The ST6Gal I is a sialyltransferase that functions in the late Golgi to modify the N-linked oligosaccharides of glycoproteins. The ST6Gal I is expressed as two isoforms with a single amino acid difference in their catalytic domains. The STcys isoform is stably retained in the cell and is predominantly found in the Golgi, whereas the STtyr isoform is only transiently localized in the Golgi and is cleaved and secreted from a post-Golgi compartment. These two ST6Gal I isoforms were used to explore the role of the bilayer thickness mechanism and oligomerization in Golgi localization. Analysis of STcys and STtyr proteins with longer transmembrane regions suggested that the bilayer thickness mechanism is not the predominant mechanism used for ST6Gal I Golgi localization. In contrast, the formation and quantity of Triton X-100-insoluble oligomers was correlated with the stable or transient localization of the ST6Gal I isoforms in the Golgi. Nearly 100% of the STcys and only 13% of the STtyr were found as Triton-insoluble oligomers when Golgi membranes of COS-1 cells expressing these proteins were solubilized at pH 6.3, the pH of the late Golgi. In contrast, both proteins were found in the soluble fraction when these membranes were solubilized at pH 8.0. Analysis of other mutants suggested that a conformational change in the catalytic domain rather than increased disulfide bond-based cross-linking is the basis for the increased ability of STcys protein to form oligomers and the stable localization of STcys protein in the Golgi.

The ST6Gal I, or β-galactoside α2,6-sialyltransferase (ST), is a glycosyltransferase that has been localized to the trans cisternae of the Golgi and the trans Golgi network (1). Within these compartments it encounters the sugar nucleotide donor, CMP-NeuAc, and functions to add terminal sialic acid residues to the N-linked oligosaccharides of glycoproteins. The ST6Gal I is expressed as two isoforms that have a single amino acid difference at position 123 in the catalytic domain (2). This single amino acid difference (Tyr → Cys) is the result of a single nucleotide change (A → G), which is likely the result of an RNA editing event (2). The STtyr isoform is found in the Golgi, at low levels on the cell surface and is cleaved and secreted from COS-1 and HeLa cells with a half time of 3–6 h. In striking contrast, the STcys isoform is found in the Golgi in moderately expressing cells and is never observed at the cell surface or cleaved and secreted into the media of COS-1 or HeLa cells (2).

Several pieces of evidence suggest that the difference observed in STtyr and STcys processing relate to differences in the localization and trafficking of these proteins in the cell. First, decreasing the temperature of cells to 20 °C prevents the cleavage and secretion of the STtyr from COS-1 cells, suggesting that this isoform is only transiently localized in the Golgi and moves beyond the trans Golgi network into a post-Golgi compartment where cleavage occurs (2). The presence of low levels of the STtyr protein at the cell surface also supports the idea that the STtyr is only transiently localized in the Golgi. In addition, it is unlikely that the presence of the Cys at position 123 eliminates cleavage per se because the major cleavage site is found in the stem region between Lys40 and Glu11 and because both isoforms are cleaved and secreted from Chinese hamster ovary cells (2, 3). This latter observation also suggests that the ST6Gal I isoforms and/or the proteases responsible for cleavage are localized differently in different cell types.

What is the basis for the differences in ST6Gal I isoform localization and processing? Two hypotheses have been formulated to explain the localization of proteins in the Golgi. The bilayer thickness hypothesis first stated by Bretscher and Munro (4) and supported by the results of Munro (5, 6) and Masi-bay et al. (7) is based on three observations. First, on average, Golgi proteins have shorter transmembrane regions than plasma membrane proteins (7, 8). Second, there is a gradient of cholesterol in membranes from the endoplasmic reticulum (ER) to the plasma membrane with the highest levels found in the plasma membrane (9). Third, in vitro liposome studies have demonstrated that increasing the levels of cholesterol in a membrane increases its width (10). From these observations, Bretscher and Munro (4) proposed that Golgi membrane proteins, with their shorter transmembrane regions, are not able to partition into transport vesicles moving to the plasma membrane and are therefore essentially retained in the Golgi. The oligomerization/kin recognition hypothesis has several origins. First, Machamer (11) proposed that proteins form oligomers in the specific microenvironments of the Golgi cisternae and that the insolubility or size of these oligomers prevents them from entering transport vesicles and trafficking to the plasma membrane. Nilsson and co-workers (12) later expanded on this idea with their kin recognition hypothesis in which they suggested that resident Golgi proteins form very large hetero-oligomers with proteins in the same cisterna and that the size of these...
oligomers prevents their partitioning into transport vesicles destined for the next compartment. Work done by Nilson et al. (13, 14) demonstrated that such hetero-oligomers were formed by the α-mannosidase II and N-acetylglucosaminyltransferase I of the medial Golgi and that their formation correlated with the localization of these two glycosyltransferases in this compartment. Similar hetero-oligomers were not observed for the β1,4-galactosyltransferase or ST6Gal I (6).

In this manuscript we demonstrate that the ability of the STTyr and STCys isoforms of the ST6Gal I to form insoluble oligomers correlates with the extent of their localization in the Golgi. In contrast, the length of the transmembrane region does not seem to impact Golgi localization or trafficking and processing of either isoform. We also investigate the effects of other ST6Gal I mutants and assess the potential role of STcys disulfide bond formation in Golgi localization. We conclude that the ST6Gal I mutants and the extent of their localization in the Golgi correlates with the extent of their localization in the Golgi.

EXPERIMENTAL PROCEDURES

Materials

Tissue culture media and reagents, including Dulbecco’s modified Eagle’s medium (DMEM), and Lipofectin were purchased from Life Technologies, Inc. Fetal bovine serum was obtained from Atlanta Biologicals (Norcross, GA). Sequenase enzyme was obtained from U.S. Biochemical Corp. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). BCIP/NBT Color Development Substrate for detection of alkaline phosphatase-conjugated antibodies was purchased from Promega (Madison, WI). FTO2B rat hepatoma cells were obtained from Dr. Carolyn Bruzdziński (University of Illinois at Chicago, Chicago, IL). Protein A-Sepharose Fast Flow and a-35S-dATP were purchased from Amersham Pharmacia Biotech. Columns for DNA purification were obtained from Qiagen Inc. (Chatsworth, CA). Protein molecular weight standards were purchased from Bio-Rad. 35S-Express protein labeling mix was purchased from NEON Life Science Products. QuickChange™ site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). SuperSignal West Pico chemiluminescence reagent, biotinyl N-hydroxysuccinimide ester, and streptavidin-agarose were purchased from Pierce. All other chemicals, including alkaline phosphatase-conjugated goat anti-rabbit IgG, were purchased from Sigma.
HCl, pH 7.4, 1 mM magnesium acetate, and 100 mM iodoacetamide). Following homogenization with a mini Dounce homogenizer, a Golgi-enriched fraction was isolated by subjecting the membrane mixtures to equilibrium sucrose density gradient centrifugation according to the method described by Xu and Shields (22). All sucrose gradient solutions contained 100 mM iodoacetamide to prevent aberrant disulfide bond formation during membrane preparation procedures. The Golgi membrane-enriched fraction was collected, washed once with 100 mM iodoacetamide, and pelleted by centrifugation at 39,000 rpm for 1 h. The pellets were resuspended in 2 ml of MNT buffer of either pH 6.3 or pH 8.0. After a 10-min solubilization on ice, one half of the membrane lysate was transferred to a Beckman SW65 Ti ultracentrifuge tube for separation into soluble and pelleted material, whereas the other half of the cell lysate was reserved as the total protein control. Following a 1-h centrifugation at 100,000 × g at 4 °C, the pellets were solubilized in Laemmli sample buffer (20) by sonication, and proteins in the supernatant and the total protein control were recovered by methanol precipitation. Briefly, four volumes of methanol were added, and the mixture was incubated at −20 °C overnight and then centrifuged for 15 min at 3000 × g. The supernatant was removed, and the protein pellet was air dried and solubilized in Laemmli sample buffer (20) by sonication. The insoluble and soluble fractions and the total protein control were electrophoresed on a 10% SDS-polyacrylamide gel, and the ST was detected by immunoblotting using the affinity purified rabbit anti-rat ST6Gal I antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG, and the SuperSignal West Pico chemiluminescence reagent (Pierce). Quantitation of the detected ST bands were done as described previously using the ImageQuant program (23).

Immunoblot Analysis of Golgi Membrane Fractions—COS-1 cells were transfected with wild type STTyr or STcys or the mutant proteins as described above. The cells were trypsinized, washed once with PBS, and suspended in five volumes of homogenization medium (0.55 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM magnesium acetate, and 100 mM iodoacetamide). Following homogenization with a mini Dounce homogenizer, membrane mixtures were subjected to equilibrium sucrose density gradients for the isolation of a Golgi-enriched fraction according to the method described by Xu and Shields (22) and as described above. To analyze the formation of disulfide bonded dimers and high molecular mass oligomers, the Golgi-enriched membrane fractions from cells expressing these proteins were treated with Laemmli sample buffer (20) with or without 5% β-mercaptoethanol at 100 °C for 10 min. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and then transferred to nitrocellulose membranes for immunoblotting. ST proteins were detecting by immunoblotting as described above.

RESULTS

Altering the Length of the Transmembrane Domain Does Not Change the Localization or Trafficking of Either Isoform of the ST6Gal I—Work from several laboratories has demonstrated a primary role for the transmembrane domain in the Golgi localization of glycosyltransferases and chimeric proteins made from glycosyltransferase sequences (reviewed in Refs. 11, 24, and 25). Elegant work from the laboratory of Munro (6) demonstrated that increasing the length of the Golgi-retained chimeric protein’s transmembrane domain led to an increase in this protein’s cell surface expression, whereas decreasing the length of the transmembrane domain of a plasma membrane protein led to an increase in its Golgi localization. Masibay et al. (7) observed similar results when the length of the transmembrane domain of the β1,4-galactosyltransferase was altered. In contrast, previous experiments in our laboratory showed that increasing the length of the STcys isoform transmembrane domain from 17 amino acids to 23 (SA23) and 29 (SA29) amino acids by replacing it with all or part of the influenza neuraminidase transmembrane region did not increase the expression of this protein on the cell surface (15). At the time these experiments were performed, we were unaware that the ST6Gal I was expressed as two isoforms and that one of these, the STTyr, is not stably retained in the Golgi but moves to a post-Golgi compartment where it is cleaved and secreted (2). When the two isoforms were identified, we hypothesized that the STTyr, which is transiently retained in the Golgi, is localized to the Golgi by a bilayer thickness mechanism based on the length of the transmembrane domain, whereas the STcys, which is stably retained in the Golgi, is localized by an oligomerization mechanism involving luminal sequences as well as its transmembrane domain.

To test our hypothesis, we have analyzed SA23 and SA29 transmembrane domain chimeras of the STTyr isoform for both increases in the rate of cleavage and secretion and increased cell surface expression (please see “Materials and Methods” for transmembrane sequences of the two chimeric proteins). In addition, we have re-evaluated the same chimeric forms of the STcys isoform for the possibility that they are cleaved and secreted (Fig. 1). STTyr-pSVL and STcys-pSVL and their SA23 and SA29 forms were transiently expressed in COS-1 cells. Expressing cells were metabolically labeled for 1 h and chased for 6 h, and ST proteins were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis as described under “Experimental Procedures.” Molecular mass standard: ovalbumin, 48.7 kDa.

![STTyr and STcys Western Blots](http://www.jbc.org/Downloaded from http://www.jbc.org/)

**Fig. 1.** The rate of STcys and STTyr cleavage and secretion is not altered by increasing the lengths of their transmembrane regions. COS-1 cells transiently expressing the wild type (WT) ST6Gal I isoforms or their SA23 and SA29 transmembrane mutants were metabolically labeled with 35S-Express protein labeling mix for 1 h and then chased in DMEM, 10% fetal bovine serum for 6 h. ST proteins were immunoprecipitated from both cell lysate (C) and medium (M) fractions, and immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described under “Experimental Procedures.” Molecular mass standard: ovalbumin, 48.7 kDa.

**Table 1.** The rate of STcys and STTyr cleavage and secretion is not altered by increasing the lengths of their transmembrane regions.

| Treatment | STTyr WT | STTyr SA23 | STTyr SA29 | STcys WT | STcys SA23 | STcys SA29 |
|-----------|----------|------------|------------|----------|------------|------------|
| C         | 100%     | 100%       | 100%       | 100%     | 100%       | 100%       |
| M         | 21%      | 42%        | 27%        | 4%       | 5%         | 7%         |

In addition, we have re-evaluated the same chimeric forms of the STcys isoform for the possibility that they are cleaved and secreted (Fig. 1). STTyr-pSVL and STcys-pSVL and their SA23 and SA29 forms were transiently expressed in COS-1 cells. Expressing cells were metabolically labeled for 1 h and chased for 6 h, and ST proteins were immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis as described under “Materials and Methods.” We found that increasing the length of the STTyr transmembrane region did not change the rate of cleavage and secretion from COS-1 cells (Fig. 1, STTyr: WT, SA23, and SA29). Forty-two percent of both the STTyr and its SA23 chimera were found in the medium after a 6 h chase, while the SA29 STTyr chimera was secreted more slowly, with only 21% of this protein found in the medium after a 6 h chase. In addition, STcys and its SA23 and SA29 chimeras were not cleaved and secreted (Fig. 2, STcys: wild type, SA23, and SA29). We previously demonstrated that the level of cell surface staining did not increase when the length of the STcys transmembrane domain was increased in the SA23 or SA29 chimeras (15). Expression of the STTyr and its SA23 and SA29 chimeras in COS-1 cells followed by indirect immunofluorescence microscopy showed that there were no differences in the intracellular Golgi or cell surface staining patterns of these three proteins (Fig. 2). We conclude from these data that the length of the transmembrane domain is not the primary determinant of stable Golgi localization and that the bilayer thickness mechanism, if used, is not the major mechanism of Golgi localization for either isoform of the ST6Gal I.

Insoluble Oligomers of Rat Liver and FTO2B ST6Gal I Are Recovered When Golgi Membranes AreLyed under Conditions of Low pH That Are Found in the Late Golgi—The observation that a difference in a single amino acid in the catalytic domain of the ST6Gal I alters the trafficking of the protein suggested that the conformation of the STTyr and STcys catalytic domains might be different. This idea and the results of the experiments described above led us to formulate a second hypothesis. We
postulated that both STtyr and STcys proteins form oligomers but that those formed from the STcys isoform are more stable and thus lead to more stable localization in the Golgi, whereas those formed from the STtyr are less stable and thus lead to its transient localization in this compartment. To test this possibility, we used an assay previously used by Schweizer et al. (17) to correlate the formation of Triton X-100-insoluble oligomers of the p63 protein with its localization in the ER-Golgi intermediate compartment. In this assay, membranes are lysed with MNT buffers of various pH values from 5.8 to 8.0 and separated into soluble and insoluble fractions by high speed centrifugation. First, we performed this assay using isolated rat liver Golgi membranes. Both isoforms are expressed in rat liver, although the exact ratio has been difficult to determine with certainty (2). These membranes were lysed with buffers of pH 5.8–8.0 and soluble and insoluble fractions separated as described in Methods. Proteins were electrophoresed on nonreducing SDS-polyacrylamide gels, and ST proteins were detected by immunoblotting with the anti-ST6Gal I antibody. The ST6Gal I exists as two forms in rat liver. Approximately 30% of the protein is a disulfide-bonded dimer, and the remaining 70% is found as monomer (19). Both the STtyr and STcys isoforms are capable of forming the disulfide-bonded dimer (see Fig. 7). The ST6Gal I proteins were found exclusively in the soluble fraction at pH 8.0, 7.4, and 6.8 (Fig. 3). However, at pH 6.3 and 5.8 a significant proportion of the ST proteins were found in the insoluble fraction, and interestingly, much of these appeared to be in the disulfide-bonded dimer form (Fig. 3).

This experiment demonstrated that a significant portion of the ST6Gal I in rat liver was stabilized as or formed Triton X-100-insoluble oligomers when Golgi membranes were lysed with buffers of pH 5.8–6.3, the pH of the late Golgi cisternae (26). This supported the hypothesis that proteins form insoluble or very large oligomers in the specific microenvironment of a Golgi cisterna that lead to their localization in that cisterna (11). Because the predicted isoelectric point of the ST6Gal I protein is 8.9 and because experimentally we have found it to be greater than 8.0,2 we were certain that the presence of oligomers at pH 5.8–6.3 did not represent the complete neutralization of the surface charges of this protein and subsequent aggregation. However, we were still concerned that lysis of membranes under conditions of low pH may lead to the nonspecific aggregation of many proteins. To evaluate this possibility, we investigated the effect of low pH buffers on the plasma membrane proteins of the FTO2B rat hepatoma cell line that also expresses detectable ST6Gal I protein. FTO2B cells were metabolically labeled for 1 h and chased for 6 h, and cell surface proteins were biotinylated as described under “Materials and Methods.” Total cellular membranes were lysed in buffers of pH 8.0, 6.3, or 5.8, and soluble and insoluble material separated as described above. Biotinylated cell surface proteins were recovered from these fractions using streptavidin-agarose (Fig. 4, Cell Surface Proteins). At the same time, ST6Gal I

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2 J. Ma and K. J. Colley, unpublished data.
proteins were immunoprecipitated from soluble and insoluble fractions (Fig. 4, ST6Gal I). We found that a small percentage of cell surface proteins were induced to form insoluble oligomers when cell membranes are lysed at pH 5.8–6.3. FTO2B cells were metabolically labeled with 35S-Express protein labeling mix for 1 h and chased with DMEM, 10% fetal bovine serum for 6 h. Top panel (Cell Surface Proteins), cell surface proteins were biotinylated with 1 mg/ml biotinyl N-hydroxysuccinimide ester as described previously (15). Cell membranes were lysed in buffers of pH 5.8, 6.3 and 8.0, and soluble (S) and insoluble (P) material was separated according to the method of Schweizer et al. (17). Biotinylated cell surface proteins were recovered from these fractions using streptavidin-agarose. Bottom panel (ST6Gal I), ST6Gal I proteins were isolated from soluble and pelleted membrane fractions by immunoprecipitation using an affinity purified rabbit anti-rat ST6Gal I antibody. Both biotinylated cell surface proteins and ST6Gal I immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Molecular mass standards: ovalbumin, 51.6 kDa; β-galactosidase, 118 kDa; bovine serum albumin, 86 kDa; ovalbumin, 51.6 kDa.

Table: Insoluble Oligomers of the STcys and the N158S STtyr Glycosylation Mutant Are Recovered When Membranes Are Lysed under Conditions of Low pH Found in the Late Golgi—

| pH   | Insoluble Oligomers (%) |
|------|------------------------|
| 5.8  | 1% (0.3%)              |
| 6.3  | 4%                     |
| 8.0  | 11%                    |

In that it is localized in the Golgi but not cleaved and secreted (Fig. 6 and Ref. 23). When Golgi membranes were solubilized using a MNT buffer of pH 8.0, we recovered no Triton X-100-insoluble oligomers containing either the STtyr or STcys proteins (Fig. 5, STtyr and STcys, pH 8.0, P). However, when Golgi membranes were solubilized at pH 6.3, the pH of the late Golgi, nearly 100% of the STcys was recovered as an insoluble oligomer, and 13% of the STtyr was recovered in this insoluble fraction (Fig. 5, STtyr and STcys, pH 6.3, P). These results strongly support our hypothesis that the STcys forms more stable oligomers than the STtyr protein in the lower pH environment of the late Golgi and that this is the basis for its more stable localization in this compartment.

Interestingly, the STtyr N158Q mutant protein demonstrated a propensity to oligomerize even at pH 8.0, with 56% of this protein found in the Triton X-100-insoluble fraction under these conditions (Fig. 5, STtyr N158Q, pH 8.0, P). The amount of STtyr N158Q protein in the Triton X-100-insoluble fraction was increased to 77% when membranes were solubilized at pH 6.3 (Fig. 5, STtyr N158Q, pH 6.3). In this way, this protein and a STser protein (Fig. 6) that also exhibits reduced cleavage and secretion (Fig. 6) behave more like the p63 protein in the pH shift Triton X-100 insolubility assay (Fig. 5, STtyr N158Q and data not shown). Schweizer et al. (17) observed that 50% of the ER-Golgi intermediate compartment protein p63 is found as Triton X-100-insoluble oligomers when membranes are solubilized at pH 8.0. As the pH of the solubilization buffer was decreased, more p63 was observed in the pellet. In contrast, mutants of p63 that were expressed on the cell surface were not found as Triton X-100-insoluble oligomers under these conditions. Based on these observations and those in this work, it is tempting to speculate that the formation of STcys-insoluble oligomers at pH 6.3 reflect its ability to oligomerize in the microenvironment of the late Golgi and its localization there, whereas the lack of strict pH dependence of the oligomerization of the STser and the STtyr N158Q mutant may lead to their oligomerization and localization earlier in the Golgi.

Other Mutations in the Early Part of the ST6Gal I STtyr Catalytic Domain Also Alter Its Trafficking in the Cell—What about the STcys protein allows it to form more stable oligomers than the STtyr protein? There are two possibilities that are not mutually exclusive. First, the STtyr and STcys proteins may have different catalytic domain conformations because of a single amino acid change in this region. Second, the introduction of an additional Cys residue to the STtyr luminal region that already contains six Cys residues that are likely to form intramolecular disulfide bonds may lead to cross-linking between monomers or with other resident Golgi proteins. To test the first possibility, that changing the amino acid at position 123 in the catalytic domain changes the conformation of this domain and its ability to form stable oligomers, we analyzed the effects of placing different amino acids at this position. We introduced a Phe at this position expecting that the mutant protein would more closely resemble STtyr and a Ser at this position expecting that the mutant protein would more closely resemble STcys but lack the ability to form disulfide bonds. We compared the cleavage and secretion of these new mutants to that of the STtyr and STcys proteins and found that they behaved as expected (Fig. 6, compare STtyr with STphe and STcys with STser). The STphe protein was catalytically active and behaved like the STtyr protein upon pulse-chase analysis with greater than 50% of both proteins found in the cell medium after a 1-h labeling and a 6-h chase (data not shown and Fig. 6, compare STtyr with STphe). Likewise, the STser behaved very similarly to the STcys. It was catalytically active and Golgi-localized (data not shown), but only 7% of this pro-
increased when membranes are solubilized at pH 6.3. The STcys isoform is quantitatively found as insoluble oligomers when Golgi membranes are lysed at pH 5.8–6.3, whereas only a small amount of the STtyr isoform is found as insoluble oligomers under these conditions. STtyr, STcys, and the STtyr N158Q mutant were transiently expressed in COS-1 cells. Golgi-enriched membranes were isolated from these cells using the method of Xu and Shields (22). One half of the isolated membranes were retained as total (T), and the other half were solubilized with MNT buffers of pH 6.3 or 8.0 and separated into soluble (S) and insoluble (P) fractions according to the method of Schweizer et al. (17). Membrane fractions were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotted using the affinity purified rabbit anti-rat ST6Gal I antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG and the SuperSignal West Pico chemiluminescence reagent (Pierce). The STtyr N158Q mutant, which lacks one of two N-linked oligosaccharides, is found as insoluble oligomers under both lysis conditions; however, the amount of oligomer recovered is increased when membranes are solubilized at pH 6.3.

When subjected to the Triton X-100 insolubility assay, the STcys and STtyr C139S proteins demonstrated intermediate levels of insoluble oligomer formation when membranes were lysed at pH 6.3. 68% of the STser was found in insoluble oligomers at pH 6.3, whereas only 32% of the STphe was found in insoluble oligomers under these membrane lysis conditions (data not shown). These results placed these proteins in between the STcys and STtyr in terms of their ability to form insoluble oligomers at the lower pH (STcys (100%) > STser (68%) > STphe (32%) > STtyr (13%)). In addition, both the STser and STphe proteins demonstrated no obvious differences in stability relative to the STcys and STtyr isoforms in either pulse-chase analyses or in the insolubility assays (data not shown). These results suggest that the nature of the amino acid at position 123 rather than its ability to form disulfide bonds can explain the increased ability of the ST6Gal I protein to form oligomers.

It was clear from the data in Fig. 3 that disulfide-bonded dimer forms of the ST6Gal I seemed to be preferentially found in the insoluble oligomers of the rat liver enzyme when membranes were lysed at pH 6.3 or 5.8. We have demonstrated that both isoforms are capable of forming dimers, so this observation should not be construed to suggest that only the STcys is found in the insoluble fraction of rat liver Golgi membranes (Fig. 7). However, it was also not unlikely that the presence of a free Cys would lead to additional disulfide bond formation between adjacent ST monomers or even other Golgi proteins. Another mutant of the STtyr, in which Cys139 is converted to a Ser (STtyr C139S), also demonstrated reduced cleavage and secretion (Fig. 6, C139S). This mutant eliminates one of the six Cys residues in the STtyr catalytic domain and therefore may also demonstrate increased disulfide bonding to itself or other proteins. To analyze these possibilities we tested the ability of the STtyr, STcys, STser, and STphe and the STtyr N158Q and STtyr C139S proteins to form disulfide-bonded dimers or higher molecular mass oligomers. The ST proteins were expressed in COS-1 cells; Golgi membranes were isolated from these cells, solubilized, and separated on SDS-polyacrylamide gels under nonreducing conditions; and ST proteins were detected by immunoblotting. We found that all of these proteins formed the disulfide-bonded dimer (Fig. 7). Notably, no additional high molecular mass forms of the STtyr or STphe proteins were detected under nonreducing conditions. However, a small proportion of both the STcys (19%) and STtyr C139S (9%) proteins were detected in heterogeneous higher molecular mass complexes (Fig. 7, STcys and STtyr C139S). These results suggested that additional disulfide bonded hetero- or homo-oligomers of STcys could exist in the Golgi, but are not found at high enough levels to account for the Triton X-100-insoluble oligomers observed in the pH shift assay. The very small amount of higher molecular mass oligomers formed by the STcys (3%) and STtyr N158Q (5%) proteins are likely to reflect SDS-resistant oligomers rather than disulfide-bonded oligomers because these proteins do not possess additional Cys residues in their luminal regions (Fig. 7, STser and STtyr N158Q). In sum, the above comparison of STtyr, STcys, and the various mutant proteins suggest that the STcys isoform has an altered conformation that allows it to form more stable oligomers in the low pH conditions found in the late Golgi. In addition, although the STcys certainly has the ability to form disulfide bonded homo- and hetero-complexes, this ability is not likely to be the major driving force behind this protein's more stable Golgi localization.

DISCUSSION

In this work, we have provided evidence that the ST6Gal I isoforms use an oligomerization based mechanism for their
localization in the Golgi. Significant amounts of rat liver and rat hepatoma ST6Gal I are found as Triton X-100-insoluble oligomers when Golgi membranes are lysed at pH 5.8–6.3, the pH of the late Golgi cisternae, but not at higher pH values. Both the STTyr and STCys isoforms of the ST6Gal I are found in rat liver, but their ratio is not definitively known, so that their contribution to the pool of insoluble oligomers is not clear from these analyses. However, individual analysis of the STTyr and STCys isoforms reveals that the STCys, which is more stably localized in the Golgi, quantitatively forms or is stabilized as Triton X-100-insoluble oligomers when Golgi membranes are lysed with buffers of pH 6.3. In contrast, the STTyr, which is transiently localized in the Golgi, demonstrates a reduced ability to form these pH-dependent insoluble oligomers.

Analysis of mutant proteins with changes at position 123 or nearby amino acids suggests that a conformational difference between the luminal domains of the two isoforms rather than a difference in their ability to form disulfide-bonded complexes can account for differences in their ability to form Triton X-100-insoluble oligomers. First, the replacement of the Cys at position 123 with a Ser residue generates a protein that behaves much like the STCys isoform in that it is not effectively cleaved and secreted (Fig. 6). In contrast, the presence of a Phe residue in that position generates a protein that is cleaved and secreted at the same rate as the STTyr. Because the STSer protein does not have an additional Cys residue in its luminal sequences, this argues that a change in conformation is sufficient for more stable oligomerization. Second, a protein lacking one of the two N-linked oligosaccharides of the ST6Gal I (STTyr N158Q) behaves like the STCys and STTyr and has a reduced level of cleavage and secretion. The absence of an oligosaccharide early in the folding process may alter the STTyr N158Q proteins conformation and, in addition, the absence of this oligosaccharide in the mature protein may promote oligomerization by exposing more hydrophobic sequences or allowing closer contact between monomers. Third, although the STCys and STTyr C139S do show some ability to form higher molecular mass disulfide-bonded complexes, it is clear that their monomeric and dimeric forms predominate, so it is more likely that changes in the conformation of their luminal domains allow increased oligomer formation. Notably, mutation of other Cys residues (at positions 181, 332, 350, 361, and 403) in the STTyr catalytic domain either markedly misfold the protein leading to its retention in the STCys or do not substantially alter its trafficking.

Results presented in this work suggest that disulfide bond-based cross-linking of ST6Gal I protein that possesses an additional Cys residue in the luminal domain is not likely to control oligomerization and ultimate Golgi localization. However, disulfide bonded dimer formation is important to consider because we observe that the majority of rat liver Golgi ST6Gal I in insoluble complexes at pH 5.8–6.3 appears to be in the disulfide-bonded dimer form (Fig. 3). All the proteins we analyzed showed the ability to form disulfide-bonded dimers, but this ability did not necessarily correlate with high levels of oligomerization and stable Golgi localization. One possibility is that ST6Gal I dimers form Triton X-100-insoluble oligomers more easily and stably than monomers and that this may be an initial step in a two-step oligomerization process. As a result, the presence of disulfide-bonded dimer in the insoluble oligomers of the rat liver Golgi enzyme may reflect a core of an oligomer that is more stable and less sensitive to the membrane solubilization conditions.

Other researchers have obtained evidence that Golgi proteins are localized in the Golgi by an oligomerization or kin recognition mechanism. Machamer and colleagues (27, 28) demonstrated that chimeric proteins containing the first transmembrane region of the coronavirus M (E1) glycoprotein are localized in the Golgi and that this is correlated with the formation of high molecular mass SDS-insoluble oligomers. Analyses by Teasdale et al. (29) and Yamaguchi and Fukuda (30) suggest that homo-dimers and perhaps hetero-oligomers of the β1,4-galactosyltransferase correlate with its Golgi localization. Work done by Nilsson et al. (13, 14) support their kin recognition model by showing that the medial Golgi enzymes, α-mannosidase II and N-acetylgalactosaminyltransferase I, form hetero-oligomers through interactions between their stem regions. Other work from the Warren laboratory (31) demonstrates that medial Golgi proteins can be isolated in detergent-insoluble complexes, providing additional support for an oligomerization-based mechanism of Golgi protein localization.

Work performed by the Munro (6) and Quasha (7) laboratories suggest a bilayer thickness mechanism of Golgi localization for ST6Gal I-based chimeric proteins and the β1,4-galactosyltransferase. These researchers found that increasing the length of transmembrane regions of ST6Gal I-based chimeric proteins or the intact β1,4-galactosyltransferase protein lead to increased cell surface expression of these normally Golgi localized proteins. Why do our results differ from those of Munro, and can these differences be reconciled? In earlier experiments, we found that chimeric proteins consisting of the ST6Gal I cytoplasmic tail and transmembrane domain plus flanking sequences fused to the ectodomains of either the transferrin receptor or the influenza neuraminidase led to the increased localization of the these proteins in the Golgi (15). However, we found that this localization was not efficient and that signifi-
cant quantities of these chimeric proteins were expressed on the cell surface (15). This was in striking contrast to what we had observed for the wild type STCys protein that demonstrates no cell surface expression and what we have more recently observed for the wild type STTyr protein, which demonstrates a much lower level of cell surface expression (compare results in Ref. 15 to those in Ref. 2). These results also suggested that the luminal sequences play an important role in efficient Golgi localization. Munro’s studies (5, 6) were performed primarily with ST6Gal I chimeric proteins that lack the latter half of the ST6Gal I stem region and the entire catalytic domain. Although we initially hypothesized that the STTyr is localized in the Golgi using a bilayer thickness mechanism and the STCys is localized in the Golgi using an oligomerization mechanism, it is clear from the results presented in manuscript that both isoforms predominantly use an oligomerization-based mechanism. This is not to say that a bilayer thickness mechanism is not playing a role in the localization of these proteins. One could envision that the shorter length of the ST6Gal I transmembrane domain may function to concentrate these proteins in a particular Golgi cisterna and that protein oligomerization occurs when the protein reaches a certain threshold concentration (please see Ref. 25 for a more detailed discussion of this possibility).

Recently, several researchers have obtained data to support a cisternal maturation model of progression of secretory proteins through the Golgi (reviewed in Refs. 32–34). In this model, the first cisterna of the Golgi forms from the fusion of transitional ER vesicles, and then this cisterna and its contents mature through the stack as resident Golgi proteins, such as the glycosyltransferases and other modification enzymes, move in a retrograde fashion in COP-I-coated transport vesicles that fuse with the earlier cisternae. The differential distribution of proteins across the Golgi stack would occur because of competition of these proteins for packaging into retrograde transport vesicles, so that a cis Golgi localized protein would be more efficiently packaged into retrograde transport vesicles than a trans Golgi enzyme (33). In contrast, the vectorial transport model suggests that proteins move through the Golgi cisternae in transport vesicles that bud from earlier cisternae and fuse with the next cisternae in the cis to trans direction (35, 36). In this view Golgi proteins would be localized via retention mechanisms that potentially are driven by the differences in cisternal microenvironments across the Golgi stack.

Our data suggesting that the ST6Gal I isoforms are localized in the Golgi via an oligomerization-based mechanism are fully compatible with either the vectorial transport/retention model or the cisternal maturation model (see Fullekrug and Nilsson’s discussion of oligomerization and cisternal maturation in their review (34)). In the context of the vectorial transport/retention model, Golgi retention could occur through the formation of very large or insoluble oligomers of the ST6Gal I STCys isoform when this protein reaches the late Golgi and the pH is relatively low. The STTyr may also form oligomers, but these may be less stable so that this isoform is only transiently retained in this compartment. Therefore in the context of the cisternal maturation model, the ability of the STCys to more stably form oligomers would allow it to more efficiently enter retrograde transport vesicles and remain associated with the Golgi, perhaps even in an earlier Golgi compartment than the STTyr. In contrast, the inability of the STTyr to efficiently oligomerize would lead to its inefficient packaging into retrograde transport vesicles and its localization later in the Golgi relative to the STCys and/or its lack of localization in this compartment and movement into a post-Golgi compartment where it is cleaved and secreted. No matter which mechanism is functioning in the transport of proteins through the Golgi apparatus, our data strongly suggest that the localization of the ST6Gal I isoforms is achieved through an oligomerization-based process. Further work will be required to determine the nature of these oligomers and whether they function in signaling retrograde transport or retention.

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REFERENCES
1. Roth, J., Taatjes, D. J., Luoqo, J. M., Weinstein, J., and Paulson, J. C. (1985) Cell 43, 287–295
2. Ma, J., Qian, R., Rausa, F. M., III, and Colley, K. J. (1997) J. Biol. Chem. 272, 672–679
3. Kitamura-Kawaguchi, S., Dohnae, N., Takio, K., Tsuji, S., and Colley, K. J. (1999) Glycobiology 9, 1397–1406
4. Bretscher, M. S., and Munro, S. (1993) Science 261, 1280–1281
5. Munro, S. (1993) EMBO J. 12, 3577–3588
6. Munro, S. (1995) EMBO J. 14, 4695–4704
7. Masliah, A. S., Balaji, P. J., Boegeman, E. E., and Quasha, P. K. (1993) J. Biol. Chem. 268, 9506–9516
8. Munro, S. (1995) Biochem. Soc. Trans. 23, 527–530
9. Orci, L., Montesano, R., Medina, P., Malaise-Lagae, F., Brown, D., Perrelet, A., and Vassalli, P. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 293–297
10. Nezil, F. A., and Bloom, M. (1992) Biophys. J. 61, 1176–1183
11. Machamer, C. E. (1991) Trends Cell Biol. 1, 141–144
12. Nilsson, T., Slusarewicz, P., Hoe, M. H., and Warren, G. (1993) FEBS Lett. 330, 1–4
13. Nilsson, T., Hoe, M. H., Slusarewicz, P., Rabouille, C., Watson, R., Hunte, F., Watzle, G., Berger, E. G., and Warren, G. (1994) EMBO J. 13, 562–574
14. Nilsson, T., Rabouille, C., Hui, N., Watson, R., and Warren, G. (1996) J. Cell Sci. 109, 1975–1989
15. Dahdal, R. Y., and Colley, K. J. (1993) J. Biol. Chem. 268, 26310–26319
16. Colley, K. J., Lee, E. U., and Paulson, J. C. (1992) J. Biol. Chem. 267, 7794–7793
17. Schweizer, A., Rohrer, J., Hauri, H.-P., and Kornfeld, S. (1994) J. Cell Biol. 126, 25–39
18. Fleischer, S., and Kervina, M. (1974) Methods Enzymol. 31, 6–41
19. Ma, J., and Colley, K. J. (1996) J. Biol. Chem. 271, 7738–7746
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. Wessèl, D., and Fluegge, U. I. (1984) J. Biol. Chem. 259, 13814–13817
22. Xu, H., and Shields, D. (1993) J. Cell Biol. 122, 1169–1184
23. Chen, C., and Colley, K. J. (2000) Glycobiology 10, in press
24. Munro, S. (1998) Trends Cell Biol. 8, 11–15
25. Colley, K. J. (1997) Glycobiology 7, 1–13
26. Anderson, R. G. W., and Orci, L. (1988) J. Cell Biol. 106, 539–543
27. Weisz, O. A., Swift, A. M., and Machamer, C. E. (1993) J. Cell Biol. 125, 1185–1196
28. Swift, A. M., and Machamer, C. E. (1991) J. Cell Biol. 115, 19–30
29. Teasdale, R. D., D’Agostaro, G., and Gleeson, P. A. (1999) J. Biol. Chem. 274, 4084–4096
30. Yamaguchi, N., and Fukuda, M. (1995) J. Biol. Chem. 270, 12170–12176
31. Slusarewicz, P., Nilsson, T., Hui, N., Watson, R., and Warren, G. (1994) J. Cell Biol. 124, 405–413
32. Glick, B. S., and Malhotra, V. (1998) Cell 95, 883–889
33. Glick, B. S., Elston, T., and Oster, G. (1997) FEBS Lett. 414, 177–181
34. Fullekrug, J., and Nilsson, T. (1998) Biochem. Biophys. Acta 1404, 77–84
35. Scheinkman, R., and Orci, L. (1996) Science 271, 1526–1533
36. Rothman, J. E., and Wieland, F. T. (1997) Science 272, 227–234
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