A Non-Golgi α1,2-Fucosyltransferase That Modifies Skp1 in the Cytoplasm of Dictyostelium*

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Skp1 is a subunit of the SCF-E3 ubiquitin ligase that targets cell cycle and other regulatory factors for degradation. In Dictyostelium, Skp1 is modified by a pentasaccharide containing the type 1 blood group H trisaccharide at its core. To address how the third sugar, fucose α1,2-linked to galactose, is attached, a proteomics strategy was applied to determine the primary structure of FT58, previously shown to copurify with the GDP-Fucose:Skp1 α1,2-fucosyltransferase. Tryptic-generated peptides of FT58 were sequenced de novo using Q-TOF tandem mass spectrometry. Degenerate primers were used to amplify FT58 genomic DNA, which was further extended by a novel linker polymerase chain reaction method to yield an intronless open reading frame of 768 amino acids. Disruption of the gene by homologous recombination resulted in viable cells, which had altered light scattering properties as revealed by flow cytometry. FT58 was necessary and sufficient for Skp1 and exhibited submicromolar Km values for both its donor and acceptor substrates, cannot be detected in the vesicular fraction of the cell, and requires the presence of a reducing agent in vitro. These properties suggest that this enzyme normally functions in the reducing environment of the cytoplasm, where substrates are expected to be less concentrated than in the secretory pathway.

A Skp1 α1,2-FTase activity (EC 2.4.1.69) has been assayed based on the transfer of [3H]Fuc from GDP-[3H]Fuc to pNP-phenyl-Galβ1,3GlcNAc (pNP-LNB) or to Galβ1,3GlcNAc-Skp1 isolated from a GDP-Fuc synthesis mutant (9, 10). Highly purified preparations of the enzyme are specific for the O-2 position of Gal that is in β1,3 linkage to an underlying HexNAc, which may be either GlcNAc or GalNAc, α- or β-linked to an aglycone. Activity varies by an order of magnitude depending on the nature of the aglycone and displays a Km for the disaccharide on Skp1 that is three orders of magnitude lower than that of the best disaccharide aglycone. The acceptor carbohydrate substrate specificity profile of the Skp1 FTase suggests a closer relationship to the vertebrate Se-type compared with the H-type α1,2-FTases found in the Golgi apparatus. However, the Skp1 FTase is present in the cytosolic fraction with little detected in the vesicular fraction of the cell and, as for the Skp1 GlcNAcTase, has biochemical characteristics of a cytoplasmic

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank1,2 EBI Data Bank with accession number(s) AF279134.

1 All sugars are in the β-configuration except for Fuc, which is in the α-configuration.

2 The abbreviations used are: GTase, glycosyltransferase; pNP-LNB, p-nitropheryl lacto-N-bioside; GlcNAcTase, UDP-GlcNAc:Skp1 GlcNAc-transferase; FTase, fucosyltransferase; CBD, chitin-binding domain; LP-1 (-2 or -3), linker primer-1 (-2 or -3); ORF, open reading frame; CAD MS/MS, collisional-activated decomposition tandem mass spectrometry; EST, expressed sequence tag; rER, rough endoplasmic reticulum.

Gala1,6Gala1(Fucα1,2Galβ1,3GlcNAc),1 which is attached in O-linkage to HyPro143 (3, 4). The HyPro attachment site is predicted to be located between a loop and an α-helix near to but projecting away from the interface with the F-box protein of the SCF-complex (5). Immunofluorescence studies localize Skp1 to the nucleus and regions of the cytoplasm (6, 7, 4), and mutant analysis suggests that at least the core disaccharide is required for concentration of Skp1 in the Dictyostelium nucleus (4).

Skp1 is predicted to be glycosylated by a novel 6-enzyme pathway, including a prolin hydroxylase and five glycosyltransferases (GTases).2 Two of the GTases, the GlcNAcTase that adds the first sugar and the α1,2-fucosyltransferase (FTase) that adds the third sugar, have been purified to near homogeneity from the cytosolic fraction of Dictyostelium cells and characterized. The GlcNAcTase transfers [3H]GlcNAc from UDP-[3H]GlcNAc to HyPro143 of a mutant Skp1 that is mostly hydroxylated but not glycosylated in vivo (8). This activity exhibits submicromolar Kms values for both its donor and acceptor substrates, cannot be detected in the vesicular fraction of the cell, and requires the presence of a reducing agent in vitro. These properties suggest that this enzyme normally functions in the reducing environment of the cytoplasm, where substrates are expected to be less concentrated than in the secretory pathway.

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rather than a Golgi protein. These properties suggest that the Skp1 FTase is also located in the cytoplasm of the cell with its acceptor substrate Skp1, not in the Golgi like the Se-FTase. The similar findings for both the GlcNAcTase and FTase suggest that the entire 6-enzyme pathway acts directly and sequentially on Skp1 in the cytoplasmic compartment.

Although eukaryotic cytoplasmic glycosylation is not as extensive and varied as glycosylation in the secretory pathway, a number of important examples are known. Dol-P-Man, Dol-P-Glc, and the Man$_{4}$GlcNAc$_{2}$-P-Dol precursor of N-glycosylation are synthesized by cytoplasmic enzymes prior to flipping into the interior of the rER (11). The sugars of phosphatidylinositol GlcNAc$_{2}$ and glucosylceramide each appear to be attached by cytoplasmically oriented enzymes (12, 13). Glycogen is the product of two glucosyltransferases (14), and the extracellular polysaccharides cellulose, chitin, and hyaluronate are synthesized by enzymes whose active sites are cytoplasmically oriented, though their polysaccharide products are co-synthetized. The low energy-content pathways are similar (22), and where protein data base library matches (OWL/SWISSPROT) failed to identify a protein precursor, these *de novo* sequences were assumed to be likely candidates for FTase primers. BLAST searches for homology matches gave moderate homology matches in some cases with GTases from other species.

**Sequencing the FT85 Gene—**Degenerate oligonucleotides were based on the peptide sequences derived from mass spectrometry and *Dictyostelium* codon usage frequencies. Multiple subsets of degenerate primers were synthesized as described in "Results," and primer lengths were 25–30 nucleotides to achieve $T_m$ values of 54–64 °C (GC + AT method) given the high AT content of *Dictyostelium* DNA. Primers were used in the touchdown (td) variation of PCR (28), in which the annealing temperature was reduced by 1 °C/cycle during each of the first 15 cycles, followed by a return to 3 °C below the initial annealing temperature for an additional 15 cycles. For a amplification of td-pcr (1 Fig. 4A), denaturation was at 94 °C for 45 s (3 min for first cycle), the initial annealing temperature was 50 °C, and extension was at 68 °C for 2 min (10 min last cycle) using a Stratagene Robocycler. Degenerate primer pools were used at 100 pmol each in a 50-μl reaction volume. CsCl-purified genomic DNA was isolated from nuclei of D. discoideum strain Ax3 (29), being careful to avoid the lower, intensely pigmented, mitochondrial region of the pellet when resuspending the nuclei.

To amplify unknown neighboring DNA, a linker-mediated PCR method was modified from a strategy originally employed by the Maron and Gene-Walker methods by CLONTECH (30). 2.5 μg of CsCl-purified DNA was digested with either BamHI, BclI, BglIII, or BsrYI, phenol/chloroform extracted, ethanol-precipitated after the addition of 2.5 μl ammonium acetate, washed in 70% ethanol and dried.

The following synthetic oligonucleotides (Integrated DNA Technologies, Coralville, IA) were used to form a double-stranded linker: 5'-GTACCTCTAGTTAGCTGGAGGTCCGGTAC-3' and 5'-H$_{4}$N-GGGGAGGTTATAG-3'. Equal molar amounts dissolved in 100-μl aliquots at 1 μl/ml, heated to 95 °C, allowed to cool slowly to 37 °C, and flash-frozen in liquid N$_{2}$. These were expected to anneal via the underlined regions. The digested genomic DNA was diluted to 5 ng/μl in ligation buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl$_{2}$, 10 μM dithiothreitol, 1 mM ATP, 25 μg/ml bovine serum albumin) with 4 units/ml of T4 DNA ligase (New England Biolabs; Beverly, MA), and 4× the calculated free DNA concentration and ethanol-precipitated to 18 °C for 16 h. 50 ng of library was used in each PCR reaction. The linker-modified libraries were typically amplified with a gene-specific primer (Fig. 4A) and LP-1, 5'-GTACCTCTAGTTAGCTGGAGGTCCGGTAC-3' using the touchdown protocol described above for 30 cycles (94 °C, 45 s; 55 °C, 45 s; 68–72 °C, 2.5–4 min) and Taq DNA polymerase. In principle, LP-1 was able to hybridize with only these DNA species that had been extended by reactions primed by the gene-specific primer. In some cases, the initial reaction was diluted 50-fold, and PCR was repeated using a nested gene-specific primer coupled with LP-1, LP-2, 5'-AGGTAGCC-CAGAAGGCGGTGTTG-3' or LP-3, 5'-GTACCTCTAGTTAGCTGGAGGTCCGGTAC-3' based on which had the best $T_m$ match. PCR products were cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and sequenced in both directions.

Nucleotide sequences were also extended by searching for matches in genomic fragments sequenced by the International *Dictyostelium* Genome Sequencing Consortium, and in cDNA/ESTs sequenced by the Japanese CNDA project at Tsukuba University, using BLAST servers at genome.imb-jena.de/dictyostelium/, dicty.sdsce.edu/, or www.csm.biol.tsukuba.ac.jp/DNAProject.html. Sequences were then confirmed on PCR amplicons full-length cDNA clones using DNA primers P5, P4, P11, P10, P12, and P6 as indicated in Figs. 4 and 5.

**FT85 Mutant Cells—**The blasticidin-S resistance (bns) cassette was excised from pBR519 (31) with PstI and ligated into the compatible NsiI site of td-PCR in pCR4-TOPO, as shown in Fig. 4B. To induce homologous recombination, the resulting DNA insert was excised with EcoRI and electroporated into *Dictyostelium* strain Ax3 cells (32).
Transformants were selected in 10 μg/ml blasticidin-S (Life Technologies, Inc.) and cloned on SM agar plates in the presence of Aerobacter aerogenes. Transformants were screened for insertion of bar into the FTS85 locus by amplification of genomic DNA using primers P9 and P10 (Fig. 4A) as described (33).

Expression of FTS85 in E. coli—The full-length coding region of FTS85 predicted from the nucleotide sequence was amplified using primers 5 and 6 on 7 and 8 (Figs. 4C and 5), cloned into pCR4-TOPO, and amplified in E. coli strain TOP10 One Shot chemically competent cells (Invitrogen). Restriction sites designed into the 5′ and 3′ ends of these primers were used to excise the PCR-amplified DNA inserts for ligation between the EcoRI and NdeI sites of pT7B1, or the SphI and PstI sites of pTYB1, creating an in-frame fusion at the N terminus or C terminus, respectively, of the IMPACT-CN chitin-binding domain (CBD) via an intein linker (Ref. 34; New England Biolabs). pMYB5, which contained the maltose-binding protein fused to the intein tag, was used as a control. The expression plasmids, pTYFT-CBD and pTYCBD-FT, were produced in TOP10 One Shot chemically competent E. coli cells and then transfected into E. coli strain ER2566 for expression, which was induced when cells achieved an A600 of 0.5, by incubation in 0.5 mM IPTG for 20 h at 15 °C. Soluble extracts were prepared by freeze-thawing cell pellets, probe sonication until the suspension became viscous, and centrifugation at 13,000 × g for 15 min at 5 °C.

Expression of FTS85 domains in D. discoideum—Predicted N-terminal and C-terminal domains of FTS85 were amplified by PCR from pTYTFT-CBD using primers 13 and 14 or 15 and 16, respectively: P13, 5′-GACCTGTTACCAAGATATATAAAAAATGGAAGTTACTCAGCAAACAAATAAAGGTGGTATTTAC and P14, 5′-CAAGATCTATTACATTATACATGACAGATATAAAATTTTGATT; P15, 5′-GACCTGTTACCGAGAAGTTATATATAAAAAATGGAAGTTACTCAGCAAACAATGTTGGTATTTAC and P16, 5′-CAAGATCTATTACATTATACATGACAGATATAAAATTTTGATT. The underlined regions correspond to FT85 DNA in Figs. 4C and 5. Primers 13 and 15 encode a 16-nucleotide sequence upstream of the start codon designed to support translation initiation, and the actin 8 terminator. The new plasmids, pVTFT85N and pVTFT85C, were cloned into TOP10 cells, purified using a QuickPrep Spin mini-prep kit (Qiagen, Valencia, CA), and electrotransfected into HW280 cells as described (4).

expression Assays in Cell Extracts—Dictyostelium cells (stationary phase) were lysed by sonic disruption with a probe sonifier in 50 mM Tris·HCl, pH 7.4, with protease inhibitors (9) and centrifuged at 13,000 × g for 1 h. The supernatant from 1.5 × 10⁶ cells (100 μg of protein) was loaded onto 13-cm long, 15–20% linear gradient polyacrylamide SDS-gels and gels electrophoresed as previously described (4). Gels were Western blotted and probed with polyclonal antibodies for Skp1 (4).

Flow Cytometry—Cells growing at a density of 1–3 × 10⁶ cells/ml in HL-5 were introduced directly into a BD Biosciences FACSCalibur flow cytometer (San Jose, CA). Forward angle (side) light scatter values were determined using illumination from an Ar ion laser (488 nm) operating at 15 milliwatts. 60,000 cells were counted in each sample, and data were analyzed using CellQuest software from BD Biosciences.

RESULTS

Sequencing the FTS85 Polypeptide—FT85 was purified nearly 1 million-fold as described previously (9). Aliquots from the final gel filtration step were assayed for FTase activity using pNP-LNB and analyzed by SDS-PAGE and silver staining. Although the present purification yielded higher levels of lower Mr contaminating bands, the staining intensity of an Mr = 85,000 band is illustrated in this spectrum where most of the band correlated with the level of FTase activity (Fig. 1). The peak fractions 27–29 were pooled and purified by SDS-PAGE followed by staining with Coomassie Blue and destaining, the gel was cut into 55–60 equal parts for scintillation counting as above.

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the signal intensity is singly charged background. However, some weak doubly charged signals are evident, as shown for m/z 461, 645, and 727, with m/z 645 expanded in the inset to illustrate the half-mass unit spacing visible within the background.

Selecting m/z 645 for MS/MS analysis produced the spectrum shown in Fig. 2B, where an excellent series of N-terminal and C-terminal (b and y') fragment ions could be readily interpreted to give the sequence MDSDL(I)SHPTR via the presence of signals shown in Fig. 2C, where Leu and Ile are of course indistinguishable in mass. The N-terminal residue mass of 148 daltons (which is actually determined in the sequencing logic from the C-terminal y'10 ion mass) could in fact be interpreted initially as either Phe or Met sulfoxide. The Met residue was assigned with certainty by tuning onto a virtually nonexistent signal at m/z 637 (corresponding to molecules 16 daltons lower), which produced a weak but identical set of y' ions with b ions 16 daltons lower on MS/MS (Met is 16 daltons lower in mass than Met sulfoxide), showing that the N terminus could not be Phe (data not shown). A BLAST (OWL) protein data base search of the MDSDDL(I)SHPTR sequence showed no hit for any known protein sequence.

MS/MS analysis of the doubly charged m/z 727 ion in Fig. 2A produced a fragment ion spectrum, which was interpreted to give a sequence YYFTL(I)L(I)DA. A search of the protein data bases showed this to be derived from Elongation Factor 1a. The sequence study of numerous peptides from two independent purifications showed many to be derived from this impurity protein or from keratin background (common when working at this level), but some 15 peptide sequences were not

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**Fig. 2. Analysis of tryptic peptides of FT85 by mass spectrometry.** A, partial mass spectrum of an FT85 in-gel tryptic digest produced by nano-electrospray MS indicating the positions of some relevant doubly charged signals (see text). The inset expands the m/z 645 ion showing the half mass isotope spacing among the singly charged background ions. B, de novo sequencing: CAD MS/MS spectrum of the m/z 645 ion on the Q-TOF showing the location and assignment of fragment ions used to determine the peptide sequence. C, the interpretation of the CAD MS/MS data showing how the N-terminal (b) and C-terminal (y') ion masses are assigned to give the sequence MDSDDL(I)SHPTR, which was then used to design forward and reverse pools of degenerate primers.
Fig. 3. MS-sequencing of peptide 2. A, CAD MS/MS spectrum of the m/z 461 ion from Fig. 2, showing the fragment ions used to deduce the peptide sequence. B, the interpretation of how the y′ ion masses were used to derive the sequence NHNPTR with high confidence, which was most probably preceded by NGLI(I) or DGL(I) (possibly resulting from deamination of N), or possibly RE (not shown). Each of these sequences was used to design pairs of degenerate primer pools for the PCR studies.

identified in database searches and were therefore presumed to derive from FT85. Of these, which included a mix of full and partial predicted sequences, the confidence limit ranged from good to very high, and from the latter, three were chosen for primer candidate sequences. Of these, two were thought suitable for design of degenerate oligonucleotides for PCR: MDSD-DL/I SHPTR and NHNPTR (peptides 1 and 2). The latter high confidence sequence was interpreted (Fig. 3, A and B) as being preceded most probably by DGL/I or NGLI(I) in MS/MS spectra of m/z 461 obtained from different fractions of the cartridge purification.

Sequencing the FT85 Gene—Oligonucleotide pools that encode candidate sequences from peptides 1 and 2 in either the forward or reverse directions were synthesized to represent all codons used in Dictyostelium at a frequency of $\geq 25\%$. Three pools of oligonucleotides corresponding to the forward and reverse reads of peptide 1 were synthesized so that the number of sequences present in each pool was 8. Similarly, 3 pools were synthesized corresponding to each direction for each of the candidate peptide 2 sequences, and the degeneracy ranged from 16- to 128-fold. For PCR, each forward direction oligonucleotide pool derived from one peptide was mixed with each reverse direction oligonucleotide pool from the other peptide and vice versa. These primer pool pairs were used to amplify DNA from genomic DNA, using the touchdown protocol described in the "Experimental Procedures" section. One combination of a forward primer pool from peptide 1 (primer 1, 5′-ATGATCTCGATATATGCW) and a reverse primer pool from peptide 2 (primer 2, based on the peptide sequence DL/I NHNPTR, 5′-RTTGGRTGRTTNNARACCATC) uniquely amplified a 1.7-kilobase DNA, td-per-1 (Fig. 4A) that was not seen with either of the primer pools alone. Its nucleotide sequence belonged to a single ORF in the forward direction. Adjacent DNA was amplified in both directions from linker-modified sublibraries of BseYI-digested genomic DNA using primers P3 and P4, and linker-specific primers, as shown in Fig. 4A.

A total of 3584 nucleotides were sequenced by PCR (Fig. 4A). The termini of this sequence overlapped with cDNA and unconfirmed genomic sequences obtained from the Dictyostelium cDNA and genome sequencing projects, which permitted tentative extension of the overall sequence to 4701 nucleotides. Conceptual translation yielded a single, long, forward-directed ORF of 2304 nucleotides in the PCR-derived sequence, with a G/C-content of 21% and codon usage typical for Dictyostelium coding regions. This main ORF was predicted to encode a 768-residue protein with a calculated Mr of 89,735 (Fig. 5), which compared favorably with the predicted Mr of FT85, 85,000 (Fig. 1B). The first Met codon of this ORF was the only ATG in the vicinity, occurred at a boundary between G/C-poor and G/C-rich sequence, and had a sequence context similar to that of other Dictyostelium genes (35). Sequences that potentially correspond to a TATA-box and an adjacent oligo(dT) box are found ~130 nucleotides upstream of the assigned start codon (Fig. 5, bold).

A second forward-directed ORF was found at the 5′-end of the PCR-derived sequence, separated from the main ORF by 262 nucleotides that have a G/C-content of 17% (Figs. 4A and 5). This ORF was extended in the upstream direction by the genome database sequences and had a length of at least 1377 nucleotides (>459 amino acids) and G/C-content of 27%. It is unlikely that the upstream ORF is a separate exon of the main ORF, as: 1) there are no consensus donor and acceptor splice sequences that can be used to create a conventional intron near the ORF boundaries, 2) its 3′-end is found in a cDNA/EST clone (see Fig. 4A) suggesting that it is a separate transcription product, and 3) a consensus polyadenylation signal (AATAAA) for the upstream ORF lies 88 nucleotides upstream of the start of the main ORF (Fig. 4A, bold). Interestingly, this suggests that the 3′-untranslated sequence of the upstream gene overlaps with the promoter and 5′-untranslated sequence of the FT85 gene.

A third, reverse-oriented ORF was found at the 3′-end of the PCR-derived sequence, which was extended an additional 541 nucleotides for an overall length of at least 652 nucleotides, using overlapping cDNA/EST sequence reads from the Japa-
A search for sequences similar to FT85 was carried out by BLAST analysis of SwissProt and conceptually translated GenBank™ sequences, yielding a large number of weak matches between its N-terminal 270 amino acids and Family 2 (23) or Family E (24) GTases. Family 2 represents a wide variety of mostly prokaryotic GTases whose catalytic domains are about 260 amino acids in length, and whose catalytic mechanisms involve inversion of the donor sugar linkage as for the Skp1 FTase (36). Similarity was highest within the cytoplasmic catalytic domain containing the Asp residue.
precipitation with trichloroacetic acid was highly enriched in the P100 fraction, as previously observed (36) and was similar between the two strains (data not shown). The apparent Mr profiles were compared after separation on an SDS-PAGE gel and quantitation of radioactivity by scintillation counting of individual gel slices. Similar profiles of incorporated [3H]Fuc were observed for the P100 fractions of each strain grown on HL-5 (Fig. 8A), and similar profiles, with slightly greater mobility, were obtained for cells starved in KP (data not shown). Thus the majority of fucosylation was not affected in the mutant. In the S100 fractions from KP-starved cells, incorporation was detected at the Skp1 position of Ax3 but not FT85 mutant HW260 cells (Fig. 8B), confirming that Skp1, which accumulated at a normal level, was not fucosylated in the FT85-mutant strain.

To test for Skp1 FTase activity directly, soluble cell extracts were initially assayed using a simple disaccharide conjugate, pNP-LNB, shown previously to be an acceptor substrate (9). In the presence of GDP-[3H]Fuc, a high level of activity was observed in normal strain Ax3 extracts (Fig. 9A). A diminished level of incorporation was seen for strain HL250, the GDP-Fuc synthesis mutant, probably because non-fucosylated Skp1 present in this strain is competitive with pNP-LNB and is not detected by the assay (9). In contrast, no incorporation was detected in the FT85 mutant strain HW260. This was not due to expression of the bsr marker, because another strain (HW264) that had incorporated this marker elsewhere in the genome expressed a normal level of activity. Negligible activity in HW260 was confirmed using Skp1(HL250), which contains only the disaccharide core, as the acceptor substrate (Fig. 9A). Thus no Skp1 FTase activity could be detected in the S13 extracts, indicating that FT85 is necessary for FTase activity.

**FT85 Exhibits Skp1 FTase Activity in E. coli**—To determine whether FT85 alone was able to fucosylate Skp1, Skp1 FTase activity was assayed in soluble extracts of *E. coli* expressing full-length Dictyostelium FT85 recombinantly. As described in “Experimental Procedures,” FT85 was fused at either its N terminus or C terminus to a chitin-binding domain via a cleavable intein linker and placed under the control of the T7 promoter (34). Induction of expression of the CBD-intein-FT85 and the FT85-intein-CBD fusions resulted in substantial levels of FTase activity using either pNP-LNB or Skp1(HL250) as acceptors (Fig. 9B). Activity was greater than that detected in wild-type extracts of Dictyostelium on a per cell protein basis.
Negligible activity was detected in extracts in the absence of pNP-LNB or Skp1(HL250), and in extracts from an induced MBP-intein-CBD expressing control strain assayed in the presence of the substrates. Detailed kinetic analyses of FT85 purified from these extracts and cleaved from its intein-CBD tag will be required to understand the basis for quantitative differences between substrates, which may result from interference by the attached intein-CBD domains. These results strongly suggest that FT85 alone, in the absence of other interacting proteins, is sufficient for fucosylation of Skp1.

The FTase Activity Maps to the C-terminal Domain of FT85—Because FT85 appears to be a multidomain protein, its N- and C-terminal regions were separately expressed in the FT85 mutant strain HW260 to determine whether either was able to reconstitute FTase activity. Coding DNA for these regions was amplified using PCR as described in Fig. 4C and cloned into a modified version of a previously described constitutive expression vector (see “Experimental Procedures”). Plasmids were electroporated into HW260 cells and transfectants were selected in 5 μg/ml G418. After 2 weeks of growth, S13 extracts were prepared and assayed for FTase activity using the pNP-LNB assay described above. Extracts from cells transfected with pVFT85C encoding the C-terminal domain exhibited a 7-fold higher level of activity than wild-type Ax3 cells (Fig. 9C). The higher activity level seen in the pVFT85C-transfected extracts was probably because of multicopy integration of pVFT85C in some of the cells and the higher activity of the plasmid’s discoidin promoter compared with the endogenous FT85 promoter. In contrast, extracts from parental HW260 cells and cells transfected with pVFT85N exhibited negligible activity. Similar results were seen in independently transfected cultures. In contrast, extracts expressing the C-terminal domain exhibited only very low activity with respect to Skp1(HL250). Thus the C-terminal domain of FT85 appears to contain the catalytic domain of the FTase, but another part of the protein is required to efficiently fucosylate Skp1.

Light Scattering Properties of FT85 Mutant Cells—FT85 mutant HW260 and normal Ax3 cells proliferated at similar rates in HL-5 growth medium and developed similar fruiting bodies (data not shown). However, HW260 cells appeared larger under
FIG. 6. Family 2 GTase sequence motifs in FT85. Sequences from five prokaryotic Family 2 GTases (in blue) are aligned to show the regions of greatest similarity (motifs A–D). In red are compared sequences from the N- and C-terminal domains of FT85, and two predicted eukaryotic Family 2 GTases. An initial alignment generated by Clustal was manually refined to optimize regions of identity or similarity across 20 enzyme sequences in this group. To facilitate comparison, hydrophobic residues are green, positively charged residues are dark red, negatively charged residues are blue, and Pro and Gly are bright red; residues that are identical in the majority of sequences are in bold, and residues that are similar in the majority of sequences are highlighted. As in hydrophobic cluster analysis, A, V, L, I, M, F, Y and W are considered similar; the other similarity groups consist of structure-breaking residues (P, Q), small residues (G, C, A, S, T), negatively charged residues or amides (D, E, N, Q), and positively charged residues (K, R). Asterisks refer to positions cited in the text that are occupied by highly conserved Asp residues. The prokaryotic sequences include a GTase involved in O-antigen synthesis (45) in Vibrio cholerae, a hypothetical GTase from Hemophilus influenzae, SpA, involved in glycosylation of the spore coat of B. subtilis (40), ExoM, a β1,4-glucosyltransferase (41) from S. meliloti involved in sucrose operon synthesis, and a β1,3-GalTase (46) from Campylobacter jejuni. The eukaryotic sequences include, in addition to the two domains of Dictyostelium FT85, hypothetical GTases from C. elegans and A. thaliana. Numbers denote positions relative to the N terminus, or number of amino acids (in parentheses) between the motifs.

A. PCR of genomic DNA

B. Western blot analysis

FIG. 7. Molecular characterization of the FT85 disruption strain. A. DNA from strains Ax3 (normal) and HW260 (FT85-1) was amplified by PCR using primers P9 and P10 and separated on a 1% agarose gel. The length of the PCR product from HW260 DNA was longer by 1.4 kilobases, corresponding to an insertion of the bar locus. As the shorter product from Ax3 DNA amplified more efficiently, lanes 3 contains 10% the amount of DNA amplified in lanes 2 and 4. B, as an indication of Skp1 FTase activity in vivo, the mobilities of Skp1s from HW260, Ax3, and HL250 (GDP-Fuc-) were compared by SDS-PAGE of their S13 fractions followed by Western blotting using mAb 3F9 (Ref. 47; upper panel). HW260 and HL250 Skp1s migrated slightly more rapidly than Ax3 Skp1, consistent with the absence of three sugars from Skp1(HL250). The slight apparent difference between Skp1s from HW260 and HL250 can be explained by a variation in total protein migration between the two lanes, as determined by postblot staining of the same gel with Coomassie Blue (lower panel).

FIG. 8. Fucosylation in FT85 mutant cells. Ax3 (normal) and HW260 (mutant) cells were metabolically labeled with [3H]Fuc. A, P100 fractions were prepared from cells labeled in HL-5 and separated on an SDS-PAGE gel run from left to right, which was fixed and cut into 54 slices to measure incorporation of label. B, S100 fractions from labeled cells starved in KP buffer were compared in the same manner. The position of a M, 20,100 marker is shown.

DISCUSSION

FT85 Is the Skp1 FTase—FT85 was originally implicated as the Skp1 FTase based on its chromatographic copurification with the enzyme activity (Fig. 1) and comigration on SDS-PAGE gels with a protein that could be photoaffinity-labeled with the donor substrate analog GDP-hexanolamine-azido-125I-salicylate (9). Tryptic peptides from gel purified FT85 were used to design degenerate oligonucleotides for use in PCR amplifications that ultimately led to the identification of FT85 genomic DNA. The in-gel digest Q-TOF-based proteomics strategy was shown to be particularly powerful here, where sample quantity was small and there is a law of diminishing returns to consider in deciding on further protein purification. Provided that sufficient doubly or triply charged peptides are studied in the tryptic mixture generated, there is a good probability, as illustrated here (Figs. 2, 3), of generating sufficient de novo sequence to initiate primer studies, and also of identifying the presence of contaminating proteins of known sequence. The unknown FT85 sequences determined were estimated to be at the few tens of femtomole level. In addition to generating de novo the peptide sequences for the
initial primer studies, the MS-MS sequence analysis confirmed 25% of the predicted amino acid sequence of FT85.

To confirm that FT85 was required for Skp1 fucosylation, the FT85 locus was targeted by insertion of the blasticidin S resistance marker into codon 276 by homologous recombination (Figs. 4B and 7A). As a result, Skp1 was expressed at a slightly lower apparent Mr (Fig. 7B), consistent with the absence of the terminal fucose-dependent trisaccharide. Metabolic labeling of

**Fig. 9.** FTase activity in FT85 mutant and recombinant FT85-expressing cell extracts. *A*, soluble (S13) extracts were prepared from normal and mutant cells, and equal amounts of protein (~0.5 mg) were incubated with 1.2 μM GDP-[3H]Fuc and 0.36 mM pNP-LNB, 0.6 μM Skp1(HL250), or no substrate for 1 h. Incorporation of radioactivity into pNP-LNB was determined using the C18-SepPak assay. Incorporation of radioactivity in the Skp1 reactions was assayed using the TCA assay. *B*, equal amounts of protein from S13 extracts of IPTG-induced *E. coli* expression strains were incubated for 2 h with substrates as in *A* (except Skp1(HL250) was at 0.3 μM). Incorporation into pNP-LNB was assayed as in *A*, and incorporation into Skp1(HL250) was assayed using the SDS-PAGE method. Data values shown represent the difference between + and − substrate and are representative of two independent trials. Note that the value for *E. coli* expressing recombinant CBD-FT85 is off-scale. CBD, chitin-binding domain; MBP, maltose-binding protein; C, S13 extracts from *Dictyostelium* strain Ax3 (labeled A3), strain HW260 (labeled 0), or HW260 cells transfected with either pVFT85N (N-a, N-b; 2 transfections) or pVFT85C (C-a, C-b; 2 transfections), encoding the N- or C-terminal domain of FT85 as shown in Fig. 4, were assayed for FTase activity as in panel *A* using pNP-LNB or Skp1(HL250). Incorporation into Skp1 was assayed using the TCA assay. Values from controls lacking added substrate have been subtracted. Data shown are averages of two experiments.
residues in motif B (see asterisks), a DXD-like motif preceded by hydrophobic residues in motif C, and a predicted catalytic aspartate in the C-terminal half of the domain (motif D). Sequences related to motifs A–D, with a similar spacing in the protein, can also be recognized in the C-terminal domain downstream of the Asn-rich stretch (Fig. 6). Motifs A–C are characteristic of the NRD2 domain (27) seen in GTase families 2, 23, and 27 according to the scheme of Henrissat and co-workers (23) (afmb.cnrs-mrs.fr/~pedro/CAZY/gtf_2.html). The NRD2 region comprises a half-domain that contains most of the residues that contact the sugar nucleotide donor, based on diffraction studies on SpsA from Bacillus subtilis (40), and most-site-specific mutagenesis of ExoM from Sinorhizobium meliloti (41). These similarities suggest that FT85 contains two domains that are each related to a large family of inverting GTases from eubacteria, archeabacteria, and eukaryotes, which can now be confirmed by site-specific mutagenesis of the asterisked residues in Fig. 6. Recent structural data suggest that these GTases belong to an ancient superfamily that includes GlcNAc-Tase-T1, β1,4GalTases, α1,3GalTases, and αGlcATases (42). Thus the sequence suggests that FT85 may be a bifunctional GTase, whose architecture resembles that of class 2 hyaluronate synthase (43) and other bifunctional prokaryotic GTases, as depicted in Fig. 4C.

This two-domain model is supported experimentally by the FTase activity studies. Previous biochemical studies showed that during purification, FT85 was susceptible to conversion to a lower Mr form of about 40,000 (FT40), based on gel filtration, that retained substantial activity (9). A time-dependent conversion of FT85 to species with apparent Mr values of 40,000 and 28,000, based on SDS-PAGE, was also observed when the protein preparation was photoaffinity-labeled with GDP-hexanolamine-azido-125I-salicylate. This suggested that the FTase activity of FT85 is mediated by a catalytic domain that may be as small as Mr 28,000, a typical size for catalytic domains of Family 2 GTases. These results are consistent with the present finding that expression of the C-terminal domain, starting immediately prior to the homopolymeric region of 32 Asn residues, reconstitutes in vitro FTase activity with respect to pNP-LNB when overexpressed in FT85 mutant cells (which have no detectable FTase activity). However, this domain exhibits only weak activity with respect to Galβ1,3GlcNAc-Skp1 (Skp1(HL250)), suggesting that another part of the protein is important for efficient recognition of a protein substrate. Recently, we have obtained evidence that purified FT85 and recombinant FT85 also encode a UDP-Gal:Skp1 β1,3-galactosyltransferase activity, and that this activity is absent from FT85 mutant cells. This activity is reconstituted by expressing the N-terminal but not the C-terminal portion of FT85. Thus the C-terminal region of FT85 downstream of the polyasparaginase stretch contains the catalytic domain of the FTase, but this region of the protein may act in concert with the rest of the protein for efficient processing of Skp1.

**Relationship to other FTases**—The specificity of the FT85 FTase for the 2-O position of Gal in Galβ1,3HexNAc disaccharides is remarkably similar to that of the human Secretor α1,2-FTase (9). The length of the proposed FTase catalytic domain of FT85, about 270 residues, is similar to that of the Se-FTase, about 330 residues. However, FT85 differs from the Se- and other mammalian α1,2-FTases in its 1) requirement for a divalent cation and a reducing agent for FTase activity in vitro, 2) greater affinities for its donor and acceptor substrates in vitro, and 3) compartmentalization in the cytoplasm rather than the Golgi lumen and absence of a membrane anchor.

**Domain Organization of FT85**—The PCR studies led to a 2304-base pair open reading frame that conceptually encodes a 768 amino acid protein with a predicted Mr of 89,735 (Fig. 5), comparing favorably with FT85’s apparent Mr of 85,000, based on SDS-PAGE (Fig. 1). Near the middle of the protein, at positions 409–441, lies a nearly homopolymeric stretch of Asn residues that might separate FT85 into two domains. BLAST analysis suggests that the N-terminal 260 amino acids is homologous to the full-length of numerous Family 2 GTases, and this is supported by the higher level of similarity at positions that are most conserved within the family. This includes a characteristic hydrophobic region at the very N terminus (motif A in Fig. 6), a pair of aspartates preceded by hydrophobic variations indicate that total absence of fucosylation has additional tentatively similar changes were seen in HL250 cells, though quantitative (normal), HL250 (GDP-Fuc

FIG. 10. Light scattering properties of FT85-mutant cells. Ax3 (normal), HL250 (GDP-Fuc–), HW260 (FT85 mutant), HW261 (FT85 mutant), and HW262 (FT85 mutant) cells were compared during logarithmic growth in HL-5 for their light scattering properties at 488 nm. Mutant), and HW262 (FT85 mutant) cells were compared during loga-

H. van der Wel and C. M. West, unpublished data.
domain (see below). Furthermore, several sequence motifs that are highly conserved in prokaryotic, microbial, and mammalian 1,2-FTases (38) are not recognizable in FT85. However, further study is required to determine whether the Skp1 FTase is related to known 1,2-FTases at the structural level.

Relationship to Other Family 2 GTases—Family 2 GTases catalyze the transfer of many kinds of sugars, including Glc-NAc, GalNAc, Glc, Gal, Rha, GlcA, abequose, and alrlose, from either purine and pyrimidine sugar nucleotides by a mechanism that involves inversion of the anomeric sugar linkage. Genetic analysis of prokaryotic Family 2 GTases suggests that they modify membrane-associated lipid-linked precursors that are oriented toward the cytoplasmic compartment (44). These GTases associate either directly or indirectly with the plasma membrane via their C termini. The product glycolipids are subsequently translocated across the plasma membrane to the extracellular space where they contribute to cell wall layers, capsules, and exopolysaccharides. The Family 2 GTase domain is also embedded in cytoplasmically exposed regions of polytopic membrane proteins that polymerize and translocate cellulose, colloso, chitin, or hyaluronan across the plasma membrane in prokaryotes and eukaryotes (15–17). In eukaryotes, this domain is also found in DOL-P-man synthase, Dol-P-Glc synthase, and ceramide glucosyltransferase; all enzymes that are oriented toward the cytoplasm and whose products are then translocated across the membrane of the rER or Golgi (11–13). The FT85-FTase has conserved the cytoplasmic compartmentalization associated with this family, but it is not apparent associated with a membrane and its product is not translocated across a membrane. This evolutionary advancement has exposed a large, new set of cytoplasmic and nuclear enzymes to potential complex O-linked glycosylation.

BLAST and motif searches of public domain data bases suggest that the protein predicted to encode by these putative genes similar to the Skp1 FTase may exist in other eukaryotes. The proteins predicted to be encoded by these putative genes lack motifs for targeting to the rER or other membranes and are not homologous to DOL-Hex synthases, ceramide glucosyltransferases, cellulose synthases, chitin synthases, or hyaluronan synthases. Examples of two of these putative genes, from Caenorhabditis elegans and A. thaliana, are shown in the alignment in Fig. 6. These sequences are not similar enough to suggest that they are FT85 orthologs. Another enzyme in the Skp1 glycosylation pathway, the Skp1 GlcNAcTase (8), is also related to Family 2 GTases in its NRD2 domain.

Role in Cell Physiology—Glycosylation is important for nuclear accumulation of Skp1 bound to insulin-like growth factors. This may affect the binding to the Abbreviation of the Flavonoid Genes (1, 2). Acknowledgments.—We thank J. Brandon Barker for his inspired efforts in purifying FT85. We are grateful to Melissa Chen and Neal Benson for their excellent assistance with flow cytometry. Frantsisk Puta generously provided pBsR519.

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A Non-Golgi α1,2-Fucosyltransferase That Modifies Skp1 in the Cytoplasm of Dictyostelium
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