Purification and Characterization of an α1,2-L-Fucosyltransferase, Which Modifies the Cytosolic Protein FP21, from the Cytosol of Dictyostelium*

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A novel fucosyltransferase (cFTase) activity has been enriched over 10^6-fold from the cytosolic compartment of Dictyostelium based on transfer of [3H]fucose from GDP-[3H]fucose to Galβ1,3GlcNAcβ-paranitrophenyl (paranitrophenyl-lacto-N-bioside or pNP-LNB). The activity behaved as a single component during purification over DEAE-, phenyl-, Reactive Blue-4, GDP-adi-pate-, GDP-hexanolamine-, and Superdex gel filtration resins. The purified activity possessed an apparent Mr of 95 × 10^3, was Mg^{2+}-dependent with a neutral pH optimum, and exhibited a K_m for GDP-fucose of 0.34 μM, a K_m for pNP-LNB of 0.6 mM, and a V_max for pNP-LNB of 620 nmol/min/mg protein. SDS-polyacrylamide gel electrophoresis analysis of the Superdex elution profile identified a polypeptide with an apparent Mr of 85 × 10^3, which coeluted with the cFTase activity and could be specifically photolabeled with the donor substrate inhibitor GDP-hexanolaminyl-azido-125I-salicylate. Based on substrate analogue studies, exoglycosidase digestions, and co-chromatography with fucosylated standards, the product of the reaction with pNP-LNB was Fucα1,2Galβ1,3GlcNAcβ-pNP. The cFTase preferred substrates with a Galβ1,3 linkage, and thus its acceptor substrate specificity resembles the human Secretor-type α1,2-FTase. Afucosyl isoforms of the FP21 glycoprotein, GP21-I and GP21-II, were purified from the cytosol of a Dictyostelium mutant and found to be substrates for the cFTase, which exhibited an apparent K_m of 0.21 μM and an apparent V_max of 460 nmol/min/mg protein toward GP21-II. The highly purified cFTase was inhibited by the reaction products Fucα1,2Galβ1,3GlcNAcβ-pNP and FP21-II. FP21-I and recombinant FP21 were not inhibitory, suggesting that acceptor substrate specificity is based primarily on carbohydrate recognition. A cytosolic location for this step of FP21 glycosylation is implied by the isolation of the cFTase from the cytosolic fraction, its high affinity for its substrates, and its failure to be detected in crude membrane preparations.

The glycosylation of proteins traversing the secretory pathway of eukaryotic cells has been well studied (1–3). Evidence has been steadily accumulating that some amino acid side chains of proteins of the cytosol and nucleoplasm are also modified, with either a single sugar, O-GlcNAC (3, 4), or oligosaccharides (5–9). The frequency of O-GlcNAC modifications of cytosolic and nucleoplasmic proteins may approach that of phosphorylation (10), and there is evidence that cytosolic glycosylation may be regulatory (5, 8, 10–12), based on the existence of glycoforms which vary with different physiological states and protein localizations, the presence of specific cytosolic glycosidases, half-lives of sugar modifications which vary relative to the host protein, and potential competition for attachment sites between O-GlcNAC and phosphate groups.

FP21 is a protein found both in the cytosol and the nucleus, and is modified by an oligosaccharide in the cellular slime mold Dictyostelium discoideum (7, 8). FP21 is a highly conserved protein whose amino acid sequence shows 68% identity, 78% similarity, and one amino acid residue difference in length between Dictyostelium and humans. FP21 has been detected in the cydin A/cdcK2 complex associated with the G1 checkpoint of the human Hela cell cycle (13), the kinetochore complex (14) of budding yeasts, and in association with certain membranes of Dictyostelium. FP21 is particularly high abundance in the inner ear organ of Corti (15–17). An FP21 gene is present in a green alga virus genome (18), and two copies are frequently found in eukaryotic genomes, including Dictyostelium. Dictyostelium FP21 possesses a single tetra- or pentasaccharide, which appears to contain Gal, Xyl, and Gal (8) and is probably O-linked based on susceptibility to release by mild base (7) or hydrazinolysis. Two FP21 isoforms have been purified which vary in their proportions of Xyl and Gal (8). Since a GDP-Fuc synthesis mutant in Dictyostelium exhibits slow growth which can be partially rescued by exogenous Fuc, we have investigated the fucosylation of FP21 for its possible involvement in this phenotype. Using afucosyl-FP21 from the fucosylation mutant as an acceptor substrate, an α-cFTase (cytosolic fucosyltransferase) activity dependent upon GDP-β-Fuc was detected in the cytosolic S100 fraction but not in the particulate or membrane fraction (7). FP21 accounted for >80% of the acceptor activity in crude S100 extracts of the

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1 P. Hieter and C. Connelly, personal communication.
2 C. M. West and H. van der Wel, unpublished data.
3 E. Kozarov and C. M. West, unpublished data.
4 The abbreviations used are: Fuc, L-fucose; pNP-LNB, paranitrophenyl β-lacto-N-bioside; GDP-Fuc, GDP-β-L-fucose; GDP-β-ASA, GDP-hexanaminyl-4-azidosalicylic acid; cFTase, cytosolic fucosyltransferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; GlcNAc, N-acetyl-D-glucosamine; Xyl, D-xylose; Gal, D-galactose; HPLC, high performance liquid chromatography; CHAPS, 3-(cyclohexylamino)propanesulfonic acid; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid; Bis-Tris propane, 1,3-bis[tetrahydroxy methyl]methyamino]propane; CHAPS, 3-[3-cholamidopropyl]dimethyammonio]2-hydroxy-1-propanesulfonic acid.

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fucosylation mutant, as if it were the primary acceptor substrate for this enzyme. This activity was also able to attach Fuc to lacto-N-biose (Galβ1,3GalNAcβ1) attached to a hydrophobic aglycone moiety, as determined by cross-competition studies. This enzyme activity appeared to mediate attachment of a peripheral Fuc, and was novel in terms of its apparent cytosolic, rather than Golgi, compartmentalization, and its submicromolar $K_m$ for GDP-Fuc. As shown here, a 1.2-million-fold purified preparation of the enzyme activity retains its high affinity for GDP-Fuc, displays a high affinity for afucosyl FP21, links Fuc in an α1,2 linkage to a β1,3-linked Gal on type 1 and 3 acceptors, and copurifies with a polypeptide which can be photoaffinity-labeled with a donor substrate analog.

**EXPERIMENTAL PROCEDURES**

**Materials**

GDP-Fuc (fucose-1-(H) (6.47 Ci/mmol) and carrier-free Na$^{23}$I were from DuPont NEN. Unlabeled GDP-β-Fuc was from Accurate or Sigma. The following were from Sigma: ATP, 3,5-cyclic GMP, GDP, 2-deoxy-GDP, GTP, and other nucleotides and nucleosides, guanine, guanosine 5'-diphosphate adenosine 3'-5'-cyclic, Octyl glucoside, sodium succinate, sodium alanine, and sodium chloride. Bovine serum albumin, yeast alcohol dehydrogenase, sweet potato arylsulfatase, tryptophanase, L-fucose, cytochrome c, and xanthine oxidase, were from Sigma. Other chemicals were the highest grades commercially available.

**Buffers**—Buffers were brought to their pH at room temperature, degassed, and then chilled. DTT or protease inhibitors were added from frozen stock solutions immediately prior to use. Buffer A was 50 mM Tris-HCl (pH 7.4), 0.25% sucrose, 10 μg/ml leupeptin, 10 μg/ml aprotonin. 50 mM phenylmethylsulfonyl fluoride (added fresh). Buffer B was 50 mM HEPES-NaOH (pH 7.4), 15% (v/v) glycerol, 5.0 mM MgCl$_2$, 0.10 mM Na$_2$EDTA, 1.0 mM DTT. Buffer C was 50 mM HEPES-NaOH (pH 7.4), 15% (v/v) glycerol, 0.25 mM Na$_2$ATP, 10 mM MgCl$_2$, 0.10 mM Na$_2$EDTA, 1.0 mM DTT. Buffer D was 50 mM HEPES-NaOH (pH 7.4), 15% (v/v) glycerol, 0.10 mM NaCl, 5.0 mM MgCl$_2$, 0.10 mM Na$_2$EDTA, 1.0 mM DTT.

**CFTase Assay**—Assay—CFTase activity was usually assayed as the transfer of [1-14C]GDP from GDP-[1-14C]Fuc to pNP-LNB, which was determined by adsorption onto a C$_8$ Sep-Pak cartridge followed by elution with methanol (20). GDP-Fuc was at a concentration 3-fold above its $K_m$ value, and pNP-LNB was at slightly below its $K_m$ value. Each assay tube routinely contained 9 μg of pNP-LNB (equivalent to a final concentration of 0.36 mM) dried from a 1 mg/ml aqueous solution in a 1.5-mL polypropylene microcentrifuge tube by vacuum centrifugation, 5 μl of 20 mg/ml BSA in buffer D, and 40 μl of enzyme sample diluted, if appropriate, in buffer D. During pilot studies, column fractions were assayed after desalting on PD-10 columns (Pharmacia) elicited in buffer D. These columns contain 9.1 ml of Sephadex G-25 medium. The reaction was initiated by the addition of 5 μl of a mixture prepared in buffer H and containing, after dilution to 50 μl, 1.0 μM GDP-Fuc (1–8 × 10$^9$ dpm), prepared from a mixture of GDP-[1-14C]Fuc and unlabeled GDP-Fuc, 15 mM MgCl$_2$, 2.0 mM MnCl$_2$, 0.10 mM EDTA, 1.0 mM DTT, and 15% glycerol. The mixture was stable for >3 days at 0°C; however, it was not stored beyond 1 day. Aliquots were frozen and thawed once, and thus divalent cation-promoted degradation of GDP-Fuc (21) did not occur. When assaying crude cell extracts and DEAE column fractions, the mixture was supplemented with sodium fluoride and ATP, at final concentrations in the assay mixture of 2.0 mM and 1.0 mM, respectively. Assays of crude extracts containing sucrose lacked glycerol. Reactions containing enzyme preparation A were supplemented with 0.05% Tween 20. The reaction mixture was incubated for 10–120 min at 30°C, and stopped by the addition of 1 ml of cold water. The reaction mixture was immediately filtered over the C$_8$ Sep-Pak cartridges, or frozen at −80°C until filtration. Cartridges were reused until flow rates became unacceptable. Samples were filtered in groups of 10 on a Visiprep manifold (Supelco), which was alternated between a waste and a receptor container set to 2 ml and 20 ml, respectively.

**Incorporation**—Incorporation was determined as described previously (7).

**Reactions involving protein acceptor substrate** were conducted similarly, except that protein acceptors were introduced from concentrated stock solutions. Tween 20 was present at 0.05% (v/v). BSA was reduced to 0.5 mg/ml, and the reaction was initiated by the addition of the cFTase. Incorporation into protein substrates was determined after SDS-PAGE of the samples, within 1 day of fixation with Coomassie Brilliant Blue and destaining, by excising the appropriate region of the gel and scintillation counting, as described (7).

**Protein Determination**—Protein concentration was determined by a commercial modification of a Coomassie Blue dye binding method (22), or from A$_{280}$ values, assuming an extinction coefficient of 1 ml/mg protein for a 1-cm path length.

**Cell Lysis**—1–2 × 10$^{11}$ cells were grown to near maximum cell density. After washing once in ice-cold water, cells were resuspended in buffer A, suctioned through a bed of glass wool, and lysed by forced passage through a 47-mm diameter Nudelnep filter with 5-μm diameter pores mounted on the end of a 60-ml syringe. The lysate was centrifuged at 4,000 × g for 2 min, and the supernatant was centrifuged at 100,000 × g for 60 min. All procedures were performed at 0–4°C.

**DEAE-Sepharose Fast Flow Chromatography**—The final S100 supernatant was pumped onto a 450-ml DEAE-Sepharose Fast Flow column (4.8 × 28 cm) pre-equilibrated in buffer B at 250 ml/h, and the column was washed with buffer B until the A$_{280}$ dropped below 1% of its initial value. dTase was eluted by a linear gradient of 50 mM NaCl, consisting of 1.25 l of starting buffer B and 1.25 l of limit buffer C. Protein was determined based on A$_{280}$ or the Coomassie Blue dye binding assay. Fractions from the main activity peak were pooled and centrifuged at −80°C.

**Phenyl-Sepharose 6 Fast Flow Chromatography**—DEAE pools from 1.2 ml of cells were thawed and pumped onto a 175-ml phenyl-Sepharose 6 Fast Flow (high-sub) column (2.6 × 31 cm) at a flow rate of 275 ml/h. The column was washed with buffer D until the A$_{280}$ dropped below 1% of its maximum. dTase was eluted by application of a linear gradient of 1 ml of buffer D and 400 ml of limit buffer E. Protein was determined based on A$_{280}$ and the Coomassie Blue dye binding assay. dTase activity was determined after 5-fold dilution of fractions with buffer D. Fractions from the activity peak were pooled and concentrated 20-fold by ultrafiltration using a PM-30 membrane, and then diluted 5-fold in buffer D.
to reduce ethylene glycol to -10% (v/v). The DEAE and phenyl columns were reused after in situ washing with 8 m urea and 0.1 M NaOH.

Dye Column Affinity Chromatography—The phenyl-Sepharose pool was diluted to 10% (v/v) ethylene glycol with buffer D and pumped onto a 25-ml column (1.6 × 25 cm) containing Reactive Blue-4 coupled to cross-linked 4% agarose beads (5.2 mg dye/ml), which had been sequentially precycled with 0.5 mM GDP-methanolamine buffer in buffer D, buffer F, buffer D, and 10% (v/v) ethylene glycol in buffer D. After sample loading, the column was washed in buffer D until the A280 dropped below 1% of its maximum, and eluted with a linear gradient of 0.1-1.5 M NaCl, consisting of 60 ml of starting buffer D and 60 ml of limit buffer F. Protein was determined based on A280, and the Tase activity was determined after 5-fold dilution with buffer D. Fractions from the activity peak were pooled, concentrated 2-3-fold on a Centricon-30 centrifugal concentrator, and diluted with buffer B to a final calculated concentration of 0.1 M NaCl.

GDP Affinity Chromatography—The Reactive Blue-4 pool was passed over a 1.2-ml GDP-adipate-agarose (1.6 μmol GDP/ml packed resin) column connected in series with a 4.0-ml GDP-hexanolamine-agarose (2.8 μmol of GDP/ml of packed resin) column. The GDP moiety of GDP-adipate is linked via the 2′- or 3′-hydroxyl of the ribose, whereas the GDP moiety of GDP-hexanolamine is attached via its β-phosphate, which corresponds to the linkage with Fuc in the native donor substrate GDP-Fuc. After washing in buffer D, the GDP-hexanolamine column was eluted with a 0.2-gradient of GDP from buffers D and G. 97% of the activity was eluted before 1 mg GDP as determined by HPLC gel filtration, and was pooled and concentrated to <1 ml in a Centricon-30 concentrator.

Superdex 200 Gel Filtration—The concentrated GDP-hexanolamine pool was applied to a 125-ml packed Superdex 200 HR 16/60 (Pharmacia) column (1.6 × 60 cm) equilibrated at 22 °C with buffer D at 0.8 mM/lmin on an LKB GTI HPLC system. Fractions were supplemented with 0.05% (v/v) Tween 20 from a 10% stock solution to stabilize activity, and stored at 4 °C. Active fractions were pooled, concentrated in a Centricon-30 ultrafiltration device, and frozen at -80 °C. M. values were calibrated based on horse spleen apoferritin (443,000), sweet potato β-amylase (200,000), yeast alcohol dehydrogenase (150,000), bovine carbonic anhydrase (58,000), and lysozyme (14,300).

The final Superdex 200 activity pool, purified 1.2 × 10-fold, is referred to as preparation A. Activity purified 317-2200-fold through DEAE, phenyl, and Superdex resins is referred to as preparation B, activity purified 106-fold through DEAE, phenyl, and Reactive Blue-4 resins is referred to as preparation C, and activity purified 27-4 fold through the DEAE-resin is referred to as preparation D.

Fucosyltransferase Characterization

Photoaffinity Labeling—GDP-hex-ASA, generously provided by Eric Hedmes, was iodinated in its salicylate moiety as described (23). Superdex 200 column fractions, 30-300 μl, were preincubated in a quartz 1-ml cuvette for 15 min in dim red fluorescent illumination in the presence of 30 μg/ml Reactive Blue-4-2SA-ASA in buffer D. Photolysis was effected by illumination at 254 nm with a 30-watt lamp, and the activity was monitored at 37 °C and 420 nm, and loss of fucosidase activity was verified by sequencing of the plasmid DNA (8). Expression host Escherichia coli BL21(DE3) cells carrying a lysogen with a copy of the T7 RNA polymerase transcription element, such that an oligo-His tag was introduced at the N-terminus. The deduced NH2-terminal sequence of rP21 is MGGHHHHHHHHSSSGHHHDDDDKHMLED following by the natural FP21 sequence, which was verified by sequencing of the plasmid DNA (8). Expression host E. coli BL21(DE3) cells carrying a lysogen with a copy of the T7 RNA polymerase gene under lacUV5 control were transformed under carbenicillin selection. After induction with isopropyl-thio-β-D-galactopyranoside, preparing colonies were examined. Incubation cultures were not observed. rP21 was isolated from the T7 RNA polymerase under nonde-naturing conditions using an affinity column consisting of nickel cations immobilized on Sepharose 6B, essentially as described (29). Purified protein was exhaustively dialyzed against 50 mM HEPES-NaOH (pH 7.4), and concentrated in Centricon prep centrifugal ultrafiltration concentrators (10-kDa molecular mass cut-off). Protein concentration was determined by amino acid composition analysis of phenylisothiocyanate-determined after acid hydrolysis, using norleucine as an internal standard (26).

Preparation of recombinant Dictyostelium FP21—The open reading frame of FP21 was amplified using Dictyostelium DNA as the template and fp15 and fp14 as primers (see Table IV in Ref. 8) in a polymerase chain reaction. These primers contained BamHI restriction sites which were used to clone the amplified DNA into a BamHI restriction site of the expression vector pET19b (Novagen, Madison, WI), downstream of the T7 RNA polymerase transcription element, such that an oligo-His tag was introduced at the NH2-terminus. The deduced NH2-terminal sequence of rP21 was MGHHHHHHHHSSSHGGHDDDDKHMLED following by the natural FP21 sequence, which was verified by sequencing of the plasmid DNA (8). Expression host E. coli BL21(DE3) cells carrying a lysogen with a copy of the T7 RNA polymerase gene under lacUV5 control were transformed under carbenicillin selection. After induction with isopropyl-thio-β-D-galactopyranoside, preparing colonies were examined. Incubation cultures were not observed. rP21 was isolated from the T7 RNA polymerase under nonde-naturing conditions using an affinity column consisting of nickel cations immobilized on Sepharose 6B, essentially as described (29). Purified protein was exhaustively dialyzed against 50 mM HEPES-NaOH (pH 7.4), and concentrated in a centrifugal ultrafiltration concentrator (10-kDa molecular mass cut-off). Purified protein (Fig. 7) was shown to be recognized in a Western blot by monoclonal antibodies 3F9 and 4E1, and anti-FP21 (68–82)/L97 (8), confirming its identity as rP21. Protein concentration was determined by amino acid composition analysis (see above).

RESULTS

Pilot Studies on Partially Purified Enzyme—Pilot studies on partially purified enzyme established the conditions of the standard assay. After DEAE-ion exchange chromatography (27-fold purified; preparation D) or a combination of ion ex-
change chromatography, phenyl-Sepharose hydrophobic interaction chromatography, and gel filtration (2200-fold purified; preparation B) enzyme activities using pNP-LNB as substrate were linear with respect to time (0–48 h) and protein concentration (data not shown). 70% of the activity maximum at pH 7.2 was retained over a broad pH range of 5.5 to 9.0, beyond which activity dropped rapidly. The activity was similar in Tris, Bis-Tris propane, MES, HEPES, or CAPSO buffers. Activity was optimal at concentrations of NaCl from 100 to 300 mM, but was stable for short times at concentrations up to at least 1.5 mM NaCl, or (NH₄)₂SO₄ at 20%. The enzyme was significantly less active or less stable in KCl. Of the divalent cations tested, as chlorides of Mg²⁺, Mn²⁺, Ca²⁺, Zn²⁺, Fe²⁺, and Co²⁺, only MgCl₂ and MnCl₂ supported activity. 0.5–2.0 mM Zn²⁺, Fe²⁺, and Co²⁺ inhibited activity from 50 to 95% in the presence of 0.5 mM MgCl₂. Optimal activity occurred in 15 mM MgCl₂ and 2.0 mM MnCl₂. Sodium fluoride and ATP stimulated activity 20–40% in crude extracts but inhibited activity 10–30% in purified preparations, and thus were included only in the former preparations. Activity was inhibited 47% by 1.0 mM N-ethylmaleimide, and only 18% by 1.0 mM pyridoxal-5-phosphate, using 0.27 μM GDP-Fuc and 0.36 μM pNP-LNB as substrates, suggesting that the active site may contain an essential cysteine (36). Glycerol (up to 30% (v/v)), 1 mM DTT, and 0.1 mM EDTA (in the presence of a concentration excess of Mg²⁺) did not inhibit activity, and thus were included as potential stabilizing agents. NaN₃ at 0.02% (w/v) led to a 25% reduction in activity and so was not used. Optimal temperature was 30 °C, with lower activities detected at 37 and 24 °C. These properties of the partially purified enzyme activity were similar to those previously determined for the unfractionated activity (7).

To test the effect of detergents, enzyme purified through the Reactive Blue-4 step (preparation C) was diluted 100-fold in buffer D and incubated in varying concentrations of 16 detergents as described under “Experimental Procedures.” Only Tween 20 and Tween 80 sustained activity at all concentrations tested. Activity losses ranged from 30 to 99% for the other detergents; in general, greater losses were observed as detergent concentrations diminished below the critical micelle concentration. At later stages of purification, 0.05% Tween 20 was superior to BSA in preserving enzyme activity at 4 °C.

Enzyme Purification—cFTase present in the S100 fraction was remarkably stable, with 90% activity remaining after 10 days at 4 °C, or after 10 freeze (−80 °C)/thaw cycles. Although activity could be recovered from desalted (NH₄)₂SO₄ precipitates, the broad range (30–70%) over which activity precipitated was not useful (data not shown). Activity quantitatively adsorbed to DEAE resins from the crude extract, and >98% of the eluted activity emerged at about 30 mM NaCl as a monodisperse peak ahead of the major protein peaks (Fig. 1A), resulting in a 25–30-fold purification (Table I). The remaining activity emerged later as a small peak. The Fast Flow resin was superior to DEAE-Sephadex and DEAE-cellulose (fibrous or granular) because of rapid flow, resistance to clogging, and resistance to shrinkage at higher ionic strengths. The column was stable to multiple cycles of use and washing in 0.1 M NaOH. Enzyme also adsorbed at pH 6.8 but enrichment was reduced 2-fold. Enrichment was also diminished by >2-fold if the cell lysate was brought to 5 mM MgCl₂ and centrifuged for 1 h at one-half the g-force, compared to the standard method. As determined by HPLC gel filtration (see below), the enzyme exhibited a single apparent Mₙ of 95 × 10⁶. The pool of the DEAE-purified enzyme was stable to storage at 4 °C and tolerated repeated freeze-thawing.

The DEAE pool adsorbed quantitatively to phenyl-Sepharose 6 Fast Flow (high sub) and >99% of the eluted activity emerged as a single peak with slight trailing close to the end of a 0–60% gradient of ethylene glycol (Fig. 1B). cFTase activity was assayed in eluted fractions after dilution in buffer D, and recovery ranged from 50 to 90%, or 53% for the trial reported (Table I). Quantitative adsorption to phenyl-Sepharose 6 Fast Flow (low sub), or to phenyl Sepharose CL-4B, required addition of 10–20% (NH₄)₂SO₄ to the DEAE pool, and thus these resins were not used.

The pool of phenyl-purified enzyme was concentrated by ultrafiltration. Recovery of activity after concentration varied from 10 to 90%, or 27% in the reported trial (Table I), and activity was not detected in the ultrafiltrate. At this stage of purification, the activity typically displayed an apparent Mₙ value of 95 × 10⁶ after HPLC gel filtration (see below), and thus poor recovery appeared to be associated with insolubility or denaturation. In early trials with the other phenyl resins involving long column residence times, substantial activity with an Mₙ value of 40 × 10⁶ was correlated with poor recovery of activity, as discussed further below.

The phenyl-pool activity adsorbed efficiently to the Reactive Blue-4 dye resin, and 97% of the eluted activity emerged as a monodisperse peak early in a gradient of 0.1–1.5 M NaCl (Fig. 1C). The remaining activity emerged later as a small peak, and overall activity recovery was 92% of Tase activity could also be adsorbed to RB-1, RB-72, RG-5, RR-120, RG-19, and CB dye resins, but the Reactive Blue-4 resin was selected on the basis of more selective binding relative to BSA. RY-3 and RY-86 dye resins did not adsorb activity. Concentration of activity by ultrafiltration resulted in a 33% loss of activity.

The Reactive Blue-4-pool activity was unretarded by GDP-adipate Sepharose CL-4B. 290 μg of protein was recovered from the column after elution with 2 mM GDP, but was not examined further. Activity in the flow-through fraction was, however, quantitatively adsorbed to GDP-hexanolamine Sepharose. The selective binding to GDP-hexanolamine was predicted based on the inhibitor characteristics of a series of GDP-Fuc analogues (see below), and is a behavior typical of mammalian α1,2-FTases (30–33). The GDP-hexanolamine column was eluted with a 0–2 mM GDP gradient, and the activity eluted in the 0–1 mM range of the gradient. 5% additional activity was eluted by various combinations of high GDP and NaCl concentrations, Tween 20 and ethylene glycol. After ultrafiltration, the enzyme activity was stable at 4 °C for at least 1 days.

The GDP affinity pool was applied to a Superdex 200 gel filtration column by HPLC. 95% of the recovered activity eluted as a monodisperse peak (peak I) centered at a Mₙ position of 95 × 10⁶ (Fig. 1D). An additional 3.7% eluted at Mₙ position 40 × 10⁶ (peak II), and the remaining activity eluted near the void volume. In pilot studies, >90% of activity applied to the GDP-hexanolamine column was recovered after gel filtration. The 35% recovery in the reported trial (Table I) did not seem to be due to retention on the column (see above), and may have been due to 3 days storage of the GDP-hexanolamine fractions at 4 °C prior to further processing. After gel filtration, activity decayed with a 12-h half-life at 4 °C, but was stable for 3 days in 0.05% (v/v) Tween 20 at 4 °C. Activity was only partially stabilized by 2.0 mg/ml BSA, and strongly destabilized by purified cytochrome c (data not shown). Finally, activity was stabilized by freezing at −80 °C.

HPLC gel filtration fractions were analyzed by SDS-PAGE and silver staining (Fig. 2). Three major silver-stained bands were visible in the fractions of highest activity (peak I), centered at 30.4-m elution volume. Only the amount of the most

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5 T. Scott-Ward and C. M. West, unpublished data.
heavily stained band, at Mr position of $85 \times 10^3$, correlated precisely with the activity of the fractions. To obtain further evidence that this polypeptide, FT85, was equivalent to the enzyme, selected HPLC gelfiltration fraction were UV-irradiated in the presence of the photoactive donor substrate analogue GDP-hex-125I-ASA, at 30 mM, in the presence or absence of 690 mM GDP-Fuc. Photolabeling under similar conditions has previously been useful in identifying fucosyl- and mannosyl-transferases (23, 35). SDS-PAGE followed by autoradiographic analysis of a fraction from peak I (30.4 ml elution volume) revealed that the most intensely photolabeled polypeptide, PL85, migrated at the position of FT85 (Fig. 3, lane e), and labeling of this band was inhibited by GDP-Fuc (lane f). PL85 was not detected in the fraction eluting at 28.6 ml (lane c), which possessed <0.2% of the peak activity, but was faintly detectable in the fraction eluting at 33.1 ml, which possessed 3.1% of the peak activity (not shown). Two additional, much less intensely photolabeled bands were also detected in the major activity peak at Mr positions 40 and 29 $\times 10^3$, and are discussed below. The relative intensities of these bands is clearer in Fig. 3, lane i, which is a shorter autoradiographic exposure. Multiple minor bands were also photoreactive in lane e, but in each case labeling was only slightly inhibited by GDP-Fuc (lane f), showing that their reactivity was nonspecific and that photoprotection by GDP-Fuc was not due to general UV absorbance by the nucleotide moiety.

The minor cFTase activity peak eluting at 37.7 ml (Fig. 1D, peak II; Mr, position of $40 \times 10^3$) contained a single, specifically-
labeled photoreactive species, referred to as PL40, at an M, value × 10^3 of 40 × 10^3 (Fig. 3, lane g). Negligible PL85 was detectable in this fraction. The amount of PL40 varied with the level of cFTase activity in neighboring fractions (data not shown). PL40 was not detectable by silver staining (Fig. 2, 37.7 ml), as expected based on its low apparent abundance relative to PL85 (Fig. 3, compare lanes e and g). PL40, together with PL29, were also observed in peak I (Fig. 3, lane e). PL40 appears to be a proteolytic fragment of PL85 based on the following observations. 1) The ratio of PL40 to PL85 in the major peak I was time dependent. After 7 days of storage at 4°C, during which activity diminished by >90%, only PL40 could be detected (Fig. 3, lanes k-o). 2) The original photolabeling of peak I described above in Fig. 3 had been performed after 4 days of storage at 4°C, during which activity had decreased by 50%. SDS-PAGE analysis of the pooled peak I fractions at this time revealed that the level of FT85 had decreased concomitant with the appearance of the new bands labeled at FT40 and FT29 (compare Fig. 3, lane a, with Fig. 2, 30.4 ml). Thus the appearance of PL40 in the main activity peak is correlated with the appearance of FT40. 3) Pilot studies on phenyl-purified enzyme showed that cFTase activity became smaller as determined by HPLC gel filtration as activity decreased,5 and that the M, 95 × 10^3 activity peak I was less stable than the M, 40 × 10^3 activity peak II.6 We conclude that PL85 is equivalent to FT85, and that FT85 is susceptible to proteolytic degradation to FT40, which is equivalent to PL40. FT40 possesses similar K_m values for its substrates and similar substrate specificity, but has a reduced specific activity compared to FT85 (see below). This model explains both the time-dependent ap-
pearedance of FT40 in the M, 95 × 10^3 activity peak after gel filtration, as well as the time-dependent appearance of the M, 40 × 10^3 activity peak II before gel filtration.

In summary, the cFTase activity was purified 1.2 million-fold at 4.3% yield. Some of the losses could be attributed to specific proteolysis, which may explain the small proportion of activity (<5%) which could be resolved from the main peak during DEAE, phenyl, Reactive Blue-4, and gel filtration chromatographies. The FT85 polypeptide represents about 30% of the total protein. The cFTase consisted of a single 95 × 10^3 M, activity after the first chromatography step and, aside from inconsistent generation of the apparently proteolytically-derived 40 × 10^3 M, activity, no other molecular species expressing this activity were detected during the purification.

Properties of the Enzyme—Fucosylation of pNP-LNB by the highly purified preparation A was linearly dependent on time and pNP-LNB concentration (data not shown). This preparation exhibited a K_m for pNP-LNB of 0.58 mM at 1 μM GDP-Fuc, compared to 0.73 mM for the unfractionated activity (7), and a V_max of 620 nmol/min/mg protein (Fig. 4). Preparation A exhibited a K_m for GDP-Fuc of 0.34 μM (Fig. 5) at 2.0 mM pNP-LNB, about 4-fold lower than the value obtained for the unfractionated activity (7). The M, 40 × 10^3 form which was generated in some trials exhibited similar kinetic properties, with K_m values for GDP-Fuc of 0.23 μM and for pNP-LNB of 0.77 mM, and was probably a degradation product of the higher M, form (see above).

Donor substrate Analogue—Nucleotides are often potent glycosyltransferase inhibitors and, similarly, GDP was a good inhibitor of the cFTase (Table II). Guanosine, GMP, and GTP were also good, but slightly weaker inhibitors, indicating that the 5'-phosphates are not important. Substituents on the 5'-phosphates are also not important, because GDP-Man and GDP-hex-ASA were good inhibitors. The ribose moiety is significant, because guanine was not a good inhibitor. The 2'-deoxy group of ribose is not required for recognition, but bulky substituents are not tolerated, as 2',3'-isopropylidene guanosine and 3',5'-cGMP were poor inhibitors. Integrity of the ribose ring is essential as the periodate-oxidized 2',3'-dialcohol was ineffective. The identity of the guanine base is important because 5'-phosphates of adenosine were not significantly inhibitory. Furthermore, the guanine moiety does not tolerate substituents at positions 6–8. Similar results were observed for the M, 40 × 10^3 form detected in some trials of preparation B.5 These observations suggested that guanine ribonucleosides modified at the 5'-position of ribose may be useful for purifying and identifying the enzyme. These predictions were validated by the affinity chromatography and photoaffinity results described above.

Acceptor Substrate Analogue—The pNP-LNB acceptor substrate consists of Galβ1,3GlcNAcβ attached to a paranitrophenyl aglycone moiety. pNP was an important recognition determinant as Galβ1,3GlcNAc at a 12-fold concentration of GDP·[3H]Fuc excess inhibited the enzyme by only 50% (Table III). Benzyl-LNB and 8-methoxy carbonyloctyl-LNB exhibited only 15 and 9% of the activity of pNP-LNB at 1 mM. These differences were attributable to changes in K_m or V_max depending on the aglycone (data not shown).

The cFTase preferred a disaccharide acceptor as negligible activity was observed with monosaccharide-pNP substrates. 2-O-methylated or α-L-fucosylation of the nonreducing terminal Gal of pNP-LNB abolished activity (Table IV). The ψ1,3-linkage of the Gal was important for enzyme recognition, as Galβ1,4GlcNAc and Galβ1,6GlcNAc were at best very weak inhibitors (Table III), and Galβ1,4GlcNAc-pNP exhibited only several percent of the activity of pNP-LNB, even above the K_m of pNP-LNB (Table IV). Finally, the enzyme was not specific for the identity or linkage of the penultimate sugar of the disaccharide, as Galβ1,3GalNAcα-benzyl was a good substrate (Table IV).

Characterization of the cFTase Reaction Product—The inability of the cFTase to fucosylate the 2-O-methylated derivative of lacto-N-biase suggested that the enzyme catalyzes the formation of a Fuc1,2Gal linkage (37). To test this possibility, the reaction product of pNP-LNB with preparation B of the enzyme, containing [3H] in its fucosyl moiety, was subjected to digestion with fucosidases. As previously observed (7), Fuc was

![Fig. 4. Kinetic properties of the cFTase with respect to pNP-LNB concentration.](Image)

FIG. 4. Kinetic properties of the cFTase with respect to pNP-LNB concentration. Approximately 125 pg of preparation A was incubated for 1 h with varying concentrations of pNP-LNB, in the presence of 1.0 μM GDP-[3H]Fuc. Inset, double-reciprocal plot of the data, which are fitted to a straight line. The apparent K_m with respect to pNP-LNB was 0.6 mM, and the apparent V_max was 620 nmol/min/mg protein.

![Fig. 5. Kinetic properties of the cFTase with respect to GDP-Fuc concentration.](Image)

FIG. 5. Kinetic properties of the cFTase with respect to GDP-Fuc concentration. Preparation A was incubated with varying concentrations of GDP-[3H]Fuc in the presence of 2.0 mM pNP-LNB for 1 h. Inset, double-reciprocal plot of the kinetic data, which are fitted to a straight line. The estimated apparent K_m with respect to GDP-Fuc was 0.34 μM, and the apparent V_max was 560 nmol/min/mg protein.
readily released with nonspecific mammalian α-fucosidases capable of cleaving Fucα1,2Gal and Fucα1,3/4/6GlcNAc linkages (Table V). α1,2-Fucosidase from A. oxidans released Fuc with similar kinetics, whereas α1,3/4-fucosidase from almond emulsin did not show any activity. Although less almond emulsin fucosidase was present in these reactions, the calculated level of activity was still in vast excess of what would be required to hydrolyze an α1,4-linkage.

The [3H]fucosyl-pNP-LNB reaction product was characterized with pNP-LNB and authentic Fucα1,2Gal and Fucα1,3/4/6GlcNAc linkages (Table V). α1,2-Fucosidase from A. oxidans released Fuc with similar kinetics, whereas α1,3/4-fucosidase from almond emulsin did not show any activity. Although less almond emulsin fucosidase was present in these reactions, the calculated level of activity was still in vast excess of what would be required to hydrolyze an α1,4-linkage.

The [3H]fucosyl-pNP-LNB reaction product was chromatographed with pNP-LNB and authentic Fucα1,2Gal and Fucα1,3/4/6GlcNAc linkages (Table V). α1,2-Fucosidase from A. oxidans released Fuc with similar kinetics, whereas α1,3/4-fucosidase from almond emulsin did not show any activity. Although less almond emulsin fucosidase was present in these reactions, the calculated level of activity was still in vast excess of what would be required to hydrolyze an α1,4-linkage.

| Inhibitor | 0 | 0.02 | 0.05 | 0.1 | 1.0 | 2.0 |
|-----------|---|------|------|-----|-----|-----|
| None      | 1.0 | 0.39 | 0.44  | 0.51 | 0.65 | 0.90 |
| Guanine   | 0.39 | 0.44  | 0.51  | 0.65 | 0.90 | 1.00 |
| GMP       | 0.51 | 0.44  | 0.51  | 0.65 | 0.90 | 1.00 |
| GDP       | 0.73 | 0.50  | 0.25  | 0.27 | 0.90 | 0.09 |
| GTP       | 0.73 | 0.50  | 0.25  | 0.27 | 0.90 | 0.09 |
| GDP-Man   | 0.58 | 0.15  | 0.15  | 0.15 | 0.15 | 0.04 |
| GDP-hex-ASA | 0.60 | 0.15  | 0.15  | 0.15 | 0.15 | 0.04 |
| AMP       | 0.60 | 0.15  | 0.15  | 0.15 | 0.15 | 0.04 |
| ADP       | 0.15 | 0.15  | 0.15  | 0.15 | 0.15 | 0.04 |
| ATP       | 0.15 | 0.15  | 0.15  | 0.15 | 0.15 | 0.04 |
| cAMP      | 0.15 | 0.15  | 0.15  | 0.15 | 0.15 | 0.04 |

**Table IV**

Relative acceptor substrate activities of synthetic disaccharide and monosaccharide conjugates.

| Acceptor substrate | Substrate concentration |
|--------------------|-------------------------|
|                    | 1.0 mM                  | 4.0 mM                  |
| 2-O-Methyl-Galβ1,3GlcNAc-pNP | 0.014 | 0.018 |
| Galβ1,3GlcNAc-pNP | 0.030 | — |
| Galβ1,4GlcNAc-pNP | 0.011 | 0.009 |
| Galβ-ppN | 0.0018 | — |
| Fucβ-ppN | 0.0041 | — |
| Glcβ-ppN | 0.00090 | — |
| GlcNAcβ-ppN | 0.0026 | — |

*Not done.*

**Table V**

Relative activity of the cFTase in the absence of inhibitor. Values are the fractions of activity relative to activity in the absence of inhibitor, for the same enzyme preparation.

| Inhibitor (mM) | 0 | 0.02 | 0.05 | 0.1 | 1.0 | 2.0 |
|---------------|---|------|------|-----|-----|-----|
| None          | 1.0 | 1.0  | 1.0  | 1.0 | 1.0 | 1.0 |
| Galβ1,3GlcNAcβ-pNP | 0.88 | 0.79 | 1.00 | 0.80 | — |
| Galβ1,4GlcNAcβ-pNP | 0.75 | 0.50 | 0.65 | 0.42 | — |
| Galβ1,6GlcNAcβ-pNP | 1.03 | 0.94 | 1.01 | 0.81 | — |
| No inhibitor | 1.00 | 1.00 | 1.00 | 1.00 | — |

GP21-II fucosylation (Fig. 8) estimated an apparent Kₘ for GP21-II of 0.21 μM, and an apparent Vₘₐₓ for 460 nmol/min/mg protein. The apparent Vₘₐₓ of the cFTase for GP21-II is about 75% of the apparent Vₘₐₓ for pNP-LNB, whereas the apparent Kₘ is over 3 orders of magnitude lower than the corresponding value for pNP-LNB. GP21-II fucosylation appeared to exhibit cooperativity with the Lineweaver-Burk plot taping to the same y intercept value and slope as GP21-II at higher substrate concentrations.

Fucosylation of the FP21 polypeptide depended on its glycosylation status. Recombinant Dictyostelium FP21 (pP21), purified under nonnondenaturing conditions from E. coli with a short oligo-His tag at its N terminus (Fig. 7), exhibited no acceptor activity with preparation A (data not shown), consistent with previous evidence that the single Fuc on FP21 is located at the nonreducing terminus of an oligosaccharide (7, 8), which is expected to be absent from pP21. FP21-I and FP21-II, isolated from normal Dictyostelium cells, were also not substrates, consistent with previous studies on crude extracts that soluble FP21 is fully fucosylated (7).

pNP-LNB and GP21 would reciprocally inhibit each other’s fucosylation if they were substrates of the same enzyme. Both GP21-I and -II inhibited pNP-LNB fucosylation in a concentr-
Recognition of GP21 by the cFTase appeared to depend primarily on carbohydrate rather than peptide determinants, based on the inhibitory potential of the different protein analogues. rP21 failed to inhibit the fucosylation of GP21-I, GP21-II, and pNP-LNB (Table VI), and in fact mildly stimulated the fucosylation of GP21-I and GP21-II. Since this stimulatory effect was not observed for pNP-LNB fucosylation, it may reflect substrate-substrate interactions, as 1) rP21 stimulated the fucosylation of GP21-I more than that of GP21-II, 2) GP21-I fucosylation was cooperative with respect to its own concentration (see above), and 3) GP21 and FP21 are known to aggregate in the concentration range examined (8). FP21-I was only a weak inhibitor (Table VI) compared to FP21-II (see below), showing an effect only at the highest inhibitor:substrate ratio tested against 0.028 μM GP21-I (Table VI). The absence of inhibitory effects by both rP21 and FP21-I suggested that the enzyme does not recognize the polypeptide backbone of FP21.

In contrast, FP21-II was a good inhibitor of the fucosylation of all three substrates, pNP-LNB, GP21-I, and GP21-II, with >50% inhibition observed at 10–100-fold concentration excess over the GP21-I and GP21-II substrate concentrations tested. The effect of FP21-II may represent product inhibition, as the reaction product, Fucα1,2Galβ1,3GlcNAcβ-pNP, was found to be an inhibitor of pNP-LNB fucosylation, when tested over a similar range of inhibitor:substrate ratios (Table VI). The failure of FP21-I to exert inhibition, except at the highest concentrations, may be due to its extra mole of Gal (8) which, if applied following fucosylation, may mask recognition of the peptide backbone of FP21.

Recognition of GP21 by the cFTase appeared to depend on the inhibitory potential of the different protein analogues. rP21 failed to inhibit the fucosylation of GP21-I, GP21-II, and pNP-LNB (Table VI), and in fact mildly stimulated the fucosylation of GP21-I and GP21-II. Since this stimulatory effect was not observed for pNP-LNB fucosylation, it may reflect substrate-substrate interactions, as 1) rP21 stimulated the fucosylation of GP21-I more than that of GP21-II, 2) GP21-I fucosylation was cooperative with respect to its own concentration (see above), and 3) GP21 and FP21 are known to aggregate in the concentration range examined (8). FP21-I was only a weak inhibitor (Table VI) compared to FP21-II (see below), showing an effect only at the highest inhibitor:substrate ratio tested against 0.028 μM GP21-I (Table VI). The absence of inhibitory effects by both rP21 and FP21-I suggested that the enzyme does not recognize the polypeptide backbone of FP21.

In contrast, FP21-II was a good inhibitor of the fucosylation of all three substrates, pNP-LNB, GP21-I, and GP21-II, with >50% inhibition observed at 10–100-fold concentration excess over the GP21-I and GP21-II substrate concentrations tested. The effect of FP21-II may represent product inhibition, as the reaction product, Fucα1,2Galβ1,3GlcNAcβ-pNP, was found to be an inhibitor of pNP-LNB fucosylation, when tested over a similar range of inhibitor:substrate ratios (Table VI). The failure of FP21-I to exert inhibition, except at the highest concentrations, may be due to its extra mole of Gal (8) which, if applied following fucosylation, may mask recognition of the peptide backbone of FP21.

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In contrast, FP21-II was a good inhibitor of the fucosylation of all three substrates, pNP-LNB, GP21-I, and GP21-II, with >50% inhibition observed at 10–100-fold concentration excess over the GP21-I and GP21-II substrate concentrations tested. The effect of FP21-II may represent product inhibition, as the reaction product, Fucα1,2Galβ1,3GlcNAcβ-pNP, was found to be an inhibitor of pNP-LNB fucosylation, when tested over a similar range of inhibitor:substrate ratios (Table VI). The failure of FP21-I to exert inhibition, except at the highest concentrations, may be due to its extra mole of Gal (8) which, if applied following fucosylation, may mask recognition of the peptide backbone of FP21.

Recognition of GP21 by the cFTase appeared to depend on the inhibitory potential of the different protein analogues. rP21 failed to inhibit the fucosylation of GP21-I, GP21-II, and pNP-LNB (Table VI), and in fact mildly stimulated the fucosylation of GP21-I and GP21-II. Since this stimulatory effect was not observed for pNP-LNB fucosylation, it may reflect substrate-substrate interactions, as 1) rP21 stimulated the fucosylation of GP21-I more than that of GP21-II, 2) GP21-I fucosylation was cooperative with respect to its own concentration (see above), and 3) GP21 and FP21 are known to aggregate in the concentration range examined (8). FP21-I was only a weak inhibitor (Table VI) compared to FP21-II (see below), showing an effect only at the highest inhibitor:substrate ratio tested against 0.028 μM GP21-I (Table VI). The absence of inhibitory effects by both rP21 and FP21-I suggested that the enzyme does not recognize the polypeptide backbone of FP21.

In contrast, FP21-II was a good inhibitor of the fucosylation of all three substrates, pNP-LNB, GP21-I, and GP21-II, with >50% inhibition observed at 10–100-fold concentration excess over the GP21-I and GP21-II substrate concentrations tested. The effect of FP21-II may represent product inhibition, as the reaction product, Fucα1,2Galβ1,3GlcNAcβ-pNP, was found to be an inhibitor of pNP-LNB fucosylation, when tested over a similar range of inhibitor:substrate ratios (Table VI). The failure of FP21-I to exert inhibition, except at the highest concentrations, may be due to its extra mole of Gal (8) which, if applied following fucosylation, may mask recognition of the peptide backbone of FP21.

Recognition of GP21 by the cFTase appeared to depend on the inhibitory potential of the different protein analogues. rP21 failed to inhibit the fucosylation of GP21-I, GP21-II, and pNP-LNB (Table VI), and in fact mildly stimulated the fucosylation of GP21-I and GP21-II. Since this stimulatory effect was not observed for pNP-LNB fucosylation, it may reflect substrate-substrate interactions, as 1) rP21 stimulated the fucosylation of GP21-I more than that of GP21-II, 2) GP21-I fucosylation was cooperative with respect to its own concentration (see above), and 3) GP21 and FP21 are known to aggregate in the concentration range examined (8). FP21-I was only a weak inhibitor (Table VI) compared to FP21-II (see below), showing an effect only at the highest inhibitor:substrate ratio tested against 0.028 μM GP21-I (Table VI). The absence of inhibitory effects by both rP21 and FP21-I suggested that the enzyme does not recognize the polypeptide backbone of FP21.

In contrast, FP21-II was a good inhibitor of the fucosylation of all three substrates, pNP-LNB, GP21-I, and GP21-II, with >50% inhibition observed at 10–100-fold concentration excess over the GP21-I and GP21-II substrate concentrations tested. The effect of FP21-II may represent product inhibition, as the reaction product, Fucα1,2Galβ1,3GlcNAcβ-pNP, was found to be an inhibitor of pNP-LNB fucosylation, when tested over a similar range of inhibitor:substrate ratios (Table VI). The failure of FP21-I to exert inhibition, except at the highest concentrations, may be due to its extra mole of Gal (8) which, if applied following fucosylation, may mask recognition of the peptide backbone of FP21.

### Table V

Fucosidase digestion of the cFtase reaction product

| Fucosidase | Units/ml | Time of digestion days |
|------------|----------|------------------------|
| α1,2-, A. oxidans F1 | 6.6 | 0.099 0.99 0.99 1.0 — |
| α1,3/4-, almond emulsin I | 0.00066 | — 0.045 0.025 — 0.031 |
| α1,3/4-, bovine kidney | 0.66 | 0.053 0.99 1.0 1.0 — |
| α1,3/4-, bovine epidymis | 0.4 | 0.025 0.095 0.039 — |

*Not done.*
DISCUSSION
Multiple FTases have been described in mammalian cell extracts and secretions which catalyze the formation of Fucα1,2Gal linkages (30–33, 40). Separate α1,2-FTases appear to be encoded by the H and Secretor loci in humans (33, 39), and DNAs encoding the H and Secretor enzymes have been cloned (40–43). Like all other known Golgi glycosyltransferases (45, 46), the Secretor and H enzymes are synthesized as type 2 membrane proteins consisting of a large C-terminal catalytic ectodomain, attached via a linker region to a transmembrane domain and a small N-terminal endodomain. The soluble, secretory forms of Golgi glycosyltransferases are apparently derived by proteolytic cleavage within the linker region (45). It is not clear whether the secretory form is physiologically meaningful. The $K_m$ values of Golgi and Golgi-derived FTases for GDP-Fuc are in the range of 10–100 $\mu$M, and apparent $K_m$ values for oligosaccharide acceptor substrates range from 0.1 to 20 $\mu$M (30–33, 40). The order of magnitude of these $K_m$ values is consistent with the expected concentrations of these substrates in the Golgi apparatus.

The Dictyostelium cFTase, which is also capable of catalyzing the formation of a Fucα1,2Gal linkage, shows both similarities to and differences from the mammalian α1,2-FTases. The cFTase occurs in soluble form after gentle cell lysis that maintained the latency of Golgi enzymes, and its activity could not be detected in particulate extracts of the cell (7). Its polypeptide could be purified to apparent homogeneity using conventional and affinity chromatography and electrophoresis methods which have been successfully applied to secreted forms of mammalian enzymes. Although the cFTase appears to be fairly hydrophobic when chromatographed on phenyl-Sepharose columns and certain detergents stabilized its activity after purification, detergents were not used during purification. The apparent $M_r$ of the cFTase polypeptide (FT85), at 85 $\pm$ 103, is approximately twice that of known Golgi enzyme cleavage products. The implication that the cFTase diverges from the Golgi type 2 membrane protein structural paradigm and resides in the cytosol must await confirmation from sequencing and cloning studies.

**FIG. 8.** Kinetic properties of the cFTase with respect to GP21 isomeric concentrations. Approximately 125 pg of preparation A was incubated for 10 min with 2.0 $\mu$M GDP-[3H]Fuc and varying concentrations of GP21-I or -II. Inset, double-reciprocal plot of the kinetic data. The data for GP21-II are fitted to a straight line, whereas the data points for GP21-I, which are nonlinear, are interpolated. The nested inset expands the plot at lower values of 1/V and 1/S. The cFTase exhibits an apparent $K_m$ of 0.21 $\mu$M with respect to GP21-II and an apparent $V_{max}$ of 460 nmol/min/mg protein.

**TABLE VI**

| Inhibitor | Inhibitor concentration | Substrate (amol/h (% inhibition)) |
|-----------|-------------------------|----------------------------------|
|           |                         | 0.36 $\mu$M pNP-LNB  | 0.028 $\mu$M GP21-I | 0.087 $\mu$M GP21-II |
| None      | 1680 (100)               | 167 (100)                        | 357 (100)                        |
| pNP-LNB   | 0.36 $\mu$M             | NA                             | 90.4 (84)                        | 239 (67)                        |
| 1.08      | NA                      | 80.2 (75)                       | 144 (40)                        |
| 3.24      | NA                      | 69.3 (65)                       | 121 (34)                        |
| 9.7       | NA                      | 63.9 (60)                       | 84.1 (23)                        |
| GP21-I    | 0.028 $\mu$M            | 1300 (77)                       | NA                              |
| 0.28      | 1030 (61)               | NA                              | NA                              |
| 0.84      | 1010 (60)               | NA                              | NA                              |
| GP21-II   | 0.087 $\mu$M            | 1365 (81)                       | NA                              |
| 0.87      | 1137 (71)               | NA                              | NA                              |
| 2.6       | 980 (58)                | NA                              | NA                              |
| rP21      | 0.33 $\mu$M             | 1560 (93)                       | 167 (156)                       | 345 (97)                        |
| 1.0       | ND                      | 189 (168)                       | 446 (125)                       |
| 3.0       | 1820 (108)              | 194 (181)                       | 519 (145)                       |
| 9.0       | 1630 (97)               | 146 (136)                       | 359 (101)                       |
| pNP-FLNB  | 0.28 $\mu$M             | 1654 (98)                       | ND                              |
| 0.84      | 1420 (84)               | ND                              |
| 2.5       | 959 (57)                | ND                              |
| FP21-I    | 0.063 $\mu$M            | 1693 (101)                      | ND                              |
| 0.17      | ND                      | 105 (98)                        | 300 (84)                        |
| 0.51      | 1625 (97)               | 101 (94)                        | 318 (89)                        |
| 1.53      | 1613 (96)               | 69.6 (65)                       | 310 (87)                        |
| FP21-II   | 0.073 $\mu$M            | 1690 (100)                      | ND                              |
| 0.22      | ND                      | 77.9 (73)                       | 235 (66)                        |
| 0.66      | 1384 (82)               | 73.6 (69)                       | 163 (46)                        |
| 1.98      | 1107 (66)               | 39.4 (37)                       | 88.3 (25)                        |

*a* Not applicable.

*b* Not determined.
The Kₘ of the purified cFTase for GDP-Fuc, at 0.34 μM, is unusually low compared to Golgi FTases, but similar to the value of a cytosolic O-GlcNAc transferase for its donor substrate UDP-GlcNAc (34). The high affinity of the cFTase for GDP-Fuc is consistent with its proposed cytosolic localization, because in the cytosol the cFTase would be in competition with the Golgi GDP-Fuc transporter (2) for this substrate. Although the cFTase differs from mammalian Golgi αFTases in its high affinity for GDP-Fuc, its nucleotide binding is more similar to α₁,2-FTases than α₁,3-FTases in its preference for GDP-hex-1,4-β-GlcNAc over GDP-α-d-glucopyranoside (31). The proportionately high sensitivity of the cFTase to inhibition by other guanine nucleotides (Table II) raises interesting regulatory questions about the effects of their intracellular pools.

The Kₘ of the purified cFTase for the type 1 disaccharide conjugate pNP-LNB (Gal|β₁,3GlcNAcβ₁-pNP) is similar to that of mammalian α₁,2-FTases. The monosaccharide conjugate pNP-Gal, and the type 2 disaccharide conjugate Gal|β₁,4GlcNAcβ₁-pNP are only very poorly if at all fucosylated, and Gal|β₁,6GlcNAc is at best a poor inhibitor. In contrast, the enzyme will fucosylate the type 3 disaccharide Gal|β₁,3GlcNAcβ₁-pNP, thus tolerating alternate sugars of opposite anomic linkage penultimate to the terminal, fucosylated Gal. The acceptor specificity preferences of the cFTase are similar to that of highly purified porcine submaxillary α₁,2-FTase (30, 44) and the Secretor-type α₁,2-FTase purified from human serum or plasma (31–33), and distinct from the H-type α₁,2-FTase, which has a broader specificity range (31–33, 40). Speculation that the Secretor-type gene preceded a gene duplication event leading to divergent evolution of the H-type gene (39) is reinforced by the present identification of a Secretor-type enzyme in the cellular slime molds. The specificity of the cFTase is compatible with what is known about the FP21 oligosaccharide, which contains Gal. However, the underlying sugar is not GlcNAc or GalNAc, as amino sugars are not detected in FP21 (8).

The cFTase exhibits varying Vₘₐₓ, and Kₘ values for different aglycone moieties attached to the disaccharide. Of the three that have been tested, pNP has the highest activity, benzyl is intermediate, and the 8-methoxycarbonyloctyl moiety exhibits an order of magnitude lower activity compared to pNP when present at concentrations near or below their Kₘ values. This suggests that acceptor substrate recognition involves more than the terminal disaccharide, which is consistent with the lower than expected inhibitory potential of free lacto-N-biose and the 3000-fold lower apparent Kₘ of the native substrate GP21. Effects of alternate aglycone moieties have also been observed for mammalian FTases (31, 33) and other Golgi glycosyltransferases (47), and polypeptide domains have been shown to be important determinants of recognition by certain glycosyltransferases (48). However, rP21 and FP21-I are not inhibitors of the cFTase, suggesting that the FP21 polypeptide is not an important determinant for cFTase recognition. The higher affinity of the enzyme for GP21-I compared to GP21-II, as determined by cross-inhibition studies (Table VI), in contrast to the higher inhibitory potential of FP21-II compared to FP21-I, may be most easily explained by glycosylation differences which have been described for the two FP21 isoforms (8). Why the different isoforms are differentially glycosylated remains to be determined. Since GP21 is the major acceptor of the reaction in crude extracts from the GDP-Fuc synthesis mutant (7), high affinity recognition of the FP21 carbohydrate implies that the carbohydrate structure is of limited distribution, and may be required because FP21 is not concentrated in a compartment. This is reminiscent of the relationship between UDP-Glc-glycoprotein Glc-1-phosphotransferase, another cytosolic glycoprotein glycosyltransferase involved in peripheral modifications, and phosphoglucomutase, which appears to be its predominant acceptor substrate (5).

The Vₘₓ of the purified cFTase with respect to pNP-LNB was 620 nmol/min/mg protein, at 1.0 μM GDP-Fuc. The Vₘₓ would be expected to extrapolate to 830 nmol/min/mg at saturating GDP-Fuc, or about 2.5 μmol/min/mg with respect to the content of FT85 in the highly purified preparation A. This value is greater than the estimated specific activity of a highly purified Se-type FTase from serum (33), but less than that of a purified porcine Golgi α₁,2-FTase (30). Projecting back to the cell, the specific activity suggests that there are on the order of 200 copies/cell of the cFTase in the cytosol. If the approximately 2 × 10⁵ copies/cell of the acceptor substrate FP21 were dissolved in the full volume of the cell, FP21 would be at a concentration of about an order of magnitude above its Kₘ for fucosylation. If the cFTase were operating at its Vₘₓ, there would be enough enzyme to be able to fucosylate all FP21 in about 1 h.

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