A Bifunctional Diglycosyltransferase Forms the Fucα1,2Galβ1,3-Disaccharide on Skp1 in the Cytoplasm of Dictyostelium*

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Skp1 is a subunit of the Skp1 cullin-1 F-box protein (SCF) family of E3 ubiquitin ligases and of other regulatory complexes in the cytoplasm and nucleus. In Dictyostelium, Skp1 is modified by a pentasaccharide with the type I blood group H antigen (Fucα1,2Galβ1,3GlcNAc-) at its core. Addition of the Fuc is catalyzed by FT85, a 768-amino acid protein whose fucosyltransferase activity maps to the C-terminal half of the protein. A strain whose FT85 gene is interrupted by a genetic insertion produces a truncated, GlcNAc-terminated glycan on Skp1, suggesting that FT85 may also have β-galactosyltransferase activity. In support of this model, highly purified native and recombinant FT85 are each able to galactosylate Skp1 from FT85 mutant cells. Site-directed mutagenesis of predicted key amino acids in the N-terminal region of FT85 abolishes Skp1 β-galactosyltransferase activity with minimal effects on the fucosyltransferase. In addition, a recombinant form of the N-terminal region exhibits β-galactosyltransferase but not fucosyltransferase activity. Kinetic analysis of FT85 suggests that its two glycosyltransferase activities normally modify Skp1 processively but can have partial function individually. In conclusion, FT85 is a bifunctional diglycosyltransferase that appears to be designed to efficiently extend the Skp1 glycan in vivo.

Skp1 has been defined in yeast, plants, and animals as a subunit of the SCF (Skp1 cullin-1 F-box protein) family of E3 ubiquitin ligases, which polyubiquitinate target phosphoproteins leading to their degradation in the 26S proteasome (1). Skp1 serves as an adaptor to link the F-box containing protein to the scaffold-like cullin-1, which is in turn linked to an E2 ubiquitin-conjugating enzyme via the ring H2 finger protein Roc1 (2). Other evidence suggests that Skp1 can also be associated with Sfn1-related protein kinases, the kinetochore CBP3 complex, the RAVE-complex linked to assembly of the vacuolar proton transporter, and another complex(es) possibly involved in membrane trafficking (3). It is not known whether these complexes all draw on the same pool of Skp1 in the cell.

Skp1 from the social amoeba Dictyostelium discoideum is modified by a pentasaccharide at a hydroxylated Pro residue at position 143 (4). The pentasaccharide consists of a Fucα1,2Galβ1,3GlcNAc-core, equivalent to the blood group H type I antigen, decorated with two α-linked Gal residues probably via the Fuc residue (5). The majority of Skp1 in both growing and developing Dictyostelium cells appears to be fully glycosylated based on Western blot M_{r} analysis of whole cell Skp1, and partial glycosylation of Skp1 is required for its normal accumulation in the nucleus (6). Assays have been developed for most of the enzymes of the Skp1 glycosylation pathway (3, 5). Analysis of subcellular fractions suggests that they modify Skp1 sequentially in the cytoplasm, rather than the secretory pathway (rough endoplasmic reticulum and Golgi) as for most glycosyltransferases that modify proteins.

We have taken a proteomics approach to define the glycosylation pathway of Dictyostelium Skp1 (3, 5). Purification of the Skp1 fucosyltransferase (Fuc-Tase) activity led to the identification of the FT85 protein and its corresponding gene (7, 8). FT85 is required for Skp1 fucosylation as judged by the absence of Fuc-Tase activity in extracts of cells whose FT85 gene was modified by the replacement of its central coding region with a blastocidin resistance marker (7). In addition, Skp1 made in these cells exhibits slightly increased mobility in SDS-PAGE consistent with the absence of Fuc and the outer, α-linked Gal residues. FT85 appears to be sufficient for Fuc-Tase activity based on expression studies in Escherichia coli (7). With 768 amino acids, the FT85 sequence harbors two potential glycosyltransferase (GTase) domains. The C-terminal domain is sufficient, when expressed in FT85 mutant cells, to fucosylate the simple disaccharide substrate Galβ1,3GlcNAc1-pNP. This corresponds to the core disaccharide of Skp1, therefore assigning Fuc-Tase activity to this domain. We report here that addition of the β1,3-linked Gal to Skp1 maps to the N-terminal half of FT85. The second and third sugars of the Skp1 glycosylation pathway are thus added by a single protein, which can act processively to efficiently extend the oligosaccharide chain. This molecular design has been previously observed for a class of glycosyltransferases involved in the biosynthesis of prokaryotic glycosaminoglycans (9), and is distinct from the mechanism of synthesis of the type I blood group H antigen in animals (10), which occurs in the Golgi apparatus using conventional type 2 membrane proteins with single GTase domains.

EXPERIMENTAL PROCEDURES

Purification of Skp1—For glycosidase digestions described in Fig. 2B, Skp1 was isolated from the FT85-null strain HW260 by DEAE

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The abbreviations used are: SCF, Skp1 cullin-1 F-box protein; CBD, chitin-binding domain; Fuc-Tase, fucosyltransferase; Gal-Tase, galactosyltransferase; GTase, glycosyltransferase; MALDI-TOF-MS, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry; pNP, para-nitrophenyl; mAb, monoclonal antibody; nt, nucleotide; TPCK, l-1-tosylamide-2-phenyl-ethyl chloromethyl ketone; UDP, uridine 5’-diphosphate.

All sugars are pyranosides and in the β-configuration except for Fuc, which is in the L-configuration.
anion exchange chromatography of the crude S100 cell fraction, as described (8). Nearly all Skp1 eluted in the pool II position as assayed by Western blot analysis using mAb 3F9 (11). For the Gal-Tase assays in Figs. 3–5, the DEAE pool of Skp1 was further purified through the phenyl-Sepharose and mAb 3F9 column steps (8). For the Fig. 6 Gal-Tase assays, the eluate from the mAb 3F9 step was applied directly to a Amersham Biosciences mini-Q column equilibrated in 50 mM HEPES-NaOH (pH 7.4), 10 mM MgCl₂, and 5 mM MnCl₂, which was mounted onto a Amersham Biosciences SmartSystem high pressure liquid chromatography unit. Skp1 was eluted with an ascending linear gradient of NaCl in the same buffer, and fractions were pooled based on A₂₈₀ values. SDS-PAGE and Western blot assays confirmed the identity and homogeneity of the pooled material. Skp1 pools were then concentrated to 15% (w/v) by addition of (NH₄)₂SO₄ followed by centrifugation, digested with endo-Lys-C (Wako) as described (4), and either 0.36 mM Galβ₁,₃GlcNAc₁₋₄Glc or a 13,000 Da peptide (cleaves Galβ₁,₄GlcNAcGal) as the donor. Amino acid mass sequence of recombinant FT85 was altered by site-directed mutagenesis of pTY(CBD-FT85) as described previously (7, 13) and described under Site-Directed Mutagenesis of Recombinant FT85. The nt sequence encoding amino acids 52–68 with a formal charge of +5 was changed from GATGAT to GACAAT; the nt sequence encoding Ser-226 was changed from CAT to CTA; the nt sequence encoding Asp-226 was changed from GAT to GAA; the nt sequence encoding amino acid Asp-508 was changed from GAT to AAC; and the nt sequence encoding amino acid Asp-653 was changed from GAT to AAC. The modified plasmids were transfected into E. coli strain ER2566, expression was induced, and cells were lysed also as before (7). Glycosyltransferase Assays—α₁-2-Fuc-Tase activity was assayed as described previously (7) using GDP-[2-3H]Fuc (17.5 Ci/mmol, diluted 5–20-fold with unlabeled GDP-Fuc; New England Nuclear) as the donor and either 0.36 mM Galβ₁,₃GlcNAc₁₋₄Glc or Skp1-GlcNAc-Gal as the acceptor. As described previously (7), 2° linked Galβ₁,₃GlcNAc and Galβ₁,₄GlcNAc were separated by in-source decay (12) in the linear mode by increasing the grid voltage.

**Results**

**Purification of FT85—Dictyostelium**

FT85 was purified from normal Dictyostelium strain Ax3 cells through the final Superdex 200 column step as described previously (7, 8). The material was subsequently adsorbed to a mini-Q ion exchange column (Amersham Biosciences) equilibrated in 50 mM HEPES-NaOH (pH 7.4), 10 mM MgCl₂, and 5 mM MnCl₂, which was mounted onto a Mini-Q column (Amersham Biosciences) equilibrated in 25 mM NH₄Ac (pH 7.1). The void volume was 20-fold with unlabeled GDP-Fuc; New England Nuclear) as the donor and either 0.36 mM Galβ₁,₃GlcNAc₁₋₄Glc or Skp1-GlcNAc-Gal as the acceptor. As described previously (7), 2° linked Galβ₁,₃GlcNAc and Galβ₁,₄GlcNAc were separated by in-source decay (12) in the linear mode by increasing the grid voltage.

**Glycosidase Digestion**

The radioactive product from the mixture of FT85, Skp1-GlcNAc purified from FT85 mutant (HW260) cells through the DEAE column step (see above), and UDP-[3H]Gal was desalted by two passages over a PD10 column (Sephadex G-25; Amersham Biosciences) equilibrated in 25 mM NH₄Ac (pH 7.1). The void volume was divided into multiple tubes each receiving 2,300 dpm, which were each taken to dryness in a vacuum centrifuge, redisolved in water, and taken to dryness. Aliquots were then dissolved in 5 μl of 0.36 M NaF, 10 mM NaCl, 0.1 mM EDTA, and 5 mM dithiothreitol, and probed with anti-

H. van der Wel and C.M. West, unpublished data.

**RESULTS**

Results of the Skp1 from FT85 Mutant Cells Lacks β-linked Gal—Skp1 from FT85 mutant cells (strain HW260) migrates slightly more rapidly than normal Skp1 on SDS-PAGE gels consistent with the loss of Fuc and 2 Gal residues thought to be attached to Fuc
Unmodified peptide (1635) as indicated in Fig. 1. B139 peptide eluted as expected between the positions of peptide 151 (4) modified at Pro143 by hydroxylation and GlcNAc. This single GlcNAc (see procedures from the peak fraction was estimated by MALDI-TOF-MS as 19,130, as described under "Experimental Procedures." Incorporation of radioactivity into Skp1-like material was measured using the SDS-PAGE assay. Where indicated, equal amounts of purified Dictyostelium FT85 were added to the reaction mixtures to test for the presence of active acceptor. B, a partially purified (DEAE column pool) sample of Skp1-GlcNAc from HW260 cells was incubated with purified FT85 and UDP-[3H]Gal as described under "Experimental Procedures" and subjected to gel filtration to separate the protein fraction from UDP-[3H]Gal. The protein fraction was trypsinized to generate low Mr peptides, which were incubated with green coffee bean α-galactosidase and either of 3 different β,1,3-galactosidases (T-III) as described under "Experimental Procedures" in the presence of trypsin inhibitors. Released radioactivity was quantitated after passage through a Dowex-2 column by liquid scintillation counting. Maximal release corresponded to 26% of dpm applied to the column.

A280 absorbance, consistent with the absence of amino acids that absorb at this wavelength. To confirm the identity of the glycopeptide, the grid voltage was increased to promote in-source decay (Fig. 1D). This yielded new ions at m/z 1652, corresponding to the loss of a GlcNAc (1651 predicted), and 1725, corresponding to the C-terminal Lys (1724 predicted). A215 peaks corresponding to alternative glycoforms were not detected (Fig. 1C). Therefore, in the absence of FT85, Skp1 was missing its β-linked Gal in addition to Fuc and the outer sugars.

FT85 Mutant Cells Lack UDP-Gal:Skp1 β,1,3-Gal-Tase Activity—The unexpected absence of the β,1,3-linked Gal in addition to the outer sugars of the Skp1 glycan might be due to an absence of Skp1 β,1,3-Gal-Tase activity in FT85 mutant cells. To test for Skp1 Gal-Tase activity, crude S100 extracts of FT85 mutant HW260 cells were incubated with UDP-[3H]Gal for 2 h, and incorporation of radioactivity into Skp1 was measured by running the extract on an SDS-PAGE gel, excising the gel band containing endogenous Skp1, and counting in a liquid scintillation counter. Negligible incorporation was detected, whereas a substantial level of radioactivity was incorporated using normal strain Ax3 extracts (Fig. 2A). Addition of purified Dictyostelium FT85 to the HW260 extract stimulated a high level of incorporation that did not occur in an aliquot of FT85 alone. Thus the low level of incorporation using the HW260 extract was not due to lack of acceptor substrate or accumulation of an inhibitor. These results suggest that a Gal-Tase activity present in S100 fraction of normal cells is missing in the FT85 mutant strain. FT85 may itself be the Skp1 Gal-Tase, which would explain why Skp1 produced in this strain is lacking its β-linked Gal.

To verify that the radioactivity was incorporated as Gal, the reaction was modified to include purified Dictyostelium FT85, UDP-[3H]Gal, and a DEAE column fraction from the HW260 S100 fraction that contained Skp1. The reaction product was separated from UDP-[3H]Gal by gel filtration and treated with trypsin to cleave the polypeptide backbone of Skp1 and to inactivate FT85. Substantial radioactivity was released by
three different β1,3-galactosidases but not an α-galactosidase (Fig. 2B). Failure to release all input dpm may have been due to inaccessibility due to incomplete digestion of Skp1 by trypsin, or possibly due to a high background of residual UDP-[3H]Gal. The results suggest that the radioactivity was incorporated as Gal in a β1,3 linkage, which corresponds to the linkage of the missing Gal that is normally attached to GlcNAc in native Skp1.

FT85 Exhibits β1,3-Gal-Tase Activity—To test directly for Skp1 Gal-Tase activity, a reaction containing only UDP-[3H]Gal, purified Dictyostelium FT85, Skp1-GlcNAc (from HW260) that had been purified through the mAb 3F9 step to near homogeneity as determined by SDS-PAGE (as in Ref. 8), and cofactors was performed. Under these conditions, FT85 exhibited a high level of Gal-Tase activity (Fig. 3A) that was specific for Skp1-GlcNAc compared with Skp1-GlcNAc-Gal isolated from a GDP-Fuc synthesis mutant (4). The level of incorporation was similar to that observed when α-Fuc-Tase activity was assayed using Skp1-GlcNAc-Gal as the acceptor substrate (Fig. 3B). No Fuc-Tase activity was detected using Skp1-GlcNAc unless UDP-Gal was also included in the reaction, confirming that fucosylation depends upon the presence of Gal to the linkage of the missing Gal that is normally attached to GlcNAc in native Skp1.

FT85 contains 768 amino acids—The N-terminal half of the protein starting at amino acid 409, which it is known to be linked in 1,3-Gal-Tase and 1,2-Fuc-Tase activities are mediated by separate domains (Fig. 5A). FT85 contains 768 amino acids divided into two regions by an Asn-rich segment from amino acid 409–440. Previously it was shown that the C-terminal half of the protein starting at amino acid 397, which was a fusion construct expressed in an Asn-linked Skp1 (Skp1myc—Gal), exhibited a high level of Gal-Tase activity (Fig. 3A). The results suggest that Gal-Tase activity depends on the presence of Gal to the linkage of the missing Gal that is normally attached to GlcNAc in native Skp1.

FT85 Exhibits β1,3-Gal-Tase Activity—To test directly for Skp1 Gal-Tase activity, a reaction containing only UDP-[3H]Gal, purified Dictyostelium FT85, Skp1-GlcNAc (from HW260) that had been purified through the mAb 3F9 step to near homogeneity as determined by SDS-PAGE (as in Ref. 8), and cofactors was performed. Under these conditions, FT85 exhibited a high level of Gal-Tase activity (Fig. 3A) that was specific for Skp1-GlcNAc compared with Skp1-GlcNAc-Gal isolated from a GDP-Fuc synthesis mutant (4). The level of incorporation was similar to that observed when α-Fuc-Tase activity was assayed using Skp1-GlcNAc-Gal as the acceptor substrate (Fig. 3B). No Fuc-Tase activity was detected using Skp1-GlcNAc unless UDP-Gal was also included in the reaction, confirming that fucosylation depends upon the presence of Gal to the linkage of the missing Gal that is normally attached to GlcNAc in native Skp1.

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360-fold greater specific activity than the S13 extract and still had negligible Fuc-Tase activity (data not shown), suggesting that the Skp1 Gal-Tase activity could be attributed directly to the expressed N-domain. Despite the presence of this activity in cell extracts, endogenous Skp1 was not fully galactosylated in vivo based on its acceptor activity in fraction 20.

Similarly, the myc-tagged C-terminal polypeptide from crude cell extracts of strain Ca was further purified over DEAE, phenyl, and Q-columns (see “Experimental Procedures”). It was expressed as two bands with apparent \( M_r \) values of 43,000 and 40,000 (data not shown) compared with the expected value of 42,933. Because the C terminus was apparently intact because of reactivity with the anti-myc mAb 9E10, it is likely that the lower \( M_r \) band was the result of processing in vivo of the 32-amino acid poly-Asn stretch at the N terminus of this construct. The purified material exhibited a 6-fold increase in Fuc-Tase-specific activity with respect to Galβ1,3GlcNAcα1-pNP (data not shown), verifying that activity in this extract could be attributed to the C-terminal polypeptide. The less than expected enrichment of specific activity correlated with an instability of the enzyme activity (data not shown). The purified activity also exhibited extremely low Fuc-Tase activity with respect to Skp1-GlcNAc-Gal (data not shown), as reported previously for the crude S13 extract (7), suggesting that inability to fucosylate Skp1 was a property of the C-terminal polypeptide rather than an inhibitor in the original extract. Mixtures of extracts containing the N- and C-terminal polypeptides did not rescue the low Fuc-Tase activity with respect to Skp1-GlcNAc-Gal (data not shown).

**Mutations in the N- and C-terminal Domains Differentially Affect β-Gal-Tase and α-Fuc-Tase Activities**—In a previous study (7), full-length FT85 was expressed recombinantly in *E. coli* as a fusion protein with a CBD linked to its N terminus via a cleavable intein linker. Soluble *E. coli* extracts containing CBD-FT85 exhibited substantial α-Fuc-Tase activity and were found here to exhibit similar β-Gal-Tase activity (see below). CBD-FT85 was subjected to site-directed mutagenesis to obtain information about the interaction of the N- and C-terminal domains in the intact protein. In the N-terminal half (Fig. 5A), four Asp residues are highly conserved among family GT2 catalytic domains (5). These include Asp-53 in the so-called DGS motif, thought to contact the sugar nucleotide donor, and Asp-226 in the EDY motif, thought to be the catalytic base. These correspond to D1 and D3 of the D1,D2,D3,(Q/R) motif seen in many family members (5, 15, 16). An alignment of the C-terminal sequence, which has low similarity, suggested that Asp-497 or Asp-508 might belong to a D1(DGS) motif, and Asp-653 to a D3(EDY) motif (see Fig. 5A and Ref. 7). The plasmid expressing CBD-FT85 was modified so that each of these Asp residues was individually changed to an Asn residue. When expression of the CBD-FT85 protein was induced in the six strains (1 normal and 5 mutant), a new protein band could be detected at the \( M_r \) position 126,000 of an SDS-PAGE gel stained with Coomassie Blue for protein (Fig. 5B), compared with the ~140,000 value expected for CBD-FT85. Similar levels of the bands were detected in all 6 strains, suggesting that the mutations did not alter the stability of the protein. The new band was also recognized by an anti-CBD antibody (Fig. 5C) and could be quantitatively bound to a chitin column (data not shown). However, elution of the column with 50 mM dithiothreitol, expected to promote self-cleavage of the intein, failed to elute FT85, as determined by silver-stained SDS-PAGE gels, and less than 0.04% of the input Fuc-Tase activity could be recovered in the eluate (data not shown). Incubation of the column in a range of pH values (3–9), time periods (up to 2 d), and temperatures (4–22°C), or in 0.1 M UDP-Gal, 2 μM GDP-Fuc, 1 μM UDP plus 1 μM GDP, or 10 mM EDTA, were also unsuccessful (data not shown). This did not appear to be due to an inability of the intein to self-cleave, as elution of the column with SDS-sample buffer with reducing agent recovered a high level of protein, about half of which was cleaved based on SDS-PAGE analysis (data not shown). Similar results were found for the expressed FT85-CBD fusion protein (7). In contrast, *Dictyostelium* FT85 did not bind chitin (data not shown), and a fusion between CBD-intein and another GTase, GnT51, could be successfully cleaved and eluted from a chitin column (13). The apparently irreversible binding of CBD-FT85 under non-denaturing conditions precluded use of the chitin column for purification.

The Gal-Tase and Fuc-Tase activities of the mutant proteins were compared by carrying out assays on the crude soluble *E. coli* extracts, and are plotted relative to activities in an extract containing normal CBD-FT85. As shown in Fig. 5D, extracts containing CBD-FT85(D53N) and CBD-FT84(D226N) exhibited no detectable Skp1 Gal-Tase activity, similar to effects of
D→A substitutions of the corresponding amino acids in the ExoM Glc-Tase of Sinorhizobium meliloti (17). In contrast, these mutants showed nearly normal levels (about 75%) of Fuc-Tase activity with respect to pNP-GlcNac-Gal, with somewhat lower activity (37–55%) exhibited with respect to Skp1-GlcNac-Gal. This was consistent with the results from the domain expression studies (Fig. 4), indicating that the N-terminal domain carries the β-Gal-Tase but not the Fuc-Tase function, and with the assays showing that the FT85 Fuc-Tase domain can function separately from the Gal-Tase domain (Fig. 3), though apparently less efficiently when Skp1-GlcNac-Gal rather than the disaccharide is used as the substrate.

In the C-terminal domain, the D497N mutation had little effect except possibly to selectively stimulate Fuc-Tase activity (Fig. 5D), though this may reflect slight differences in the level of FT85 expression (Fig. 5C). The D508N mutation mildly suppressed both activities (39–60%), whereas the D653N mutation selectively inhibited the Fuc-Tase activity (1.6–14% remaining) relative to the Gal-Tase activity (22% remaining). The measurable Fuc-Tase activity remaining in these mutant proteins indicates that the targeted residues do not perform exactly the same functions predicted for the corresponding residues in the N-terminal domain. The strong inhibitory effect of the D653N mutation on the Fuc-Tase activity is consistent with the domain expression results assigning this activity to the C-terminal domain, and the considerable degree of inhibition of Gal-Tase might result from an interaction between the two domains.

**FT85 Modifies Skp1 Processively in Vitro**—A kinetic analysis of the Gal-Tase activity of FT85 in the presence of UDP-[14C]Gal yielded a hyperbolic dependence on Skp1-GlcNac (Fig. 6) that, when plotted in double-reciprocal fashion, yielded an apparent \( K_m \) of about 0.8 \( \mu M \). When GDP-[3H]Fuc was included in the reaction, the reaction rate was stimulated over 2-fold, suggesting that reaction efficiency was promoted when both transferase activities could act coordinately. In the tandem reaction, fucosylation occurred at about 75% of the rate of galactosylation. This suggests that FT85 acts processively on Skp1-GlcNac, as the fucosylation rate would have lagged far behind the galactosylation rate if it depended on the solution concentration of Skp1-GlcNac-Gal derived from the first reaction. FT85 is able to directly fucosylate Skp1 that has been already galactosylated with similar kinetics to that of galactosylation in the tandem reaction. Thus the reduced rate of fucosylation in the tandem reaction is not due to an inefficient Fuc-Tase activity per se, but may result from inefficient transfer from the Gal-Tase domain to the Fuc-Tase domain.

**DISCUSSION**

Various strategies have evolved to ensure the efficient, sequential processing of target substrates that are modified by multi-enzyme glycosylation pathways. For example, lipid-linked acceptors are concentrated with GTases by association with the same lipid bilayer of discrete organelles or the plasma membrane. There is evidence for multi-protein enzyme complexes in the extension of N-linked (18) and O-linked (19) glycans of proteins in the lumen of the Golgi apparatus, which also confines donor and acceptor substrates. For the cytoplasmic/nuclear glycoprotein Skp1, whose glycosylation does not appear to be associated with organelles (3, 5), it is reported here that two of the processing steps, addition of β1,3-linked Gal and α1,2-linked Fuc, appear to be mediated by separate domains of the same polypeptide.

A previous study (7) documented that FT85 is both necessary and sufficient for the fucosylation of Skp1 in vitro and in vivo. Here it is shown that FT85 is also both necessary (Figs. 1 and 2) and sufficient (Fig. 3) for the addition of β1,3-Gal, the sugar to which Fuc is subsequently added in α1,2-linkage. An explanation for how a single polypeptide could perform both functions emerges from an inspection of its sequence (see Fig. 5A). With 768 amino acids, FT85 is unusually long considering that glycosyltransferase catalytic domains typically consist of 250–300 amino acids. Near the middle of the protein is a homopolymeric stretch of 32 Asn residues, found occasionally in Dictyostelium proteins. Upstream in the N-terminal half of the protein is a sequence related to the full-length of family GT2 GTases that includes key Asp residues of the so-called D1,D2,D2,Q/R/KXXRW signature seen in some family members (5). D2 is equivalent to the DXD motif of many metal-binding GTases, and, like the family GT2 GTases, the β-Gal-Tase inverts the anomeric linkage of Gal as it is transferred from UDP-Gal to Skp1. A previous study showed that the C-terminal half of FT85 can fucosylate the Skp1 disaccharide pNP-GlcNac-Gal in vitro (7), and a provisional alignment with family GT2 sequences also suggested the tentative existence of family GT2 motifs in this domain, which is also metal-dependent, possesses an apparent DXD motif and inverts the anomalous configurational function of Fuc (5, 7).

The N-terminal half of FT85, expressed in FT85-null Dictyostelium cells together with the middle Asn-32 stretch of amino acids and an additional 12 downstream amino acids resulting from the cloning strategy (see diagram in Fig. 5A), appears to exhibit normal Skp1 β,3-Gal-Tase activity in vitro (Fig. 4). Consistent with this result, the conservative replacement by Asn of either of two key Asp residues (from the D1 and D3 motifs), predicted to be associated with binding the sugar nucleotide or catalysis as interpreted from the crystal structure of Spa3 (21), abolishes the Gal-Tase activity of the full-length protein with minimal effect on the Fuc-Tase activity (Fig. 5). These Asp residues lie in the first 270 amino acids, which are likely to constitute the catalytic Gal-Tase domain. The role of the next 135 amino acids C-terminal to this is not known.

The C-terminal half of FT85 (including the middle N\_\_ segment and 10 upstream amino acids), although able to efficiently fucosylate pNP-GlcNac-Gal, was very inefficient at modifying Skp1-GlcNac-Gal in both crude extracts (7) and after further purification (data not shown). The region of FT85

4. P. M. Coutinho and B. Henrissat (1999) Carbohydrate-Active Enzymes server afmb.cnrs-mrs.fr/~cazy/CAZy/index.html.
from amino acids 270–400, between the two catalytic domains and upstream of the N27 stretch, may be important for recognition of the Skp1 protein, though it is possible that the 10 amino acid C-terminal myc-tag may selectively interfere with utilization of the Skp1 substrate. The tentative prediction based on sequence alignment (7) that Asp-653 is involved in catalysis was not supported by the failure of the D653N substitution to abolish Fuc-Tase activity (Fig. 5). Activity was reduced to 1.6–14%, compared with 22% for Gal-Tase activity. The selective effect on Fuc-Tase activity is consistent with the association of this region of the protein with the Fuc-Tase catalytic domain as revealed by the domain expression study (7), so the effect on the Gal-Tase activity is assumed to be secondary. This correlates with the observed reduction in Gal-Tase activity when Fuc-Tase activity is blocked by withholding GDP-Fuc (Fig. 6). The other two mutations, D497N and D508N, had lesser and non-selective effects on Fuc-Tase activity, also seemingly to disqualify the hypothesized roles of these amino acids in binding GDP-Fuc. However, changes that are less conserved than the D→N substitutions may be more informative. The ability of the two domains to function independently indicates that the sugars are separately transferred to the non-reducing terminus of the growing glycan chain rather than involving a disaccharide intermediate.

Though the two domains are able to function independently in a catalytic sense, they appear to be interdependent for optimal processing of the natural substrate Skp1. The results suggest a model in which the N-terminal domain possesses a docking site for Skp1, possibly in the region of amino acids 270–400, which is located between the two catalytic domains. This is the predicted location for acceptor substrate docking in family GT2 GTases (22), which are related to family GT2 GTases to which the N-terminal catalytic domain belongs (5). This would explain why the free C-terminal domain poorly fucosylates Skp1-GlcNAc-Gal (7), and sharing of the same Skp1 docking site could explain why mutational or biochemical reduction of Fuc-Tase activity in intact FT85 inhibits β-Gal-Tase activity (Figs. 5 and 6). A structural association of the proposed Skp1-docking site with the Gal-Tase catalytic domain might explain why pNP-α-GlcNAc or pNP-β-GlcNAc are not β-Gal-Tase acceptors,¹ whereas the Fuc-Tase can modify synthetic acceptors in vivo.

The kinetic analyses suggest that Skp1 in this site is equally available to either GTase activity as long as the correct sugar acceptor structure is present (Fig. 6). However, the proposed docking site exhibits selectivity because a 5-fold concentration excess of Skp1-GlcNAc-Gal does not inhibit the galactosylation of Skp1-GlcNAc (Fig. 3A). Though further kinetic, mutagenic, and structural studies will be required to address the mechanism of action of this interesting enzyme, it is clear from the present results that its design promotes the efficient extension of the Skp1 glycan from the monosaccharide to the trisaccharide. This would avoid the accumulation of a β-Gal-terminated glycan that might interact with β-galactoside-binding lectins in the cytoplasm, such as discoids (23).

The one enzyme-one sugar linkage paradigm for glycan chain synthesis (10, 24) is generally upheld, but there are numerous exceptions including the FT85 example described here. Glycans with repeat disaccharides are in many instances extended by single polypeptide di-GTases, which add sugars incrementally in repeat fashion. There appear to be two distinct mechanisms (9). In the synthesis of cellulose, chitin, and hyaluronic acid in eukaryotes, a single catalytic domain, related to that of conventional family GT2 GTases, appears to catalyze the addition of each sugar of the disaccharide repeat from conventional sugar nucleotide donors (15, 25). A second mechanism is found in the synthesis of certain prokaryotic glycosaminoglycans, where two GT2 family GTase domains are linked together in the same polypeptide (9) as concluded here for FT85. Processive GTases have also been described for the extension of non-repeating glycans in glycolipids in prokaryotes (26, 27) and of glycosaminoglycans in animals (28), though the mechanisms have not been delineated. FT85 appears to represent a novel adaptation of the two-domain di-GTase model to the extension of a non-repeating disaccharide on a protein-linked glycan in the cytoplasm of a eukaryote. The cytoplasmic localization of this enzyme is consistent with the family GT2 classification of its β-Gal-Tase domain, as GT2 family GTases are, so far, variably oriented toward the cytoplasm (5, 20). Unlike most family GT2 GTases, however, FT85 is not associated with a membrane, and its reaction product is not ultimately translocated across the membrane (5, 16, 20).

Strain HW260 has an insertion of the bar-resistance marker in the coding region of its FT85 gene, resulting, as shown here and previously (7), in complete loss of the two biochemical activities associated with the protein, both in vitro and in vivo (Figs. 1 and 2). Therefore this may be a null allele. Previously it was observed that HW260 cells have distinct optical properties including smaller size and grow to a higher saturation density in growth medium (7). Biochemical analyses are consistent with the model that Skp1 is the only acceptor substrate for FT85 action (5), suggesting that glycosylation of Skp1 is important for global cellular regulation. The more complete biochemical understanding of the biochemical lesion in FT85-null cells provided by this study provides a new tool for future investigations of the role of Skp1 glycosylation, including assessing whether β-galactosylation is important. For example, a previous study (6) showed that glycosylation appears to be required for the nuclear accumulation of Dictyostelium Skp1, and, although the GlcNAc-Gal disaccharide is sufficient for this effect, the importance of β-Gal is not known.

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