Glycosylation of proteins and lipids occurs in the endoplasmic reticulum and the Golgi apparatus and is mediated by glycosyltransferases and glycosidases that are resident there. The enzymes are ordered so that carbohydrates can be sequentially added to and removed from proteins and lipids (1-3). The mechanism for retention of the enzymes in the appropriate subcompartment and the domains of the enzymes that direct this localization are not clear. At present there are two proposed mechanisms for Golgi glycosyltransferase localization, kin recognition (4, 5), where like glycosyltransferases form aggregates within the Golgi, and the lipid bilayer model, where the hydrophobic transmembrane domain retains the transfersases in the Golgi (6). Neither the kin-recognition nor the lipid bilayer models fully explain the specific and functional localization of transferases to discrete sites within the Golgi; we now demonstrate that the cytoplasmic tail is a key element in the specific localization of α1,2-fucosyltransferase.

Glycosyltransferases are type II integral membrane proteins, with the catalytic domain residing in the lumen of the Golgi (7) and the N terminus in the cytoplasm (cytoplasmic tail). Several glycosyltransferases have been localized in compartments of the Golgi by electron microscopy (for review see Ref. 8). Studies of glycosyltransferases have demonstrated the importance of the transmembrane domain and its flanking sequences (9-14) and the luminal domain (15) for localization.

We examined two glycosyltransferases that compete for the same acceptor, N-acetyllactosamine (NAcLac), and showed that when there is expression of α1,3-galactosyltransferase (GT) together with α1,2-fucosyltransferase (FT) within a cell, the FT predominates, to produce H substance rather than Galα1,3Gal on the cell surface (16). Furthermore, switching the cytoplasmic tails of these transferases altered the dominant effect of FT indicating that the cytoplasmic tail affects the localization sites of the enzymes (17).

Here we examine the role of the cytoplasmic tail of FT for functional localization in the Golgi using the model system of competition with GT for acceptor and show in functional assays by microscopy that the cytoplasmic tail is essential for FT localization as the ability to compete with GT was abolished when the tail was removed, the enzyme moved from the Golgi to the trans Golgi network, and its localization site. When the tail was removed, the enzyme moved from the Golgi to the trans Golgi network, suggesting that the transmembrane is responsible for retention and that the cytoplasmic tail is responsible for localization. The cytoplasmic tail of α1,2-fucosyltransferase contains 8 amino acids (MWGPSRRH), and mutating these to alanine indicated a role for amino acids 3 to 7 in localization with a particular role of Ser5. Mutagenesis of Ser5 to amino acids containing an hydroxyl (Tyr and Thr) demonstrated that the hydroxyl at position 5 is important. Thus, the cytoplasmic tail, and especially a single amino acid, has a predominant role in the localization and thus the function of α1,2-fucosyltransferase.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs—** Untagged GT (18) and FT (19) were as described. The cytoplasmic tail FT mutants used the following oligonucleotides as the 5' PCR primer (the mutated amino acid is lowercase): FTyr2ala, 5'-GGCGATCACATG GetsgtCCACCGCCACCCACTCCTGCTG; FTVai3ala, 5'-GGCGATCACATGTG G getsgCCCACCGCCACCTCCTGCTG; FTPro4ala, 5'-GGCGATCACATGTG G getscAGCGCCACCTCCTGCTG; FTHis8ala, 5'-GGCGATCCATCTG GCTCCACCGCGCGACCTCCTGCTG; FTHis8ala, 5'-GGCGATCCACATG GCTCCACCGCGCGACCTCCTGCTG; FTHis8ala, 5'-GGCGATCCACATG GCTCCACCGCGCGACCTCCTGCTG; FTser5ala, 5'-GGCGATCACATGTG GCTCCACCGCGCGACCTCCTGCTG; FTser5ala, 5'-GGCGATCACATGTG GCTCCACCGCGCGACCTCCTGCTG; FTPos5ala, 5'-GGCGATCACATGTG GCTCCACCGCGCGACCTCCTGCTG; and FTPos5ala, 5'-GGCGATCACATGTG GCTCCACCGCGCGACCTCCTGCTG.

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1 The abbreviations used are: NAcLac, N-acetyllactosamine; GT, α1,3-galactosyltransferase; FT, α1,2-fucosyltransferase; IB4, Griffonia (Bandeiraense) simplicifolia lectin 1; UEA-1, Ulex europaeus agglutinin 1; FITC, fluorescein isothiocyanate; TGN, trans Golgi network; mfu, median fluorescence units.

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the untagged pig α1,3-galactosyltransferase (18) as template. The oligonucleotides were 5′-GCGGATCCATGTGGGTCCCCAGCCGCACCA-
CGTTGCTGCAATATCGTGTCGTC-3′ and 5′-GCTAGGAGCGTC
AGATGTTATTCTAACAAATATTAC-3′. All DNA was digested with BamHI and XbaI and ligated into pcDNA1.

Cell Culture and Transfection—DNA was purified using Qiagen products and transfected into COS-7 cells using DEAB-dextran (20) or LipofectAMINE Plus (Life Technologies, Inc.) as recommended by the manufacturer. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (CSL) supplemented with 10% fetal calf serum (CSL).

Immunofluorescence and Confocal Microscopy—COS-7 and Chinese hamster ovary cells transformed with large T antigen (21) were stained with Becton Dickinson FACSCalibur. For confocal microscopy counting was performed blind, and experiments were repeated at least three times; flow cytometry was performed at least three times using a Becton Dickinson FACSCalibur. For confocal microscopy, cells were passaged into chamber slides and cultured overnight prior to incubation for 4 h in cycloheximide (100 μM) to inhibit protein synthesis, followed by a 15-min incubation in Brefeldin A (5 μg/ml). Cells were washed three times using 0.5% saponin/phosphate-buffered saline followed by permeabilization and incubation in 0.5% saponin/phosphate-buffered saline containing diluted antibodies or lectins. The FLAG epitope was detected with anti-FLAG monoclonal antibody M2 (Sigma) followed by sheep anti-mouse Ig conjugated to FITC (Silenus). The cells were mounted in Prolong (Molecular Probes). Grifonia (Bandeira) simplicifolia lectin 1 (IB4) and Ulex europaeus agglutinin 1 (UEA1) (Sigma) were conjugated to FITC and used to stain for the products of GT, Galα1,3Gal, and FT (H substance), respectively.

Enzyme Assays—The assays for α1,2-fucosyltransferase and α1,3-galactosyltransferase were as described (16).

RESULTS

Removal of the FT Cytoplasmic Tail Reduces the Ability of the Enzyme to Compete for Acceptor—The strategy used was to alter FT and perform the following two studies: (a) measure the function of FT by its effect on Galα1,3Gal expression on the cell surface and (b) examine cells by confocal microscopy to localize the enzymes. When GT (which attaches Galα1,3Gal linkages to NAcLac) and FT (which attaches α1,2-fucose to NAcLac creating H substance) are coexpressed, FT fucosylates NAcLac, in preference to GT, and this reduces the level of Galα1,3Gal (the product of GT) on the cell surface (16).

To establish the functional assays, DNA constructs (Fig. 1) were transfected into Chinese hamster ovary cells transformed with large T antigen that were analyzed by flow cytometry (Fig. 2, A–J) for Galα1,3Gal staining (the enzymatic product of GT), using IB4–FITC (Fig. 2, A, C, E, G, and I) or for H substance (the enzymatic product of FT), using UEA1–FITC (Fig. 2, B, D, F, H, and J).

Several important findings were apparent from these co-transfection experiments. Cells transfected with GT led to expression of Galα1,3Gal where the positive cells (shown by the marker on each panel) had 250 median fluorescence units (mfu; see Fig. 2A); these cells were nonreactive with UEA-1 lectin detecting H substance (Fig. 2B). Cells transfected with FT gave reciprocal results, negative for Galα1,3Gal (with IB4; see Fig. 2C) and positive for H substance (281 mfu with UEA1; see Fig. 2D). When the two transferases were present simultaneously, the level of Galα1,3Gal was markedly reduced (63 mfu; see Fig. 2G) compared with GT expressed alone (250 mfu; see Fig. 2A); there was no decrease observed in the level of H substance (297 mfu; see Fig. 2H versus 281 mfu; see Fig. 2D). These findings were previously interpreted as being due to the localization of FT in an earlier compartment of the Golgi than GT (16, 17). Thus the system was in place to examine alterations in the localization of the FT after removing the cytoplasmic tail.

When the cytoplasmic tail was removed from FT (FTΔcyt) there was reduced but not absent expression of H (158 mfu; see Fig. 2F) compared with full-length FT (281 mfu; see Fig. 2D) and no expression of Galα1,3Gal (Fig. 2E); this was not because of different levels of the transferases expressed, which were similar as shown by Western blot (data not shown). However, there was appreciable surface staining for H substance indicating that FTΔcyt was functional. Clearly the lack of the FT cytoplasmic tail was affecting FT function and perhaps its localization. This was confirmed in coexpression studies with GT, as FTΔcyt was no longer able to decrease Galα1,3Gal expression (173 mfu; see Fig. 2I) to the same extent as full-length FT (63 mfu; see Fig. 2G). Therefore FTΔcyt is present (as H is present), but as it no longer affects Galα1,3Gal expression, it is unlikely to be acting on the acceptor before GT, suggesting it has moved elsewhere. Thus the two enzymes, FT and GT, no longer have the same relationship, and FTΔcyt is no longer acting on the acceptor prior to GT. Indeed, GT appears to act before FTΔcyt, as the amount of H substance on the surface of the cells when GT and FTΔcyt were coexpressed (92 mfu; see Fig. 2J) was reduced compared with FTΔcyt alone (158 mfu; see Fig. 2F) showing that GT is now acting on the acceptor before FT.

What has happened to the FT after removal of the tail? It is certainly present in the Golgi (see also below) as it is able to fucosylate proteins (UEA1-positive staining). We suspect it has moved to a post-Golgi compartment as the level of UEA1 staining is less than when the tail is present and GT, which is in the trans Golgi, is able to act on the acceptor before the FTΔcyt. It was therefore important to directly examine the localization of FT after removal of the tail (FTΔcyt).

The Intracellular Distribution of FT Is Changed after the Cytoplasmic Tail Is Removed—Fluorescent microscopy was used on cycloheximide-treated cells, which were subsequently stained for the transferases. The appearance of the Golgi and TGN can be distinguished using Brefeldin A treatment (22). When cells are stained for Golgi prior to Brefeldin A treatment, they have a juxtanuclear staining pattern, which is lost after Brefeldin A treatment, to become diffuse cytoplasmic staining indicative of endoplasmic reticulum (22). However, the TGN, which also has a juxtanuclear staining pattern without Brefeldin A, collapses around the microtubule organizing center with Brefeldin A and appears as a bright area of staining, similar to that observed for the microtubule organizing center (22). When FT-FLAG was examined, the juxtanuclear staining pattern which also has a juxtanuclear staining pattern without Brefeldin A, collapses around the microtubule organizing center with Brefeldin A and appears as a bright area of staining, similar to that observed for the microtubule organizing center (22). When FT-FLAG was examined, the juxtanuclear staining pattern which also has a juxtanuclear staining pattern without Brefeldin A, collapses around the microtubule organizing center with Brefeldin A and appears as a bright area of staining, similar to that observed for the microtubule organizing center (22). When FT-FLAG was examined, the juxtanuclear staining pattern which also has a juxtanuclear staining pattern without Brefeldin A, collapses around the microtubule organizing center with Brefeldin A and appears as a bright area of staining, similar to that observed for the microtubule organizing center (22).
because Brefeldin A causes the TGN to collapse around the microtubule organizing center (22), and in our study this is the appearance observed in the cells. No cell surface staining for the FLAG tag was observed in either FT- or FTΔcyt-transfected cells (data not shown). Thus after removing the FT tail, FT has moved from the Golgi to the TGN. The microscopic and functional studies show that the cytoplasmic tail is responsible for the specific localization of FT in the Golgi, and after its removal, the enzyme is found in the TGN.

**Ser5 of the FT Cytoplasmic Tail Is Essential for Functional Golgi Localization**—Having shown the importance of the FT cytoplasmic tail, we sought to determine which of the 8 amino acids (MWVPSRRH) in the cytoplasmic tail sequence of FT are important for its localization. Using the same functional readout each amino acid of the cytoplasmic tail was mutated and was examined for function. Thus, it would be expected that mutation of an amino acid in the FT tail, which is important for localization, would result in a reduced ability of FT to compete for acceptor with GT and result in a subsequent increase in the level of Galα(1,3)Gal. Each amino acid of the cytoplasmic tail

**Fluorescence**

**FIG. 2. Determination of the ability of FTΔcyt to compete with GT for acceptor.** Chinese hamster ovary cells transformed with large T antigen were cotransfected with DNA encoding the following: A and B, vector + GT; C and D, vector + FT; E and F, vector + FTΔcyt; G and H, GT + FT; and I and J, GT + FTΔcyt. The cells were analyzed by flow cytometry after surface staining for (i) the products of GT and Galα(1,3)Gal detected with IB4-FITC and (ii) the products of FT and H substance detected with UEA-1-FITC. The marker set for the population of positively stained cells is shown with the mfu.
was substituted by Ala to produce constructs FTTrp2ala, FTVal3ala, FTPro4ala, FTSer5ala, FTArg6ala, FTArg7ala, and FTHis8ala. These constructs, full-length FT, or vector were cotransfected with GT into COS-7 cells, and the number of cells showing surface staining for IB4-FITC or for UEA-1-FITC was counted (Fig. 4A). In cells transfected with FT or each of the FT mutants, 60% of cells were stained with UEA-1, showing that FT was present and functional. However, differences in the ability of the mutants to inhibit GT activity (as measured by cell surface IB4 staining) were found. Using FT alone, 50% of cells were positive for Galα1,3Gal, and this was reduced to 20% by coexpression of FT. The FTVal3ala and FTHis8ala mutants also inhibited Galα1,3Gal expression to 20%, i.e. these mutations had no effect on FT function and therefore localization and were unlikely to be involved in the localization of the enzyme. However, transfection of the FTTrp2ala, FTPro4ala, FTArg6ala, and FTArg7ala mutants with GT resulted in 40% of cells expressing Galα1,3Gal, indicating some alteration of FT localization. However, the Ser5 to Ala mutation removed the inhibitory effect of FT on GT as staining for Galα1,3Gal was on 50% of cells, and therefore there was no competition between the enzymes. Thus of the 8 amino acids, positions 4–7 are involved in localization of the enzyme. Ser5 is the most important amino acid; for position 3, a Val to Ala substitution is not a major structural change and could be involved in FT localization.

To confirm that these differences were not because of changes in the enzymatic activity caused by mutation to the cytoplasmic tail, enzyme assays were performed. We have previously shown that changes in the levels of Galα1,3Gal and H substance on the cell surface were not because of changes in enzymatic activity (16) and that chimeric glycosyltransferases did not exhibit altered kinetics of enzyme function after switching of cytoplasmic tails between GT and FT (17). However, the issue of expression level of the FT mutants in the current experiments needed to be addressed. Cells were transfected as described for Fig. 4A, and lysates were assayed (Table I). There were no significant differences in the enzymatic rates between any of the FTs or the rate of GT (this was expected as the same amount of GT encoding DNA was transfected into each dish of cells). In particular, the S5A mutant had an enzyme rate of 1216.4 ± 206.7 pmol/hr/mg compared with that of FT, which was 1212.7 ± 206.1 pmol/hr/mg. Thus, the increase in Galα1,3Gal is not because of a decrease in the level of enzyme activity of the FT mutant.

The mutations in the cytoplasmic tail could lead to subtle shifts in FT localization. However, the assay described above (Fig. 4A) could not detect subtle differences as the number of cells expressing H substance was similar for all the mutants (Fig. 4A). To investigate possible subtle changes a new system was developed that is vulnerable to minor alterations in GT function. A construct was produced, fGT, which encodes the 8 amino acid cytoplasmic tail of FT, but not the transmembrane and luminal domains of GT. Based on earlier work using the human FT cytoplasmic tail (17), fGT would be positioned in the same subcompartment as GT and would compete for acceptor equally as both transferases have the same cytoplasmic tail. Using coexpression of fGT and the FT mutants, any physical shift of FT mutants from the functional site in the Golgi would reduce their ability to compete for acceptor with fGT and therefore result in a loss of UEA-1 staining. fGT was cotransfected with the Ala mutant FTs, wild-type FT, or vector alone, and cell surface staining for IB4 and UEA-1 was determined (Fig. 4B). FT did not inhibit the level of Galα1,3Gal after cotransfection with fGT (60% of cells were positive), but rather both enzymes were able to function equally as they are probably located in the same subcompartment. Similarly, none of the mutants altered the level of cell surface H substance (60% of cells) except for FTSer5ala, which reduced the level of cell surface staining from 60 to 30%. This indicates that FTSer5ala has not been retained in the appropriate site within the Golgi but moved to a more distal compartment. By contrast, the fGT is retained in the original FT subcompartment and adds Galα1,3Gal to NAcLac (high IB4 levels), and therefore there is less acceptor remaining for the FTSer5ala that has shifted to a later subcompartment (reduced UEA-1 levels). As mutations of the other amino acids did not affect UEA-1 staining, it is clear that Ser5 is the most crucial amino acid for functional localization in COS-7 cells.

An Hydroxyl Group at Amino Acid Position 5 Is the Essential Component of FT Localization—The S5A mutation had a crucial effect on FT function although Ser and Ala differ by an hydroxyl group. To determine whether this hydroxyl group was the essential element in FT localization, Thr and Tyr (which also contain an hydroxyl group) were also substituted at position 5. FT and FTSer5tyr and FTSer5thr were cotransfected with GT, and the surface expression of Galα1,3Gal and H substance on COS-7 cells was determined (Fig. 5). When FT was transfected, 60% of cells were stained with UEA-1 (Fig. 5A), and when GT was transfected 60% of cells were stained with IB4 (Fig. 5B). When FT and GT were coexpressed the level of UEA-1 staining remained at 60% (Fig. 5C), and the level of IB4 staining was reduced to 20% (Fig. 5D). As shown above, FTSer5ala was cotransfected with GT, and the level of staining for both UEA-1 (Fig. 5E) and IB4 (Fig. 5F) was 60%, showing again that the mutant lacking the hydroxyl group could no longer compete with GT. However, coexpression of FTSer5tyr or FTSer5thr (both containing an hydroxyl group) with GT gave high levels of staining (60% of cells) with UEA-1 (Fig. 5, G and I, respectively) but not IB4 (Fig. 5, H and J, respectively, 20% of cells) showing that an hydroxyl group at position 5 is important for the ability of FT to compete with GT. This was confirmed using FTSer5phe and GT in cotransfections that gave similar IB4 and UEA-1 staining.
staining patterns to FTser5ala and GT (data not shown), demonstrating that the hydroxyl group rather than the ring structure of Tyr is important for FT localization.

DISCUSSION

The addition of carbohydrate moieties to proteins and lipids occurs in a specific order, however the current models of resident Golgi protein localization do not adequately describe the mechanism of retention and localization of the glycosyltransferases that perform the glycosylation. Here we analyzed the involvement of the cytoplasmic tail of FT in localization in the Golgi. When FT and GT are coexpressed, FT first contacts the NAcLac acceptor and utilizes it prior to GT, leaving little acceptor for GT, which results in a reduction in Galα(1,3)Gal on the cell surface (16, 17). Removal of the cytoplasmic tail of FT reversed the ability of FT to compete with GT, and Galα(1,3)Gal expression was restored (Fig. 2). In addition,

FIG. 4. Determination of the ability of FT mutants to compete with GT or ftGT for acceptor in COS-7 cells. COS-7 cells were cotransfected with wild-type or mutant FT and (A) GT or (B) ftGT. The percentage of cells expressing (i) the products of GT and Galα1,3Gal and detected with IB4-FITC and (ii) the products of FT and H substance and detected with UEA1-FITC are shown. DNA transfection combinations are shown.
The hydroxyl residue is important for the correct localization and function of FT. This is evidenced by the fact that the cytoplasmic tail of FT is necessary for the localization of the enzyme in the Golgi so that it can perform its function with respect to competition with GT (17). Heterodimers of the hydrophobic transmembrane domain of trans Golgi enzymes (9) or to the luminal domain of the Golgi enzymes mannosidase II and N-acetylgalactosaminyltransferase I (4, 24). However, although aggregation may contribute to retention, it is not clear how this aggregation could contribute to Golgi localization to a specific site. Our data are consistent with the kin-recognition model, as aggregation may be a prerequisite for the interaction of the FT cytoplasmic tail with some localizing agent on the cytoplasmic surface of the Golgi such as glycosyltransferase receptors or with a Golgi matrix (25). Nonreducing SDS polyacrylamide gel electrophoresis showed that FT is present as dimeric and larger molecular weight forms, possibly tetrameric (data not shown); others have found it as a dimer (15). This raises the possibility that multiple cytoplasmic tails brought close together by dimer formation may be important for localization.

The second, lipid bilayer model (6) is reliant upon the general property of hydrophobicity and length of the transmembrane domains rather than upon specific sequences in the transmembrane domain for retention within the Golgi. Aggregation of homo- and heterodimers into large complexes because of the hydrophobic transmembrane domain allows preferential utilization of NAcLac. This is consistent with the lipid bilayer model, as the transmembrane domain may retain the enzyme and play a role as an anchor, without which the transferase would be secreted. As we have not observed the “tailless” version of FT in any vesicular structures or on the cell surface, we presume it is the length and hydrophobicity of the transmembrane domain of FT that leads to its retention in the TGN. Thus the characteristics of the transmembrane domain are such that FT cannot be transported beyond the TGN, whereas the tail acts to keep it in a specific site in the Golgi.

Thus, it is likely that a number of different factors dictate the precise localization of glycosyltransferases. Aggregation of the enzymes and their transmembrane domains may be involved in general Golgi retention, whereas retention sequences in glycosyltransferases may allow localization in specific functional subcompartments of the Golgi. The concept of a second glycosyltransferase signal for specific functional localization has also recently been suggested by others (26) who carefully analyzed the biosynthetic products of glycosyltransferases in vivo so that changes in their localization leading to changes in product could be monitored.
The cytoplasmic tail is part of the general flanking sequence of the transmembrane domain along with the stem region in the luminal domain, which has been considered important for localization of Type II membrane proteins (9–14). This study shows that the role of the cytoplasmic tail of FT in Golgi localization is far more substantial than previously thought and that there are only a few amino acids involved in this phenomenon. The nature of the molecular interaction of the FT tail with the cytoplasmic surface of the Golgi remains to be determined, especially in light of recent findings of the importance of the cytoplasmic portions of giantin (27) in Golgi localization.

Acknowledgments—We thank Dr. S. Russell of Peter MacCallum Cancer Institute for advice and helpful comments.

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