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

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Skp1 is an adaptor-like protein in E3SCF-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 Mn²⁺ 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/β-galactosidase. Skp1 exhibited an almost 1000-fold lower Km advantage, but still much less active than Skp1 itself because of a Km 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.

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

α-Galp 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 xenotaxenogens and might contribute to specific recognition determinants. With the ultimate goal of understanding the role of peripheral α-galactosylation in the cytoplasm, possessing its phylogenetic urge, 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 HEPES-NaOH (pH 7.4), 5 mM MgCl2, 0.1 mM NaEDTA, 15% (v/v) glycerol, 1 mM DTT, and the protease inhibitors. Buffer B was 50 mM HEPES-NaOH (pH 7.4), 5 mM MgCl2, 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), 50% (v/v) saturated (NH4)2SO4, 15% (v/v) glycerol, 5 mM MgCl2, 1 mM DTT. Buffer D was 25 mM ammonium acetate (pH 7.4), 15% (v/v) glycerol, 5 mM MgCl2, 1 mM MnCl2, 15% (v/v) glycerol, 1 mM DTT. Buffer F was 50 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 1 mM MnCl2, 15% (v/v) glycerol, 1 mM 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 and aliquots were assayed for GalT activity (see below). The enzyme was diluted into the following buffers (100 mM): MES-NaOH (pH 6.6), MOPS-NaOH (pH 6.4–6.8), and HEPES-NaOH (pH 7–8).4

GalT Assays

Assay Conditions—GalT activity was assayed by the transfer of [3H]Gal from UDP-[6-3H]Gal (American Radiolabeled Chemicals) to synthetic glycossides or Skp1. Unless indicated otherwise, reactions (15–60 μl) contained 1–5 μM UDP-[3H]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 MnCl2, 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 [3H]-labeled acceptor substrates (see below), UDP-[14C]-Gal (300 μCi/ml; 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 6–6.6), MOPS-NaOH (pH 6.4–6.8), and HEPES-NaOH (pH 7–8).4

Synthetic Acceptor Substrates—Fuc1,2Galβ1,3GlcNAcß1-pNP (21), Galβ1,3GlcNAcß1-pNP (22), and Galβ1,3GlcNAcß1-Bn (23) were synthesized previously. Galβ1,3GlcNAcß1-Bn was synthesized according to the method for the preparation of Galβ1,3GlcNAcß1-Bn (23). Fuc1-αMe, Fuc1-αallyl, and Fuc1-ßBn were obtained by the reaction of α-fucose and alcohol in the presence of acidic resin Dowex 50X-8 (H+). For example, α-fucose on treatment with benzyl alcohol under these conditions yielded Fuc1-αMe-Bn. The supernatant was loaded at 4 °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% (v/v) (NH4)2SO4, 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 (Amersham Biosciences) equilibrated at 4 °C in buffer B, and the column was washed with buffer B until the A280 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 A280 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, onto a 5-ml Hi-Trap Q-Sepharose column (Amersham Biosciences) equilibrated in buffer E. The column was washed with buffer E until the A280 was less than 0.01. The column was eluted at 21 °C, with a 50-m1 linear gradient of 0–0.2 M NaCl in buffer E, followed by a 25–0.5 M NaCl gradient of 10 min.

Superdex 200 Chromatography—The pooled GalT activity from the third Q-Sepharose column was concentrated in a Centricon 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. Fractions were assayed for GalT activity separately, at 4 °C, by integrated absorbance at 29051 for 2 min and 334/750 nm (NP glycosides). UDP-[3H]-Gal (300 μCi/ml; 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 6.4–6.8), and HEPES-NaOH (pH 7–8).4

Protein Determination—Protein concentration was determined by the Coomassie Blue dye binding method (Pierce Coomassie Plus), or calculated from A280 values, assuming A280 = 1.0.

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peak based on $A_{280}$ and confirmed by scintillation counting of aliquots of the fractions. Concentration was determined from the specific activity of the incorporated $[3H]$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 mixture of mononanosegalactoses that is 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 $Skp1$.

SmartSystem HPLC, and eluted with a 3-ml linear gradient of 0–20% acetonitrile in the same buffer at 22°C. The column was eluted in a gradient of 0–40% acetonitrile, and the sample was subjected to liquid scintillation counting. For the native $Skp1$, which is almost homogeneously glycylated (9), the assay mixture contained UDP-$[3H]$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 $Skp1 b1.3-Galf/iol,1,2-FucT$ (20) and GlcNAcT (11). A major peak of activity was detected centering at 0.16 m NaCl of the salt gradient (Fig. 1A), which overlapped with the early eluting minor pool of $Skp1$ (17), but eluted later than the $Skp1 b1.3-Galf/iol,1,2-FucT$ (20). Minor peaks of apparent activity were reproducibly detected at fractions 52–72 and elsewhere. The major activity peak could also be assayed using $Fuc1,2Gal1-Bn$ as the acceptor substrate (data not shown). $Fuc1,2Gal$corresponds to the non-reducing end disaccharide of one of the activity eluted as a symmetrical peak slightly ahead of the $Skp1A1(HW120)-myc$ glycoforms, suggesting that this may be $Fuc1,2Gal$ that 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). A $Fuc1,2Gal$corresponds to the non-reducing end disaccharide of one of the $Skp1A1(HW120)-myc$ glycoforms, suggesting that this may be $Fuc1,2Gal$ activity predicted by structure studies on the native $Skp1$ glycans (8). Based on the assay using $Fuc1,2Gal1-Bn$, 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$_4$)$_2$SO$_4$ and loaded onto a phenyl-Sepharose column (Fig. 1B). Nearly all activity, using $Fuc1,2Gal1-Bn$ 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 $Fuc1-Bn$ as the acceptor (Fig. 1C), resulting in a 3.7-fold purification with 92% yield (Table I). Similar elution profiles were seen using either $Fuc1-Bn$ or $Fuc1,2Gal1-Bn$ (data not shown), indicating that the GaIT 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, 66,000) (Fig. 1D). 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 $α$-GaIT activity appeared to be homogeneous chromatographically and with respect to its acceptors based on a

RESULTS

Detection and Purification of the GaIT Activity—The initial screen for $Skp1 α$-GaIT 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 αGal residues (8, 9). This heterogeneity does not occur in wild-type $Skp1$, which is almost homogeneously glycosylated (9). The assay mixture contained UDP-$[3H]$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). $Fuc1,2Gal$corresponds to the non-reducing end disaccharide of one of the $Skp1A1(HW120)-myc$ glycoforms, suggesting that this may be $Fuc1,2Gal$ activity predicted by structure studies on the native $Skp1$ glycans (8). Based on the assay using $Fuc1,2Gal1-Bn$, the activity was purified 31-fold at a 72% yield (Table I).

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Gal1,2PN or Gal1,3PN (data not shown). After incubation at 37°C for varying times, aliquots were applied to a C$_8$ Sep-Pak in water. Non-hydrolyzed substrate was eluted with MeOH and quantitated by counting as above.
competition experiment. Fuca1,2Galβ1-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 Fuca1,2Galβ1-pNP was linear with respect to time (data not shown) and enzyme concentration (Fig. 2A). Dilution in the absence of the reducing agent DTT led to a loss of activity that could be rescued by 1 mM DTT (Fig. 2B), as for the other

**Dictyostelium** Skp1 GTs. Dilution in the absence of divalent cations reduced activity and EDTA abolished activity (Fig. 2C). 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. 2D). The enzyme was most active from pH 6.4 to 7.4 (Fig. 2E). 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. 3A), which yielded an apparent $K_m$ of 3.5 μM based on analysis of the data by the Lineweaver-Burk method (Fig. 3B).

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, Fuca1,2Galβ1-Bn (see below). The fractions were freeze-thawed and treated with 0.1% Tween 20 to permeabilize the vesicles. Incorporation into Fuca1,2Galβ1-pNP 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. 4A). 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-[3H]Gal by endogenous UDP-Gal, parallel reactions were supplemented with equal amounts of purified GalT. As shown in Fig. 4B, full α-GalT activity was recovered from the microsomal preparation compared with activity in the

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**TABLE I**

| Sample          | Protein Activity | Specific Activity | Yield | Purification step |
|-----------------|------------------|-------------------|-------|-------------------|
| S100            | 25.520 mg        | 757 units         | 0.030 | 100               |
| DEAE-FF         | 590 mg           | 542 units         | 0.92  | 72                |
| Phenyl-Sepharose| 56 mg            | 260 units         | 4.6   | 34 (48)           |
| Q-Sepharose     | 14 mg            | 240 units         | 17    | 32 (92)           |
| Superdex 200    | 3.8 mg           | 274 units         | 72    | 36 (114)          |

Note: Data are shown as 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. GaT activity was assayed under standard conditions, using 1.5 mM UDP-[3H]Gal (13,200 dpm/pmol), for 1 h. 1 unit = 1 nmol/h.

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**FIG. 1. Chromatographic purification of the α-GaT.** 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-GaT 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$_4$)$_2$SO$_4$, followed by a 0–70% ethylene glycol gradient. Fractions were assayed with 0.23 mM Fuca1,2Galβ1-pNP. 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-GaT activity was assayed using 1.8 mM Fuca1-pNP. 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-GaT 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 $M_s$ standards are shown at the top.
Fig. 2. Characterization of the \(\alpha\)-GalT. The purified enzyme pool shown in Fig. 1D was diluted 10-fold in the solutions described, in the presence of 3 \(\mu\)M UDP-Gal and 0.3 mM Fuc1,2Gal1-\(p\)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.
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. 5A. Fuc1,2Galβ1-Bn was the best acceptor followed by Fuc1Bn and Fuc1,2Galβ1,3GlcNAcβ1-Bn, which corresponds to the full-length glycan. A kinetic analysis showed that Fuc1,2Galβ1-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. 5A). A similar difference was observed for the analogous series conjugated to pNP in place of Bn (Table II). Fuc1-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. 5B), demonstrating specificity for α-linked Fuc. β-Linked β-Fuc had no measurable activity.
activity (Fig. 5B), signifying stereospecificity. The reducing terminal disaccharide Gal\(\alpha\)1,3GlcNAc\(\beta\)1-Bn and its anomer Gal\(\beta\)1,3GlcNAc\(\alpha\)1-Bn were also not acceptors (Fig. 5A), consistent with the interpretation that the \(\alpha\)-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 Fuc\(\beta\)1-pNP. 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 Fuc\(\beta\)1-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 \(\beta\)-galactosidase and \(\alpha\)-galactosidase from \(X.\) manihotis, but not \(E.\) coli \(\beta\)-galactosidase (Fig. 6).

Therefore, Gal appears to be transferred in \(\beta\)-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 \(\alpha\)-, \(\beta\)-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

### Table II

| Substrate                        | \(K_m\) | \(V_{\text{max}}\) | Relative catalytic efficiency* |
|----------------------------------|---------|------------------|------------------------------|
| Fuco1-Bn                         | 3.8     | 0.21             | 1                            |
| Fuco1,2Gal\(\beta\)1-Bn          | 0.84    | 12               | 260                          |
| Fuco1,2Gal\(\beta\)1,3GlcNac\(\alpha\)1-Bn | 2.2    | 0.12             | 0.99                         |
| Fuco1-pNP                        | 3.7     | 0.31             | 1.5                          |
| Fuco1,2Gal\(\beta\)1-pNP         | 0.82    | 45               | 990                          |
| Fuco1,2Gal\(\beta\)1,3GlcNac\(\beta\)1-pNP | 1.2    | 0.32             | 4.8                          |
| Fuco1,2Gal\(\beta\)1,3GlcNac\(\alpha\)1-Skp1 | 0.006  | 7.0              | 21,000                       |

* Catalytic efficiency \((V_{\text{max}}/K_m)\) 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 Fuco1-Bn = 1.
FIG. 6. Sensitivity of the reaction product to α-galactosidase treatment. Fucol-pNP was quantitatively modified by the purified GalT preparation in the presence of UDP-[3H]Gal, purified on a C18 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 C18 Sep-Pak.

a single radioactive sugar had been added by the GalT.

To confirm the position of Gal substitution, Galol1–2Fucol-Bn, Galol1,3Fucol-Bn, and Galol1,4Fucol-Bn were synthesized, purified, and characterized by NMR.2 These isomers were baseline-separated when chromatographed on a C18 reversed-phase column in a gradient of acetonitrile at pH 4.0 (Fig. 7). The radiolabeled GalT reaction product with Fucol-Bn coeluted precisely with the Galol1,3Fucol-Bn standard, confirming the galactosidase results indicating that the enzyme forms a Galol1–3Fuc 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 Fucol1,2Galβ1,3GlcNAc 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-[3H]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, Fucol1,2Galβ1-pNP, 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 Fucol1,2Galβ1-pNP, 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 Skp1A11(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 Galol1,3Fuc 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 1-Fuc, (b) sensitivity of the transferred Gal to removal by an α1,3/6-galactosidase, and (c) co-chromatography of the Gal-Fucol-Bn reaction product with authentic Galol1,3Fucol-Bn but not other positional isomers of this glycoside. The Galol1,3Fuc 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 a 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 Fucol1-Bn, other synthetic
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 α,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 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 Dicyostelium 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.

Dicyostelium Skp1 Is a Natural Substrate for α-GalT1—α-GalT1 was first detected in a screen for GalT activities that excluded. Given the ability of e.g. α-1,2Galβ1,3GlcNAc1-Skp1, one of the two peripheral Gal residues was susceptible to release by the enzyme interaction. This is further supported by evidence that the GTs that add these sugars are at least transiently associated with the Golgi apparatus as type 2 integral membrane proteins (32), unless they are proteolytically cleaved from their membrane anchors.

Second, the full-length Skp1 acceptor glycan, Fuc1,2Galβ1,3GlcNAcα1-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 Fuc1,2Galβ1,3GlcNAcα1-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.

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 Pro143 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 (Pro143).
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-GaIT1 has multiple domains supporting recognition of separate regions of Skp1. Dependence of α-galactosylation on Skp1 folding is consistent with evidence that α-GaIT1 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 [3H]Fuc (35), (b) Skp1 is the only Skp1 FucT acceptor that accumulates in the absence of GDP-Fuc (35), and (c) the Skp1 β-GaIT (which adds the β-Gal to which Fuc is attached) also displays dependence on the Skp1 polypeptide (13, 35). If the Fucol1,2Galβ1,3GlcNAc trisaccharide is unique to Skp1, why are multiple determinants in the Skp1 polypeptide also necessary for efficient modification by the α-GaIT? 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 Man9GlcNAc moiety of nascent glycoproteins with respect to the UDP-Glc:GlcNAc GlcTase is strongly stimulated by an unfolded carrier polypeptide (36, 37), although for Skp1 we propose the opposite effect that folding stimulates acceptor activity.

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

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