THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 28, Issue of July 9, pp. 29050–29059, 2004
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Printed in U.S.A.

Specificity of a Soluble UDP-Galactose:Fucoside α1,3-Galactosyltransferase That Modifies the Cytoplasmic Glycoprotein Skp1 in *Dictyostelium**

Received for publication, December 18, 2003, and in revised form, April 20, 2004
Published, JBC Papers in Press, May 3, 2004, DOI 10.1074/jbc.M313858200

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Skp1 is an adaptor-like protein in $E3^{SCF}$-ubiquitin ligases and other multiprotein complexes of the cytoplasm and nucleus. In *Dictyostelium*, Skp1 is modified by an unusual pentasaccharide containing a Galα1-Fuc linkage, whose formation is examined here. A cytosolic extract from *Dictyostelium* was found to yield, after 2400-fold purification, an activity that could transfer Gal from UDP-Gal to both a Fuc-terminated glycoform of Skp1 and synthetic Fuc conjugates in the presence of Mn^{2+} and dithiothreitol. The microsomal fraction was devoid of activity. The linkage formed was Galα1,3Fuc based on co-chromatography with only this synthetic isomer conjugate, and sensitivity to α1,3/6-galactosidase. Skp1 exhibited an almost 1000-fold lower K_m and 35-fold higher V_{max} compared with a simple α-fucoside, but this advantage was abolished by denaturation or alkylation of Cys residues. A comparison of a complete series of synthetic glycosides representing the non-reducing terminal mono-, di-, and trisaccharides of Skp1 revealed, surprisingly, that the disaccharide is most active owing primarily to a V_{max} advantage, but still much less active than Skp1 itself because of a K_m difference. These findings indicate that α-GalT1 is a cytoplasmic enzyme whose modification of Skp1 requires proper presentation of the terminal acceptor disaccharide by a folded Skp1 polypeptide, which correlates with previous evidence that the Galα1,3Fuc linkage is deficient in expressed mutant Skp1 proteins.

Skp1 is a small protein that has been found in multiple distinct heteromeric protein complexes in the cytosolic and nuclear compartments of eukaryotic cells (1–6). The best characterized complex is the family of $E3^{SCF}$-ubiquitin ligases that target modified proteins for polyubiquitination and subsequent degradation via the 26 S proteasome (6). In this complex, Skp1 serves as an adaptor to link the catalytic E2-ligase via cullin-1 to the F-box protein specificity factor (7). In the lower eukaryote *Dictyostelium*, Skp1 is nearly quantitatively modified by a pentasaccharide at a hydroxyproline near its C terminus, in a region of the protein that associates with the F-box partner (8). The function of glycosylation is not known, but partial glycosylation is necessary for nuclear concentration of Skp1, and cells that are genetically unable to extend the core monosaccharide are smaller and grow to higher saturation densities (9, 10).

The first 3 sugars are added sequentially to Hyp-143 of Skp1 by polypeptide GlcNAcT1[1] (11, 12) and a processive bifunctional β1,3-GalT/α1,2-FucT (10, 13). These sugar nucleotide-dependent enzymes are novel in their soluble nature and absence of rough endoplasmic reticulum targeting sequences or membrane anchor motifs. Therefore, at least the early steps of Skp1 glycosylation are mediated by a novel pathway of cytoplasmic enzymes (5, 14). Bioinformatics studies suggest that a related modification pathway occurs in the cytoplasm of other lower eukaryotes including a diatom and an oomycete (15). The Fuc terminus of the core trisaccharide of *Dictyostelium* Skp1 is further modified by two α-linked Gal*p* residues whose linkages have not been established (8, 14). The mechanism of α-galactosylation is not known but it seems likely that it will involve novel sugar nucleotide-dependent enzymes in the cytoplasm or nucleus. It is not known whether the Skp1 enzymes will be more related to α-GalTs of the eukaryotic Golgi or those that extend lipopolysaccharides and other glycoconjugates in the prokaryotic cytoplasm (16).

Unlike expressed wild-type Skp1, two mutant Skp1s containing amino acid substitutions in their N-terminal regions are poorly glycosylated *in vivo* (9). Mass spectrometric analysis of one, Skp1A1(HW120)-myc, showed that it consists of a mixture of the unmodified protein and glycoforms terminated with GlcNAc, Fuc, or one or two α-Gal residues (8). Incomplete peripheral galactosylation might result from inefficient addition of the sugar or increased susceptibility to its removal. These intermediate glycoforms of Skp1A1(HW120)-myc are potentially useful acceptor substrates for assaying the activity of the GalTs whose substrates are accumulated, as found for the Skp1 GlcNAcT activity (11). Although the function of the pe-

* This work was supported in part by National Institutes of Health Grant R01-GM37539. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GlcNAcT, *N*-acetylglucosaminyltransferase; Bn, benzyl; DTT, dithiothreitol; Fuc, L-fucose unless specified as D-; FucT, fucosyltransferase; Gal, D-galactose (pyranose); GalT, galactosyltransferase; GT, glycosyltransferase; *p*NP, *para*-nitrophenyl; β-Gal, β-galactosidase; HPLC, high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

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ripheral α-Gals is not known, they appear to be heterogeneous between two normal cellular pools of Skp1 (17).

α-Gal*p* residues are non-reducing terminal modifications of *O*- and *N*-linked glycans of selected secretory glycoproteins in subhuman animals and microorganisms (18, 19), where they constitute potent xenoantigens and might contribute to specific recognition determinants. With the ultimate goal of understanding the role of peripheral α-galactosylation in the cytoplasm and assessing its phylogenetic range, we have undertaken an investigation of the enzymatic basis of this modification on Skp1. A screen of an ion exchange fractionation of a cytosolic extract of *Dictyostelium* cells using mutant Skp1A1(HW120)-myc as an acceptor yielded a prominent α-GalT activity that was partially purified and characterized. α-GalT1 appears to be a cytoplasmic GT like the earlier enzymes in the pathway. Based on studies of model acceptor compounds, α-GalT1 modifies the blood group H (type 1) trisaccharide of Skp1 by the addition of an α-Gal to the 3-position of Fuc. The modification is greatly potentiated by normally folded Skp1 in a manner that suggests the importance of conformation of the acceptor sugar structure. These findings provide an explanation for why mutant Skp1 is incompletely α-galactosylated *in vivo*.

EXPERIMENTAL PROCEDURES

Buffers

Buffers were adjusted to their pH values at 22 °C, filtered, degassed, and stored at 5 °C. DTT and protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin (final concentrations), were added just before use. Buffer A was 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, and the protease inhibitors. Buffer B was 50 mM HEPES-NaOH (pH 7.4), 5 mM $MgCl_2$, 0.1 mM NaEDTA, 15% (v/v) glycerol, 1 mM DTT, and the protease inhibitors. Buffer C was 85 mM ammonium acetate (pH 7.4), 20% (v/v) saturated $(NH_4)_2SO_4$, 15% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM DTT. Buffer D was 25 mM ammonium acetate (pH 7.4), 15% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM DTT. Buffer E was 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM $MnCl_2$, 15% (v/v) glycerol, 1 mM DTT. Buffer F was 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM $MnCl_2$, 15% (v/v) glycerol, 0.15 M NaCl, 1 mM DTT.

Purification of α-GalT1

Cell Growth and Lysis—Dictyostelium discoideum strain HW302 (9), which overexpresses a normal copy of Skp1B, was grown in 2 30-liter batches of HL-5 axenic growth medium at 22 °C to maximum cell density (~10^7 cells/ml), collected by centrifugation at 3,000 × *g* for 1 min, resuspended in H_2O, centrifuged again, and resuspended in buffer A at 2 × 10^8/ml. Cells were immediately filtered through a bed of glass wool, lysed by forced passage through a 5-μm pore diameter Nuclepore filter (20), and successively centrifuged at 3,000 × *g* for 2 min and 100,000 × *g* for 70 min to yield the cytosolic supernatant S100 fraction that was chromatographed immediately as described below. For small scale preparations, the glass wool prefilter step was skipped, and the pellet from the 100,000 × *g* centrifugation was resuspended in buffer B, recentrifuged, and resuspended in buffer B again. The resulting S100 and washed P100 fractions were frozen at −80 °C.

DEAE-Sepharose Chromatography—Each S100 fraction (from 30 liters) was pumped onto a 450-ml column of DEAE-Sepharose Fast Flow (Amersham Biosciences) equilibrated at 4 °C in buffer B, and the column was washed with buffer B until the A_{280} returned to near baseline. The column was eluted with a 2.5-liter linear gradient of 0–0.25 M NaCl in buffer B, followed by 400 ml of 0.25 M NaCl in buffer B. Column fractions were frozen at −80 °C and aliquots were assayed for GalT activity (see below).

Phenyl-Sepharose Chromatography—Fractions from the main GalT activity peak from both DEAE-Sepharose runs were pooled, adjusted to 20% (w/v) $(NH_4)_2SO_4$, and centrifuged at 12,000 × *g* for 30 min. The supernatant was loaded at 4 °C onto a 2.6 × 20-cm column of phenyl-Sepharose Fast Flow (high sub) column (Amersham Biosciences) equilibrated in buffer C. The column was washed with buffer C until the A_{280} returned to baseline level, and eluted with a descending linear 750-ml gradient of buffer C to buffer D. The column was washed with buffer D until the A_{280} returned to near baseline level, and a 750-ml ascending linear gradient from 0 to 70% ethylene glycol in buffer D, followed by 200 ml of 70% ethylene glycol in buffer D was then applied.

Q-Sepharose Chromatography—The active fractions from the phenyl-Sepharose column were divided into three pools, and each loaded separately, at 4 °C, onto a 5-ml Hi-Trap Q-Sepharose column (Amersham Biosciences) equilibrated in buffer E. The column was washed with buffer E until the A_{280} was less than 0.01. The column was eluted at 21 °C, with a 50-ml linear gradient of 0–0.2 M NaCl in buffer E, followed by a 10-ml gradient of 0.25–0.5 M NaCl in buffer E.

Superdex 200 Chromatography—The pooled GalT activity from the three Q-Sepharose columns was concentrated in a Centriprep 30 ultrafiltration device (Amicon) to 2.2 ml at 4 °C, and loaded onto a 16/60 Superdex 200 column (Amersham Biosciences) equilibrated in buffer F. The column was eluted with buffer F at 1 ml/min at 21 °C. M_r values of the calibration standards were 200,000, sweet potato β-amylase; 66,000, bovine serum albumin; and 29,000, carbonic anhydrase.

Protein Determination—Protein concentration was determined by the Coomassie Blue dye binding method (Pierce Coomassie Plus), or calculated from A_{280} values, assuming $A_{280}^{0.1\%} = 1.0$.

GalT Assays

Assay Conditions—GalT activity was assayed by the transfer of [^{3}H]Gal from UDP-[6-^{3}H]Gal (American Radiolabeled Chemicals) to synthetic glycosides or Skp1. Unless indicated otherwise, reactions (15–60 μl) contained 1–5 μM UDP-[^{3}H]Gal (13,200–64,000 disintegrations/min/pmol), acceptor substrate as described below, 50 mM HEPES-NaOH (pH 7.4), 50 mM NaCl, 2 mM $MnCl_2$, 5 mM DTT, 0.1 mg/ml bovine serum albumin, and 0.1% (v/v) Tween 20, and were incubated at 22 °C for 30–120 min. In some tests containing ^{3}H-labeled acceptor substrates (see below), UDP-[U-^{14}C]Gal (300 mCi/mmol; American Radiochemical Corp.) was used in place of the tritiated compound. Early purification fractions (up through Q-Sepharose) were assayed in the presence of 1 mM ATP and 1 mM NaF. To determine the pH optimum, the enzyme was diluted into the following buffers (100 mM): MES-NaOH (pH 6.0–6.6), MOPS-NaOH (pH 6.6–7.4), and HEPES-NaOH (pH 7.4–8.0).

Synthetic Acceptor Substrates—Fucα1,2Galβ1,3GlcNAcβ1-*p*NP (21), Galβ1,3GlcNAcβ1-*p*NP (22), and Galβ1,3GlcNAcβ1-Bn (23) were synthesized previously. Galβ1,3GlcNAcα1-Bn was synthesized according to the method described for the preparation of Galβ1,3GlcNAcβ1-Bn (23). Fucα1-Me, Fucα1-allyl, and Fucα1-Bn were obtained by the reaction of L-fucose and alcohol in the presence of acidic resin Dowex 50-X (H^+). For example, L-fucose on treatment with benzyl alcohol under these conditions yielded Fucα1-Bn (24). Synthesis of Fucα1,2(Galα1,6)Galβ1-Bn and Fucβ1,2(Galα1,6)Galβ1-Bn will be described later.[2] Concentrations were determined spectrophotometrically using an extinction coefficient for Bn glycosides of 3.4 × 10^2 M^{-1} cm^{-1} at 255 nm, and for *p*NP glycosides of 1.15 × 10^4 M^{-1} cm^{-1} at 300 nm. Concentrations of the other glycosides were determined by sugar composition analysis based on hydrolysis in 2 M trifluoroacetic acid for 4 h at 100 °C, followed by chromatography in 17 mM NaOH on a PA-10 column (Dionex) and quantitation by integrated pulsed amperometry as described (25). Other *p*NP glycosides were from Sigma.

Fucα1,2Galβ1,3GlcNAcα1-Bn and Fucα1,2Galβ1,3GlcNAcβ1-Bn were produced by enzymatic fucosylation of Galβ1,3GlcNAcα1-Bn and Galβ1,3GlcNAcβ1-Bn, respectively, using soluble recombinant Skp1 FT85 FucT purified from *Escherichia coli* strain ER2566 transfected with pTY(CBD-FT85) (13). After induction, harvest, and lysis, the bacterial extract was centrifuged at 12,000 × *g* for 30 min. At 4 °C, the supernatant was made 15% saturated in $(NH_4)_2SO_4$ and centrifuged again. The supernatant was loaded onto a 35-ml column of phenyl-Sepharose Fast Flow (high sub) (Amersham Biosciences) equilibrated in 15% $(NH_4)_2SO_4$ in buffer C. Elution was performed with a 140-ml linear gradient of buffer C to buffer D. After a 35-ml wash with buffer D, a second, 170-ml gradient of 0–70% (v/v) ethylene glycol in buffer E was applied followed by 35 ml of 70% ethylene glycol in buffer E. Fractions were assayed for α1,2-FucT using Galβ1,3GlcNAcβ1-*p*NP as described (25). Active fractions were recovered from the second gradient and loaded onto a 3.3-ml DEAE 5PW column (Toso Haas) equilibrated in buffer B at 22 °C. The column was washed with buffer B, eluted with a linear gradient of 0–0.5 M NaCl in buffer B, and active fractions were pooled. The disaccharide-Bn compounds were incubated for 24 h at 21 °C in 50 mM HEPES-NaOH (pH 7.4), 15 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM DTT, and 0.1 M NaCl, with a 4-fold excess of GDP-[^{3}H]Fuc and an aliquot of purified Skp1 FucT. The reaction mixture was applied to a C_{18} reversed phase column and eluted as described below. The fucosylated derivative eluted in a baseline separated

[2] R. D. Locke, Sirajud-Doulah.k, and K. L. Matta, unpublished data.

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peak based on A_{254} and confirmed by scintillation counting of aliquots of the fractions. Concentration was determined from the specific activity of the incorporated [^{3}H]Fuc.

Skp1A1(HW120)-myc—Skp1A1(HW120)-myc was purified through the monoclonal antibody 3F9 step as previously described (13) from *Dictyostelium* strain HW120 (9). Skp1A1(HW120)-myc, which contains 2 missense mutations (I34T, D71G) in its N-terminal region, consists of a mixed population of glycoforms (8, 9) as summarized in the text.

Fucosylated Skp1 from Strain HL250—Skp1 was purified from *Dictyostelium* strain HL250 as described above for Skp1A1(HW120)-myc. This strain, unable to synthesize GDP-Fuc, accumulates Skp1 containing the Galβ1,3GlcNAc-disaccharide (8). The disaccharide on Skp1(HL250) was fucosylated using soluble recombinant Skp1 FT85 FucT as described above for the disaccharide-Bn compounds. Skp1(HL250) was incubated for 48 h at 21 °C with a 4.4-fold excess of GDP-fucose and a 5-fold excess of purified Skp1 FucT needed for complete fucosylation as determined in parallel aliquots supplemented with GDP-[^{3}H]Fuc (data not shown). Fucosylated Skp1 was diluted 2-fold with water and applied to a 0.24-ml mini-Q column (Amersham Biosciences) equilibrated in buffer B at 22 °C on an Amersham Biosciences SmartSystem HPLC, and eluted with a 3-ml linear gradient of 0–0.6 M NaCl in buffer B. Skp1-containing fractions were identified by SDS-PAGE and Western blotting with monoclonal antibody 3F9 (9).

Quantitation of Reaction—Reactions containing the Skp1 substrates were terminated by the addition of 5 μl of 20 mg/ml bovine serum albumin and 700 μl of ice-cold 10 mM sodium pyrophosphate in 10% (w/v) trichloroacetic acid. After incubation on ice for 1 h, samples were vacuum-filtered through GF/C glass fiber filters pre-wetted with ice-cold 10% (w/v) trichloroacetic acid. The reaction tube was rinsed with 10 mM sodium pyrophosphate in 10% (w/v) trichloroacetic acid that was also applied to the filter. The filter was rinsed 3 times with 1 ml of ice-cold 10% (w/v) trichloroacetic acid and 4 times with 1 ml of ice-cold acetone, and counted in 10 ml of Scintiverse BioHP (Fisher) with a Beckman LS6500 scintillation counter. Alternatively, reaction mixtures were diluted in 2× sample buffer and separated on 7–20% SDS-PAGE gel, stained with Coomassie Blue, followed by excision of the region of the gel containing Skp1 as described (12). Negative control samples were either quenched at time 0, contained no acceptor substrate, or no enzyme, and subtracted as background as indicated.

Reactions containing synthetic glycosides were terminated by the addition of 1 ml of ice-cold water or 1 mM EDTA. For the Bn and *p*NP derivatives, the mixture was filtered over a C_{18} Sep-Pak cartridge (Millipore) mounted on a 10-position Visiprep vacuum manifold (Supelco). After seven 5-ml water washes, each Sep-Pak was eluted with 5 ml of MeOH into a scintillation vial. 15 ml of Scintiverse LS was added and the sample was subjected to liquid scintillation counting. For methyl and allyl derivatives, the mixture was applied to a 0.5-ml column of Dowex-1 (Sigma) prewashed with 5 column volumes of 1 N HCl and 20 column volumes of water. The 1-ml flow-through fraction and three 1-ml water washes were collected in a 20-ml scintillation vial, diluted with 4 volumes of Scintiverse LC, and analyzed for disintegrations/min as above.

Product Characterization—To identify the position of substitution by Gal, Fuc1α-Bn was reacted with the purified pool of α-GalT1 in the presence of 1 mM UDP-[^{3}H]Gal for varied periods of time, to compare partially and fully modified preparations. The reaction product was recovered by elution from a C_{18} Sep-Pak with MeOH. The dried material was dissolved in 10 mM ammonium formate (pH 4.0) and applied to a C_{18}-reversed phase column (4.6 × 250 mm, TSK ODS-120T, 5 μm) according to Ref. 26. The column was eluted in a gradient of 0–40% acetonitrile in the same buffer at 22 °C at 1 ml/min. The eluate was monitored by measuring A_{254} and liquid scintillation counting of fractions.

Positional isomers of Galα1-Fucα1-Bn were synthesized in a two-step process that included: 1) condensation of the appropriately blocked benzyl-1-*O*-α-L-fucoside with methyl-2,3,4,6-*tetra*-(*O*-4-methoxybenzyl)-1-thio-β-D-galactopyranoside as the glycosyl donor, and 2) deprotection of the resulting disaccharide.[2] Products were characterized by NMR and MS.[2]

To determine sensitivity of incorporated radioactivity to digestion with α-galactosidase, Fucα1,2Galβ1-*p*NP (0.6 mM) was reacted in the presence of the purified pool of α-GalT1 and UDP-[^{3}H]Gal (1.8 mM) until completion as determined by incorporation of [^{3}H]Gal. The reaction product was recovered by elution from a C_{18} Sep-Pak with MeOH as above and taken to dryness in a vacuum centrifuge. This material was dissolved in a preparation of α1–3/6-galactosidase from *Xanthomonas manihotis* (New England Biolabs; Ref. 27), α-galactosidase from green coffee beans (Calbiochem), or β-galactosidase from *E. coli* as described (8). Enzyme activities were qualitatively verified colorimetrically using Galα1-*p*NP or Galβ1-*p*NP (data not shown). After incubation at 37 °C for varying times, aliquots were applied to a C_{18} Sep-Pak in water. Non-hydrolyzed substrate was eluted with MeOH and quantitated by counting as above.

RESULTS

Detection and Purification of the GalT Activity—The initial screen for Skp1 α-GalT enzyme activities was modeled after the assay for the Skp1 GlcNAcT (11). The acceptor substrate was a purified recombinant mutant form of Skp1, Skp1A1(HW120)-myc, which previous studies showed consisted of multiple structures including 2 glycoforms lacking one or both of the outer αGal residues (8, 9). This heterogeneity does not occur in wild-type Skp1, which is almost homogeneously glycosylated (9). The assay mixture contained UDP-[^{3}H]Gal, 1 μM Skp1A1(HW120)-myc, divalent cations Mg^{2+} (5 mM) and Mn^{2+} (2 mM), 5 mM DTT, and 50 mM NaCl, at pH 7.4. A cytosolic (S100) fraction from growing cells was initially tested for activity as this was a source of the other known Skp1 modification enzymes (11, 20). Substantial incorporation of radioactivity into the Skp1 band was observed after SDS-PAGE analysis of this reaction, relative to the level found in the absence of added Skp1A1(HW120)-myc (data not shown).

The S100 preparation was fractionated on DEAE-Sepharose using the method that had been previously employed for the purification of the Skp1 β1,3-GalT/α1,2-FucT (20) and GlcNAcT (11). A major peak of activity was detected centering at 0.16 M NaCl of the salt gradient (Fig. 1*A*), which overlapped with the early eluting minor pool 1 of Skp1 (17), but eluted later than the Skp1 β-GalT/α-FucT (20). Minor peaks of apparent activity were reproducibly detected at fractions 52–72 and elsewhere. The major activity peak could also be assayed using Fucα1,2Galβ1-Bn in place of Skp1A1(HW120)-myc as the acceptor substrate (data not shown). Fucα1,2Gal corresponds to the non-reducing end disaccharide of one of the Skp1A1(HW120)-myc glycoforms, suggesting that this may be the fucoside α-GalT activity predicted by structure studies on the native Skp1 glycan (8). Based on the assay using Fucα1,2Galβ1-Bn, the activity was purified 31-fold at a 72% yield (Table I).

The major activity pool from the DEAE-Sepharose column was adjusted to 20% saturated $(NH_4)_2SO_4$ and loaded onto a phenyl-Sepharose column (Fig. 1*B*). Nearly all activity, using Fucα1,2Galβ1-Bn as acceptor, eluted sharply near the end of the ethylene glycol gradient, partially overlapping with a large protein peak, and still copurifying with Skp1 (data not shown). This resulted in an additional 5-fold purification, with a step yield of 48% (Table I).

The pooled activity peak was subjected to a second anion exchange separation. The enzyme eluted sharply at 0.1 M NaCl when assayed using Fucα1-Bn as the acceptor (Fig. 1*C*), resulting in a 3.7-fold purification with 92% yield (Table I). Similar elution profiles were seen using either Fucα1-Bn or Fucα1,2Galβ1-Bn (data not shown), indicating that the GalT adds Gal directly to Fuc. A second small peak was found partially overlapping with the later eluting Skp1 peak as determined by dot-blot analysis.

The Q-Sepharose activity pool was concentrated and loaded onto a Superdex 200 gel filtration column. The great majority of activity eluted as a symmetrical peak slightly ahead of the bovine serum albumin standard (M_r 66,000) (Fig. 1*D*). This resulted in a 4.2-fold purification with 114% yield (Table I).

The four chromatographic steps resulted in an activity that was 2400-fold purified, with a 36% yield, that was fully separated from the potential endogenous acceptor substrate Skp1. The α-GalT activity appeared to be homogeneous chromatographically and with respect to its acceptors based on a

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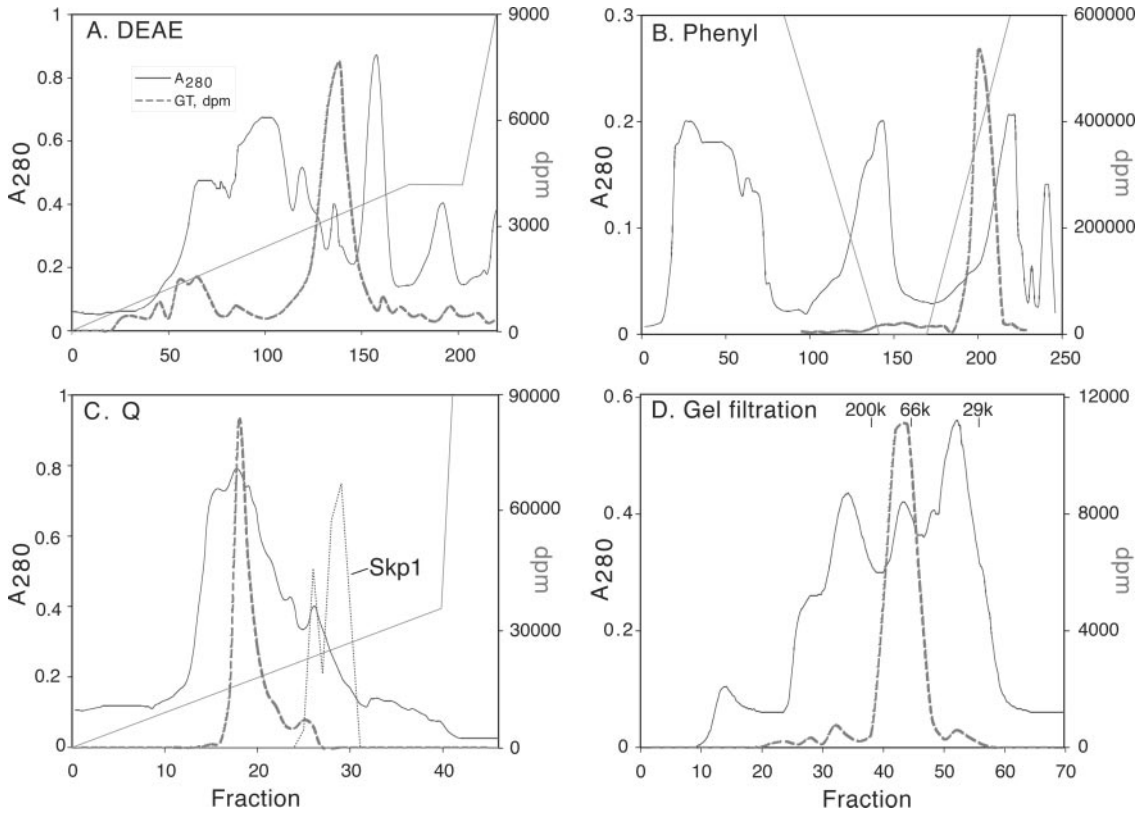


FIG. 1. **Chromatographic purification of the α-GalT.** *A*, the S100 fraction from a 30-liter *Dictyostelium* cell culture was adsorbed to a DEAE Fast Flow-Sepharose column and proteins were eluted with a 0–0.25 M NaCl gradient as represented by the *thin line*. Protein was monitored by A_{280} nm (*solid thick line*), and fractions were assayed for α-GalT activity with 0.1 μg of Skp1A1(HW120)-myc as acceptor (*dashed line*). The flow-through fraction contained no detectable activity (data not shown). *B*, active fractions from 2 DEAE purifications were bound to a phenyl-Sepharose column and eluted with a descending gradient (20–0% saturation) of $(NH_4)_2SO_4$, followed by a 0–70% ethylene glycol gradient. Fractions were assayed with 0.23 mM Fucα1,2Galβ1-*p*NP. *C*, the active phenyl-Sepharose pool (one-third) was applied to a Hi-Trap Q-Sepharose column and eluted with a 0–0.2 M NaCl gradient. α-GalT activity was assayed using 1.8 mM Fucα1-*p*NP. The elution position of Skp1 as assessed qualitatively using a dot-blot assay with monoclonal antibody 3F9 is shown as a *dotted line*. Active fractions from the three runs were pooled and concentrated by ultrafiltration. *D*, the Q-Sepharose α-GalT pool was concentrated at 90% yield and applied to a Hi-Load Superdex-200 gel filtration column. Fractions were assayed as in *panel C*. Elution positions of the M_r standards are shown at the *top*.

TABLE I
Purification of the Skp1 α-GalT

Results are shown for the purification of the enzyme from 3.9×10^{11} *Dictyostelium* cells, as described under "Experimental Procedures" and "Results." Chromatographic elution profiles are shown in Fig. 1. GalT activity was assayed under standard conditions, using 1.5 mM Fucα1,2Galb1-Bn and 3.3 μM UDP-[^{3}H]Gal (13,200 dpm/pmol), for 1 h. 1 unit = 1 nmol/h.

Sample	Protein	Activity	Specific activity	Yield	Purification step
	mg	*units*	*units/mg*	*%*	*-fold*
S100	25,520	757	0.030	100	
DEAE-FF	590	542	0.92	72	31
Phenyl-Sepharose	56	260	4.6	34 (48)	153 (5)
Q-Sepharose	14	240	17	32 (92)	567 (3.7)
Superdex 200	3.8	274	72	36 (114)	2400 (4.2)

competition experiment. Fucα1,2Galβ1-Bn, at 1 (near its K_m, see below) and 4 mM, inhibited incorporation into Skp1A1(HW120)-myc by 37 and 61%. In addition, the purified preparation exhibited no autoincorporation activity based on trichloroacetic acid precipitation of a reaction conducted with no added acceptor substrate (data not shown).

General Properties of the GalT—The partially purified enzyme was most active at 27 °C, at the upper end of the growth temperature range for *Dictyostelium* (data not shown). Activity toward Fucα1,2Galβ1-*p*NP was linear with respect to time (data not shown) and enzyme concentration (Fig. 2*A*). Dilution in the absence of the reducing agent DTT led to a loss of activity that could be rescued by 1 mM DTT (Fig. 2*B*), as for the other *Dictyostelium* Skp1 GTs. Dilution in the absence of divalent cations reduced activity and EDTA abolished activity (Fig. 2*C*). The presence of either $MnCl_2$ or $MgCl_2$ sustained activity, but $MnCl_2$ at 2 mM was optimal and 4-fold better than $MgCl_2$. The enzyme preferred salt around 50 mM and NaCl yielded higher activity than KCl (Fig. 2*D*). The enzyme was most active from pH 6.4 to 7.4 (Fig. 2*E*). Addition of Tween 20 to 0.1–1.0% did not affect activity (data not shown). The enzyme exhibited typical hyperbolic dependence on UDP-Gal concentration (Fig. 3*A*), which yielded an apparent K_m of 3.5 μM based on analysis of the data by the Lineweaver-Burk method (Fig. 3*B*).

To determine whether GalT activity was also present in the microsomal fraction, a washed P100 fraction, which contains microsomes, and the S100 fraction, from which the GalT was purified, were compared using the optimized conditions and the most active synthetic acceptor substrate, Fucα1,2Galβ1-Bn (see below). The fractions were freeze-thawed and treated with 0.1% Tween 20 to permeabilize the vesicles. Incorporation into Fucα1,2Galβ1-Bn by the P100 fraction was <1% that of the S100 fraction, based on a comparison of disintegrations/min recovered from C_{18} Sep-Pak eluates of reactions containing or lacking the acceptor (Fig. 4*A*). In contrast, a conventional Golgi type 2 membrane GT was retained with the particulate fraction under these conditions (31). To control for potential inhibition or dilution of UDP-[^{3}H]Gal by endogenous UDP-Gal, parallel reactions were supplemented with equal amounts of purified GalT. As shown in Fig. 4*B*, full α-GalT activity was recovered from the microsomal preparation compared with activity in the

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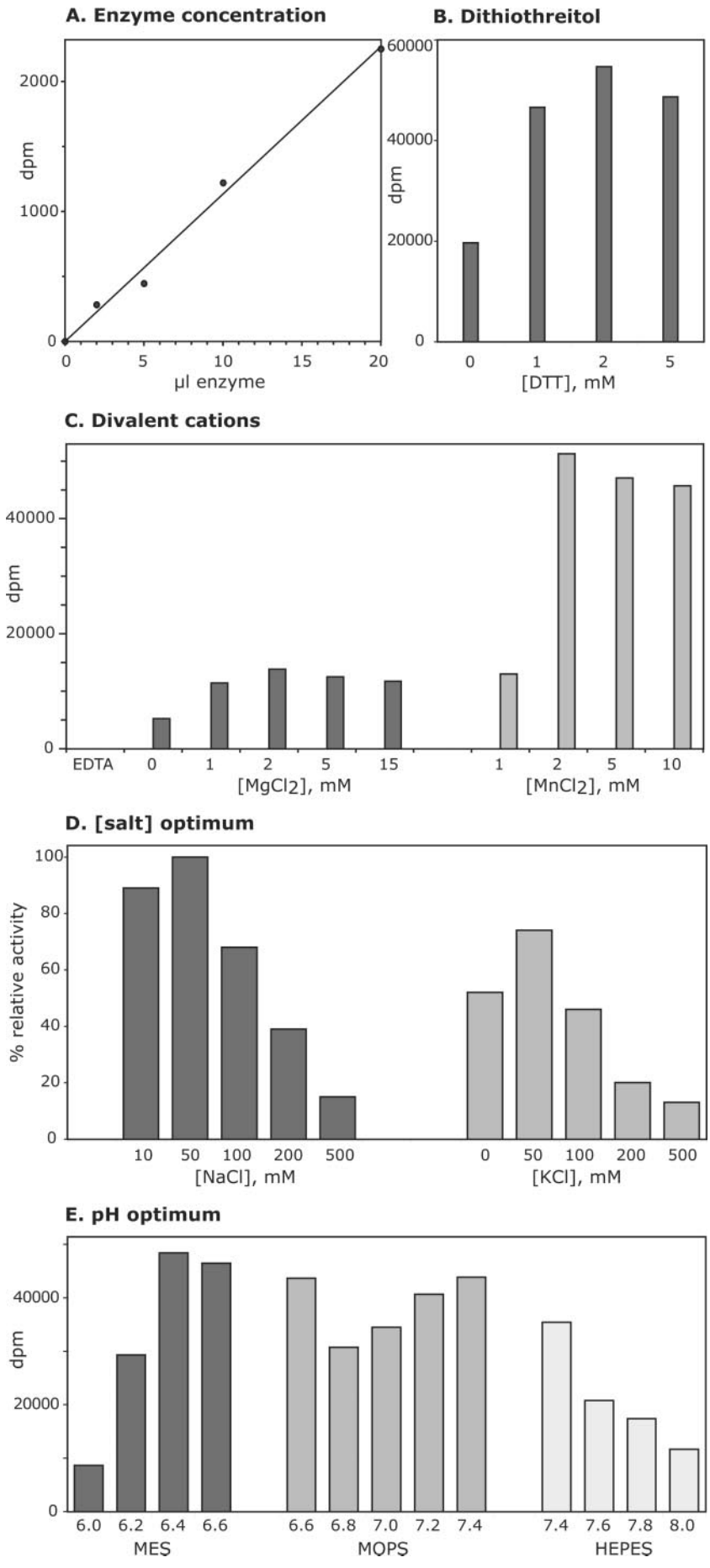


FIG. 2. **Characterization of the α-GalT.** The purified enzyme pool shown in Fig. 1*D* was diluted 10-fold in the solutions described, in the presence of 3 μM UDP-Gal and 0.3 mM Fucα1,2Galβ1-*p*NP. *A*, activity depended linearly on enzyme amount, and time (data not shown). *B*, activity was maximally stimulated by the addition of 1 mM DTT. *C*, activity was abolished by the addition of 6 mM EDTA. Addition of $MnCl_2$ at 2 mM or higher maximally stimulated activity. 2 mM $MgCl_2$ only partially substituted. *D*, activity was maximally stimulated by NaCl at 50 mM. 50 mM KCl only partially substituted. *E*, activity exhibited a broad pH optimum of 6.4–7.4, and declined outside this range.

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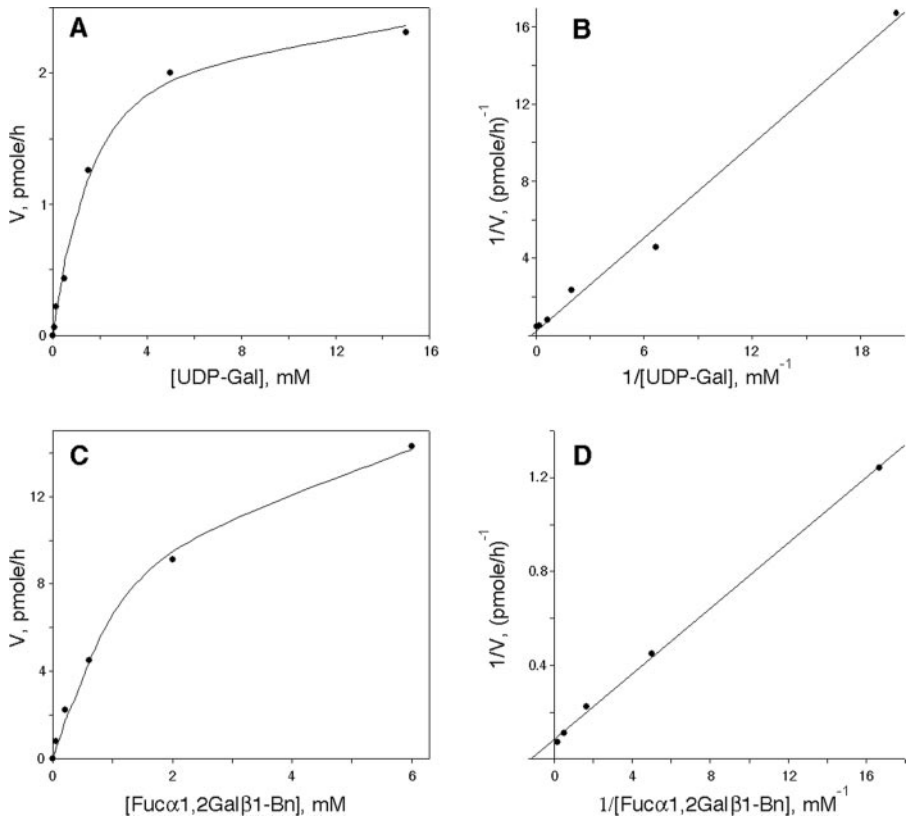


FIG. 3. **Kinetic analysis of the α-GalT.** The purified enzyme pool was assayed under optimal conditions defined in the legend to Fig. 2 at different substrate concentrations. *A*, UDP-Gal concentration was varied in the presence of 0.3 mM Fucα1,2Galβ1-Bn. *B*, double-reciprocal replot of the data in *panel A*. *C*, the concentration of Fucα1,2Galβ1-Bn was varied in the presence of 3 μM UDP-Gal. *D*, double reciprocal replot of the data in *panel C*. Curves in *A* and *C* represent best fits of the data as hyperbolic relationships; *lines* in *B* and *D* represent linear least squares best fit of the data.

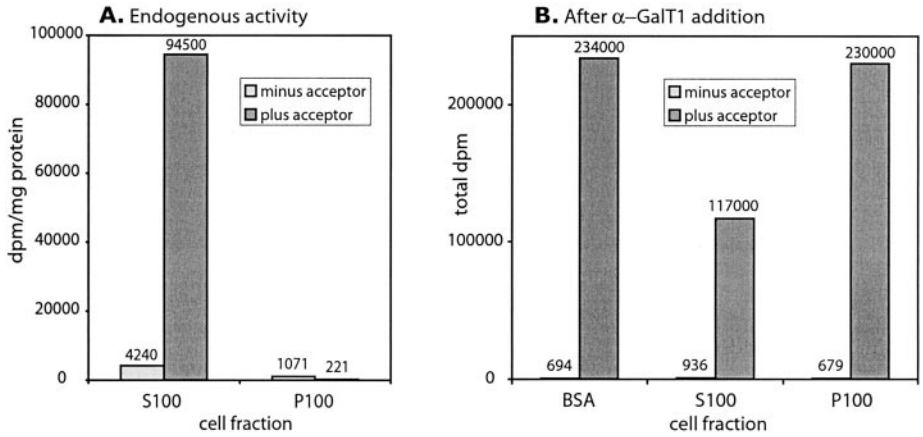


FIG. 4. **Absence of α-GalT1 activity in the particulate fraction.** *A*, cytosolic and particulate fractions were prepared as described under "Experimental Procedures," freeze-thawed, and assayed in the presence of 1 μCi of UDP-Gal (2.2×10^6 disintegrations/min), 0.1% Tween 20, and ±5 mM Fucα1,2Galβ1-Bn as indicated, for 2 h in a volume of 40 μl. *B*, parallel assays were supplemented with equal aliquots of purified α-GalT1. *BSA*, 1 mg/ml bovine serum albumin.

presence of bovine serum albumin, whereas only partial activity was recovered from the S100 fraction. Because the apparent absence of activity from the P100 fractions does not result from inhibition, it is likely that the GalT is not associated with the particulate cell fraction in cells. This is consistent with dependence of the activity on a reducing agent, a property that better matches the reducing environment of the cytoplasm than the oxidizing environment of the secretory pathway.

Activity of the GalT toward Synthetic Acceptors—Various synthetic glycosides were compared to help define the acceptor substrate specificity of the enzyme. These were either chemically synthesized and verified for purity by HPLC, NMR, and MS, or synthesized chemo-enzymatically and purified by HPLC, as described under "Experimental Procedures." Bn-conjugates corresponding to the non-reducing terminal mono-, di-, and trisaccharides of the Skp1 glycan are compared in Fig. 5*A*. Fucα1,2Galβ1-Bn was the best acceptor followed by Fucα1Bn and Fucα1,2Galβ1,3GlcNAcα1-Bn, which corresponds to the full-length glycan. A kinetic analysis showed that Fucα1,2Galβ1-Bn (Fig. 3, *C* and *D*) had a 2.5–4-fold lower K_m and 50–100-fold higher V_{max} than the other two compounds, resulting in a 260-fold higher relative catalytic efficiency (Table II). Therefore, the purified α-GalT exhibited a marked preference for the distal disaccharide relative to the native full-length glycan. It did not matter whether the full-length glycan was attached in α- or β-linkage to Bn (Fig. 5*A*). A similar difference was observed for the analogous series conjugated to *p*NP in place of Bn (Table II).

Fucα1-Bn exhibited similar catalytic efficiency relative to the full-length trisaccharide (Table II) indicating that this is the minimal acceptor and that the Gal is linked directly to an unknown position on the Fuc. Decreasing the number of carbons in the aglycon resulted in improved acceptor activity (Fig. 5*B*) that did not, however, attain that of the disaccharide. Substitution of the β-Gal moiety of the disaccharide (Fucα1,2Galβ1-Bn) with α6-linked Gal partially compromised but did not abolish acceptor activity (Fig. 5*A*), suggesting that the enzyme tolerates glycan branching. β-Linked Fuc was a poorer acceptor either as a monosaccharide conjugate (Fig. 5*B*) or in the branched trisaccharide (Fig. 5*A*), demonstrating specificity for α-linked Fuc. β-Linked D-Fuc had no measurable

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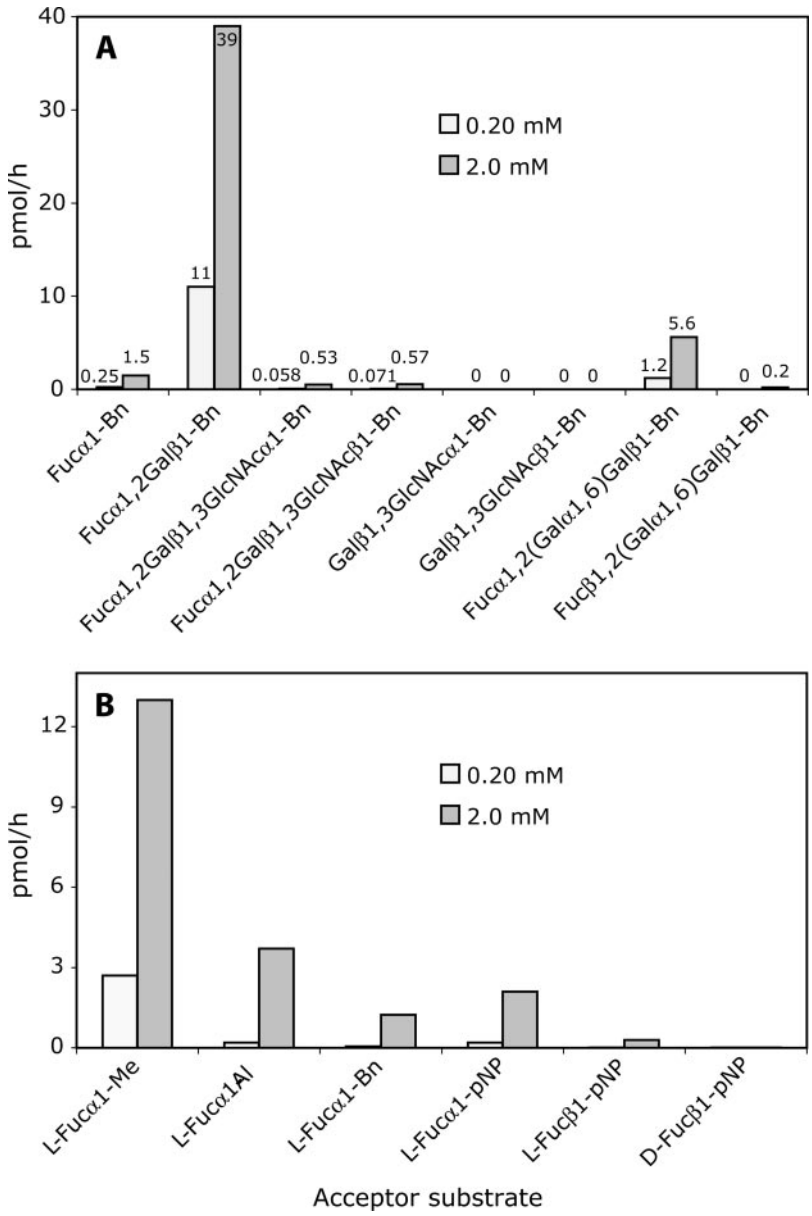


FIG. 5. **Acceptor specificity of the α-GalT.** The purified enzyme pool was incubated in either 0.2 or 2.0 mM synthetic glycoside for 1 h in the presence of 3 μM UDP-[^{3}H]Gal. Transfer of [^{3}H] to the glycoside was assayed by adsorption to a C_{18} Sep-Pak cartridge and elution with methanol, or as the flow-through fraction of a Dowex-1 column (for the methyl and allyl conjugates). *A*, a series of Bn conjugates. *B*, a series of Fuc monosaccharide conjugates. Levels <0.02 pmol/h are reported as 0.

TABLE II
Kinetic constants for galactosylation of synthetic glycosides and Skp1

Acceptors were assayed under standard conditions with the 2400-fold purified enzyme preparation. Apparent K_m and V_{max} values were calculated from double reciprocal plots using linear least squares analysis to generate the best fit line as in Fig. 3*B*.

Substrate	K_m	V_{max}	Relative catalytic efficiency[a]
	mM	*pmol/h/μl*	
Fucα1-Bn	3.8	0.21	1
Fucα1,2Galβ1-Bn	0.84	12	260
Fucα1,2Galβ1,3GlcNAcα1-Bn	2.2	0.12	0.99
Fucα1-*p*NP	3.7	0.31	1.5
Fucα1,2Galβ1-*p*NP	0.82	45	990
Fucα1,2Galβ1,3GlcNAcβ1-*p*NP	1.2	0.32	4.8
Fucα1,2Galβ1,3GlcNAcα1-Skp1	0.006	7.0	21,000

[a] Catalytic efficiency (V_{max}/K_m) is calculated as the apparent second-order rate constant with respect to the acceptor substrate. Values were determined at the same enzyme concentration and are reported relative to Fucα1-Bn = 1.

activity (Fig. 5*B*), signifying stereospecificity. The reducing terminal disaccharide Galβ1,3GlcNAcα1-Bn and its anomer Galβ1,3GlcNAcβ1-Bn were also not acceptors (Fig. 5*A*), consistent with the interpretation that the α-Gal is linked to Fuc.

Characterization of the Gal-Fuc Linkage—Time course analyses of the GalT reaction with UDP-[^{3}H]Gal showed that, at completion, 0.88–1.07 mol of Gal was incorporated per mol of Fucα1-*p*NP. Parallel HPLC analysis on a C_{18} column (see below) or a Dionex PA-10 column indicated the existence of only a single radioactive species throughout the time course, confirming that only a single Gal was added and indicating that this occurred at a single position (data not shown).

To verify transfer of radioactivity to Fucα1-Bn as Gal, the susceptibility of [^{3}H] in the reaction product to galactosidase digestion was calculated from the binding of non-hydrolyzed radioactivity to a C_{18} Sep-Pak cartridge. [^{3}H] was released by both green coffee bean α-galactosidase and α1,3/6-galactosidase from *X. manihotis*, but not *E. coli* β-galactosidase (Fig. 6). Therefore, Gal appears to be transferred in α-linkage to possibly the 3-position of Fuc (because Fuc lacks a 6-OH substituent). One of the peripheral Gal residues of the Skp1 glycopeptide is susceptible to *X. manihotis* α1,3/6-galactosidase (8). HPLC analysis of partially hydrolyzed samples did not reveal any radioactive species in addition to the starting compound (data not shown), consistent with the evidence above that only

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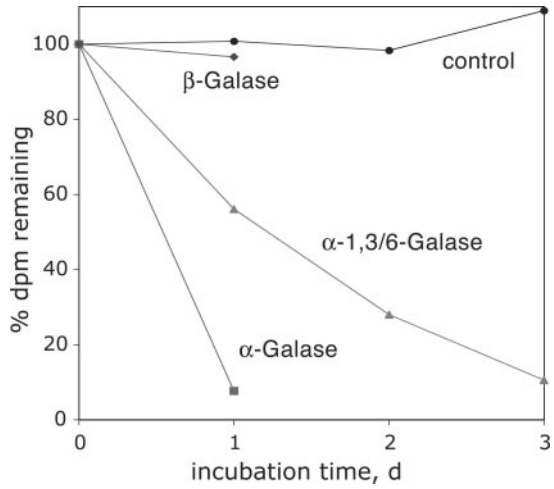


FIG. 6. **Sensitivity of the reaction product to α-galactosidase treatment.** Fucα1-*p*NP was quantitatively modified by the purified GalT preparation in the presence of UDP-[^{3}H]Gal, purified on a C_{18} Sep-Pak, and subjected to green coffee bean α-galactosidase (*squares*), *X. manihotis* α1,3/6-galactosidase (*triangles*), β-galactosidase from *E. coli* (*diamonds*), or buffer alone (*circles*). At daily intervals, non-hydrolyzed material was determined by recovery on a C_{18} Sep-Pak.

a single radioactive sugar had been added by the GalT.

To confirm the position of Gal substitution, Galα1–2Fucα1-Bn, Galα1,3Fucα1-Bn, and Galα1,4Fucα1-Bn were synthesized, purified, and characterized by NMR.[2] These isomers were baseline-separated when chromatographed on a C_{18} reversed-phase column in a gradient of acetonitrile at pH 4.0 (Fig. 7). The radiolabeled GalT reaction product with Fucα1-Bn coeluted precisely with the Galα1,3Fucα1-Bn standard, confirming the galactosidase results indicating that the enzyme forms a Galα1–3Fuc linkage.

Activity of the GalT toward Skp1—Skp1 isolated from strain HL250, which is unable to synthesize GDP-Fuc from GDP-Man, contains the Galβ1,3GlcNAc disaccharide (8) and is not a substrate for the GalT (Fig. 8). To generate the Fucα1,2Galβ1,3GlcNAc trisaccharide, a recombinant form of FT85, the Skp1 β-GalT/α-FucT, was partially purified from extracts of *E. coli* and used to quantitatively fucosylate Skp1(HL250) based on incorporation in a parallel reaction trace labeled with GDP-[^{3}H]Fuc (data not shown). Fucosylated Skp1 was a superior substrate for the GalT (Fig. 8), with a K_m that was over 2 orders of magnitude lower than that of the best synthetic glycan found, Fucα1,2Galβ1-*p*NP, although its V_{max} was 6–7-fold lower (Table II). Nevertheless, its overall catalytic efficiency toward Skp1 was 21-fold higher than that of Fucα1,2Galβ1-*p*NP, suggesting that the Skp1 polypeptide is an important determinant for enzyme recognition.

To confirm the importance of Skp1 tertiary structure for GalT recognition, fucosylated Skp1 was subjected to the denaturing treatments shown in Fig. 8. Heating, treatment with 6 M urea, and reduction and alkylation with iodoacetamide each greatly impaired the acceptor activity of Skp1. Thus, the native structure of Skp1 appears to be important for recognition by α-GalT, which provides an explanation for the incomplete α-galactosylation of mutant Skp1A1(HW120)-myc *in vivo* (8).

DISCUSSION

α-GalT1 Is a Cytoplasmic α1,3-GalT—The GalT activity defined in this study catalyzes the formation of the novel Galα1,3Fuc linkage, and will be referred to as α-GalT1. The assignment of this linkage is based on (*a*) the ability of the enzyme to modify acceptors containing only the sugar L-Fuc, (*b*) sensitivity of the transferred Gal to removal by an α1,3/6-galactosidase, and (*c*) co-chromatography of the Gal-Fucα1-Bn

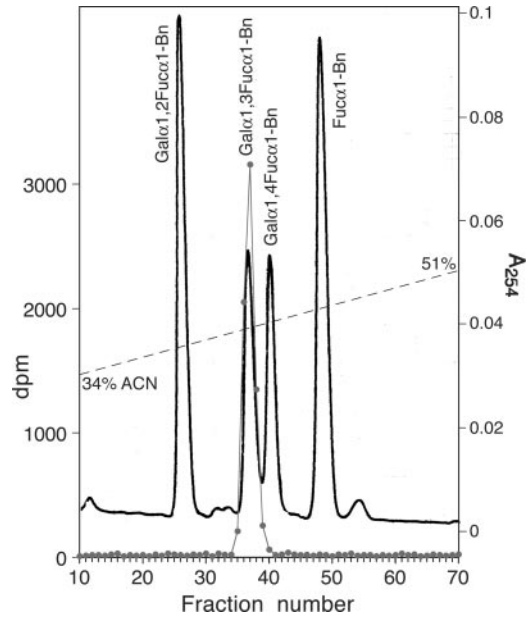


FIG. 7. **Co-chromatography of reaction product with Galα1,3Fucα1-Bn.** The GalT reaction product formed from UDP-[^{3}H]Gal and Fucα1-Bn was mixed with ~100 nmol of the synthetic glycosides shown, and separated on a C_{18} reversed phase HPLC column in an ascending gradient of acetonitrile (*ACN*) at pH 4. The reaction product was detected by scintillation counting of fractions and the glycosides were detected by absorbance at 254 nm. The region of the chromatogram in which the glycosides eluted (36–51% ACN) is shown.

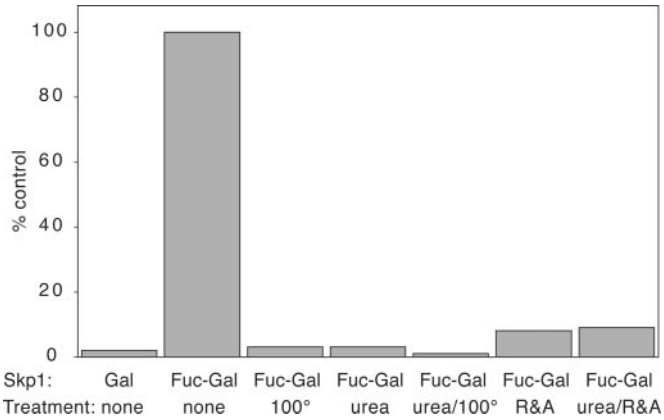


FIG. 8. **Acceptor substrate activity of Skp1.** Native Skp1(HL250) isolated from a GDP-Fuc synthesis mutant was quantitatively fucosylated using recombinant FT85 Skp1 αFucT, and are referred to as *Gal* and *Fuc-Gal*. Aliquots of fucosyl-Skp1 were heated at 100 °C for 3 min, treated with 6 M urea for 15 min, and/or reduced with DTT and alkylated with iodoacetamide (*R&A*) (8), as indicated. Samples were diluted 100-fold in standard buffer and assayed as acceptors for the purified α-GalT1 in the presence of UDP-[^{3}H]Gal. Incorporation of disintegrations/min was determined by trichloroacetic acid precipitation.

reaction product with authentic Galα1,3Fucα1-Bn but not other positional isomers of this glycoside. The Galα1,3Fuc linkage has not been reported previously on glycoconjugates in either prokaryotes or eukaryotes, although internal Fuc residues capped by β4-linked Gal or β3-linked GlcNAc have been seen in an *N*-linked glycan from octopus rhodopsin (28) and in epidermal growth factor modules (29), respectively.

Although only 2400-fold purified, the enzyme activity is chromatographically homogeneous by ion exchange, hydrophobic interaction, and size exclusion criteria, and has been separated from detectable endogenous substrates and degradases. In addition, the activity is kinetically homogeneous with respect to individual substrates, and forms a unique product based on characterization of the reaction with Fucα1-Bn, other synthetic

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glycosides and Skp1 glycoforms, and competition studies with Skp1A1(HW120)-myc. Therefore, the enzyme is purified enough to yield reliable information yet, based on recovery yield (36%), sufficiently intact so as to exhibit native properties. Information about the protein composition of the enzyme must await further purification or cloning of its gene.

This α1,3-GalT resembles traditional GalTs in its dependence on UDP-Gal as the sugar donor (16, 30). Its apparent K_m for UDP-Gal, 3.5 μM, is at the low end of the range for Golgi-associated GTs but higher than that of some cytoplasmic GTs (14). The dependence of the enzyme on Mn^{2+} or, less efficiently, Mg^{2+}, is common for GTs especially of the GT-A (SpsA) superfamily (5). Its pH optimum range of 6.4–7.4 is physiological.

However, the enzyme is unusual as it is found in the cytosolic fraction of the cell extract after gentle cell lysis and behaves as a soluble protein throughout the purification. No activity was detected in the microsomal (particulate) fraction of the extract (Fig. 4). In addition, The enzyme depends on the presence of a reducing agent for activity, and has a very high catalytic efficiency for a cytoplasmic/nuclear protein acceptor, Skp1 (see below). Therefore, α-GalT1, like 2 other GTs that modify Skp1 (11, 14, 20), appears to be a cytoplasmic (or nuclear) protein. This is unlike conventional GTs that modify glycoprotein substrates, which, like *Dictyostelium* pp αGlcNAcT2 (31), are usually associated with the Golgi apparatus as type 2 integral membrane proteins (32), unless they are proteolytically cleaved from their membrane anchors.

Dictyostelium Skp1 Is a Natural Substrate for α-GalT1—α-GalT1 was first detected in a screen for GalT activities that could modify a recombinant underglycosylated mutant Skp1 isolated from *Dictyostelium* (Fig. 1*A*). After 2400-fold purification of the enzyme, Fucα1,2Galβ1,3GlcNAcα1-Skp1 (containing the wild-type polypeptide backbone) is an excellent substrate with a catalytic efficiency that is 14,000-fold better than that of Fucα1-*p*NP and 21-fold better than Fucα1,2Galβ1-*p*NP (Table II). Gal appears to be added to Fuc because Galβ1, 3GlcNAcα1-Skp1 is not an acceptor, and Fucα1,2Galβ1-Bn is a competitive acceptor. Finally, the Galα1,3Fuc linkage formed by α-GalT1 on model substrates is likely to be present in the pentasaccharide on native Skp1. The glycopeptide carrying the pentasaccharide was analyzed previously by mass spectrometry after treatment with galactosidases or mild acid (8). Like the Gal added by α-GalT1, one of the two peripheral Gal residues was susceptible to release by *X. manihotis* α1,3/6-galactosidase but not a recombinant α1,3-galactosidase (from Glyko), whereas both were released by a nonspecific α-galactosidase. Both Gal residues appear to be attached via Fuc because of simultaneous loss of all 3 sugars from the glycopeptide during a mild acid hydrolysis time course, and their absence from a mutant that does not attach Fuc. Although the differential susceptibility to α1,3/6-galactosidase and α1,3-galactosidase suggested that the first Gal is α1,6-linked to Gal because of the absence of a 6-OH group on Fuc, the alternative explanation that the first Gal forms a Galα1,3Fuc linkage that is simply not cleaved by the α1,3-galactosidase (whose specificity had been defined on different model substrates) for other reasons (*e.g.* steric hindrance by the second Gal) could not be excluded. Given the ability of α-GalT1 to form the Galα1,3Fuc linkage on small compounds and its competitive high catalytic efficiency for Skp1, it is now likely that the alternative explanation is correct and that the first Gal is α1,3-linked to Fuc in native Skp1. It is therefore likely that the second Gal is α-linked elsewhere on Fuc or the αGal, although attachment to an underlying sugar via a linkage with identical acid liability to that of the Fucα1,2Gal linkage, by an enzyme that depends on prior addition of Fuc, cannot be excluded. The enzyme that forms this linkage may be represented by one of the minor GalT peaks in Fig. 1*A*.

Based on the strong preference of α-GalT1 for Skp1 relative to the synthetic glycan conjugates, the evidence that native Skp1 contains the Galα1,3Fuc linkage, and the co-compartmentalization of the two proteins in the cytosol, it is likely that Skp1 is a natural substrate for the enzyme *in vivo*. Cloning and disruption of a candidate cytoplasmic α-GalT coding region[3] present in the nearly fully sequenced *Dictyostelium* genome may provide a test of this model.

α-Galactosylation of Skp1 Depends on Its Tertiary Structure—As summarized above, the catalytic efficiency of α-GalT1 toward a presumptive natural acceptor substrate, Fucα1,2Galβ1,3GlcNAcα1-Skp1, is much greater than that of the simple synthetic glycosides (Table II), indicating an important contribution of the Skp1 polypeptide to processing by α-GalT1. The greatest factor in improved catalytic efficiency is decreased K_m, so the polypeptide may contribute toward better initial binding. This may occur by two separate mechanisms as indicated by three lines of evidence. First, pretreatment of Fucα1,2Galβ1,3GlcNAcα1-Skp1 with heat, urea, and/or an agent, which alkylates Cys residues (iodoacetamide), essentially abolished reactivity (to <10%) (Fig. 8). These effects, which are expected to selectively alter the polypeptide, indicate that normal polypeptide folding is important for recognition by α-GalT1.

Second, the full-length Skp1 acceptor glycan, Fucα1,2Galβ1, 3GlcNAc-, if α-linked to Bn, was a 21,000-fold inferior acceptor (based on catalytic efficiency, Table II) compared with when it was attached to the Skp1 polypeptide, where it is also thought to be α-linked (15, 32). The β-linked trisaccharide was an equally poor acceptor when attached to either Bn or *p*NP. Surprisingly, the trisaccharide was no better than simple fucosides of Bn or *p*NP. The aglycons do not appear to be inhibitory *per se* as the non-reducing terminal disaccharide Fucα1,2Galβ1-*p*NP was more than 200-fold more efficient than the trisaccharide conjugate, primarily because of an improvement in V_{max}. A possible explanation for this phenomenon comes from NMR studies on the solution structure of lacto-*N*-fucopentaose-1 and a Lewis b tetrasaccharide (which both contain the trisaccharide), that find that the Fuc ring prefers to stack over the GlcNAc allowing a hydrophobic interaction between the 6-methyl of Fuc and the *N*-acetyl group of GlcNAc (33, 34). We propose that this fold-back interaction sterically interferes with access of the trisaccharide to the active site of α-GalT1, and that the native conformation of Skp1 inhibits the fold-back conformation.

The third line of evidence relates back to the original observation that a mutant Skp1, which has two point mutations (I34T, D71G) in the N-terminal half of the protein, is incompletely α-galactosylated at Pro-143 when expressed *in vivo*. In contrast, no glycoforms lacking the underlying αFuc or β-Gal residues were observed, indicating that the GTs that add these sugars are not affected by the mutations. The possibility that the outer Gal deficiency is attributable to inefficient synthesis rather than action of a galactosidase is supported by the partial co-purification of α-GalT1 with the previously described minor Skp1 pool I (17) through the anion exchange and hydrophobic interaction chromatographic steps (Fig. 1), suggesting a frustrated substrate-enzyme interaction. This is further supported by evidence that pool 1 Skp1 is incompletely galactosylated (25). Therefore, the mutations may affect recognition by α-GalT1 at a site separate from the sugar addition site (Pro-143).

These results suggest that α-GalT1 recognition of Skp1 in-

[3] H. van der Wel, A. Ercan, and C. M. West, unpublished studies.

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volves multiple determinants, including one in the N-terminal half associated with the mutant residues in Skp1A1(HW120)-myc, and a second that is associated with presentation of the non-reducing terminal disaccharide that in turn presumably depends on folding in the C-terminal region. These determinants may not be completely independent. It is therefore predicted that α-GalT1 has multiple domains supporting recognition of separate regions of Skp1. Dependence of α-galactosylation on Skp1 folding is consistent with evidence that α-GalT1 appears to be selective for Skp1 *in vivo*. This is based on observations that (*a*) Skp1 is the most prominent soluble cellular protein metabolically labeled with the acceptor sugar [^{3}H]Fuc (35), (*b*) Skp1 is the only Skp1 FucT acceptor that accumulates in the absence of GDP-Fuc (35), and (*c*) the Skp1 β-GalT (which adds the β-Gal to which Fuc is attached) also displays dependence on the Skp1 polypeptide (13, 35).

If the Fucα1,2Galβ1,3GlcNAc trisaccharide is unique to Skp1, why are multiple determinants in the Skp1 polypeptide also necessary for efficient modification by the α-GalT? Because the polypeptide determinants are associated with tertiary structure, these results raise the possibility that α-galactosylation functions as a quality control step for Skp1 folding that must be executed before Skp1 is subject to the final modification step of the pathway, addition of the second α-linked Gal residue. A precedent for this model exists in the rough endoplasmic reticulum, where the acceptor activity of the $Man_9GlcNAc_2$ moiety of nascent glycoproteins with respect to the UDP-Glc:*N*-glycan GlcTase is strongly stimulated by an unfolded carrier polypeptide (36, 37), although for Skp1 we propose the opposite effect that folding stimulates acceptor activity.

Acknowledgments—We are grateful to Scott McMillen and the UF ICBR Protein Chemistry Core Lab for use of the Dionex HPLC system, and Dr. Ana Maria Garcia for technical assistance.

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J. Biol. Chem. 2004, 279:29050-29059.
doi: 10.1074/jbc.M313858200 originally published online May 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313858200

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