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Molecular Characterization of a Novel UDP-galactose:Fucoside α3-Galactosyltransferase That Modifies Skp1 in the Cytoplasm of *Dictyostelium*[*]

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Skp1 is a nucleocytoplasmic protein that is post-translationally modified by a pentasaccharide, Galα1,Galα1,3Fucα1,2Galβ1,3GlcNAcα1*O*-, at a 4-hydroxylated derivative of Pro-143 in the amebazoan *Dictyostelium discoideum*. An enzymatic activity that catalyzes formation of the Galα1,3Fuc linkage by transfer of Gal from UDP-αGal to Fucα1,2Galβ1,3GlcNAcα1*O*-benzyl, or the corresponding glycoform of Skp1, was described previously in cytosolic extracts of *Dictyostelium*. A protein GT78 associated with this activity has been purified to chromatographic homogeneity. In-gel tryptic digestion followed by nano-liquid chromatography-mass spectrometry on a quadrupole time-of-flight geometry instrument with data-dependent tandem mass spectrometry acquisition yielded a number of peptide fragmentation spectra, nine of which were manually *de novo* sequenced and found to map onto a predicted 3-exon gene of unknown function on chromosome 4. GT78 is predicted to comprise 648 amino acids with an N-terminal glycosyltransferase and a C-terminal β-propeller domain. Overexpression of GT78 with a His_6-tag resulted in a 120-fold increase in GalT-activity in cytosolic extracts, and purified His_6-GT78 exhibited α3GalT-activity toward a synthetic acceptor substrate. Expression of the truncated N-terminal region confirmed the predicted catalytic activity of this domain. Disruption of the GT78 gene led to a loss of enzyme activity in extracts and accumulation of the non-galactosylated isoform of Skp1 in cells. GT78 therefore represents the Skp1 α3GalT, and its mechanism conforms to the sequential model of Skp1 glycosylation in the cytoplasm shown for earlier enzymes in the pathway. Informatics studies suggest that related catalytic domains are expressed in the Golgi or cytoplasm of plants, other protozoans, and animals.

Skp1 is best known as an adaptor in the SCF-family of E3[3]-ubiquitin ligases, whose subunits include Skp1, cullin-1, and an F-box protein that selects target proteins for polyubiquitylation by the E2 catalytic subunit (1–3). These ligases are responsible for the degradation of a large number of cell cycle regulatory proteins and transcriptional factors and therefore perform key roles in a wide variety of developmental and physiological pathways in all eukaryotes. Specific E3(SCF)ubiquitin-ligases are subject to regulation by NEDDylation, other protein subunits, and assembly disassembly cycles that appear to accompany catalysis. Skp1 also performs less understood functions in the assembly of other protein complexes at membrane surfaces and in related E3-ubiquitin ligases.

In the social amebazoan *Dictyostelium discoideum*, Skp1 is subject to modification at Pro-143 by 4-hydroxylation and subsequent glycosylation, resulting in occurrence of Galα1,Galα1,3Fucα1,2Galβ1,3GlcNAcα1-linked to 4-HyPro143 of the major cellular isoform (4). The enzymes responsible for Pro-hydroxylation and the first three sugars have been expressed recombinantly and characterized as soluble proteins that accumulate and operate in the cytoplasm of the cell (5). They are evolutionarily distantly related to known prolyl 4-hydroxylase and glycosyltransferase (GT) genes but lack N-terminal targeting sequences for the secretory pathway. Disruption of the Skp1 prolyl 4-hydroxylase gene confirms its biosynthetic role *in vivo* (6), and reveals a requirement for fruiting body formation during development.[4] The second and third sugars are added by PgtA, a di-GT whose biosynthetic function *in vivo* has also been established by gene disruption (7). PgtA appears to make more subtle contributions to growth and development (8).[4] The properties of these enzymes suggest a model in which Skp1 is post-translationally modified by independent proteins in a sequential manner (5). The relationship between E3(SCF)ubiquitin-ligase assembly and post-translational modification of Skp1 remains to be explored.

To determine the linkages and function of the peripheral αGal residues on Skp1, we have investigated the enzymes responsible for their attachment. An enzyme activity that adds one of the outer αGals has been partially purified and characterized to show that it is a UDP-Gal-dependent enzyme that catalyzes formation of a Galα1,3Fuc linkage by a mechanism that depends on proper presentation of the underlying Skp1 trisaccharide (9). Inspection of the *Dictyostelium* genome sequence (10) iden-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) DQ340632

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[3] The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; E2, ubiquitin carrier protein; Bn, benzyl-; Fuc, L-fucose; Gal, D-galactose; GT, glycosyltransferase; pNP, *para*-nitrophenyl; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MS, mass spectrometry; nt, nucleotide.

[4] C. M. West, H. van der Wel, and Z. Wang, unpublished data.

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tified two predicted but uncharacterized soluble GTs (11) expected to retain the anomeric linkage of the sugar nucleotide donor in the resulting glycosidic linkage formed. However, they exhibit no obvious sequence similarity to known α3GalTs. Here we describe the further purification of the activity as a single protein GT78. Molecular characterization shows that GT78 is one of the unidentified GT-like sequences from the genomic analysis and is required for modification of Skp1 with α3Gal *in vivo*. GT78 is a novel two-domain protein with an N-terminal catalytic domain and a C-terminal domain, not required for activity, that is predicted to form a β-propeller-like protein-protein interaction domain. The findings predict that a large number of unassigned sequences in the genomes of plants, protists, and animals with sequence similarity to the this catalytic domain will have retaining GT activity.

EXPERIMENTAL PROCEDURES

Purification of α3GalT1—Three 32-l batches of strain Ax3 were grown, lysed, and centrifuged. The soluble fraction from each was purified over a DEAE-column as described (9), and enzyme-active fractions were stored at −80 °C. Active fractions were subsequently pooled for purification over phenyl- and Q-columns as described (9), except that the latter was a 60-ml Q-Sepharose high performance column (Pharmacia).

Active fractions (3 × ~80 ml) from the Q-Sepharose column were loaded onto a 2-ml UDP-hexanolamine-Sepharose column (~20 μmol/ml gel, kindly provided by Dr. G. W. Hart) pre-equilibrated at 22 °C with 50 mM HEPES-NaOH (pH 7.5), 10 mM NaCl, 2 mM $MnCl_2$, 0.01% Tween 20 (v/v), and 2 mM dithiothreitol. After return of the A_{280} value to baseline level, the column was eluted with a 10-ml gradient from 10 mM NaCl to 500 mM NaCl, 2 mM UDP in the same buffer on a Pharmacia SmartSystem HPLC. A commercial preparation of UDP-hexanolamine with a ~10-fold lower substitution density did not efficiently retain activity (data not shown). Fractions with α3GalT activity were concentrated from 2 ml to 45 μl in a Microcon-30 centrifugal ultrafiltration cartridge (Amicon). The sample was applied to a Superdex200 3.2/30 column pre-equilibrated at 22 °C with 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 15% glycerol, 0.01% Tween 20, and 1 mM dithiothreitol and eluted isocratically in 50-μl fractions using the SmartSystem HPLC. Aliquots from the Superdex200 fractionation were analyzed on a 7–20% polyacrylamide SDS-gel and silver-stained as described previously (6).

α3GalT Assay—α1,3GalT activity was assayed as described previously (9) in the presence of 2.5 μM UDP-Gal and 5 mM Fucα1-pNP (Sigma) in 40 μl. UDP-Gal was a 1:1 mixture of UDP-[6-^{3}H]Gal (20 Ci/mmol, American Radiochemical Corporation) and unlabeled UDP-αGal (Sigma). After a 0–2-h incubation at 22 °C, reactions were diluted with 1 ml of ice-cold 10 mM Na-EDTA (pH 8.0) and applied to a C_{18}-SepPak solid extraction device (Waters). After washing with water, MeOH-eluted material was subjected to liquid scintillation counting in the presence of BioSafe-II (Fisher). To determine the linkage formed, chromatographic elution on a C_{18} reversed phase column of the Sep-Pak-purified product of a reaction with Fucα1-Bn was compared with that of the six anomeric and positional isoforms of synthetic Gal-Fucα1-Bn (12), as described (9).

Protein Assay—Protein was assayed based on Coomassie Blue dye binding using a commercial kit (Bio-Rad).

Sequence Determination of GT78—Superdex fractions 30 and 31 were diluted with LDS sample buffer (Invitrogen catalog number NP007) with reducing agent (Invitrogen catalog number NP004), heated to 70 °C for 10 min, and run on a 10% BisTris precast gel (Invitrogen, NP0301 BOX). The M_r standards were the Bio-Rad broad range marker (shown in Fig. 1*C*). The Coomassie Blue-stained gel band corresponding to GT78 was subjected to in-gel digestion with trypsin, and the released peptides were captured and desalted on a C_{18} microcartridge and subjected to nano-liquid chromatography-MS and data-dependent MS/MS analysis on a Q-TOF geometry (Q-STAR) instrument as described (8, 13, 14). A selection of nine MS/MS spectra produced were manually *de novo* sequenced and six high confidence sequences used for the data base search.

Cloning GT78 gDNA—GT78 coding DNA was amplified in a PCR using CsCl-purified gDNA from strain Ax3 (6) and primers agtc, 5′-GG*GCTAGC*GAAAAAAAAGTTGAATATATTAAAGAAAATG-ATAAG, and agth, 5′-TT*GGATCC*TTATAAATCCCATGATTT-GATTTGTTTATCG (Invitrogen). See Figs. 2 and 3 for primer locations. 5′-end NheI and BamHI restriction sites for subcloning are italicized. The 50-μl reaction volume, containing 20 pmol of each primer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 5 units of *Taq* polymerase (Eppendorf), 0.5 units of *Pfu* polymerase (Stratagene), and 50 ng of gDNA, was reacted for 34 cycles (94 °C, 45 s; 53 °C, 45 s; 68 °C, 6 min). The desired product was purified on a 0.8% (w/v) SeaKem GTG-agarose gel (6), cloned into pCR4TOPO (Invitrogen) yielding pTOPOagtA, and sequenced on a ABI3730 capillary sequencer at the OMRF DNA Sequencing Facility using primers M13(F), M13(R), agtd, and agti. The sequence matched exactly that of chromosome 4:146830-144665 (ID DDB0185318).

Cloning GT78 cDNA—First copy cDNA was synthesized from total RNA of *D. discoideum* strain Ax3 developed for 13 h, using Moloney murine leukemia virus reverse transcriptase (New England Biolabs) and primer agth (15). The product was then amplified using agtc and agth as described above. The resulting cDNA was cloned into pCR4TOPO, yielding pTOPOcagtA. Sequencing revealed two random point mutations, A1401G and T1851C; the former was corrected by site-directed mutagenesis (as in Ref. 7), and the latter, which does not affect the amino acid sequence, was retained.

Homologous Expression and Purification of His_6-GT78—pVS(His)spy was a gift from A. M. Garcia and consists of the *Dictyostelium* expression vector pVS4 (16) whose KpnI-BamH1 fragment was deleted and replaced by a DNA fragment consisting of a KpnI site, 15 nt of A/T-rich DNA, start codon, His_6-tag, TEV-protease cleavage site, NheI cloning site containing *spyA* sequence, and a BamH1 site.[5] pTOPOcagtA was digested with NheI and BamHI and the insert was gel-purified and ligated into pVS(His)spy similarly digested with NheI and BamH1. This plasmid, pVS(His)agtA, creates the following nt sequence surrounding the start codon: *ggtacc*aaataaaataaaaaaatgggcagcagccatcatcatcatcatca-cagcagcggcagagaaaacttgtatttccagggccatat*ggctagc*GAAAAAAAAGTT-GAA (uppercase refers to GT78 DNA, italics refer to restriction sites, underline refers to new start codon) and is expected to encode His_6/TEV site-tagged GT78 with the N-terminal amino acid sequence: *MGSSHHHHHHSSGRENLYFQGHMAS*EKKVE (italics refer to new sequence which replaces the original start Met). The C terminus of this GT78 isoform is native. For expression of the N-terminal half of GT78, cDNA was amplified from pTOPOcagtA using primers agtc and agti, 5′-GG*GGATCC*TTATTCTTTATTATTTTCATCATCATTATTAA-TATTGC, cloning into pCR4TOPO as above, recovered by digestion with NheI and BamHI, and ligated into similarly digested pVS(His)spy to yield pVS(His)NagtA. pVS(His)agtA and pVS(His)NagtA were used to transform strain Ax3 as described (6), and transformants were selected in the presence of 10 μg/ml G418. G418-resistant cells were passaged axenically in the presence of 10 or 100 μg/ml G418 and subsequently cloned by growth on agar plates with *Klebsiella aerogenes*. Extracts from individual clones were assayed for αGalT enzyme activity.

[5] A. G. Garcia, and C. M. West, unpublished data.

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Recombinant His_6-GT78 (full-length) was purified over DEAE- and phenyl-columns as described for native GT78 (9) based on SDS-PAGE and Western blot analysis for the protein. Nitrocellulose blots were blocked in 3% (w/v) bovine serum albumin in phosphate-buffered saline, probed with a 1:2000 dilution of anti-His antibody (Novagen) in the same solution followed, after washing, by a 1:10,000 dilution of Alexa Fluor 680-conjugated goat anti-mouse IgG (Molecular Probes) in 5% (w/v) nonfat dry milk in Tris-buffered saline, and imaged as described previously (6). Active fractions were pooled and purified on a 1-ml HisTrap HP column (Amersham Biosciences) as described previously (6), and fractions eluting in 0.5 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9), and 5 mM dithiothreitol were analyzed after dilution.

Disruption of the GT78 Locus—A 1024-bp 5′-fragment of the GT78 locus (see Figs. 2 and 3) was PCR-amplified from gDNA using primer agtb, 5′-GG*TCGCGA*TAATGATGCCAACACAACTTAG, and agtj, 5′-GG*AAGCTT*AATGAAATAGAAACCAGCACAAATATCGT and cloned into pPCR4TOPO as described above to yield pTOPO(5′-agtA). A blasticidin S resistance marker, floxed-*bsr* (*fbsr*), was released from pBluescriptKS using Hind III and NotI (17) and ligated into pTOPO(5′-agtA) predigested with HindIII and NotI to create pTOPO(5′-agtA)fbsr. A 1064-bp 3′-fragment of GT78 was generated by PCR using agte, 5′-GG*GATCC*GGAAATCAAGGTGGATGGATTAGAA, and agtg, 5′-GG*GTTAAC*TGGAAAAGTTTTAATGAGAGTAAGTCT (see Figs. 2 and 3) and cloned into pCRTOPO. This was digested with BamHI and NotI, and the released 3′-GT78 insert was subcloned into pTOPO(5′agtA)fbsr digested with BamHI and NotI, yielding pTOPO(5′agtA)fbsr(3′agtA). The sequence of each PCR-generated fragment was verified as described above. The disruption DNA was released with NruI and HpaI and used to transform strain Ax3 cells as above. Blasticidin S-resistant clones were examined by PCR to screen for the expected double cross-over event that would result in replacement of 1197 nt of GT78 coding DNA with 1.5 kb of *fbsr* DNA, as described under "Results." For template DNA, 10^6 logarithmically growing cells were recovered from HL-5 growth medium by centrifugation in a 1.5-ml microcentrifuge tube at 16,000 × *g* for 15 s and washed twice by resuspension in cold H_2O and recentrifugation. The cell pellet was resuspended in 100 μl of 0.1% Nonidet P-40 in 1× *Taq* polymerase buffer (containing 1.5 mM $MgCl_2$) supplemented with 5.26 μl of 5 mg/ml proteinase K, freshly diluted in 1× *Taq* buffer from a 50 mg/ml stock solution stored at −20 °C, and incubated at 56 °C for 45 min and 100 °C for 10 min (to inactivate proteinase K). 6 μl was used immediately (or after as many as three freeze-thaw cycles) as a template per 50-μl PCR. The bsre primer used to test for the presence of *fbsr* DNA was 5′-AAGATAAAGCTGACCCGAAAGCTC.

RESULTS

Purification of α3GalT Protein—The Skp1 αGalT was previously purified 2400-fold over DEAE (anion exchange), phenyl (hydrophobic), Q (anion exchange), and Superdex (gel filtration) columns at 36% yield (9). The purified preparation modified agalactosyl Skp1 and catalyzed formation of a Galα1,3Fuc linkage on Fucα1-Bn and is referred to as αGalT1. To identify the responsible protein, new material was purified 330-fold (*versus* 570-fold previously) through the Q-column step at 7% yield (*versus* 32% previously), which separates the activity from Skp1. Activity eluted at each step as a single peak based on incorporation of ^3H from UDP-[^3H]Gal into Fucα1-pNP (data not shown) essentially as described previously (9). Most activity was adsorbed to a UDP-hexanolamine column, and eluted as a single peak with slight trailing in a gradient of UDP and NaCl (Fig. 1*A*). UDP-hexanolamine may retain the activity by binding the enzyme at its UDP-Gal substrate binding site as for other UDP-sugar-dependent GTs (18). For the final column purification step, the UDP-hexanolamine activity pool was concentrated by centrifugal ultrafiltration and subjected to size-exclusion chromatography. Enzyme activity eluted as a symmetric peak in a pattern that superimposed over the total protein profile based on absorbance at 280 nm (Fig. 1*B*). Activity was purified ~16,000-fold from the original cell extract. An analysis of fractions by SDS-PAGE revealed a predominant band migrating at a position corresponding to an M_r of 78,000 (Fig. 1*C*),

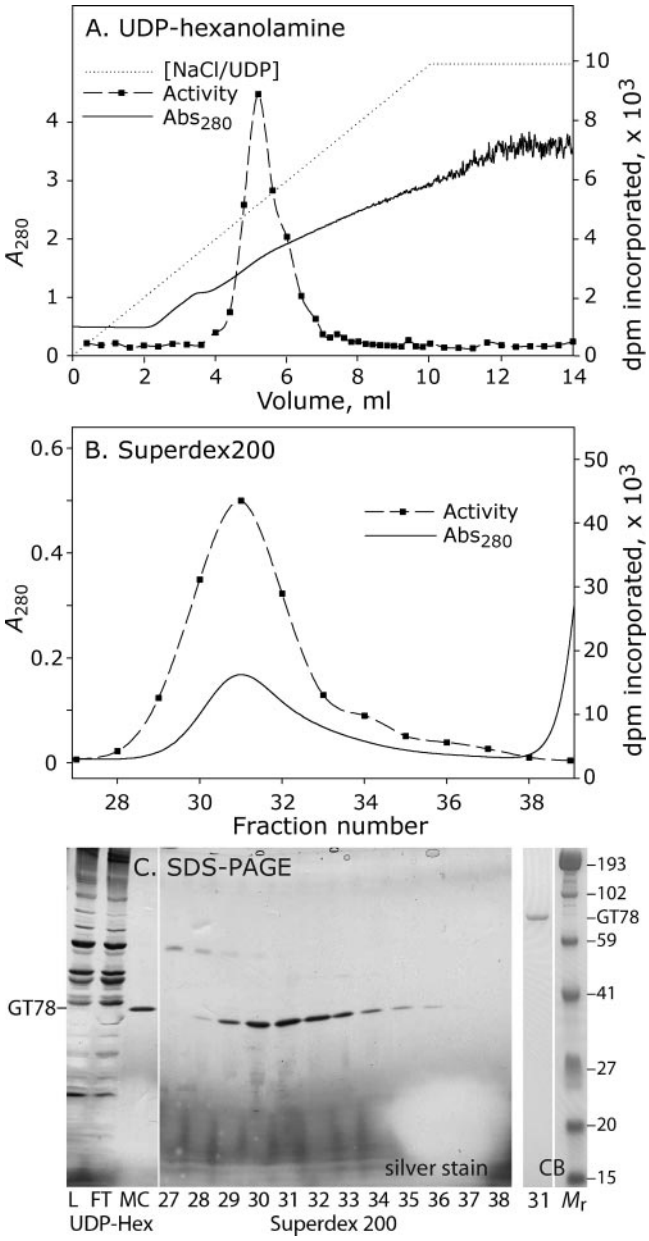


FIGURE 1. **Purification of the α3GalT activity.** The activity was partially purified from a soluble S100 extract of *Dictyostelium* cells over DEAE-, phenyl-, and Q-columns. *A*, active fractions from the Q-Sepharose column were applied directly to a UDP-hexanolamine-Sepharose column and eluted with an ascending gradient of NaCl (10–500 mM) and UDP (0–2 mM) as shown by the *dotted line*. A_{280}, contributed by protein and UDP, is shown by the *solid line*. 1 μl from each fraction was assayed for activity in the presence of 2.5 μM UDP-[^3H]Gal and 5 mM Fucα1-pNP for 2 h. *B*, active fractions from *A* were concentrated on a Microcon-30 ultrafiltration cartridge and chromatographed on a Superdex200 column. A_{280} is shown by the *solid line*. 1 μl of each 200-μl fraction was assayed for activity. *C*, SDS-PAGE analysis of fractions from the UDP-Hexanolamine and Superdex columns. *Left panel* (silver-stained): *L*, 40 μl of 80 ml of total load volume applied to the UDP-Hexanolamine column; *FT*, flow through (44 μl of 85 ml) from the UDP-Hexanolamine column; *MC*, SDS-rinse from Microcon-30 membrane (1 μl of 44 μl); *27–39*, fractions (2 μl of 50 μl) from the Superdex200 eluate shown in *B*. *Right panel* (Coomassie Blue-stained): *31*, fraction 31 (2.5 μl) from the Superdex200 eluate; M_r, marker from the same gel.

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estimated based on comparison to known M_r standards (*right panel*). This protein, referred to as GT78, also coeluted with activity. Comparison with the sample applied to the UDP-hexanolamine column shows that GT78 constituted a minor component before affinity chromatography, consistent with the high degree of purification indicated by the enzyme activity measurements. These results suggest that the GT78 polypeptide functions alone as αGalT1.

Sequencing GT78—The GT78 band from fractions 30 and 31 run on a SDS gel and stained with Coomassie Blue was excised and subjected to trypsinization. Peptides were recovered and characterized on a Q-Star mass spectrometer to obtain amino acid sequence data. The following high confidence peptide sequences were obtained: YSENAE, NNDYGFEN, (L/I)FTTS(L/I)DK, FWD(L/I)NSGR, PNGTNY, NTSGND.

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                gaaataaattattttaaaaagtggtttctcgataaaatattattattg     -661 agta
atatattaaatgaaaaaaaaaaataaaaaaaaaataaaaaaaaaaaaaaaaaaaataaaaa     -601
aaaaattaaaattaaaattaaaattaaaaataaaaatttaattaaaataaaaattaaaat      -541
aaaattaaaagaattgttttttttttataaggctttttttacctgaaaaaacgataat       -481 agtb
gatgccaacacaacttagctacatgataaaagatttttttaataatgttttttaacaaa      -421
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aatacaatttttttttttttttttttttttttttgataaaaatttttattttagaatt        -61
taattgtttatcatttttttgtaattgttttttttttttttttttatacccaaaaaac        -1

M  E  K  K  V  E  Y  I  K  E  N  D  K  I  V  L  M  C  N  Y        20
ATGGAAAAAAAAGTTGAATATATTAAAGAAAATGATAAGATTGTATTAATGTGTAATTAT     60 agtc

G  F  R  D  M  T  L  N  L  L  K  C  F  E  K  L  S  I  D  K        40
GGATTTAGAGATATGACATTGAATTTATTGAAATGTTTTGAAAAGTTATCAATTGATAAA     120

S  R  Y  I  L  Y  C  M  D  D  K  A  Y  Q  F  F  A  E  F  K        60
AGTAGATACATTTTATATTGTATGGATGATAAAGCATATCAATTCTTTGCAGAGTTTAAA     180

G  I  E  C  Q  R  F  S  R  D  D  I  I  N  S  S  T  S  S  T        80
GGTATTGAGTGTCAAAGATTCTCAAGAGATGATATTATAAATTCTTCAACATCATCAACA     240

Q  L  F  H  D  N  N  T  N  D  N  K  G  I  Y  S  E  N  A  E       100
CAACTTTTCATGATAATAATACAAACGATAATAAAGGAATTTATAGTGAAAATGCCGAG      300

S  Y  G  D  I  G  F  R  A  I  C  N  E  K  P  L  V  V  L  D       120
TCATATGGTGATATTGGATTTAGAGCGATTTGTAATGAAAAACCATTAGTAGTATTGGAT     360

V  L  K  K  G  Y  N  V  L  W  T  D  T  D  I  V  W  K  R  D       140
GTTTTAAAAAAAGGTTATAATGTATTATGGACTGATACTGATATAGTTTGGAAAAGAGAT     420

P  F  I  H  F  Y  Q  D  I  N  Q  E  N  Q  F  T  N  D  D  D       160
CCATTCATTCATTTCTATCAAGATATTAATCAAGAAAATCAATTCACTAACGATGACGAC     480

I  D  L  Y  V  Q  Q  D  D  D  D  I  C  A  G  F  Y  F  I  R       180
ATTGATTTATACGTTCAACAAGATGATGACGATATTTGTGCTGGTTTCTATTTCATTCGT     540 agtj

S  N  Q  R  T  I  K  F  I  Q  D  S  I  N  F  L  N  P  C  I       200
TCAAATCAAAGAACAATTAAATTTATTCAAGATTCAATCAATTTCTTAAACCCATGTATT     600

D  D  Q  I  A  M  R  L  F  L  K  S  Q  G  I  N  I  K  S  K       220
GATGATCAAATTGCAATGAGATTATTTTTAAAATCTCAAGGTATTAATATTAAATCAAAA     660

N  I  L  L  S  L  S                                              227
AATATTTTATTATCATTATCTgtaagttttattttttcattatttaattaatttattatt     720 PacI

taattaattaatttattattattattattattattattattattattattattattatta     780 PacI

                        E  N  D  K  K  D  K  I  R  Y  R  L        239
ttattattattattattatagGAAAATGATAAAAAAGATAAAATTAGATATAGATTA        840

L  D  K  K  L  F  P  N  G  T  N  Y  F  N  L  K  I  T  Q  R       259
TTAGATAAAAAGTTATTTCCAAATGGTACAAATTATTTTAATTTAAAAATTACTCAAAGA     900

D  N  I  T  P  F  I  I  H  N  N  C  I  I  G  H  R  S  K  K       279
GATAATATTACACCATTCATTATTCATAATAATTGTATAATTGGTCATAGAAGTAAAAAA     960 agtd

D  R  F  I  E  Y  G  L  W  Y  I  N  D  D  E  I  D  I  N  S       299
GATAGATTCATTGAATATGGATTATGGTATATTAATGATGATGAAATTGATATTAATAGC     1020

N  I  N  N  D  D  E  N  N  K  E  I  K  L  F  K  N  V  L  K       319 WD40-1
AATATTAATAATGATGATGAAAATAATAAAGAAATAAAATTATTTAAAAATGTGTTGAAA     1080 agti
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FIGURE 2. **Predicted nucleotide and protein sequence of GT78.** The six peptide sequences obtained from the GT78 gel band shown in Fig. 1C mapped to a single predicted gene (DDB0185318 at the *Dictyostelium* database). The nt sequence associated with this locus was used to create oligonucleotides (*underlined* nt), which were used in PCR experiments to confirm the gDNA sequence of GT78 in strain Ax3 and in reverse transcription and PCR of total cellular RNA to determine intron/exon boundaries. The sequenced peptides are *bold underlined*, and additional peptide sequences that could be identified retrospectively from the MS data are *underlined*. Coding nt are in *uppercase*; key restriction endonuclease sites are in *italics*; predicted polyadenylation nt sequences are in *bold* or *underlined*; and the stop codon of the downstream reverse-oriented gene is in *bold italic*. *Italicized* amino acid sequences represent WD40-like motifs, with the beginning of each in *bold*. This sequence has been deposited at GenBank™ with accession number DQ340632.

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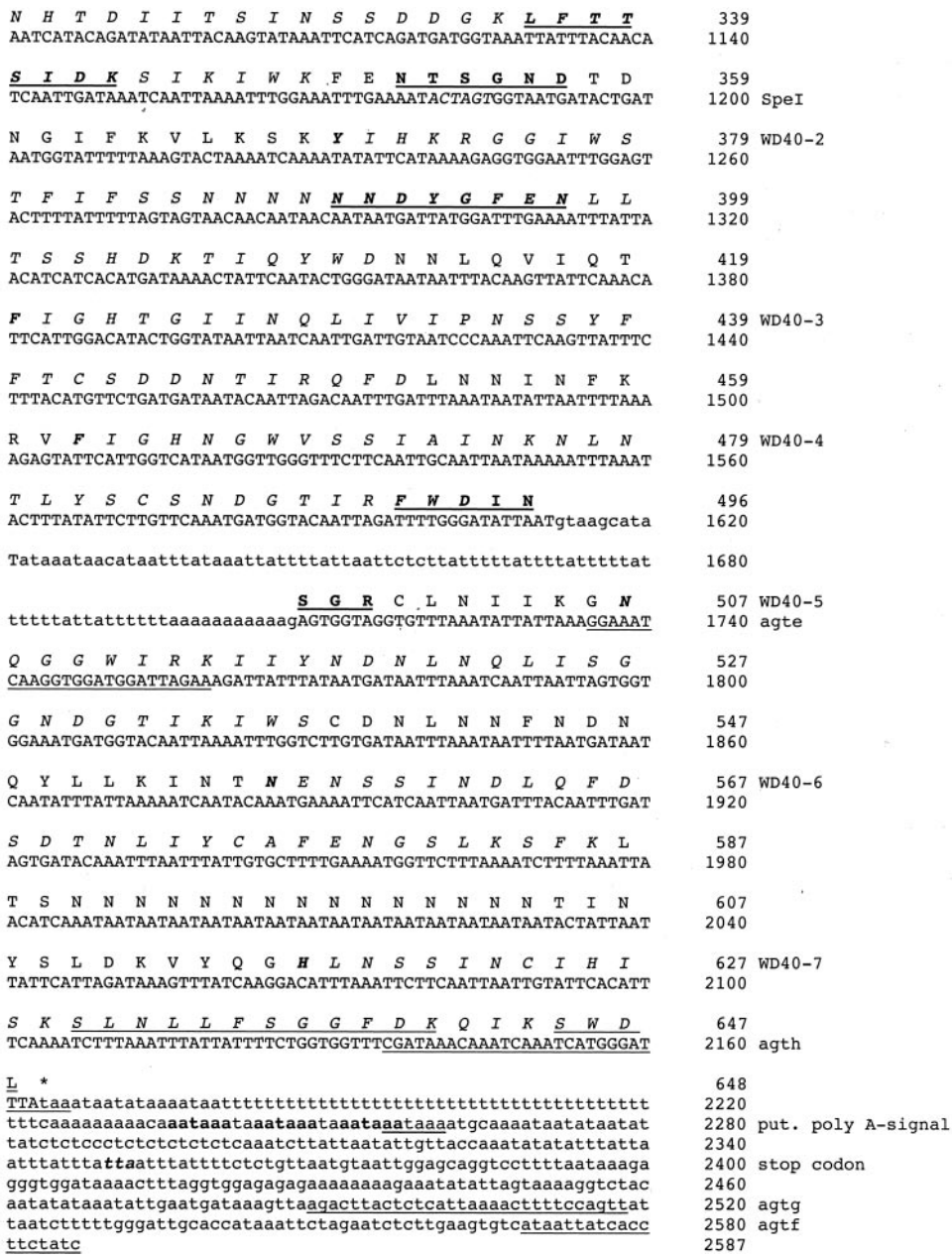


FIGURE 2—*continued*

A search of predicted *Dictyostelium* protein sequences at the *Dictyostelium* database showed that all six peptides were present in a predicted gene model, DDB0185318, on chromosome 4 at nt 144665–146830. The nt sequence of DDB0185318 and flanking DNA was confirmed by a PCR method (see below) and is shown with the predicted protein sequence in Fig. 2. Positions of the sequenced peptides are underlined in bold. Three additional ions detected in the MS analysis match tryptic peptides from the sequence and are underlined. DDB0185318 encodes a predicted protein of 648 amino acids and M_r 75,245 in approximate agreement with the apparent 78,000 M_r of GT78 and with the elution of the α3GalT1 activity just prior to bovine serum albumin (M_r 66,000) in size-exclusion chromatography (9). The gene consists of 3 predicted exons with introns of 123 and 96 nt (see Fig. 3*A*), and the second intron is confirmed by the peptide FWD(L/I)NSGR, which spans the exon 2/3 junction. Both introns were confirmed by RT-PCR using primers agth and agtc, which yielded a DNA sequence lacking the predicted intronic DNA. The predicted C terminus of the protein is confirmed by the peptide SWDL. Amino acids 5–9 of the predicted N terminus were confirmed by the peptide VEYIK. The predicted N-terminal peptide, EKK after Met cleavage, would not have been detected by the MS method because of its small size and hydrophilicity. However, the putative start codon is likely correct, because the 1.3 kb of predicted intergenic DNA extending upstream to the reverse-oriented predicted upstream gene is relatively AT-rich with homopolymeric stretches of A and T, characteristic of intergenic and intronic DNA. Furthermore, there are no candidate exon sequences that have an ATG start codon and are terminated by a gt intron start sequence, and not by a stop codon, which can be spliced in-frame to the proposed exon 1 upstream of VEYIK. Finally, the predicted start codon is preceded by a favorable A residue at position −3 as for most other *Dictyostelium* genes.

Protein Sequence Analysis—BLAST analysis of the GT78 sequence against publicly available databases reveals that the C-terminal 330

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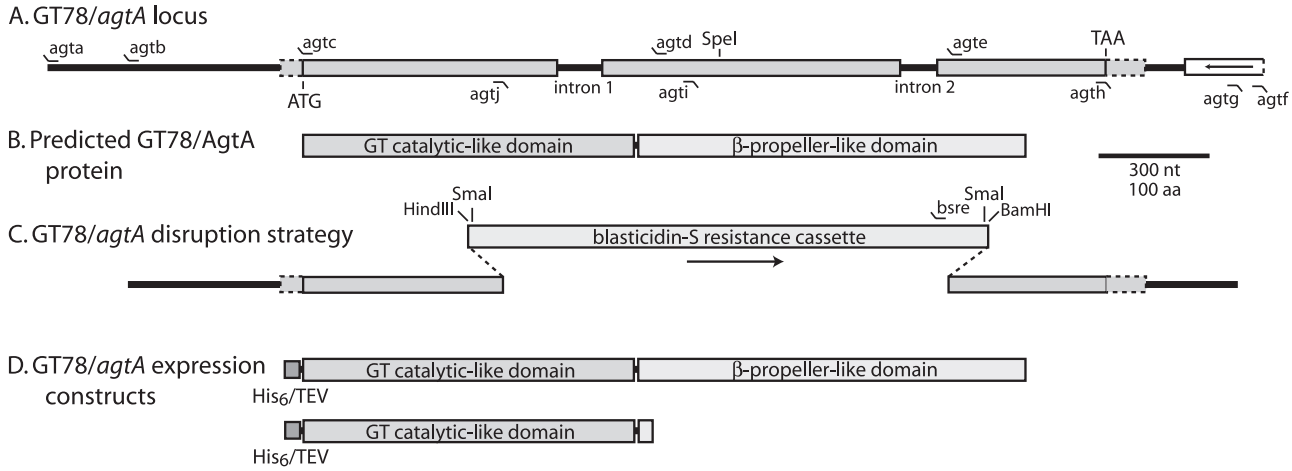
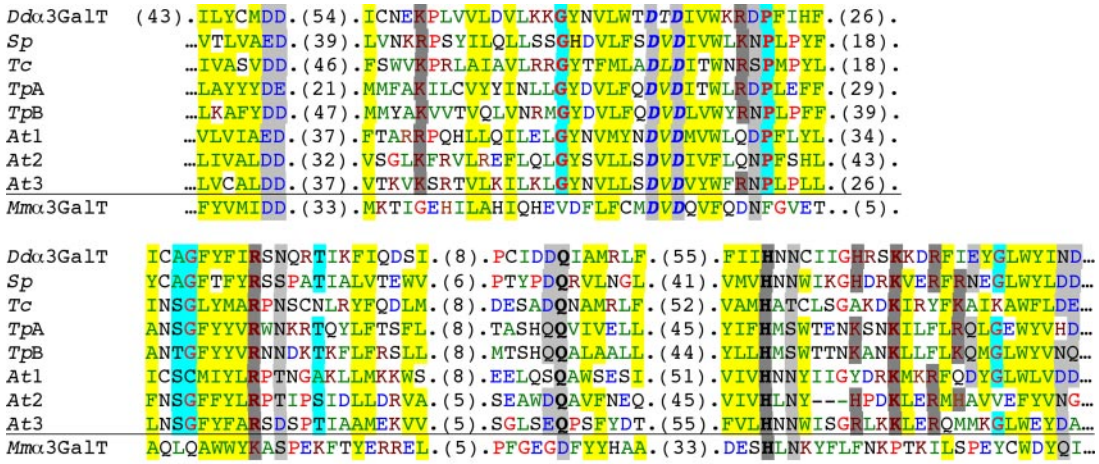


FIGURE 3. **Domain diagram of the GT78 locus.** *A*, exon structure of DDB0185318, also referred to as GT78/*agtA*, as confirmed in Fig. 2. *B*, predicted protein domains. *C*, construct for disruption of the GT78 locus. *D*, constructs for expression of His_6-tagged full-length GT78 and the N-terminal region of GT78. Positions of primers and key restriction sites are noted.



Dd, *Dictyostelium discoideum*; gi:66811205; soluble protein
Sp, *Strongylocentrotus purpuratus*; gi:72008043; predicted soluble protein
Tc, *Trypansoma cruzi*; Tc00.1047053508461.84; at http://www.genedb.org/genedb/tcruzi/; predicted type 2 membrane protein
Tp, *Thalassiosira pseudonana*; at http://genome.jgi-psf.org; predicted type 2 membrane protein
*At*1, *Arabidopsis thaliana*; gi:49617789; predicted type 2 membrane protein
*At*2; gi:18410771; predicted type 2 membrane protein
*At*3; gi:42563115; predicted soluble protein
Mm, Mus musculus; gi:15419874; type 2 membrane protein

FIGURE 4. **Alignment of GT78 with related sequences from other organisms.** Sequences related to the N-terminal half of GT78 were retrieved from genomic DNA databases using BLAST. Amino acid names are colored according to shared characteristics, and similarities between sequences (above the line) are highlighted in *bold* when identical across the group or *shaded* when shared according to amino acid type, as in Ref. 5. The putative D*X*D motif is in italics. The most closely related sequences are above the line, and the sequence of murine α3GalT is below. Origin of the sequences are listed below, and whether the encoded protein is predicted to be soluble (cytoplasmic) or a type 2 transmembrane protein (Golgi) is indicated.

amino acids of the protein contains 7 WD-40-like motifs (italicized amino acids in Fig. 2) predicted to fold as a β-propeller domain. This model was supported by a sequence-based algorithm (19). β-Propeller domains are found in a variety of proteins and generally mediate protein-protein interactions (19). The sequence of the N-terminal 290 amino acids does not match known glycosyltransferase catalytic domains at an Expect value of below E = 10. However, it does align with a number of sequences of unknown function from plants, other protozoans and an animal with Expect values in the range of 10^{-18} to 10^{-10}. One of these, *At*1 (Q9ZSJ2) from *Arabidopsis thaliana*, was predicted to encode a Golgi GT possibly involved in pectin biosynthesis in a predicted structure-based bioinformatics search for GTs (20). Clusters of similarity, including a conserved D*X*D motif embedded between hydrophobic amino acid clusters that is typical of superfamily A GTs, are distributed throughout the N-terminal region, as shown in Fig. 4. An alignment with a murine Golgi α3GalT shows that these sequences are more closely related to one another than to a known α3GalT. The N-terminal half of GT78 is therefore a candidate for encoding the predicted α3GalT activity of GT78. The sequence of GT78 lacks classical targeting motifs for compartmentalization in the rER or nucleus, consistent with its presence in the cytosolic extract of the cell (9).

Recombinant Expression of GT78—Initial attempts to express undegraded GT78 in *Escherichia coli* to confirm its α3GalT enzymatic activity were unsuccessful (data not shown). To ensure likelihood of successful expression, GT78 was overexpressed in *Dictyostelium*. The full-length coding region was ligated into a modified pVS expression vector, which attaches a His_6-tag followed by a TEV protease cleavage site at the N terminus and directs expression under control of the strong, semiconstitutive discoidin-1γ promoter. pVS lacks autonomous replication sequences and typically integrates in tandem arrays (21), and

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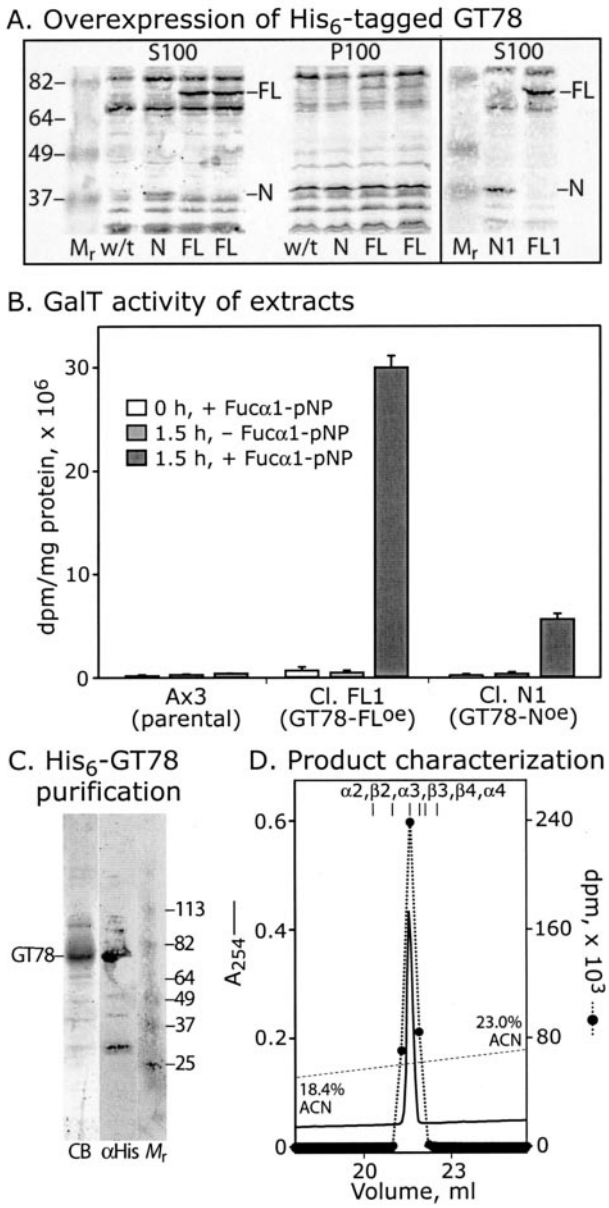


FIGURE 5. **Overexpression of GT78 protein.** Full-length GT78 (*FL*) or its N-terminal region (*N*), as described in Fig. 3, was overexpressed in Ax3 (wild-type (*w/t*) or normal) cells. *A*, Western blot analysis of cytosolic (*S100*) and total particulate (*P100*) cell fractions (100 μg of protein) from stationary phase cultures. Blots were probed with anti-His_6 antibody. *Left*, primary cultures; *right*, clones N1 and FL1. *B*, S100 fractions (140 μg of protein) from Ax3 and clones FL1 and N1 were assayed for GalT activity in the presence of 2.5 μM UDP-[^{3}H]Gal and ±5 mM Fucα1-pNP for 0 or 1.5 h as indicated. *Error bars* refer to S.E. for results from two independent cell extracts. *C*, His_6-GT78 was purified from an S100 preparation of clone FL1 over DEAE-, phenyl- and Ni^{+2}-columns, based on Western blot detection using anti-His antibody. A peak fraction from the Ni^{+2}-column was subjected to SDS-PAGE and either stained with Coomassie blue (*CB*) or Western blotted and probed with anti-His antibody (*αHis*). M_r = protein size standards from the same gel. *D*, the GT78 preparation shown in *C* was assayed for GalT activity toward Fucα1-Bn in the presence of UDP-[^{3}H]Gal, and the reaction product was chromatographed together with synthetic Galα1,3Fucα1-Bn on a C_{18} reversed phase column. Elution of the standard was monitored by its absorbance at 254 nm, and radioactivity was determined by liquid scintillation counting of fractions. Elution positions of synthetic isomers are indicated in order of the linkage types listed.

cells with higher copy number are enriched at higher (≥100 μg/ml) concentrations of G418. Cells from non-clonal cultures selected at 100 μg/ml were initially fractionated into particulate and soluble fractions. Western blot analysis with an anti-His_6-tag Ab revealed in the S100 fraction a novel band with an apparent M_r of 76,000 (Fig. 5*A*) corresponding to the expected position of full-length His_6-GT78 (M_r 77,844). Only a faint band was detected in the P100 fraction suggesting that the majority of GT78 was soluble as expected for α3GalT1 (9). After cell cloning, clone FL1 was selected for further analysis based on high expression of this band. Using the assay based on the acceptor substrate Fucα1-pNP, the S100 extract from clone FL1 exhibited 117-fold higher GalT specific activity than the parental strain Ax3 (Fig. 5*B*). The increased activity appeared attributable to expressed GT78 because His_6-GT78 and GalT activity copurified over DEAE-, phenyl-columns, and Ni^{+2}-columns (data not shown). SDS-PAGE and Western blot analysis for the His_6-tag showed the preparation to be highly enriched in GT78 (Fig. 5*C*). This preparation was highly active (data not shown) and confirmed to be an α3GalT based on formation of a radiolabeled reaction product from Fucα1-Bn, which coelutes on a C_{18}-reversed phase column with synthetic Galα1,3Fucα1-Bn (Fig. 5*D*). This method was previously shown to separate Galα1,2Fucα1-Bn, Galα1,3Fucα1-Bn, Galα1,4Fucα1-Bn, Galβ1,2Fucα1-Bn, Galβ1,3Fucα1-Bn, and Galβ1,4Fucα1-Bn (9, 12).

To test whether the N-terminal region of GT78, which exhibits remote sequence similarity to a predicted GT (see above), is catalytically active, amino acids 2–310 were expressed with an N-terminal His_6-tag as for the full-length protein. An anti-His_6-reactive band accumulated in the S100 fractions at a position corresponding to M_r 38,000, comparable to its predicted M_r of 39,482 (Fig. 5*A*). An S100 extract of clone N1 exhibited GalT activity toward Fucα1-pNP that was 23-fold greater than that of parental cells (Fig. 5*B*). The lower activity relative to the full-length protein may be because of lower level of expression based on comparative anti-His_6-tag labeling (Fig. 5*A*), although the C-terminal domain may also be required for optimal activity.

Disruption of the GT78 Locus—To confirm that GT78 is required for endogenous α3GalT activity and for α3-galactosylation of Skp1, the GT78 locus was targeted by a double crossover gene replacement event intended to exchange half of the catalytic domain and most of the β-propeller-like domain with a blasticidin S-resistance cassette (*fbsr*) as depicted in Fig. 3*C*. The desired exchange was tested by PCR using GT78-associated primers (agta and agtf in Fig. 3*A*), which hybridize outside of the disruption DNA. As shown in Fig. 6*A*, these primers amplified a 3.3-kb DNA fragment from strain Ax3 (not shown) and clone 2 (containing a randomly integrated copy of *fbsr*), as expected for wild-type DNA (3295 nt), and a 3.6-kb band from clone 1, as expected for the modified locus (3556 nt). Ax3 and clone 2 DNA were cut once by SmaI but not SpeI, as expected (see Fig. 3*A*), whereas clone 1 DNA was cut twice by SmaI but not SpeI, as expected (see Fig. 3*C*). Because these long PCR products did not yield strong bands, strains Ax3 and clone 1 were examined further using primers specific for either the deleted (agtd) or replacement DNA (bsre), and the downstream primer agtf. As expected, a 1.6-kb fragment was amplified using agtd and agtf from Ax3 but not clone 1 DNA, whereas a 0.9-kb band was amplified using bsre and agtf from clone 1 but not Ax3 DNA. Therefore, gene targeting resulted in the desired replacement of the central region of the GT78 gene, including parts of both the catalytic region and the β-propeller-like region, by foreign DNA encoding the drug-resistance marker.

To compare total GalT activity, S100 extracts from clone 1 and Ax3 were assayed using Fucα1-pNP as above. As previously reported (9), the Ax3 S100 exhibited activity that was readily detected over background defined by either absence of acceptor substrate or at zero time (Fig. 6*B*). In comparison, the activity of clone 1 was reduced ~80% on a per protein basis. Residual GalT-like activity may be because of contamination from Golgi vesicles during cell lysis or possibly represent the predicted αGalT activity that adds the second αGal residue to Skp1, potentially also to the Fuc moiety (4). It is unlikely to represent residual activity of the GT78 locus because part of the catalytic domain coding region is

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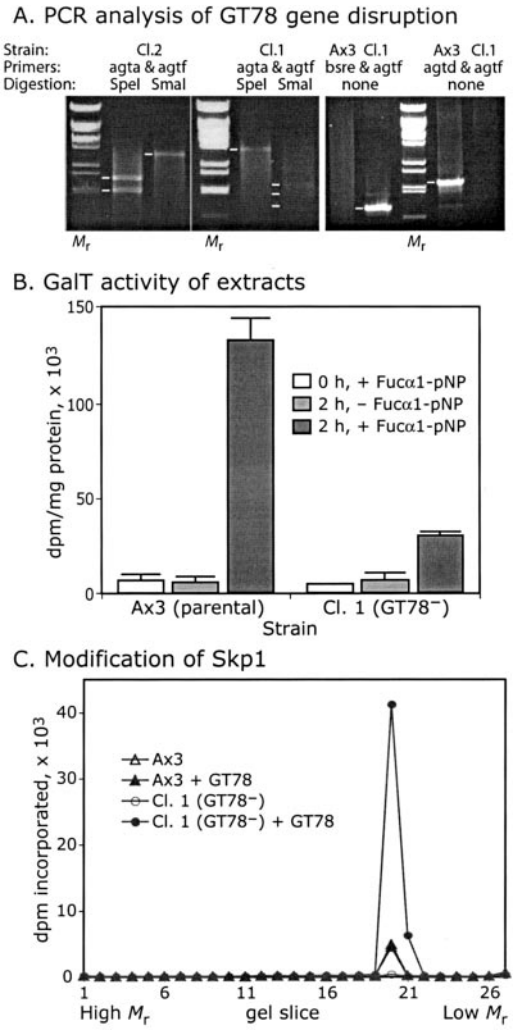


FIGURE 6. **Disruption of the GT78 locus.** The gene disruption DNA shown in Fig. 3 was electroporated into the normal strain Ax3 and blasticidin-resistant clones were isolated. *A*, DNA associated with the GT78 locus was PCR-amplified from extracts of strain Ax3 and two blasticidin-resistant clones using the indicated primers (shown in Fig. 3), resolved on a 1% agarose gel, and visualized with ethidium bromide. In some cases, amplified DNA was digested with SpeI or SmaI. Bands are indicated by *white bars*. *B*, S100 fractions were prepared from Ax3 and clone 1 from *A*, desalted on a Sephadex (PD10) gel filtration cartridge, and analyzed for GalT activity in the presence of 2.5 μM UDP-[^{3}H]Gal and ±5 mM Fucα1-pNP for 0 or 2 h as indicated. *Error bars* show the S.E. from two separate assays. *C*, S100 extracts (200 μg of protein) were incubated with or without 2400-fold purified αGalT1 (from Fig. 1 in Ref. 9) in the presence of 2.5 μM UDP-[^{3}H]Gal for 1 h and then separated by SDS-PAGE. The gel was sliced, and incorporation was measured by scintillation counting. Results are typical of two independent trials.

deleted and is unlikely to be because of the existence of a closely related protein, because BLAST analysis of the genome sequence at the *Dictyostelium* genome sequence database (10) does not reveal sequences related to that of the catalytic domain with an Expect < 0.1, compared with the 10^{-168} value of the sequence to itself. These results support the evidence from the overexpression studies above that GT78 encodes αGalT1 and, in addition, reveal that GT78 is not required for growth. GT78 mutant cells exhibit a normal growth rate in HL-5 medium and form fruiting bodies after starvation (data not shown).

To determine whether the α3Gal modification of Skp1 was affected by the mutation *in vivo*, the level of unmodified Skp1 was measured in a biochemical reconstitution assay. S100 extracts of Ax3 and clone 1 were supplemented with UDP-[^{3}H]Gal and parallel reactions were supplemented with equal aliquots of purified GT78. All four reactions were resolved by SDS-PAGE and individual lanes were cut into 27 slices. Normal strain Ax3 extracts incorporated a modest amount of radioactivity (4500 dpm) into a band at the position of Skp1 and negligible radioactivity into any other proteins, irrespective of the addition of exogenous GT78 (Fig. 6*C*). In contrast, clone 1 incorporated no detectable radioactivity into Skp1, as expected because of disruption of the gene. The addition of partially purified GT78 to the clone 1 extract resulted in a high level of incorporation of radioactivity (47,000 dpm), 10-fold greater than that seen in Ax3, into Skp1. This indicates that an unmodified isoform of Skp1 that serves as a substrate of GT78 accumulates in the absence of GT78 *in vivo*, indicating that Skp1 depends on GT78 for its α3-galactosylation. Furthermore, absence of incorporation into unmodified Skp1, shown by this result to be present in the mutant extract, in the −GT78 trial confirms the interpretation from Fig. 6*B* using Fucα1-pNP that α3GalT activity is absent from GT78$^-$ mutant extracts. Finally, Skp1 is the only substrate of GT78, which accumulates in the absence of the enzyme, that can be detected using this approach.

DISCUSSION

GT78 appears to be the previously described α3GalT that forms the Galα1,3Fuc linkage on Skp1. GT78 catalyzes formation of the linkage after purification from *Dictyostelium* extracts to apparent homogeneity over 5 columns including a UDP affinity step (Fig. 1, *B* and *C*). Therefore the GT78 polypeptide appears to function alone in catalysis. The catalytic role of GT78 is supported by the observation that overexpression of His_6-GT78 in *Dictyostelium* results in a >100-fold increase in specific activity (Fig. 5*B*). The new activity copurifies with His_6-GT78 over ion exchange, hydrophobic interaction, and Ni^{+2}-affinity columns (Fig. 5*C*) and exhibits α3GalT activity based on cochromatography of the reaction product with an authentic standard (Fig. 5*D*). Finally, GT78 is required for the great majority of αGalT1-like activity in extracts using either Fucα1-pNP or endogenous Skp1 as an acceptor, based on analysis of a strain in which the GT78 gene was targeted by gene replacement (Fig. 6, *B* and *C*). Furthermore, GT78 is required for the α3-galactosylation of Skp1, based on accumulation of unmodified Skp1 in the GT78-disrupted strain (Fig. 6*C*). Finally, the sequence of the N-terminal half of GT78 bears remote resemblance to other predicted GTs (20), and homologous overexpression studies show that this domain exhibits α3GalT activity toward Fucα-Bn as for the full-length protein (Fig. 5*B*). Based on these observations, we conclude that GT78 is the Skp1 αGalT1, and the gene locus is named *agtA*. Detection of the other predicted αGalT (αGalT2) can now be approached using the αGalT1-null strain and purified αGalT1 to prepare an acceptor substrate.

GT78/αGalT1 is a soluble protein in a cytosolic extract (Fig. 5*A*), and its sequence confirms that it lacks targeting motifs for accumulation in other compartments such as the secretory pathway, mitochondrion, or nucleus. The earlier enzymes in the Skp1 modification pathway are also soluble cytoplasmic proteins that can function independently *in vitro* to mediate their enzymatic functions (5, 6). Therefore the properties of GT78/αGalT1 support the general model that Skp1 is sequentially modified in stepwise fashion, as occurs for other *O*-glycosylation pathways in the Golgi apparatus (22). However, in this case the enzymes all appear to reside in a single compartment, the cytoplasm, in contrast to sequential distribution along subcompartments of the Golgi. A second difference is that in the Skp1 pathway, two of the GT activities (β3GalT and α2-fucosyltransferase) reside in the same protein, which is able *in vitro* to processively extend the glycan chain (7).

A previous study showed that modification of Skp1 by αGalT1 is

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sensitive to Skp1 folding *in vitro* and *in vivo* based on effects of denaturants and missense mutations (9). In addition, studies of model sugar acceptors suggest that folding of the glycan chain is also important for recognition. Interestingly, the biochemical reconstitution results of Fig. 6*C* suggest that a subpopulation (~10%) of Skp1 is available as an αGalT1 substrate only after cell lysis. Together the results suggested that αGalT1 processing of Skp1 is conditional upon proper glycan presentation that might in turn depend on Skp1 folding or possibly complex formation. Although the biochemical basis for conditionality is not known, the β-propeller-like domain on GT78/αGalT1, not required for catalytic activity *per se*, may play a pivotal role when Skp1 is the substrate. β-Propeller domains mediate protein-protein interactions in other proteins (19) and in one example targets a *Dictyostelium* protein kinase to its target substrate (23). Although in another example a β-propeller domain serves as a fucose lectin (24), it seems more likely that in the case of GT78 the catalytic domain may recognize the Fuc acceptor site and the C-terminal domain may recognize a second region of Skp1 as previously hypothesized (9). This may explain why GT78/αGalT1 copurifies with Skp1 through two column purifications. In addition, Skp1 and GT78/αGalT1 appear to have similar abundance in cells, as each required ~15,000-fold purification to homogeneity (see above and Ref. 25). This raises the possibility that the two proteins interact not only catalytically but also stoichiometrically.

αGalT1-null cells grow axenically with a normal doubling time and develop to form typical fruiting bodies. This contrasts with the culmination-defective phenotype of the first enzyme in the pathway,[4] the prolyl 4-hydroxylase, which is required for attachment of αGlcNAc to Skp1 by GnT1. The role of GnT1 in culmination is not known because attempts to disrupt the *gntA* locus in a wild-type background have been unsuccessful.[4] Disruption of *pgtA*, required for addition of the next sugar to GlcNAc, results in decreased cell size and increased cell density of axenically grown cells (8) and a delay but not blockade of development.[4] Further comparative studies are required to determine whether the αGalT1-null strain exhibits these more subtle phenotypes. These findings show that the early and later steps of the pathway have distinct functions. Biochemical reconstitution studies like those shown in Fig. 6*C* suggest that Skp1 is the only protein in the cell subject to this post-translational modification pathway (8).[4] However, further studies are required to rigorously rule out the existence of distinct target proteins that may mediate the different functions.

Searches of sequence databases using BLAST have identified a phylogenetically diverse cluster of deduced amino acid sequences that are remotely related to and share key amino acids with GT78 (Fig. 4). Some of these are classified together as family GT77 in the CAZy GT data base (26), based on a predicted structural similarity to known GTs (20). The discovery that GT78 is an α3GalT supports this model, although the sequences are so diverged that only retention of the linkage of the postulated UDP-sugar donor can be predicted. Most of the sequences lie in predicted type 2 transmembrane proteins suggesting that they function in the Golgi apparatus, whereas others are predicted to encode cytoplasmic proteins, which, however, lack β-propeller-like domains. Because the latter genomes appear to lack other Skp1 GT-like genes, the GT78-like proteins may glycosylate other cytoplasmic proteins in their organisms.

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Molecular Characterization of a Novel UDP-galactose:Fucoside α 3-Galactosyltransferase That Modifies Skp1 in the Cytoplasm of *Dictyostelium*

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