity of Activated Human Lymphocytes Influences Production of IFN-Gamma

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Modulation of the sialic acid content of cell surface glycoproteins and glycolipids influences the functional capacity of cells of the immune system. Although four genetically distinct mammalian sialidases (Neu1-4) have been identified, the expression of only lysosomal Neu1 and plasma membrane-associated Neu3 is detected in freshly isolated and activated human T lymphocytes. Activation of lymphocytes *in vitro* by exposure to anti-CD3 and -CD28 Abs resulted in a 6-fold increase in Neu1 activity in cells grown in culture for 24 h. After growth in culture for 5 days, activated lymphocytes expressed Neu1 sialidase activity at a level 9-fold greater than in freshly isolated cells. In contrast, the activity of Neu3 changed minimally during a 5-day culture period. The increase in Neu1 enzyme activity correlated with increased synthesis of Neu1-specific RNA as determined by real-time RT–PCR. Although localized predominantly in lysosomes in freshly isolated cells, Neu1 was detected within 24 h of cell activation on the outer surface of CD4+ and CD8+ T lymphocytes using Neu1-specific Abs and flow cytometry. Compared with freshly isolated lymphocytes, activated cells expressed an increased number of cell surface binding sites for the lectins *Erythrina cristagalli* (ECA; binds to Galb1-4GluNAc-) or *Arachis hypogaea* (PNA; binds to Galb1-3GalNAc-). The increase in amount of ECA bound to the surface of activated cells was reduced when activated cells were grown in culture in the presence of competitive sialidase inhibitors 2,3-dehydro-

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2-deoxy-*N*-acetylneuraminic acid (DANA) or 4-guanidino-2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Zanamivir), suggesting that sialidase activity in activated cells was partly responsible for the hyposialylation of specific cell surface glycoconjugates. Growth of cells in the presence of DANA or Relenza did not down-regulate the expression of either Neu1 or Neu3. Sialidase activity in activated lymphocytes was associated with the production of IFN-γ. In activated lymphocytes, IFN-γ expression was decreased from 842 ng/mL in the medium of control cells to 341 and 514 ng/mL when cells were maintained in culture in the presence of DANA or Relenza, respectively. The down-regulation of IFN-γ occurred at the level of RNA synthesis. Thus, sialidase activity in stimulated T lymphocytes contributes to the desialylation of specific cell surface glycoconjugates and influences lymphocyte involvement in the immune response.

Session Topic: Glycans in Disease

(219) Differences in *O*-GlcNAc Modifications of the Major Transcription Factor NFkB in Tumor Cells Variants Which Differ in Their Malignant and Metastatic Capacity

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Posttranslational modifications (PTM) of proteins are common, and significant processes in eukaryotic cells which are known to play a pivotal role in various major cellular events. In transcription processes, PTMs are most important for protein–protein interactions and also play a determinative role in the regulation of the activation, formation, and organization of transcription complexes, that is, the transcriptosomes. Recently, an additional form of posttranslational modification of intracellular proteins by O-linked *N*-acetylglucoseamine (*O*-GlcNAc) has been described by C.R. Torres and G.W. Hart. This form of PTM was found to be abundant and almost exclusively restricted to the cytoplasm and nucleus. In the presently reported investigation, an attempt was made to explore the possible existence of *O*-GlcNAc modifications in NFkB transcription complexes in two cloned variants of the murine T10 Fibrosarcoma, namely IE7 and IC9. These clones differ in their malignant and metastatic capacities and also differ in their expression of major MHC class I encoded cell membrane associated glycoproteins which was found to be directly linked to their metastatic capacity. In our previously presented work, we demonstrated the existence of differences in *O*-GlcNAc modifications of major transcription factors complexes such as NFkB and AP1 in the aforementioned clones using several different methods of analysis, for example, Emsa and lectin-affinity chromatography. The results obtained from our current investigation using β-*N*-acetylglucosaminidase to perform an enzymatic deglycosylation strongly supports the assumption that the p-65 subunit of NFkB could posses alternate patterns of *O*-GlcNAc modification, presumably because of the glycosylation of different Ser/Thr residues in the aforementioned variant clones. Our preliminary results demonstrated the existence of differences in the trimerization of the enzyme OGT between these variant clones. These differences in trimerization of the enzyme could function as a fine tuning mechanism which regulates the structural feature of OGT in these cells and as the result may alter the transcriptional activity of NFkB and its transcription complexes owing to PTMs. Indeed, preliminary results using transient transfection of the aforementioned clones with an expression vector which induces a promoter possessing a specific NFkB consensus sequence and firefly luciferase as a reporter gene demonstrate the existence of profound differences in luciferase activity in these cells. These results may indicate the biological significance and the total effect of the observed differences in *O*-GlcNAc modification of the P-65 subunit on NFkB-dependent transcription activities and regulation, which could determine the observed differences in the malignant phenotype of these variant tumor clones.

(220) Detection of Differentially Expressed Glycogenes in Trabecular Meshwork of Primary Open-Angle Glaucoma Eyes

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To identify differentially expressed glycogenes in trabecular meshwork (TM) of primary open-angle glaucoma (POAG) eyes. Total RNA was isolated from TM of cadaver eyes derived from donors diagnosed with glaucomas of different etiologies and from normal controls. RNA was amplified and hybridized to the GLYCOv2 oligonucleotide microarray that contains probes for carbohydrate-binding proteins, glycosyltransferases, and other genes involved in the regulation of glycosylation. Statistical analysis was used to identify differentially expressed genes between normal and POAG samples. This study revealed that POAG TM has a distinct gene expression profile that sets it apart from normal TM. Of the 2001 genes on the array, 19 genes showed differential expression of >1.4-fold in POAG. Mimecan and activin βA which have previously been shown to be up-regulated in model systems of glaucoma were both found to be elevated in POAG TM. Many genes were identified for the first time to be differentially regulated in POAG. Among the up-regulated genes were (1) cell adhesion molecules including intercellular adhesion molecule –1, platelet endothelial cell adhesion molecule –1, and P-selectin all of which are targets of NF kappa B which has been shown to be activated in glaucomatous TM; (2) lumican, a core protein of keratan sulfate proteoglycans; and (3) the receptor for IL6, a cytokine that has been shown to be up-regulated in TM in response to elevated intraocular pressure. Among the down-regulated genes were (1) chondroitin-4-*O*-sulfotransferase involved in the synthesis of chondroitin sulfate chains and (2) the receptor for PDGFβ, a growth factor that has been shown to stimulate both TM cell proliferation and phagocytic activity. Using microarray technology, we show for the first time that POAG TM has a distinct glycogene expression profile that sets it apart from normal TM. Differentially expressed glycogenes identified in this study have not been previously investigated for their role in the pathogenesis of POAG and, thus, are novel factors for further study of the mechanism of the disease.

(221) The Role of Differential Glyco-Gene Expression in Metastasis

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Metastasis involves local invasion, intravasation, transport, arrest, extravasation, growth at a secondary site, and angiogenesis. Carbohydrate- and lectin-mediated adhesion reactions may be involved in these mechanisms. To study metastasis and carbohydrates, we used four closely related murine mammary carcinoma cell lines. These cell lines include the highly metastatic 4T1 mouse mammary carcinoma line, and three lines derived from it; first line that has changed metastatic properties (4T1-Int), and other two lines which are not metastatic (67NR and 4T07). In clustering experiments, as expected, the cells with altered metastatic properties were found to be more closely related to the original metastatic line than the nonmetastatic cell lines. We have previously shown that antibody to TF-Ag (Galbeta1-3GalNAc) inhibits the ability of 4T1 cells to metastasize to the lung and enhances survival. This indicates that TF-Ag is involved in the metastatic process. Abnormal glycosylation patterns and changes in cell surface antigens also may cause or be the result of metastatic transformation. Looking at the relative expression levels of RNA for carbohydrate-related genes between metastatic and nonmetastatic cell lines may reveal some of the changes in cell surface carbohydrates that are responsible for metastasis. We used the help of the Consortium for Functional Glycomics to compare the carbohydrate-related genes in the four cell lines on a Glyco-gene chip. Isolated total RNA from each cell line was sent to the Consortium for Functional Glycomics for Glyco-gene chip analysis. The Glyco-gene chip is a microarray capable of detecting over 1800 different genes related to cell surface carbohydrate expression. Several sialyltransferases showed higher expression levels on the Glyco-gene chip in the nonmetastatic cell lines, whereas several galactosyltransferases and GalNAc transferases were found to be at higher levels in the metastatic cells. Sialic acid transport protein (LAMP-2) also showed increased expression in the nonmetastatic cell lines on the Glyco-gene chip. Galectin-9 showed higher expression in the metastatic line. These chip data will be validated using real-time quantitative RT–PCR for the following genes: ST3Gal1, ST6Gal1, b4GalT4, ST3Gal6, ST3Gal5, CD34, VCAM-1 (CD106-Long trans), glypican-6, Siglec-10, galectin-9, galectin-9 iso, sialic acid transport protein (LAMP-2), protective protein for beta-galactosidase, b4GalNAcT2 (GM2_GD2), Galnt-12, and ST3Gal2. So far, the expression levels of seven of the genes have been validated by real-time quantitative RT–PCR. For the following genes, ST3Gal1, ST3Gal2, ST3Gal5, ST3Gal6, ST6Gal1, galectin-9, and galectin-9 iso, similar ratios in gene expression between cell lines were observed with quantitative RT–PCR as those seen on the Glyco-gene chip. The level of surface carbohydrate expression will be confirmed using flow cytometry to detect several of the products of these genes, as well as TF-Ag, on cells from each carcinoma cell line.

(222) Development and Characterization of a Peptide Mimic of TF-Antigen

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The Thomsen–Friedenreich antigen (TF-Ag) is a carbohydrate tumor-associated antigen found in high amounts on the surface of several types of tumor cells, including breast, lung, prostate, and ovary. TF-Ag expression contributes to the process of cancer cell adhesion and metastasis. Metastatic sites, such as liver, bone, and lymph nodes, contain lectins that bind to TF-Ag. A highly specific, well-characterized IgG3 monoclonal antibody has been developed to TF-Ag. This antibody can interfere with TF-Ag binding to the vascular endothelium and thus block a primary step in tumor metastasis, and, in addition, antibodies to cell surface antigens can be cytotoxic. Thus, the development of a vaccine that causes patients to generate antibodies toward TF-Ag would have great clinical value. However, carbohydrate antigens generate T-cell-independent responses in the body. More effective are T-cell-dependent responses, generated by peptides and proteins. Therefore, identifying peptide mimics of TF-Ag is desirable. Other groups have developed antibodies to saccharide antigens in response to peptide mimics and demonstrated that animals immunized with peptide mimics have a memory response when immunized with the saccharide. In response to carbohydrate–epitope-bearing tumor cells, current research shows that T cells primed by peptide mimics can then react with carbohydrate molecules to produce cellular responses, and MHC molecules on antigen presenting cells normally displaying peptides can display glycopeptide moieties. This leads us to the hypothesis that vaccinations using a unique peptide mimic of TF-Ag will be able to generate immune responses to TF-Ag epitopes on tumor cells which will be clinically useful in active immunotherapy of many cancers. Our laboratory has identified peptide sequences able to mimic TF-Ag, as demonstrated first by the ability of antibody to TF-Ag (F11) to bind the peptide mimics in immunoblotting experiments. The peptides can block F11 binding to TF-Ag as seen by inhibition ELISA experiments. To measure the affinity of F11 for the peptides versus TF-Ag, Biacore analysis was performed. The peptides were also shown to block rolling and stable adhesion of cancer cells to the vascular endothelium in an *in vitro* model system, showing specificity of the interaction and potential of the peptides to block an important step in metastasis. The peptide mimics were conjugated to carrier proteins and used to immunize rabbits and mice with limited success thus far. Continuing experiments will use multiple antigenic peptides for immunizations followed by serum analysis using ELISA for the production of antibodies reactive to TF-Ag, and spleen cells for reactivity to TF-Ag bearing cells by proliferation and cytotoxicity assays. It was determined using MHC prediction databases that the peptide mimic sequences can be presented by MHC molecules. Molecular modeling of the peptide and F11 is currently underway. X-ray crystallography and peptide sequence modifications will be performed to generate better mimics if future immunizations are unfavorable. This research has the potential to be used to decrease tumor cell burden of cancer patients by specifically targeting TF-Ag positive cancer cells as well as aid in the blockage of newly forming tumors.

(223) Glycodynamics of Human Osteoblastic Cells

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Bone tissue is rich in glycoproteins involved in all aspects of bone biology. However, the biosynthesis and role of the glycan chains of bone glycoproteins are largely unknown. Our goal is to determine the pathways of glycosylation in bone cells and to examine how the cytokines present in osteoarthritis bone tissue regulate these processes. The mechanism of action of tumor necrosis factor (TNFalpha) and transforming growth factor (TGFbeta) was examined in primary cultures of human osteoblasts from osteoarthritis patients, as well as in osteosarcoma cells, SJSA-1, and prostate cancer cells metastasizing to the bone, PC-3, as models for bone cells. Cell proliferation was assessed by [3H]thymidine incorporation, apoptosis by TUNEL staining, and cell differentiation by assaying alkaline phosphatase activities. Cell surface carbohydrates were determined by lectin-binding assays and the biosynthesis of glycoproteins by assaying glycosyltransferase activities. We demonstrated that each bone cell type has characteristic levels of enzyme activities synthesizing complex Asn-linked and Ser/Thr-linked carbohydrate chains (*N*-glycans and *O*-glycans, respectively) and that specific glycans were exposed on cell surfaces. Treatment of cells with TNFalpha or TGFbeta caused multiple alterations of cell surface lectin-binding patterns and activities of several glycosyltransferases. We conclude that apoptosis and cell proliferation or differentiation of primary human osteoblasts, as well as of specific bone cancer cells, is associated with alterations in glycosylation. These changes can result in altered cell surface functions which may be of importance in osteoarthritis. This work has been supported by Materials and Manufacturing Ontario.

(224) Structural Studies of the Cholera Toxin-Binding Epitopes in the Lipopolysaccharide Fractions of *Campylobacter jejuni*

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Elevation of anti-GM1 ganglioside antibodies is frequently a representative event of Guillain-Barre syndrome (GBS). Although the etiological mechanism for the generation of anti-GM1 antibodies has not been clarified, *Campylobacteriosis* is a potential risk factor for the induction of GBS, causing the production of high titer anti-GM1 antibodies. Molecular mimicry between the carbohydrate structures of GM1 and lipopolysaccharides (LPS) derived from a certain strain of *Campylobacter jejuni* has been proposed as the trigger of the antibody production. We have previously shown that a LPS fraction from serotype HS:19 of *C. jejuni* is composed of two components (XXXX, 2003), characterized as fast and slow moving bands (LF and LS) on the TLC plate as revealed by cholera toxin (CT) overlay method. We immunized Lewis rats with the LPS fraction from serotype HS:19 of *C. jejuni* and were successful in generating cross-reactive antibody between LPS and GM1. This antibody was reactive to both of LS and LF based on TLC-overlay. To facilitate structural analysis, LF and LS were purified by silica gel column chromatography and CT-affinity chromatography. After mild acid hydrolysis of LS, the oligosaccharide was recovered from an aqueous fraction and converted to *p*-aminobenzoic acid octyl ester (ABOE) and 1, 2-diamino-4, 5-methylenedioxybenzene (DMB) derivatives. ABOE- and DMB-derivatives were analyzed by high-performance liquid chromatography (HPLC) using a fluorescence detector. ABOE- or DMB-labeled oligosaccharide was recovered and subject to MALDI-TOF mass spectrometric analysis. Structural analysis of the oligosaccharide confirmed that it has the following structure Gal-GalNAc-Gal-(NeuAc)Gal-Hep-(Glc; PO_3H)Hep-Kdo. Validation of the GM1-like epitope was shown by the presence of a terminal pentasaccharide within this structure. On the other hand, no GM1-like epitope was validated in LF, which was unexpectedly characterized as lipid A, following alkaline phosphatase treatment, chloroform partitioning, hydrazinolysis, and then hexane partitioning. Structural analysis of LF revealed that it is a novel lipid A with the following structure 1,4′-bisphosphorylated glucosamine disaccharide N,N′-acylated by 3-(2-hydroxytetracosanoyloxy)octadecanoic acid at 2- and 2′-positions. The affinity of LF to CT required the presence of phosphate groups in the glucosamine disaccharide residue, because LF was unable to bind CT after alkaline phosphatase treatment. Based on the above data, we propose that binding of LF to CT may require electrostatic interaction. We conclude that GM1 and LF may bind to different sites on the CT molecule.

(225) Standardizing Lectin-ELISA for Routine Screening of Serum Transferrin Glycosylation

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Glycans are involved in genesis, development, and processing of many diseases, and a routine tool to study glycosylation changes would be of significant help to both diagnose and understand underlying pathophysiological changes in these diseases. Glycan structures are very complex and methods to analyze them are generally prohibitively demanding and time consuming for routine screening. ELISA-type assays of glycosylation might be a solution, but because of numerous technical difficulties, this type of assays never made their way into clinical practice. Aiming to establish such a method, we are developing a lectin-based ELISA for the analysis of serum transferrin glycosylation. Transferrin is a serum glycoprotein that has two N-linked oligosaccharide chains consisting of bi- or triantennary structures, each possibly terminating in sialic acid. Immobilization of antibodies against transferrin enabled us to perform simple, in the well, purification of transferrin from serum. *In situ* treatment with 20 mM periodate for 30 min at +4°C completely abrogated binding of lectins to carbohydrates on immobilized antibodies without significantly affecting antibody's affinity for transferrin. The method was tested by analyzing glycosylation of transferrin in sera obtained from healthy individuals, chronic obstructive pulmonary disease (COPD) patients, CDG patients, and alcoholics. Repeated analysis of the same sample showed good within plate and between plates reproducibility. Expected decrease of transferrin sialylation was observed in alcoholics and CDG patients, and statistically significant decrease of sialylation was also found in COPD. Our lectin-ELISA method for transferrin glycosylation appears to reliably quantitate changes in glycosylation, and because it uses a very small amount of serum and allows rapid screening of multiple samples it promises to be a useful tool to screen glycosylation changes in disease.

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(226) The Significance of Altered Alpha-1-Acid Glycoprotein Glycosylation in Breast Cancer

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In humans, alpha-1-acid glycoprotein (AGP) is an acute phase protein that increases in concentration in the plasma 2- to 5-fold in certain pathophysiological states. It is extensively glycosylated (45%) with five asparagine-linked complex oligosaccharide chains. In normal serum, AGP exists as a heterogeneous population of glycoforms. Heterogeneity arises through structural differences in monosaccharide sequence and linkages, degree of branching, and extent of sialylation. The glycosylation of AGP is also altered in many disease conditions including breast cancer. Thus, unique alterations in AGP glycosylation could indicate the early presence of breast cancer, before it can be observed by existing detection methods or physical symptoms. AGP is known to bind drug molecules in the plasma including tamoxifen, the anti-oestrogen drug used in treating breast cancer. If bound, tamoxifen remains in the plasma, therefore unable to reach its target site to elicit the desired anti-tumour effect. In breast cancer plasma, the level of albumin (which binds 99% of tamoxifen) decreases while that of AGP increases considerably inferring that AGP becomes the major binder of tamoxifen. Additionally, the affinity of breast cancer AGP may be influenced by disease-specific alteration in glycosylation that could alter the conformation of, and thus access to, the binding site. Increased binding would reduce the amount of free tamoxifen available for therapeutic effect. This project will determine whether disease-specific alterations in the glycosylation of AGP could be diagnostic for the detection of breast cancer at an earlier point, closer to its initial development, than existing methods thus improving survival rates and whether any changes in glycosylation alter the extent of binding to tamoxifen and therefore affect therapeutic effectiveness. Plasma samples were obtained from patients on either adjuvant or postsurgery tamoxifen therapy. AGP concentration in each sample was measured by immunoturbidity. After quantification, AGP was isolated from each sample by low pressure chromatography and the glycosylation pattern determined using high pH anion-exchange chromatography. The binding capacity of AGP for tamoxifen was determined using a microtitre assay based on the intrinsic fluorescence of AGP which is reduced in a concentration-dependent manner by binding to tamoxifen. The levels of AGP in serum of breast cancer populations were, on average, increased in comparison with healthy plasma, thus confirming that breast cancer elicits an acute phase type response. Initial analysis of AGP glycosylation revealed that there is a noticeable difference between healthy and breast cancer populations. Furthermore, there are differences in the degree of binding to tamoxifen which appear related to glycosylation pattern. The identification of disease-specific alterations in AGP glycosylation could be used to detect the onset of early stage breast cancer before other methods, would correlate in earlier detection of the disease, closer to its initial development, drastically improving survival rates. Correlating altered glycosylation to changes in tamoxifen binding could identify a mechanism that could be used to proactively determine whether prescribing tamoxifen to an individual patient would result in the desired therapeutic benefit.

(227) Pre-mRNA Splicing Factor and Annexin A1 Mediate Carbohydrate-Dependent Hematogenous Cancer Cell Colonization in the Mouse Lung

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Apical surfaces of epithelial cells are covered by a variety of carbohydrates attached to membrane proteins and lipids. When epithelial cells are transformed, the repertoire of cell surface carbohydrates alters significantly. Many studies suggest that *O*-glycans with terminal structures such as sialyl Lewis x (sLeX) play an important role in cancer metastasis. Because sLeX is the ligand for selectins, the selectins are thought to be responsible for sLeX-dependent cancer metastasis. To investigate carbohydrate-dependent cancer metastasis *in vivo*, we developed a sLeX-dependent experimental cancer metastasis model in the mouse. Although mouse melanoma B16 cells are negative for sLeX-antigen, they acquire sLeX-antigen after transfection with fucosyltransferase III (FTIII) cDNA. When sLeX-positive B16-FTIII-M cells were injected intravenously into mice, B16-FTIII-M cells colonized the lung, whereas sLeX-negative B16 cells did not (Ohyama *et al*., 1999). Previously, we identified several peptides that interact with selectins as ligand mimicry (Fukuda *et al*., 2000). When one such peptide, IELLQAR, was injected intravenously into mice, it bound to lung vasculature and inhibited lung colonization of B16-FTIII-M cells. However, this peptide inhibited cancer colonization in mutant mice lacking both E- and P-selectins, suggesting the existence of a carbohydrate-binding receptor or IELLQAR peptide receptor (IPR) distinct from selectins (Zhang *et al*., 2002). The IPR proteins were isolated by IELLQAR peptide affinity chromatography from rat lung membranes. Proteomic analysis identified the major IPR protein as a pre-mRNA splicing factor (SF). Although SF proteins do not have a signal sequence typical of a membrane protein, a biotinylation reagent injected intravenously into mice-labeled SF protein, which was immunoprecipitated with an anti-SF antibody. Upon intravenous injection into mice, anti-SF antibodies inhibited lung colonization of B16-FTIII-M cells *in vivo*. We have also identified another IPR as Annexin A1 (Axn1). Carbohydrate-binding activity of SF and Axn1 was confirmed by recombinant SF and Axn1 proteins expressed in bacteria. Given that galectin-1 and -3 exhibit RNA-splicing activity (Wang *et al*., 2004) and that Axn-1 and -2 bind RNA (5), carbohydrate- and RNA-binding activities may overlap in these group of proteins.

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(228) MUC1 Membrane Trafficking is Modulated by Multiple Interactions

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MUC1 is a mucin-like transmembrane protein found on the apical surface of many epithelia. Because aberrant intracellular localization of MUC1 in tumor cells correlates with an aggressive tumor and a poor prognosis for the patient, experiments were designed to characterize the features that modulate MUC1 membrane trafficking. By following [35S]Met/Cys-labeled MUC1 in glycosylation-defective Chinese hamster ovary cells, we previously found that truncation of *O*-glycans on MUC1 inhibited its surface expression and stimulated its internalization by clathrin-mediated endocytosis (Altschuler *et al*., 2000). To identify signals for MUC1 internalization that are independent of its glycosylation state, the ectodomain of MUC1 was replaced with that of Tac, and chimera endocytosis was measured by the same protocol (Kinlough *et al*., 2004.). Endocytosis of the chimera was significantly faster than for MUC1 indicating that features of the highly extended ectodomain inhibit MUC1 internalization. Analysis of truncation mutants and tyrosine mutants showed that Tyr20 and Tyr60 in the 72-amino acid cytoplasmic tail were both required for efficient endocytosis. Mutation of Tyr20 significantly blocked coimmunoprecipitation of the chimera with AP-2 indicating that Y20HPM is recognized as a YXXf*f*nmotif by the mu2 subunit. The tyrosine-phosphorylated Y60TNP was previously implicated as an SH2 site for Grb2 binding, and we found that mutation of Tyr60 blocked coimmunoprecipitation of the chimera with Grb2. This is the first indication that Grb2 plays a significant role in MUC1 endocytosis. Palmitoylation of transmembrane proteins can also affect their membrane trafficking, and the context of the MUC1 sequence CQC between the transmembrane domain and a cluster of basic residues fits the minimal consensus for protein S-acylation. [3H]Palmitate labeling of CHO cells expressing MUC1 or mutants with one or two Cys changed to Ala revealed that MUC1 is dually palmitoylated at the CQC motif. Mutation of both Cys (AQA) blocked palmitoylation but did not affect either the detergent solubility profile of the Tac-MUC1 chimera or the rate of chimera delivery to the cell surface. Using cell surface biotinylation and calculation of rate constants from membrane trafficking profiles of wild type and mutant AQA chimera indicates that palmitoylation is required for recycling from endosomes to the cell surface, but not for endocytosis. In contrast, mutation of tyrosine in Y20HPM reduces the rate constants for both endocytosis and recycling consistent with an additional role for this Yxxf motif. Our data suggest that palmitoylation of MUC1 could mediate its exit from endosomes by enhancing interaction of the YHPM motif with an adaptor such as AP-1.

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(229) Simultaneous Specific Quantification of Dermatan Sulfate and Heparan Sulfate in Urine

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Dermatan sulfate (DS) and heparan sulfate (HS) are glycosaminoglycans (GAG) that accumulate in cellular lysosomes of patients with mucopolysaccharidosis (MPS) storage disorders because of genetic defects in key enzymes of GAG degradation. A simple method for quantification of DS and HS, with no interference from other GAG including chondroitin sulfate A (ChS A) and chondroitin sulfate C (ChS C), is important for diagnosis of different types of MPS and the evaluation of new therapeutic treatments for these disorders. Here we demonstrate selective enzymatic degradation with chondroitinase ABC (ABC), chondroitinase B (B) and heparinases (H), and chemical depolymerization with nitrous acid (NA) of urine GAG, coupled with a microtiter plate-based, dimethylene blue dye-binding method (DMB) (Wiederschain *et al*., 2003) to quantify DS and HS in urine. The validated method consists of (1) quantification of total GAG in a sample using DMB, (2) NA- and B-treatment of two equal sample aliquots, (3) quantification of residual GAG in each sample aliquot after treatment using DMB, and (4) calculation of DS and HS concentration by subtraction using the before/after treatment GAG values. Specific lyases are well-known tools in the structural identification of GAG. The use of ABC, B, and H was investigated to sequentially degrade GAG and to quantify DS and HS. ABC was stable and fully degraded DS from pig skin and intestinal mucosa, but also degraded ChS A and ChS C, whereas degrading <5% of HS. On the other hand, B specifically degraded up to 80% DS within a 2-h incubation and was inactive with ChS A, ChS C, and HS as substrates. A mixture of heparinases Types I, II, and III (HI + II + III) degraded HS (1 mg/mL) much more effectively (~90%) than each enzyme alone. NA at pH ~1.5 is known to selectively depolymerize HS by deaminative cleavage of glycosidic bonds of N-sulfated glucosamine residues, whereas those of N-acetylated aminosugars found in other GAG are resistant. Treatment with NA was found to depolymerize up to 90% HS with no effect on DS. NA treatment demonstrated similar HS results compared with a specific monoclonal antibody HS ELISA method and sample digestion with HI + II + III, in a simpler and cost-effective manner. This method demonstrates good precision, with inter- and intra-assay variability <12% relative standard deviation (RSD). Between analysts variability is <7%, whereas between run variability is <2%. Assay performance is monitored using internal HS and DS controls, as well as urine from known MPS patients.

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(230) Biological Significance of Cancer-Associated Sialyl-Tn Antigen: Modulation of Malignant Phenotype in Gastric Carcinoma Cells

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The abnormal expression of Sialyl-Tn antigen (Neu5Acα2,6GalNAc-α-*O*-Ser/Thr) is a common phenotypic change observed in cancer and is associated with cancer aggressiveness and poor prognosis. Sialyl-Tn is rarely observed in normal tissues but highly expressed in a variety of carcinomas, including gastric carcinoma. We have recently characterized the role that the human glycosyltransferases ST6GalNAc-I and ST6GalNAc-II play in the biosynthesis of the Sialyl-Tn antigen. We evaluated the carbohydrate antigens expression profile induced by each enzyme and observed that ST6GalNAc-I is the major Sialyl-Tn synthase, whereas the ST6GalNAc-II induces expression of Sialyl-6T structure (Marcos *et al*., 2004). This study characterizes the biological behavior of gastric carcinoma cell line MKN45 stably transfected with the full-length of human ST6GalNAc-I (MST6-I) or ST6GalNAc-II (MST6-II). Slow aggregation assay showed a markedly reduced homotypic cell–cell adhesion of MST6-I cells, with most cells remaining solitary, and moderate reduction of MST6-II cells, presenting few loose aggregates, when compared with mock cells that form tight cell clusters. The MST6-I cells, expressing Sialyl-Tn, presented a significant increase in adhesion and migration on extracellular matrix (ECM) substrates, such as fibronectin and collagen I. These motility alterations were correlated with cytoskeletal alterations such as the formation of lamellipodia and filopodia, in contrast to a round cell and the lack of actin extensions observed in mock-transfected cells. The MST6-I cells expressing Sialyl-Tn showed a 2.5-fold increased invasion capability in matrigel invasion assays when compared with mock cells. Incubation in the presence of the blocking monoclonal antibody anti-Sialyl-Tn reverted the invasive behavior. MST6-II showed a moderate increase in adhesion, migration, and invasion capabilities when compared with mock. Putative Sialyl-Tn carriers in MST6-I and MST6-II cells are currently being identified by immunoprecipitation studies. In conclusion, we found that *de-novo* expression of Sialyl-Tn leads to major morphological and cell behavior alterations in gastric carcinoma cells which were reverted by specific antibody blockage. Sialyl-Tn antigen is able to modulate *per se* a malignant phenotype inducing a more aggressive cell behavior, such as decreased cell–cell aggregation and increased ECM adhesion, migration, and invasion. These findings strongly suggest that Sialyl-Tn antigen plays a crucial role in carcinoma progression. Supported by FCT (POCTI/CBO/44598/02) and AICR (grant 05-088). Both S.P. and N.T.M. contributed equally to this work.

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(231) The Effect of Glycosylation on the Drug-Binding Ability of AGP

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Human alpha-1-acid glycoprotein (AGP) is a hepatically produced plasma glycoprotein, the plasma level which increases 2- to 5-fold as a result of the hepatic acute phase reaction. The glycosylation of AGP can also be subject to marked changes in the branching of its oligosaccharide chains and in the degree of fucosylation and sialylation resulting in expression of disease-specific glycoforms. The exact biological function of AGP is still controversial; however historically, it has been recognized as a major plasma binder of drugs. Therefore, its increased level in various disease states will considerably influence the free plasma level of drug, and therefore the effectiveness *in vivo*. Although the drug-binding site of AGP is peptide in nature, altered glycosylation may influence drug-binding capacity. The size and surface location of the oligosaccharide chains of AGP influences binding by affecting the conformation of, and thus access to, the binding site. Thus alterations in both the levels and glycosylation of AGP could influence the extent to which AGP is able to bind particular drugs. Such an alteration could represent a significant, novel form of resistance to the disease; represent an apparent mechanism of *in vivo* resistance to depletion of the drug in the plasma and potentially would necessitate dose adjustment according to plasma AGP expression. This is of particular significance in tuberculosis (TB) due to the emergence of multi-drug resistance. To determine the extent to which drugs used to treat TB are bound by AGP and the effect of glycosylation on this interaction. The degree of AGP binding to TB drugs isoniazid, rifampicin, *p*-aminosalicylic acid, and pyrazinamide was determined by analyzing the fluorescence spectra (300–400 nm) of AGP, because the intrinsic fluorescence of the protein is reduced (quenched) by drug binding; the fluorescent emissions of tryptophan (and to a lesser extent tyrosine) residues are masked by the binding of a drug. AGP was isolated from a normal plasma population using a three-stage low pressure column chromatography process. The branching pattern of the oligosaccharide chains in each sample was established using a concanavalin (con) A ELISA, then an affinity column of this lectin was used to produce three glycoform fractions which were assessed by drug binding to determine the effect of glycosylation differences. High pH anion exchange chromatography (HPAEC) was used to characterize the oligosaccharides profile of each glycoform population. A consideration of the fluorescent spectra of AGP before and after the addition of the various drugs indicated that certain drugs, especially isoniazid, were bound. Therefore, the drug concentration in plasma would be decreased. The separation of the normal population into fractions based on the degree of chain branching resulted in differences in drug binding, compared with whole AGP. The ability of AGP to bind TB drugs is correlated with glycosylation and may represent a significant and novel form of resistance which could necessitate dose adjustment according to plasma expression.

(232) Requirement of Golgi GDP-Fucose Transporter for Notch Signaling in *Drosophila*

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Congenital disorder of glycosylation IIc (CDG IIc), also termed leukocyte adhesion deficiency II (LADII), is a recessive syndrome characterized by

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slowed growth, mental retardation, and severe immunodeficiency. Recently, the gene responsible for CDG IIc was found to encode a guanosine diphosphate (GDP)-fucose transporter. Here, we investigated the possible cause of the developmental defects in CDG IIc patients using a *Drosophila* model. Biochemically, we demonstrated that a *Drosophila* @homolog of the GDP-fucose transporter, the Golgi GDP-fucose transporter (*Gfr*), specifically transports GDP-fucose *in vitro*. To understand the function of the *Gfr* gene, we generated null mutants of *Gfr* in *Drosophila*. The phenotypes of the *Gfr* mutants were rescued by the human GDP-fucose transporter transgene, suggesting that *Gfr* is an ortholog of this human gene. Our phenotype analyses revealed that Notch signaling was deficient in these *Gfr* mutants. GDP-fucose is known to be essential for the fucosylation of N-linked glycans and for O-fucosylation, and both fucose modifications are present on Notch. Our results suggest that Gfr is involved in the fucosylation of N-linked glycans on Notch and its O-fucosylation, as well as those of bulk proteins. However, despite the essential role of Notch O-fucosylation during development, the *Gfr* homozygote was viable. Thus, our results also indicate that the *Drosophila* genome encodes at least one other GDP-fucose transporter that is involved in O-fucosylation of Notch. Finally, our results implicate the reduction of Notch signaling in the pathology of CDG IIc.

(233) Detection of Differentially Expressed Glycogenes in Healing Mouse Corneas: Comparison of Galectin-3-Deficient and Wild-Type Mice

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(1) To identify differentially expressed genes in healing mouse corneas by using glycogene microarrays and (2) to identify differentially expressed glycogenes in the corneas of galectin-3-deficient (gal3 –/–) and wild-type (gal3 +/+) mice. Transepithelial excimer laser ablations were performed on gal3 –/– and gal3 +/+ mouse corneas, and the wounds were allowed to heal partially *in vivo* for 18–22 h. Total RNA was isolated from both normal and healing corneas. RNA was amplified and hybridized to the GLYCOv2 oligonucleotide microarray that contains probes for carbohydrate-binding proteins, glycosyltransferases, and other genes involved in the regulation of glycosylation. Statistical analysis was used to identify differentially expressed genes. In wild-type gal3 +/+ mice, of the 2001 genes on the array, the expression of 37 genes was up-regulated and 40 genes was down-regulated >1.5 fold in healing corneas compared with the normal, unwounded, corneas. Among the up-regulated glycogenes were C-type lectins, mincle, and dectin-1; glycosyltransferases, b3GalT5, b4GalNAcT2, sialyltransferases 4A, and 4C; extracellular matrix proteoglycan, serglycin; and MUC-1 mucin. Among the down-regulated genes were fibulin 5, IL-1 delta, VEGF-B, sialyltransferases-7, 8, and 9, and lumican. Comparison of glycogene expression pattern of unwounded corneas of gal3 –/– and gal3 +/+ mice revealed that largely galactosaminyltransferases were up-regulated in the gal3 –/– mice. Of particular significance is the finding that a galactosyltransferase, b3GalT5, which is markedly up-regulated in healing gal3 +/+ corneas, is in contrast down-regulated in healing gal3 –/– corneas. Glycogene microarray technology was used to identify for the first time many genes that are differentially regulated during corneal wound healing in galectin-3-deficient and wild-type mice. These differentially expressed glycogenes have not previously been investigated in the context of wound healing and represent novel factors for further study of the mechanism of wound healing. Our findings that the expression of b3GalT5 is markedly down-regulated in healing gal3 –/– corneas, supports our hypothesis that galectin-3 modulates the expression of key glycosyltransferases, which in turn, regulate glycosylation of proteins, which serve as counterreceptors of the lectin itself.

(234) Modeling the Pathomechanisms Underlying Protein-Losing Enteropathy in Post-Fontan Patients

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Patients born with univentricular hearts survive because of surgical interventions including the Fontan procedure. However, months to years after the surgery, 3–10% of the post-Fontan patients develop protein-losing enteropathy (PLE) which is defined as the enteric loss of plasma proteins. Half of these patients die. Why some post-Fontan patients develop this complication is unknown and so is the molecular basis of PLE itself. The Fontan surgery causes venous hypertension which might predispose for PLE. PLE onset is then often correlated with viral infections (elevated IFNgamma) and inflammation (elevated TNFalpha). Intestinal biopsies revealed the loss of heparin sulfate (HS) proteoglycans (HSPG) from the basolateral surface of intestinal epithelial cells during PLE episodes. These four factors (increased pressure, IFNgamma, TNFalpha, and HS loss) have been observed in other seemingly unrelated diseases associated with PLE, and we hypothesize that they all directly or indirectly contribute to the development of PLE. We established the first tissue culture model of PLE to investigate the contributions of the four factors alone and in combination. Increased pressure, HS loss, and TNFalpha alone, but not IFNgamma induced protein leakage. Both HS loss and TNFalpha amplified pressure-induced protein leakage. HS loss and IFNgamma amplified TNF-mediated protein leakage. FACS analysis revealed that IFNgamma up-regulates TNFalpha receptor 1. Both effects of IFNgamma treatment were further enhanced to a similar extent when cell-associated HS was removed. IFNgamma and TNFalpha bind to HS, and our results suggest that loss of cell-associated HS makes more IFNgamma and TNFalpha available to interact with their receptors inducing protein leakage. Addition of soluble HS or heparin completely reversed the effects of cell-associated HS depletion. A combination of all four factors caused the most pronounced protein leakage, which again could be reversed by the addition of soluble HS or heparin. Leakage was not only observed from the basolateral to the apical side of the epithelial monolayer, but also from the apical to the basolateral side, suggesting that our results have implications not only for PLE, but also for the translocation of LPS or bacteria from the lumen into the blood in inflammation, sepsis, and possibly autoimmune diseases. We are now investigating whether the synergisms between pressure, HS loss, IFNgamma, and TNFalpha also occur *in vivo* using a mouse model mimicking PLE. Mice genetically deficient in syndecan-1, the predominant HSPG on the basolateral surface of intestinal epithelial cells, have increased intestinal protein loss which can be further aggravated by TNFalpha injections. Loss of Syndecan-1 and TNFalpha alone or in combination increase pressure-induced leakage through mucosal explants. Ongoing experiments aim to elucidate the role of IFNgamma and the reversal of protein leakage with heparin. Our models aim to clarify the pathomechanisms of PLE and may help to guide therapeutic studies using heparin and heparin-like molecules to treat or even prevent this life-threatening condition not only for post-Fontan patients but also for patients with other diseases associated with PLE, for example, congenital disorders of glycosylation or inflammatory bowel disease. Supported by NIH (R01 DK 065091, R21 HL 078997), Children's Hearts Fund, and Deutsche Forschungsgemeinschaft (DFG BO 2488/1-1).

(235) A *Caenorhabditis elegans* Model of Insulin Resistance: An OGT-1 Knockout Shows Altered Macronutrient Storage and Dauer Formation

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N-Acetylglucosaminyl transferase, OGT, catalyzes the terminal addition of *N*-acetylglucosamine (GlcNAc) to serine and threonine residues. This type of O-linked glycosylation occurs on nuclear pore proteins, transcription factors, and signaling kinases. There is evidence that this type of modification is dynamic and can occur in opposition to phosphorylation. In addition to modulating the function of other proteins, OGT mediates the last step in a nutrient-sensing "hexosamine-signaling pathway" which may be deregulated in diabetes. The OGT null phenotype in mouse is embryonic lethal. In contrast, the *Caenorhabditis elegans* OGT null is viable and fertile. Here, we used a strain of OGT null *C. elegans* to examine the effects of loss of OGT function on nuclear import and several outputs of the insulin-signaling pathway. We found that the nuclear import of several transcription factors were unaffected by the loss of OGT. Interestingly, we found striking alterations in macronutrient storage and changes in dauer formation. Specifically, we saw an ~3-fold elevation in trehalose levels and glycogen stores with a concomitant ~3-fold decrease in triglyceride levels. In addition, we found that loss of OGT suppresses dauer formation induced by the temperature sensitive allele *daf-2(e1370)*, an insulin-like receptor gene. Our results indicate that OGT modulates macronutrient storage and dauer formation in *C. elegans*.

(236) Differential Glycosylated Patterns of Intracellular Chaperone Protein gp96 is a Determinant of Prostate Cancer Phenotype

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Intracellular endoplasmic resident gp96 is a conserved glycoprotein that associates with cellular peptides, and the gp96-peptide complex has clinical significance in cancer and in viral and bacterial infections. Tissue-specific peptides associated with gp96 impart the uniqueness to gp96; however, neither gp96 nor the peptides individually impart the clinical features related to gp96-mediated tumor regression. The biochemical and biological characteristics of gp96 associated

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with glycosylation is an understudied area. In this study, we examined the cancer-specific glycosylation patterns of tissue-purified gp96 that was obtained by a series of column chromatography and confirmed by a single band by SDS–PAGE. Neutral and amino sugar were released by TFA and HCl hydrolysis and sugar moieties measured and quantified in a HPAEC-PAD machine. A comparative analysis of monosaccharide compositions of gp96 between normal rat prostate and two cancerous rat prostate tissues, nonmetastatic/androgen-dependent Dunning G and metastatic/androgen-independent MAT-LyLu was undertaken. Marked differences were observed between the gp96 monosaccharide compositions of the normal and cancerous tissues. Furthermore, gp96 molecules from more aggressive cellular transformations were found to carry decreasing quantities of several monosaccharides as well as sum total content of neutral and amino sugars. The sugar composition of gp96-affected peptide binding as determined by using a defined viral peptide VSV8 of the following sequence RGYVYQGL. We believe that the unique glycosylation patterns contribute to cellular phenotype and that the posttranslational modifications of gp96 may affect its functional attributes. These data have a notable relevance for the development of autologous gp96-based cancer vaccines (as well as vaccines for infectious and bacterial pathogens). The contribution of the glycosyl residues on gp96 as determinant of cancer phenotype is being examined both structurally and functionally in a prostate cancer model. Both A.B. and R.K.T. contributed equally to this work.

(237) Synthesis and Characterization of Fragments from the Mucin-Like Region of α-Dystroglycan

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Electron microscopy shows that the α-dystroglycan glycoprotein is organized as two globular domains separated by an extended region that likely corresponds to the mucin-like sequence in the middle of the protein (Brancaccio *et al*., 1995). This region of extended secondary structure appears to be important in organizing the dystrophin protein complex in skeletal muscle. The Ser and Thr residues in this region are highly glycosylated, including at least a majority of carbohydrates linked to the amino acid residues in an unusual way via an α-O-linked mannose (Martin, 2003). Characterization of the epitopes indicate the presence of some α-O-linked GalNAc-based epitopes as well (Sasaki *et al*., 1998). Defects in the posttranslational O-glycosylation of the mannose-linked glycans of this region, attributed to mutations in the enzymes that mediate this process, result in a variety of forms of muscular dystrophy. In addition to the primary structure of the epitopes, their specific disposition dictated by the extended organization is likely to be important in the interactions of this middle segment of the molecule with other proteins in the complex, and thus in organizing the complex. In the typical mucin architecture, the α-O-linked GalNAc residue attached to the amino acid is crucial in stabilizing the extended structure (Coltart *et al*., 2002). The arrangement of functional groups on mannose, however, would require different intramolecular interactions to achieve the same global molecular features. To address the role of the mannosylated residues in conformational preferences of α-dystroglycan and to determine whether only this initial residue can initiate the extended secondary structure, we are examining a series of mannosylated glycopeptides derived from the α-dystroglycan sequence. Glycosyl amino acid building blocks, *N*-(9-fluorenylmethoxycarbonyl)-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-L-serine/threonine pentafluorophenyl esters, were stereoselectively synthesized based on reaction of 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl fluoride, activated by boron trifluoride etherate, with *N*-(9-fluorenylmethoxycarbonyl)-L-serine/threonine pentafluorophenyl esters, in yields of 50 and 46%, respectively. The α stereochemistry of the glycosidic linkage was established from the couplings of the anomeric proton and carbon 1JC1,H1Ser = 172.2 Hz, 1JC1,H1Thr = 173.2 Hz. These building blocks have now been used in solid-phase synthesis of the first two glycopeptide fragments from mucin-like region of α-dystroglycan in our series, Ac-Val-Glu-Pro-Thr(α-D-Manp)-Ala-Val-NH2 and Ac-Val-Ser(α-D-Manp)-Thr(α-D-Manp)-Pro-Lys-NH2, which were obtained upon cleavage from the resin and deprotection. The corresponding aglycone peptides, namely, Ac-Val-Glu-Pro-Thr-Ala-Val-NH2 and Ac-Val-Ser-Thr-Pro-Lys-NH2 were also synthesized as reference molecules. Building blocks and products were characterized by normal-phase and/or reversed-phase high-performance liquid chromatography, and electrospray ionization mass spectrometry. Additional molecules in this series are being synthesized, and we will describe their conformational properties.

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(238) *O*-GlcNAc Levels Modulate Adipocytokine Secretion Under Diabetic Conditions

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Increases in intracellular glycosylation, via *O*-GlcNAc modification, induce insulin resistance in rodent adipocytes. It has been demonstrated that the induction of insulin resistance, the hallmark of Type II diabetes, in adipose tissue induces resistance in other tissues as well as the whole animal. Furthermore, it has become increasingly clear over the last decade that adipocytes, besides being an energy storage depot, are an endocrine tissue that secrete a variety of signaling proteins, termed adipocytokines. Given that adipocytokines are regulated primarily at the level of transcription, the emerging role of *O*-GlcNAc in modulating transcription including leptin, and the established role for *O*-GlcNAc in inducing insulin resistance, we hypothesized that elevation in *O*-GlcNAc-modified proteins would modulate the profile of secreted proteins from adipocytes. Using differentiated 3T3-L1 and primary adipocytes, we induced insulin resistance classically (hyperglycemia and chronic insulin exposure) or through the pharmacological elevation of global *O*-GlcNAc levels. We then compared the secreted proteome of these cells with control adipocytes grown under insulin-responsive conditions (euglycemia). Using offline HPLC and tandem mass spectrometry (2D-LC-MS/MS) approaches, we have identified >150 proteins in the secreted proteome of adipocytes. Given that the majority of secreted proteins are glycosylated, we have used beta-elimination/Michael addition (BEMAD) and PNGaseF in the presence of 18-O water methodologies to map O- and N-linked sites, respectively. We have quantified changes in the secreted proteome when shifted from insulin-responsive to insulin-resistance conditions using percent protein coverage and our recently developed method for covalently tagging alkylated cysteine residues with isotope heavy and light DTT via the BEMAD methodology. This approach has enabled us to identify novel biomarkers for insulin resistance, including several that are regulated by global *O*-GlcNAc levels.

(239) Evaluation of the Glycosylation Status of Alpha-Dystroglycan in Hereditary Inclusion Body Myopathy

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Hereditary inclusion body myopathy (HIBM) is an adult onset autosomal recessive neuromuscular disorder characterized by slowly progressive myopathic weakness and atrophy. HIBM is caused by mutations in UDP-GlcNAc 2-epimerase (GNE)/*N*-acetylmannosamine kinase (MNK), the bifunctional and rate-limiting enzyme in sialic acid biosynthesis. We developed individual GNE and MNK enzymatic assays and determined reduced activities in cultured fibroblasts of patients with HIBM harboring missense mutations in either or both GNE and MNK enzymatic domains. To assess the effects of individual mutations on enzyme activity, normal and mutated GNE/MNK enzymatic domains were synthesized in a cell-free *in vitro* transcription-translation system and subjected to the GNE and MNK enzymatic assays. This revealed that mutations in one enzymatic domain affected not only that domain's enzyme activity but also the activity of the other domain. This loss of enzyme activity impairs sialic acid production, which may interfere with proper sialylation of glycoconjugates. We investigated how this would lead to muscle pathology. First, we demonstrated normal isoelectric focusing (IEF) patterns of transferrin in HIBM patients' serum, suggesting normal N-linked glycosylation. Next, we performed immunohistochemistry on HIBM muscle employing antibodies against components of the dystrophin–glycoprotein complex. Beta-dystroglycan and laminin-alpha 2 showed normal patterns, but antibodies recognizing O-linked glycan epitopes of alpha-dystroglycan (α-DG) showed reduced staining. α-DG contains both *O*-GalNAc and *O*-mannose-linked glycans; the latter are rare in mammals. Finally, we showed normal IEF patterns of apolipoprotein C-III, which contains only *O*-GalNAc-linked glycans, suggesting that

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O-GalNAc glycosylation is unaffected in HIBM. These findings suggest a defect in O-mannosylation of α-DG in HIBM. Understanding the function and regulation of the O-linked mannose pathway is essential for developing diagnostic tests and therapies for HIBM and other muscular dystrophies with similar pathology.

(240) Consequences of Mutations in UDP-GlcNAc 2-Epimerase/ManNAc Kinase for Pathology of Hereditary Inclusion Body Myopathy

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Hereditary inclusion body myopathy (HIBM) is a unique group of neuromuscular disorders characterized by adult onset, slowly progressive distal and proximal muscle weakness, and a typical muscle pathology with cytoplasmic "rimmed vacuoles" and cytoplasmic or nuclear inclusions composed of tubular filaments. A single homozygous missense mutation was first identified in Persian and other Middle Eastern Jewish patients in the gene encoding UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE), the key enzyme in the biosynthetic pathway of sialic acid. Furthermore, >40 different missense mutations in this same gene have been identified in quadriceps sparing HIBM cases diagnosed in several isolated families of non-Jewish origin worldwide. To biochemically characterize the HIBM mutations of GNE, we recombinantly expressed GNE proteins with 13 different mutations in both domains of the bifunctional enzyme. All mutant enzymes still displayed UDP-GlcNAc 2-epimerase as well as ManNAc kinase activities, but compared with the wild-type enzyme either one or both enzyme activities were reduced. The extent of reduction strongly differs among the mutants, ranging from 20 to 80%. To get a more detailed view into potential structural effects of the HIBM mutations, we generated models of the three-dimensional structures of the epimerase and the kinase domains of GNE and determined the localization of the HIBM mutations within these proteins. Whereas in the kinase domain, most of the mutations are localized inside the enzyme, mutations in the epimerase domain are mostly located at the protein surface. We further analyzed GNE in patient-derived muscle cell lines and found reduction of UDP-GlcNAc 2-epimerase activity between 30 and 70%. Nevertheless, analysis of overall glycoconjugate sialylation in these cells revealed no differences, although cells with GNE mutations which cause residual activities of <50% showed reduced expression of polysialic acid. It is therefore likely that subtle changes in sialylation contribute to the pathological mechanism of HIBM. Otherwise, the different mutations result in different enzymatic activities but not in different disease phenotypes, therefore do not suggest a direct role of the enzymatic function of GNE in the disease mechanism.

(241) Interleukin-4 Induces Specific pp-GalNAc-T Expression and Altered Mucin O-Glycosylation in Colonic Epithelial Cells

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One of important unresolved questions in the mucin glycosylation is the presence and cell-specific roles of a large number of UDP-*N*-acetylgalactosamine (GalNAc) : polypeptide *N*-acetylgalactosaminyltransferases (pp-GalNAc-Ts). The expression of some pp-GalNAc-Ts depends on cell types. Also, the expression seems to be because of physiological status of the cells. On the mucous epithelia, such as intestinal walls, mucins secreted by epithelial cells protect tissue from infectious parasites, allergens, and chemical or physical irritants. Biological properties of mucins important in such protective functions could be determined at least in part by the density of *O*-glycans resulted from the function of pp-GalNAc-Ts. However, it is not known whether immune responses influence O-glycosylation of mucins in the intestinal epithelial cells. A colonic epithelial cell line LS174T was used to assess the effect of interleukin (IL)-4 on the levels of mRNA corresponding to eight pp-GalNAc-Ts. Competitive RT–PCR analysis was performed to quantitate transcript of pp-GalNAc-T1, T2, T3, T4, T6, T7, T8, and T9. The levels of pp-GalNAc-T1, T4, and T7 was found to increase 3- to 4-fold 6 h after exposure of LS174T cells to IL-4 (20 ng/mL), whereas pp-GalNAc-T2, T3, T6, and T9 did not change. pp-GalNAc-T8 was not detected in LS174T cells. The expression levels of MUC2, MUC5AC, MUC5B, and MUC6 mRNA were also elevated by IL-4 treatment under the same conditions. These results indicate that colonic epithelial cells are transcriptionally stimulated by IL-4 to produce mucins which are likely to be uniquely glycosylated. Lysates of untreated or IL-4-treated cells were examined for their ability to transfer GalNAc residues into a polypeptide corresponding to the tandem repeat portion of MUC2 under cell-free environments. A peptide, GTQTPTTTPITTTTTVTPTPTPTG, was chemically synthesized, labeled with FITC, and used as the acceptor substrate. Microsome fractions extracted from LS174T cells after exposure to IL-4 for 24 h were prepared and used as the source of pp-GalNAc-Ts. The reaction products were separated by reverse-phase HPLC and analyzed on MALDI-TOF mass spectrometer to determine the number of GalNAc residues attached to the substrates. The results show that glycopeptides with an increased number of incorporated GalNAc residues were synthesized when lysates of IL-4-treated cells were used. Finally, the culture supernatants of untreated and IL-4-treated LS174T cells were obtained and examined for their reactivity with peanut agglutinin (PNA), *Vicia villosa* agglutinin-B4 (VVA-B4), anti-sialyl Tn monoclonal antibody TKH2, and anti-sulfo Lea monoclonal antibody 91.9H after SDS–polyacrylamide gel electrophoresis. The results indicate that IL-4-treated cells secreted a larger quantity of PNA and VVA-B4-reactive mucin-like high molecular weight glycoproteins than untreated cells did. The increase might be because of elevated mucin core polypeptide and/or to increased density of *O*-glycans on mucins. In conclusion, colonic epithelial cells seem to respond to IL-4 and secrete mucins with altered characteristics.

(242) Dynamic O-Linked Glycosylation of Bnip-3 Regulates Bnip-3 Translocation and Pro-Apoptotic Activity During Ischemia

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Intracellular proteins modified with O-linked *N*-acetylglucosamine (*O*-GlcNAc) have been identified in every cellular compartment, except the mitochondria. The lack of *O*-GlcNAc-modified proteins in the mitochondria is especially puzzling given the identification of an *O*-GlcNAc transferase (OGT) isoform targeted to the mitochondrial inner membrane. In this study, we show that the pro-apoptotic activity of Bnip-3, a hypoxia-inducible BH3-only mitochondrial protein, is regulated by O-linked glycosylation. Under basal conditions, mitochondrial Bnip-3 is O-linked glycosylated in a cardiomyocyte cell line (HL-1 cells) and in the mouse heart. In low oxygen/low glucose conditions (ischemia), Bnip-3 is deglycosylated, translocates to the cytosol, and induces caspase-dependent apoptosis. Bnip-3 is constitutively deglycosylated in HL-1 cells transfected with OGT siRNA. In addition, OGT knockdown enhances Bnip-3 translocation during ischemia. Conversely, PugNAc treatment prevents Bnip-3 deglycosylation and translocation during ischemia. PugNAc treatment also blocks ischemia-induced cell death. Here, we present findings that suggest modification of Bnip-3 by *O*-GlcNAc may act to integrate oxygen and nutrient signaling at the mitochondrial membrane, thereby regulating mitochondrial function and apoptosis. Our current studies are focused on determining the sites of Bnip-3 glycosylation and on identifying other mitochondrial proteins regulated by O-linked glycosylation during ischemic insults in cardiomyocytes.

(243) Tn Syndrome is Caused by a Somatic Mutation in the Molecular Chaperone Cosmc

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The genetic basis for the expression of Tn antigen in individuals with Tn-related disorders is not known. These disorders include Tn syndrome, IgA nephropathy, and Henoch–Schönlein purpura, but the Tn antigen is also found in many cancers. The Tn antigen is a truncated *O*-glycan GalNAc-Ser/Thr normally modified by core 1 β3 GalT (T-synthase) in the Golgi apparatus to generate core 1 *O*-glycan or T-antigen, Gal-GalNAc-Ser/Thr. Recently, we found that the expression of the active T-synthase requires coexpression of the X-linked, single exon-encoded molecular chaperone Cosmc (Ju and Cummings, 2002). In patients with Tn syndrome, the Tn antigen is expressed on blood cells of all lineages, suggesting that the condition is clonal and somatic and due to loss of T-synthase activity. Here, we report that Tn-syndrome is caused by a somatic mutation in the Cosmc. We performed genetic and biochemical analyses of DNA and glycoproteins on blood cells from two male donors with Tn syndrome and 25 normal donors. Sequencing of Cosmc was performed on genomic DNA from each donor following amplication by the polymerase chain reaction. Glycoproteins in blood cell extracts were analyzed by western blotting to probe for expression of the Tn and sialyl Tn antigens. The Tn antigen was expressed in multiple glycoproteins from leukocytes and erythrocytes in donors with Tn syndrome, but not in normal donors. In contrast to the normal sequence of Cosmc from healthy donors, DNA from donors with Tn syndrome had specific somatic mutations in Cosmc, one with a premature stop codon and

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the other with a nonconservative amino acid change. Expression of recombinant Cosmc from these mutated genes showed that both mutations resulted in loss-of-function for Cosmc leading to an inactive T-synthase. These studies demonstrate that a somatic mutation of X-linked Cosmc originally occurring in multipotential hematopoietic stem cells in patients with Tn-syndrome results in a deficiency in its chaperone activity leading to an inactive T-synthase and expression of the Tn-antigen on blood cells. The identification of Cosmc mutations in individuals with Tn syndrome provides the first insight into the genetic basis for this disease and has substantial implications for understanding other Tn-related disorders that may also result from acquired, somatic mutations in Cosmc.

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(244) Control of Metabolism by the Hexosamine and *N*-Glycan Processing Pathways

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Epithelial-to-mesenchymal transition (EMT) polarizes the cytoskeletol assembly to allow cell migration of invasive cancer cells, and this requires Ras/Erk, PI3 kinase/Akt activation, and TGF-b/Smad2/3 signaling. Polyomavirus middle T (PyMT) Mgat5–/– tumor cells fail to undergo EMT, due to an insufficient in autocrine TGF-b/Smad2/3 signaling, yet continue to grow as solid, poorly invasive tumors. The PyMT Mgat5–/– tumor cells display a general impairment in cytokine responsiveness due to the increased residency of receptors in the early endosomes (Partridge *et al.*, 2004). However, the PyMT oncoprotein directly activates Shc/Ras/Erk and p85/PI3K, stimulating glucose metabolism and cell proliferation. Reactive oxygen species (ROS) (superoxides and hydrogen peroxide) from metabolic sources can be a potential intrinsic stimulus for autonomous growth. H_2O_2 titrates the activity of redox-sensitive proteins and, notably, oxidizes an essential thiol group in the active site of all protein tyrosine phosphatases. Here, we report that upon serum withdrawal, PyMT Mgat5–/– tumor cells fail to arrest glycolysis, mitochondrial ROS production, Akt and Erk activation, and cell cycle progression. FCCP-mediated uncoupling of oxidative respiration blocked excess ROS production in the Mgat5–/– tumor cells and normalized Erk and Akt activation. Furthermore, Mgat5 re-expression or stimulation of the hexosamine pathway in Mgat5–/– cells restored metabolic regulation and ROS production. Golgi UDP-GlcNAc is rate limiting for GlcNAc- branching of *N*-glycans, and GlcNAc salvage into the pathway increased the triantennary fraction of *N*-glycans, restored galectin-3 binding to ERF and TGF-b receptors. This enhanced surface receptor expression and sensitivity to ligands and restored metabolic and cell cycle control in PyMT Mgat5–/– tumor cells. Our results suggest that metabolic homeostasis is dependent on nutrient flux through hexosamine and the *N*-glycan processing pathways.

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(245) GNE is Involved in Cell Growth via Modulation of Ganglioside Metabolism

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The UDP-GlcNAc 2-epimerase/ManNAc 6-kinase bifunctional enzyme, known as GNE, is a key regulator of metabolic flux into the sialic acid biosynthetic pathway. Consequently, single amino acid mutations in GNE that occur in the human disease hereditary inclusion body myopathy (HIBM) were initially expected to reduce sialic acid biosynthesis in these patients. Clinical evidence, however, has not borne out that a significant decrease in overall levels of sialic acid occurs in HIBM. In this study, we sought alternative explanations for the cellular basis of HIBM by overexpression of wild-type and HIBM-mutant forms of recombinant GNE in HEK293 (human embryonic kidney) and SJCRH30 (human skeletal muscle) cells. These studies showed a link between GNE and ganglioside metabolism (specifically, changes to ganglioside GD3 were observed). The connection between GNE and GD3 was further probed by the addition of exogenous GD3 to these cells, as well as by the inhibition of GNE with short interfering RNAs (siRNA). These experiments verified that metabolic links exist between upstream (GNE) and downstream (GD3) pathway elements. In addition, effects on cell behavior, specifically, changes to cell growth trends were observed. One implication of these findings is that the cellular distribution of gangliosides provides a new molecular mechanism to consider toward elucidating the molecular basis of HIBM. A second key finding was that the changes that occurred in HEK293 and SJCRH30 cells were consistently opposite to each other, which may explain why the effects of HIBM are selective for skeletal muscle cells.

(246) Design of a Quantitative Method for Detection of Allele-Specific RNA Expression

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Hereditary inclusion body myopathy (HIBM) and sialuria are two distinct disorders resulting from mutations in the same gene, GNE, coding for the bifunctional, rate-limiting enzyme in sialic acid biosynthesis, that is, UDP-GlcNAc 2-epimerase (GNE)/ManNAc kinase (MNK). Sialuria is caused by dominant mutations in the allosteric site of GNE/MNK, leading to a loss of feedback inhibition and increased sialic acid excretion. In contrast, HIBM is caused by recessive GNE mutations outside the allosteric site, resulting in decreased GNE/MNK enzyme activity and decreased sialic acid production. Both sialuria and HIBM exhibit variable clinical phenotypes. We examined whether mutation-dependent variations in allelic expression of GNE could account for the variable disease phenotypes. We developed a real-time RT–PCR method that rapidly and accurately detects and quantifies allele-specific expression. The procedure is based on the use of a combination of two allele-specific fluorescent reporter probes and real-time amplification kinetics. We first tested the validity of each allele-specific assay across a concentration range obtained by mixing cell-free transcribed normal and mutated GNE RNA. Each of the assays proved to be accurate and mutation specific, allowing us to study allelic expression of GNE. Next, we applied the assays to RNA obtained from fibroblasts of sialuria or HIBM patients. No patient showed a significant difference in mutation-dependent allelic GNE expression, indicating that allelic expression did not cause the variable phenotypes in these patients with sialuria or HIBM. This novel, allele-specific RNA quantifying method is convenient and rapid and requires minimal concentrations of RNA (<25 ng). The procedure is attractive for various applications, including validation of si-RNA silencing experiments. In fact, we have demonstrated the method's validity for allele-specific RNA gene silencing using si-RNAs in sialuria fibroblasts. In addition, this method can be employed for studies of X-chromosomal inactivation, genetic imprinting, and epigenetics.

(247) Identification of Novel Pathways in Hereditary Inclusion Body Myopathy

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Hereditary inclusion body myopathy (HIBM) is a unique group of adult onset neuromuscular disorders characterized by slowly progressive distal and proximal muscle weakness and typical muscle pathology including rimmed vacuoles and filamentous inclusions. The prototype form of HIBM, particularly frequent in Middle Eastern Jews, is an autosomal recessive disorder related to mutations in the gene encoding the bifunctional enzyme UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE), the limiting enzyme in the biosynthetic pathway of sialic acid. Many different missense mutations were identified in GNE in HIBM patients worldwide, whereas a unique homozygous mutation, M712T, was found in all Middle Eastern patients. Although the overall sialylation is not significantly impaired in muscle cells of these patients, the underlying myopathological pathways leading from GNE mutations to the disease phenotype is poorly understood. To elucidate the first occurring downstream events of this pathway, we have analyzed the gene expression patterns of muscle specimens from 10 HIBM patients, carrying the M712T mutation, presenting with mild histological changes, and 10 healthy individuals, using the Affymetrix U133A microarrays. About 374 genes were identified as significantly differentially expressed in affected versus healthy individuals. Among those, a large number of transcripts encoding proteins (18%) are involved in various processes occurring in the mitochondria. For further insights in this pathophysiology, we have established a cellular model of human myoblasts from HIBM patients and identified cellular and molecular characteristics of these cells. HIBM and control muscle cells showed a similar growth pattern of actively proliferating cells with no significant differences between the two cell types. However, the differentiation pattern of the cell cultures was heterogeneous among HIBM cultures compared with a uniform pattern in the

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control muscle cultures, as assessed by western blot and immunocytochemistry. Furthermore, the apoptotic pattern of HIBM cell cultures as well as the signal transduction pathway initiated by insulin stimulation were highly impaired, resulting in changes of various known crucial players of the apoptotic cascade, as well as of several not yet identified phosphorylated tyrosine kinases. Altogether these data point to a possible involvement of the mitochondrial apoptosis pathway in the pathophysiology of HIBM. Whether the impairment of this pathway is caused by a direct effect of defective GNE, or through the impaired sialylation of a specific effector protein, remains to be elucidated. To date, GNE has been related solely to the sialic acid biosynthetic pathway, and no clear understanding of the cause/effect relationship between GNE mutations and HIBM phenotype could be provided. The identification of a mitochondrial expression signature in HIBM-affected muscles could disclose new functions for GNE in muscle cells.

(248) Gene Expression Profile in *Helicobacter pylori (felis)*-Induced Inflammation and Gastric Cancer: Early Expression of Inflammation-Associated Genes

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Helicobacter pylori is a spiral-shaped, Gram-negative microaerophilic bacterium that infect over half the world's population. The infection of this organism leads to chronic active gastritis, mucosa-associated lymphoid tissue formation, atrophy, and intestinal metaplasia which are generally regarded as conditions that predispose to gastric cancer. However, the mechanism by which this organism induces inflammation and carcinogenesis is not fully understood. Insulin–gastrin (INS-GAS) transgenic mice have a human gastrin gene under the influence of rat insulin promoter. These mice express human gastrin which stimulates gastric acid secretion and growth of acid-secreting parietal cells. It has been reported that these mice develop gastric adenocarcinoma over 20 months of age, and *Helicobacter felis*, which are closely related to *H. pylori*, infection accelerates (<8 months) the development of gastric adenocarcinoma (Wang *et al*., 2000). In this study, INS-GAS mice were infected with *H. felis*, and the stomachs were examined periodically. Time course analysis of gene expression by using gene microarray revealed that several inflammation-associated genes including chemokines, adhesion molecules, and the molecules associated with *H. pylori* infection such as surfactant protein D and CD74 were up-regulated compared with uninfected controls. Immunohistochemical analysis demonstrated that CD74 is strongly associated with adenocarcinoma and, to some extent, associated with *H. felis* infection. Histological examination revealed that some mice developed gastric adenocarcinoma 2 months after the infection. All mice developed adenocarcinoma by 8 months after the infection. After 6 months of infection, more than half of those mice developed invasive adenocarcinoma. It has been shown that NCC-ST-439 and HECA-452 antibodies recognize 6-sulfated and nonsulfated sialyl Lewis x on core 2 branch and that on core 2 branch and/or extended core 1 *O*-glycans, respectively (Kobayashi *et al*., 2004). We found that NCC-ST-439 stains adenocarcinoma cells, and HECA-452 additionally stains those cells negative for NCC-ST-439. Lymphocytic infiltration predominantly to submucosal layer was observed in most *H. felis*-infected mice, and the lymphocytic infiltration was associated with the formation of PNAd on HEV-like vessels detected by MECA-79. Because HECA-452 and NCC-ST-439 antigens represent nonsulfated and sulfated sialyl Lewis x capping structures on extended core 1- and core 2-branched *O*-glycans, we determined the expression of a family of core 2 branching enzymes. Semi-quantitative RT–PCR demonstrated that both C2GnT1 and C2GnT2 transcripts were increased with time, but the transcription level of C2GnT2 was >~1000 times that of C2GnT1, and C2GnT3 expression was not detected. These results as a whole indicate that *H. felis* induced inflammation, assessed by the expression of MECA-79 antigen, and inflammation-associated chemokines precedes adenocarcinoma formation, detected by NCC-ST-439 and CD74. Supported by NIH grant CA33000.

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(249) Comparative Analysis of Sialomucin and Glycolipid E-Selectin Ligand Activities: Effects of HCELL Knockdown

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An increased understanding of the molecular basis of colon cancer metastasis is needed to devise novel therapies for prevention of late-stage disease. The critical first step in metastatic invasion of target organs is the "tethering and rolling" attachment of circulating cancer cells onto vascular endothelium under the shear conditions of blood flow. The endothelial molecule E-selectin is a principal effector of these shear-resistant adhesive events, and there is strong evidence that E-selectin receptor–ligand interactions contribute to the formation of metastasis. We recently identified the sialofucosylated HCELL glycoform of CD44 on the LS174T colon carcinoma cell line and demonstrated its function as a high affinity glycoprotein E-selectin ligand using the novel blot rolling assay. However, the relative contribution(s) of HCELL in adhesion of colon carcinoma cells to E-selectin relative to other potential glycoprotein and glycolipid ligands remains to be determined. To address this issue, we investigated the binding of LS174T cells under physiological flow conditions to 6-h IL-1β stimulated human umbilical vein endothelial cells (HUVECs) expressing E-selectin. Attachment of LS174T cells to HUVECs was entirely E-selectin dependant, as evidenced by complete abrogation of tumor cell binding when HUVECs were incubated with anti-E-selectin antibody. Protease treatment of tumor cells allowed separation of the glycoprotein versus glycolipid contributions to E-selectin-mediated adhesion, whereas transduction with anti-CD44 small interfering RNA (siRNA) to knockdown CD44 expression (>90% relative to vector control and untreated cells) allowed direct assessment of HCELL-binding activity. At shear stresses >1 dyn/cm^2, anti-CD44 siRNA transduction led to =50% decrease in the number of tumor cells tethering to stimulated HUVECs relative to negative control cells. In addition, protease treatment of anti-CD44 siRNA transduced cells failed to further significantly influence tethering to HUVECs, and the number of cells that bound was approximately equal to the number of protease-treated control cells that bound. Thus, HCELL is the principal glycoprotein E-selectin ligand at shear stresses >1 dyn/cm^2, and glycolipid ligand(s) account for the remainder of E-selectin binding activity. Rolling velocity, a measure of binding strength, was also dependent on HCELL expression: anti-CD44 siRNA transduced LS174T cells rolled significantly faster on HUVECs than vector control or untreated cells. However, at 1 dyn/cm^2 and below, additional glycoprotein ligand(s) other than HCELL may be operational, as tethering of anti-CD44 siRNA-transduced, vector control, and untreated cells was approximately equal, and protease treatment of these three cell types led to an equivalent increase in the rolling velocity on HUVECs. It has not been previously recognized that colon cancer cell adherence to E-selectin is mediated predominantly by the HCELL glycoform of CD44 and, in particular, that this dominance is shear stress dependant. Taken together, our data demonstrate the complex regulation of colon cancer/endothelial adhesive interactions mediated by the interplay of glycoprotein and glycolipid E-selectin ligands under hydrodynamic shear conditions and reveal a critical role for HCELL in the initial attachment of LS174T cells on endothelial E-selectin at higher shear stress.

(250) Carbohydrate-Based Small Molecules as Anti-Cancer Drugs: Short Chain Fatty Acid–Hexosamine Hybrids

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Here, we report the design and synthesis of SCFA-hexosamine hybrid molecules, exemplified by the 2-acetamido-1,3,4,6-tetra-*O*-butanoyl-2-deoxy-β-D-mannopyranose (But4ManNAc). The rationale behind the design of these molecules was to synergistically enhance the anti-cancer properties of both the sugar and SCFA moieties by simultaneously targeting the two hallmarks of this disease, which are aberrant protein production and glycosylation. Specific aberrations in protein synthesis inactivate the cell cycle checkpoints resulting in uncontrolled cell growth. It has long been known that butyrate, a short chain fatty acid produced in the gut by friendly bacteria from dietary fiber, can prevent colon cancer. Butyrate acts in various ways including, inhibition of histone deacetylase (HDAC) and a consequent induction of overexpression of a cell cycle checkpoint protein p21—a cyclin dependent kinase (CDK) inhibitor. In spite of the discovery of potent HDAC inhibitors like phenylbutyrate, tirchostatin A,

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suberoyl hydroxamic acid (SAHA), and so on, butyrate may be the choice because of its physiological production and minimal side effects. Efforts are underway to improve the bioavailability of sodium butyrate as in the form of prodrugs as esters, like tributyrin, AN-9 (pivaloyloxymethyl butyrate), and mannose butyrate. It has been known for several decades that abnormal glycosylation, where the patterns of complex carbohydrates found on the surfaces of cancer cells are dramatically different than those on healthy cells. In particular, aberrations in specific patterns of sialic acid-containing glycoforms expressed on the cell surface glycans are found in many disease states. Sialic acids are biosynthesized from *N*-acetyl-D-mannosamine (ManNAc), and the flux through the pathway can be influenced by exogenously supplied natural or nonnatural analogs, providing the impetus for the use of ManNAc as the monosaccharide core in our SCFA-hexosamine hybrids. The prototypic hybrid, But4ManNAc, successfully enhanced cellular responses associated with both the butyrate and sugar. The butyrate activity was studied by monitoring the up-regulation of endogenous p21 by flow cytometry and recombinant p21 expression by using a luciferase reporter plasmid (Luc-p21$^{WAF1/CIP1}$). Results showed that But4ManNAc is at least 20–50 times more efficient than either sodium butyrate alone or a combination of ManNAc and (4x) sodium butyrate at supporting an increase in p21-driven gene expression. At the same, as previously reported (Kim *et al*., 2004), the ManNAc core gained access to the sialic acid pathway ~2100 times more efficiency that its free monosaccharide counterpart. The biological responses elicited by SCFA-sugar hybrids are under further investigation. As butyrate is known to activate certain specific sialyl transferases, results of the studies of the gene expression levels of enzymes involved in ganglioside biosynthesis will be reported. We have also observed that the butyrate activity depends on the sugar carrier used. For example, the mannose per-butyrate was found to be nontoxic, whereas the *N*-acetyl-D-glucosamine perbutyrate increased the levels of protein *O*-GlcNAc levels.

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(251) Selective Targeting of BNCT Reagents by Differences in Sialic Acid Expression

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Boron neutron capture therapy (BNCT), initially developed in 1950s, has attracted increasing attention in recent years. The effort has been largely oriented toward the eradication of glioblastoma multiforme (GBM) and melanoma with interest in other types of malignancies as well. Selective targeting of BNCT reagents to tumors is one of the key components of this cancer therapy procedure. Tumor cell surfaces exhibit abnormal glycosylation in the form of overexpressed naturally occurring oligosaccharides as well as glycoforms that are normally expressed only during fetal development. Many tumor-associated carbohydrate antigens possess the monosaccharide sialic acid, and indeed, the overexpression of sialic acid has been correlated with the malignant and metastatic phenotypes in epithelial-derived cancers from gastric, colon, pancreatic, liver, lung, prostate, and breast tissue, and in several types of leukemia. The collective display of multiple sialylated antigens on a tumor cell can result in the presentation of up to 109 sialic acid residues per cell and can account for the broad distribution of the high sialic acid phenotype across many different types of cancers. Diagnostic strategies that target cells on the basis of sialic acid expression may therefore find broad utility in cancer therapy. Here, we present a strategy for the selective delivery of BNCT reagents to tumor cells that exploits intrinsic differences in sialic acid expression. The approach capitalizes on the unnatural substrate tolerance of the enzymes in the sialoside biosynthetic pathway, which allows the metabolic conversion of boron-containing unnatural sialic acid to the corresponding sialosides in human cells. The synthesis of unnatural sialic acids and the initial biological studies will be presented.

(252) O-Glycosylation in *Toxoplasma gondii*

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The UDP-GalNAc : polypeptide *N*-acetylgalactosaminyltransferase (ppGalNAc-T) family of enzymes initiates mucin-type protein O-glycosylation in the secretory pathway by catalyzing the transfer of the monosaccharide, *N*-acetylgalactosamine (GalNAc), to specific threonine and serine residues on target proteins. Multiple ppGalNAc-T isoforms were identified in vertebrates, insects, and worms. This redundancy complicates studies of the biological roles and detailed substrate specificities of individual isoforms. Therefore, we selected a single-celled parasite, *Toxoplasma gondii*, as a model to study mucin-type O-glycosylation. This parasite causes toxoplasmosis in a wide range of animals, including humans. Although toxoplasmosis is asymptomatic in individuals with a normal immune system, it is a major cause of opportunistic infections, such as encephalitis, in immunosuppressed patients, particularly those with AIDS and transplant recipients. In addition, *T. gondii* causes congenital infections leading to birth defects or abortion in humans and domestic animals. *T. gondii* has a complex life cycle; rapidly replicating, virulent tachyzoites responsible for acute infection can transform into slowly replicating, quiescent, encysted bradyzoites in response to attack by the host immune system. The cyst wall not only maintains the integrity of the cyst, but also prevents penetration by antibiotics and soluble products of the host immune system. In addition, the *T. gondii* cyst wall contains a glycoprotein(s) that is recognized by the GalNAc-specific *Dolichos biflorus* lectin, thus emphasizing the clinical importance of studying O-glycosylation in this parasite. To this end, using both a PCR approach with degenerate primers and BLAST searches of the ongoing *T. gondii* genome sequencing project (ToxoDB: the toxoplasma genome resource), we identified a family of five ppGalNAc-Ts. The predicted open-reading frames encode Type II transmembrane proteins that share characteristic features with their mammalian, insect, and *Caenorhabditis elegans* orthologs. We cloned three isoforms, ppGalNAc-T1, T2, and T3, which are expressed by both tachyzoites and bradyzoites. Recombinant, soluble forms of these proteins were stably expressed in *Drosophila melanogaster* S2 cells. Two isoforms, ppGalNAc-T1 and T3, exhibited a "follow-up" type of enzyme activity by preferentially incorporating GalNAc into pre-glycosylated EA2 and MUC5AC synthetic glycopeptides. Tagged full-length forms of ppGalNAc-T1, T2, and T3 that are expressed in transfected *T. gondii* will also be used for a comparative study of their substrate specificity. Thus, we need to identify native toxoplasmal proteins that contain acceptor sequences for each *T. gondii* ppGalNAc-T isoform. Using flow cytometry, we demonstrated that live, nonpermeabilized tachyzoites bind jacalin lectin, which recognizes *O*-glycans. In addition, an O-glycoprotein-enriched fraction was obtained from tachyzoite lysate by lectin affinity chromatography and analyzed by two-dimensional electrophoresis followed by MALDI-TOF MS peptide mass mapping. These studies identified several candidate toxoplasmal *O*-glycoproteins expressed during the tachyzoite life cycle stage. Cataloging the *T. gondii* O-glycoproteins will help validate the biochemical phenotype of various ppGalNAc-T gene knockout organisms. It will also support the claim that mucin-type O-glycosylation is an important, evolutionarily conserved modification that appeared early during evolution with the appearance of single-celled eukaryotes.

(253) Targeting Tumor Lectins, Galactmannan Derivative Shows Promising Results in Preclinical Studies and Phase I Clinical Trial With 5-FU Refractory Patients

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Galactomannan derivative (DAVANAT), enhances 5-fluorouracil (5-FU), irinotecan, and bevacizumab in colon and mammary tumor models. It is hypothesized that DAVANAT interacts with carbohydrate-specific lectins on tumor surface, facilitating transport of chemotherapeutics into the tumor cells. The lectins present in large amounts on tumor surfaces, and known to mediate cell association, and involved in tumor apoptosis and metastasis. The soluble galactomannan, made of a poly (1-4)-linked β-D-mannopyranose backbone with branched (1-6)-linked α-D-galactopyranose, has substantiated its safety and tolerability when coadministrated with 5-fluorouracil as third line therapy in phase I clinical trial, with over 50% of patients stabilized. A galactomannan with molecular weight of ~50 kDa has been selected from a variety of modified and purified galactomannans. The polysaccharides were screened for enhancement of chemotherapeutics in *in-vitro* and *in-vivo* models. Animal model studies were designed to evaluate compatibility and anti-cancer synergistic activity, simulating proven clinical regimens with anticancer drugs like 5-FU, irinotecan, oxaliplatin, and bevacizumab. The GMP process has been scaled up to 10 kg, and the polymeric structure has been validated using 13C and 1H nuclear magnetic resonance (NMR) and size exclusion chromatography with multi angle laser light scattering (SEC-MALLS) with over 98% purity. Coadministration of galactomannan with mono and combinational chemoregimens have been shown to give enhancement in animal models. The phase I human trial founds DAVANAT in combination with 5-FU (500 mg/m^2) is well tolerated at the highest dose tested of 280 mg/m^2. Of 28 patients, with advanced disease and average tumor load of over 100 mm, 26 patients had measurable disease, of which 14 had stable (50%) and 11 had progressive disease (40%) (by RECIST criteria). Three patients had serious adverse events (dehydration, dyspnea, and

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thrombocytopenia) thought to be at least possibly related to drug treatment. Animal's models have shown galactomannan has broad-spectrum enhancement of anticancer activity when coadministrated with cytotoxic or antiangiogenic agents. DAVANAT tolerability and potential for enhancement of anticancer therapy has been established in animal's models and in human when administered with 5-FU. The stabilization of patients warrants further testing of this combination for safety and efficacy in a phase II clinical trial.

(254) Expression of Polysialylated Neural Cell Adhesion Molecules in Human Head and Neck Cancer

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The structurally unique α2,8-linked polysialic acid (polySia) chains that covalently modify NCAMs are oncodevelopmental, tumor-associated cell surface antigens that regulate cell–cell adhesive interactions. As an antiadhesive glycotope, the re-expression of polySia on several human cancers attenuates the adhesive property of NCAM, thus facilitating cell detachment and tumor metastasis. Expression of polySia-NCAM in lung and pancreatic cancer, for example, correlates with malignant behavior and poor prognosis. The aim of this study was to investigate the extent of polySia expression in H & N cancers to determine the extent of polysialylation on these tumors and its potential clinical significance. PolySia and NCAM expression was analyzed in 47 H & N cancer patients. The anti-polySia polyclonal antibody, H.46, and monoclonal anti-NCAM antibody, 123C3, were used in western blot experiments. The diagnostic enzyme endo-*N*-acylneuraminidase was used to confirm the presence of α2,8-linked polySia chains. PNGaseF was used to release *N*-glycans from NCAM, thus allowing the NCAM isoform to be determined. The following results were obtained as (1) PolySia was expressed in 29 of the 47 H & N cancers examined (61.7%), whereas NCAM was expressed in 34 cases (72.3%); (2) PolySia was expressed only in NCAM positive tumors; (3) PolySia-NCAM expression was increased in nasal cavity cancers, where 83% (5 of 6 cases) were in the paranasal sinus and 69% (9 of 13 cases) were oral-oropharyngeal cancers. This increase was in contrast to laryngeal cancers, where only 19% (2 of 7 cases) were positive. None of the five parotid cancers expressed polySia-NCAM; (4) PolySia-NCAM expression was not correlated with tumor stage, nodal metastasis, or smoking history but was expressed on a number of different tumors including basal and squamous cell carcinomas, nasopharyngeal angiofibroma, malignant histiocytoma, chondrosarcoma, neuroblastoma, melanoma, chondrosarcoma, synovial sarcoma, Schwannoma, neurofibroma, lymphoma, teratocarcinoma, and adenocystic, papillary, verrucous, and Hurthle cell carcinomas; (5) unexpectedly, 92% of all NCAM positive cancers expressed the 140 kDa NCAM isoform. (1) PolySia-NCAM was re-expressed in ~2/3 of the 47 H & N cancers studied and comprised a number of different cancers. (2) PolySia-NCAM appeared to be differentially expressed according to anatomical site, with the highest level of expression in the nasal cavity. (3) Tshe 140 kDa isoform of NCAM was the predominately polysialylated NCAM associated with H & N cancers. (4) Correlation of these findings with perineural invasion and patient outcome is currently under study.

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