A single amino acid difference in the catalytic domain of two isoforms of the Δ2, 6-sialyltransferase (ST6Gal I) leads to differences in their trafficking, processing and oligomerization. The STTyr isoform is transiently localized in the Golgi and is ultimately cleaved and secreted, while the STCys is stably localized in the Golgi and is not cleaved and secreted. The stable localization of the STCys is correlated with its enhanced ability to oligomerize. To test the hypothesis that multiple signals can mediate Golgi localization and further evaluate the role of oligomerization in the localization process, we evaluated the effects of individually and simultaneously altering the cytosolic tail and transmembrane region of the STCys isoform. We found that the localization, processing and oligomerization of the STCys was not substantially changed when either the core amino acids of the cytosolic tail were deleted or the sequence and length of the transmembrane region was altered. In contrast, when these changes were made simultaneously, the STCys was converted into a form that was processed, secreted, and weakly oligomerized like the STTyr. We propose that STCys oligomerization is a secondary event resulting from its concentration in the Golgi via mechanisms independently mediated by its cytosolic tail and transmembrane region.

The glycan structures that modify proteins and lipids play a variety of roles during development, in the normal adult animal, and in various disease states (for review see (1)). The enzymes that add the terminal monosaccharides to both O-linked and N-linked glycans reside in the cisternae of the Golgi apparatus in an overlapping pattern that reflects the order in which they act on the growing carbohydrate structures. This pattern of glycosyltransferase localization allows the access of these enzymes to their sugar nucleotide donors, which are transported from the cytosol into the Golgi lumen by specific transporters, and their glycoconjugate substrates, which are generated in the same or earlier cisternae. Experimental manipulation of glycosyltransferase compartmentation disrupts the access of enzymes to their donors and substrates and alters the glycan structures synthesized by a cell (2,3). These observations highlight the importance of the sequential location of glycosylation enzymes throughout the Golgi apparatus for the efficient and accurate biosynthesis of cellular glycoconjugates.

The ST6Gal I is an Δ2, 6-sialyltransferase that adds terminal Δ2, 6-linked sialic acid to N-linked oligosaccharides of glycoproteins (4). This enzyme has been localized to the trans Golgi and the trans Golgi network of hepatocytes (5) and in post-Golgi compartments in other cells (see (6) for references). Like other glycosyltransferases, the ST6Gal I is also found as a soluble form in various body fluids such as colostrum and serum (for a review, see (6)). The ST6Gal I has previously been shown to exist as two naturally occurring isoforms that differ by one amino acid at position 123 in the catalytic domain (7). This amino acid change, from Tyr to Cys, is the result of a single A to G nucleotide change. The STTyr isoform is encoded by genomic DNA, while both the STTyr and the STCys isoforms are encoded by liver RNA, suggesting that the STCys RNA is generated by an RNA editing event (7).
Interestingly, the single amino acid change in the enzyme catalytic domain does not significantly impact the catalytic activity of the two ST6Gal I isoforms (8). Instead, the STtyr and STcys show dramatic differences in their trafficking and localization. The STtyr is transiently localized in the Golgi, cleaved in a post-Golgi compartment, and secreted from COS-1 cells with a half-time of approximately 6 hours (7). In contrast, the STcys is more stably localized in the Golgi, and is not rapidly cleaved and secreted, but is found in the endoplasmic reticulum (ER) when highly expressed (7,8). The stable Golgi localization of the STcys is correlated with its ability to form pH dependent, insoluble oligomers (9). We found that while the majority of the STcys isoform is recovered as insoluble oligomers at pH 6.3, the pH of the late Golgi, less than 20% of STtyr was found as insoluble oligomers under these conditions (9). These results suggest that the single amino acid difference in the catalytic domain of the ST6Gal I isoforms leads to subtle differences in the conformation of this region that allow the STcys to form more stable oligomers than the STtyr. This in turn correlates with the differences in the efficiency of Golgi localization of the two proteins.

There are currently two models for the transit of cargo proteins through the Golgi that also offer differing views of Golgi protein localization. In the vesicular transport model originally proposed by Rothman and colleagues (10,11), resident proteins such as glycosyltransferases, are stably localized (retained) in the Golgi cisternae, while the cargo proteins (those that are destined for the plasma membrane, secretory vesicles, and lysosomes) are transported in an anterograde direction to each successive cisterna in small COPI coated vesicles. In the context of this model, Golgi proteins were envisioned to be retained via a membrane partitioning (bilayer thickness) mechanism or by microenvironment specific oligomerization. The bilayer thickness model proposed by Bretscher and Munro (12) suggested that Golgi proteins, by virtue of their relatively short transmembrane regions, are unable to partition into transport vesicles destined for later compartments with “wider” membranes that are rich in cholesterol. While other laboratories provided evidence for this model (13,14), we found that increasing the length of the 17 amino acid transmembrane region of either ST6Gal I isoform did not enhance their Golgi exit (15). Alternatively, other investigators (16-18) proposed that, in the microenvironment of a particular Golgi cisterna, resident proteins form insoluble and / or very large homo- or hetero - oligomers that prevent their movement into adjacent compartments. The oligomerization of Golgi enzymes has been demonstrated by various laboratories, and in some cases, enzyme oligomerization is correlated with the localization of specific glycosyltransferases (9,19-21).

More recently, the cisternal maturation model has been proposed and supported by the work of several investigators (22-29). In this transport model, proteins exit the ER in COPII coated vesicles and fuse with, or coalesce to form, a vesicular tubular compartment or ER-Golgi intermediate compartment. At the same time, resident cis Golgi proteins, including cis Golgi glycosylation enzymes, are transported in a retrograde fashion in either COPII coated vesicles or other types of carriers, such as tubules (20, 21), from the cis Golgi cisternae to this compartment. The net result of this coordinated anterograde transport of cargo and retrograde transport of cis Golgi enzymes is the creation of a new cis Golgi cisterna, the contents of which are progressively matured by the retrograde transport and introduction of medial Golgi enzymes and then trans Golgi enzymes from more mature cisternae. In cisternal maturation model, the localization of Golgi enzymes is maintained by continuous retrograde transport, in contrast to the retention of enzymes predicted by the vesicular transport model.

As evidence accumulates in support of the cisternal maturation model, the role of lipid partitioning and oligomerization need to be reconsidered in these contexts, and other possibilities examined. In particular, the possibility that the cytosolic sequences of Golgi proteins may mediate incorporation into retrograde transport carriers via their interactions with cytosolic coat proteins, is worthy of investigation. Interestingly, while enzyme transmembrane regions are required for the Golgi localization of all glycosyltransferases
studied to date (6,18,30), and lumenal sequences have been found to be involved in the location of some (7,9,20,31,32), the role of enzyme cytosolic tails in Golgi localization has been less clear (31-36).

Our early studies did not support a role for the ST6Gal I cytosolic tail in its Golgi localization, but rather suggested important roles for the enzyme’s transmembrane and luminal sequences (15,37). Interestingly, analysis of chimeric proteins demonstrated that the ST6Gal I cytoplasmic tail and transmembrane region were able to only transiently localize reporter sequences in the Golgi, and this contrasted with the more stable localization of the STcys (15,37). These observations raised the possibility that Golgi localization is mediated by different simultaneously acting signals and mechanisms. We pursued the current studies in an effort to evaluate the possibility that multiple regions of the ST6Gal I isoforms can independently mediate Golgi localization, and to reevaluate the role of the cytosolic tail and enzyme oligomerization in this process. Our data demonstrate that the cytosolic tail and transmembrane region of the ST6Gal I both play roles in Golgi localization, and that the ability of the STcys to form pH dependent, insoluble oligomers is compromised only when both the cytosolic tail and transmembrane region are altered. These data strongly suggest that oligomerization of the STcys isoform is a secondary event that occurs only after the enzyme is concentrated in the Golgi by mechanisms that involve its transmembrane region and cytosolic tail.

MATERIALS AND METHODS

Tissue culture media and reagents, including Dulbecco’s modified Eagle’s Medium (DMEM), Opti-MEMI, Lipofectin, LipofectAMINE and fetal bovine serum (FBS), as well as oligonucleotides, Taq polymerase supermix, and restriction endonucleases were purchased from Invitrogen Corporation (Carlsbad, CA). SuperSignal chemiluminescence reagents were from Pierce. Prestained protein molecular mass standards (myosin 203 kDa; Δ-galactosidase, 109 kDa; Bovine serum albumin, 78 kDa; ovalbumin, 51 kDa; carbonic anhydrase, 34 kDa) were from Bio-Rad. Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA). Quick-Change site directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). [35S]-S-Express protein label and [Δ-35S] dATP were obtained from Perkin Elmer Life Sciences. Protein A-Sepharose was purchased from Amersham Biosciences. Fluorescein isothiocyanate-conjugated (FITC) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit and mouse secondary antibodies were obtained from Jackson Immunoresearch (West Grove, PA.). Other chemicals and reagents were purchased from Sigma (St. Louis, MO) and Fisher (Hanover Park, IL).

Construction of ST6Gall mutants- STTyr SA23 and STcys SA23 (9,15) cDNA constructs in the pSVL vector were used as templates for PCR construction of Δtail SA23-pSVL cDNAs of both ST6Gal I isoforms. The ΔtailSA23 mutants of both isoforms were amplified from their SA23-pSVL cDNAs using the 5' primer 5'-GCT CTA GAT GAA GAA AAA GAT CTT AAC C-3' and the general 3' pSVL primer 5'-GGG GTA CCG GTT TAG AGC AGA AG-3'. In these constructions, the 5' primer used for amplification effectively deletes amino acids 2-6 of the cytoplasmic tail to leave Met-Lys-Lys-Lys at the amino terminus and introduces a Xba I site in the 5' untranslated region. The products were digested with XbaI and SstI (found in the original pSVL polylinker region) and recloned into the pSVL vector cut with the same enzymes. The 5’ primer used for amplification effectively deletes amino acids 2-6 of the cytoplasmic tail to leave Met-Lys-Lys-Lys at the amino terminus and introduces an Xba I site in the 5’ untranslated region. The products were digested with XbaI and SstI (found in the original pSVL polylinker region) and recloned into the pSVL vector cut with the same enzymes. The tail deletion mutant for the STTyr was generated by digesting the existing STcys Δtail-pSVL cDNA and wild type STTyr-pSVL with Bgl II and inserting the portion of the catalytic region of STTyr (containing the one amino acid change) into the STcys Δtail-pSVL construct.

In order to generate stably expressing cell lines, the STTyr and STTyr Δtail SA23 were cloned into the pClneo vector (Promega Corp.). Coding sequences were amplified by PCR from the corresponding pSVL constructs. For the STTyr, 5’-CGC TCG AGA TGA TTC ATA CCA AC-3’ was used as the 5’ primer and generated a Xho I site in the 5’ untranslated
region, while 5'-CCT CTA GAT CAA CAA CGA ATG TTC CG-3' was used as the 3' primer and generated a Xba I site in the 3' untranslated region. The STtyr coding sequence was inserted into pCIneo using the XhoI and XbaI sites in the plasmid. For the STtyr Δtail SA23, 5'-GCT CTA GAT GAA GAA AAA GAT CTT AAC C-3' was used as the 5' primer and 5'-GAC TCT AGA TCA ACA ACG AAT GTT-3' was used as the 3' primer. These primers generated XbaI sites on both the 5' and 3' ends. The STtyr Δtail SA23 sequence was cloned into the pCIneo vector using the XbaI site in the polylinker. All sequences were confirmed by Sequenase 2.0 DNA sequencing.

Transfection of COS-1 cells- COS-1 cells were maintained in DMEM, 10% fetal bovine serum (FBS) and grown in a 37°C, 5% CO₂ incubator until 50-70% confluent in 100-mm tissue culture dishes or in 24 well plates containing 12-mm glass coverslips. Lipofectin transfections were performed according to the manufacturer's protocol. Briefly, 30 µl Lipofectin and 20 µg of DNA were incubated separately in 1.5 ml Opti-MEM in polystyrene culture tubes at room temperature for 35 min. They were then mixed together and then incubated for another 15 min at room temperature. The transfection mixtures were then added to cells washed with 2 x 5 ml Opti-MEM. The cells were then incubated in the 37°C, 5% CO₂ incubator for 6 h and then 4 ml DMEM, 10% FBS was added to the plates and the incubation continued for another 16 h. For coverslip transfections, 0.5 µg DNA and 3 µl Lipofectin were used in 150 µl Opti-MEM per coverslip.

Immunofluorescence Localization of ST6GalI mutants- COS-1 cells were plated on glass coverslips and transiently transfected with the ST6GalI cDNA expression constructs, as described above. Sixteen to 20 h post-transfection, the cells were stained as described previously (7). For immunolocalization of ST6GalI and its mutants, cells were fixed for 8 min in −20°C methanol for staining of internal structures. Cells were washed with 2 x 1 ml PBS, blocked for one hour with 5% goat serum in PBS, and incubated with a 1:100 dilution of an affinity purified rabbit anti-ST6GalI antibody in 5% goat serum in PBS for one hour. After again washing the coverslips with PBS (4 x 1 ml), the cells were incubated with a goat anti-rabbit IgG FITC-conjugated secondary antibody. The cells were washed a second time with in PBS (4 x 1 ml) washes and mounted on glass slides using 20 µl of mounting media (15% (w/v) Vinol 205 polyvinyl alcohol, 33% (v/v) glycerol, 0.1% sodium azide, pH 8.5, in PBS). Cells were visualized and photographed Leica DMRB microscope equipped with a Hamamatsu CCD camera driven by the OpenLab imaging program (Improvision, Coventry, England).

Generation of cell lines stably expressing ST6GalI proteins- HeLa cell lines stably expressing the STtyr and STtyr Δtail SA23 proteins were generated. The STtyr-pCIneo and STtyr Δtail SA23-pCIneo were linearized using the BamH I site in the vector and transfected into HeLa cells using Fugene 6 lipid mediated transfection according to the manufacturer's procedure. After 24 hours, the cells were passaged 1:4 into DMEM supplemented with 10% FBS and 500 µg/ml Geneticin. Cells were grown in selective media for 2 weeks and then clones were isolated and analyzed for stable protein expression by immunofluorescence microscopy (see above) and immunoblotting (38).

Metabolic Labeling of Cells – Sixteen to 20 h following transfection, cells were incubated with 10 ml cysteine/methionine free-DMEM for 1 h. After incubation, the media was replaced with 4 ml fresh cysteine/methionine free-DMEM containing 100 µCi/ml 35S-Express protein labeling mix (Dupont/NEN). Cells were radiolabeled for 1 h in a 37°C, 5% CO₂ incubator. After labeling, medium was removed and the labeled cells were washed with 10 ml PBS and chased for the indicated times in 4 ml unlabeled DMEM, 10% FBS. The media was collected at the end of the chase, the cells were washed with 10 ml PBS and then lysed with 1 ml of immunoprecipitation buffer 2 (50mM Tris-HCl, pH7.5, 150mM NaCl, 5mM EDTA, 0.5% Nonidet p-40, 0.1% SDS). Cell lysates and media samples were stored at −20°C overnight.
Immunoprecipitation of ST6Gal I - ST6Gal I isoforms and mutant proteins were immunoprecipitated from the cell lysates and the media using 5 µl of the purified rabbit anti-ST6Gal I antibody and protein A-Sepharose, as previously described (7). The immunoprecipitation beads were resuspended in 50 µl of Laemmli sample buffer containing 5% DME, boiled for 10 min and the entire sample, including Sepharose beads, was directly loaded into the gel well. The proteins were separated on a 10% SDS-polyacrylamide gel. Radiolabeled proteins were visualized by fluorography using 10% 2,5-diphenyloxazolone in dimethyl sulfoxide and gels were exposed to Kodak Biomax MR film at –80°C.

pH Dependent Oligomerization Assay- The pH dependent insolubility assay is based on the protocol of Schweizer et al. (39). Golgi-enriched membranes were isolated according to the method of Xu and Shields (40). Membranes were centrifuged for 1 h at 39,000 rpm in an SW41 rotor and solubilized with the pH 6.3 or pH 8.0 MNT buffers (20 mM MES, 30 mM Tris, 100 mM NaCl, 1.25 mM EDTA, 1 mM EGTA, 100 mM iodoacetamide, 1% Triton X-100). After a 30 min solubilization on ice, the lysates were transferred to a SW65 Ti (Beckman) ultracentrifuge tube and MNT buffers of the proper pH were used to fill the tubes. Following a 1 h centrifugation in the SW65 Ti rotor at 100,000 × g at 4°C, the pellets were solubilized in Laemmli buffer by sonication in a sonicating water bath for 5 min. The proteins in the supernatant were recovered by methanol precipitation. Briefly, four volumes of methanol were added to the samples and the mixtures were stored at –20°C overnight. The precipitated proteins were centrifuged at 5,000 rpm for 15 min and the pellets were solubilized in Laemmli sample buffer by sonication. The samples were separated on a 10% SDS-PAGE gel and visualized by immunoblotting with an anti-ST6Gal I antibody. Immunoblotting was performed as previously described (38).

RESULTS

The Golgi localization of some glycosyltransferases is found to be mediated by transmembrane region length (13,14,41) or sequence (42-44). However, we found that changing both the length and the sequence of the transmembrane region of either ST6Gal I isoform did not change their trafficking or localization in the Golgi (9,15). These observations led us to question whether another region of the proteins was acting as an additional localization signal. If glycosylation enzymes are maintained in the Golgi by continuous retrograde transport, as predicted by the cisternal maturation model, then one would predict the enzymes’ cytosolic tails would mediate the clustering of enzymes into coated membrane regions prior to transport via interactions with cytosolic coat proteins. With this in mind, we considered the possibility that the cytosolic tail of the ST6Gal I was acting as a redundant signal for Golgi localization.

Either the cytosolic tail or the transmembrane region, in conjunction with the luminal sequences, are sufficient to maintain the stable Golgi localization of the STcys isoform. To evaluate the role of the cytoplasmic tail and transmembrane sequences in STcys Golgi localization, we constructed a STcys tail SA23 protein in which the five core amino acids of the nine amino acid cytoplasmic tail were deleted and the 17 amino acid transmembrane region was replaced with 23 amino acids of the transmembrane region of the influenza neuraminidase, a cell surface protein (Figure 1). We compared the localization, processing and oligomerization of the STcys Δtail SA23 mutant to the wild type STcys, the STcys Δtail and STcys SA23 mutant proteins, and the STtyr protein (see Figure 1 for schematic representations of all ST6Gal I isoforms and mutant proteins).

In order to ensure that the STcys Δtail SA23 mutant was properly folded and exited the ER, we compared the localization of this mutant to that of the STcys Δtail, STcys SA23, and STtyr and STcys proteins. This analysis was also important to do in light of recent work by Giraudo and Maccioni (45) who demonstrated...
that Golgi glycosyltransferases possess signals for export from the ER in their cytoplasmic tails. These signals are comprised of Arg and Lys residues in the cytoplasmic tail adjacent to the membrane-spanning region. For the ST6Gal I, it is likely that the three Lys residues immediately adjacent to the membrane-spanning region represent its ER export signal. Following expression in COS-1 cells, ST6Gal I proteins were localized by indirect immunofluorescence microscopy using an affinity purified anti-ST6Gal I polyclonal antibody and a FITC-conjugated secondary antibody. As seen in Figure 2, the STcys, STcys Δtail and STcys SA23 proteins exhibited both Golgi and ER staining, while the STtyr appeared to be more tightly Golgi localized. Interestingly, the localization of the STcys Δtail SA23 protein was very similar to that of the STtyr with more pronounced Golgi localization and less ER localization. This was the first suggestion that altering both the cytoplasmic tail and transmembrane region of the STcys had altered its trafficking. In addition, our localization analysis confirmed our predictions that the three Lys residues adjacent to the membrane-spanning region are sufficient to mediate export of enzyme from the ER.

To determine whether the STcys Δtail SA23 protein was trafficking like the STtyr protein, we evaluated the processing of the STcys protein and STcys mutant proteins and compared them to the STtyr. The ST6Gal I proteins in the pSVL vector were transiently expressed in COS-1 cells. Expressing cells were labeled for 1 h with 35S-Met/Cys and chased with unlabeled medium for 6 h. ST6Gal I proteins were immunoprecipitated from both cell lysates and medium fractions and the immunoprecipitates analyzed by SDS-PAGE and fluorography. We found that while approximately 40% of the STtyr was found in the medium after a 6 h chase period, less than 20% of the STcys, STcys Δtail and STcys SA23 were found in the medium after this chase period (Figure 3). Interestingly, the STcys Δtail SA23 was cleaved and secreted to a greater extent than the other STcys proteins. Like the STtyr, approximately 40% of the STcys Δtail SA23 protein was also found in a cleaved form in the medium after a 6 h chase.

To more carefully evaluate the kinetics of this cleavage and secretion, we performed a more extensive pulse-chase analysis of the STcys, STcys ΔtailSA23 and STtyr (Figure 4). We found that after 2 h of chase, approximately 10% of all three proteins had exited the cell or been degraded. After that point, the amount of the STcys remaining in the cell greatly exceeded that of either the STtyr or STcys Δtail SA23. Since the amount of STtyr and STcys Δtail SA23 proteins recovered from the media after 2, 6, and 12 h chase did not substantially differ, it was likely that some proportion of these proteins were being diverted for degradation, possibly in the lysosome. A similar observation was made in our earlier work (7). Nevertheless, whether secreted or degraded, the STtyr and STcys Δtail SA23 proteins were being trafficked in a similar fashion and leaving the Golgi with the same kinetics.

Taken together these data suggest that the cytoplasmic tail or the transmembrane region, in conjunction with the STcys catalytic domain, are sufficient to prevent this protein from moving beyond the Golgi apparatus. Only after both regions are altered can the STcys move beyond the Golgi for cleavage and secretion or degradation. These results also point to the fact that the STcys catalytic domain alone is not sufficient for stable localization. STcys pH-dependent, insoluble oligomer formation is compromised only when both the cytosolic tail and transmembrane region are altered. Besides their differences in trafficking and secretion, another difference between the two ST6Gal I isoforms is found in their ability to form oligomers. Previous work correlated the formation of pH dependent insoluble oligomers of the STcys with its stable localization (9). Since the only difference between the two isoforms is a single amino acid in the catalytic domain, we surmised that the conformation of the STcys catalytic domain is permissive for oligomer formation, while that of the STtyr catalytic domain is not. Based on work done previously by ourselves and others that showed that the cytoplasmic tail and transmembrane regions of glycosyltransferases were sufficient to
localize reporter sequences in the Golgi with varying levels of efficiency (for example (15, 46)), and the data presented in Figures 3 and 4 that show that the STcys catalytic domain alone does not allow stable enzyme localization, we predicted that the tail and transmembrane regions of the ST isoforms may be mediating events that concentrate the isoforms in the Golgi. In the case of the STcys isoform, this concentration could in turn allow oligomerization.

To evaluate whether the cytoplasmic tail and/or transmembrane region of the enzyme are necessary for oligomerization, we subjected the STtyr, STcys, STcys Δtail, STcys SA23 and STcys Δtail SA23 to the pH-dependent insolubility assay previously used to distinguish differences in the abilities of the STtyr and STcys to form oligomers (9). ST6Gal I proteins were transiently expressed in COS-1 cells and Golgi-enriched membranes isolated. Membranes were solubilized in buffers of pH 6.3 and 8.0 containing 1% Triton-100. Soluble (S) and insoluble, pelleted material (P) was separated by high-speed centrifugation and analyzed by SDS-PAGE and immunoblotting (Figure 5). We found that 70% of the STcys, 78% of the STcys Δtail, and 81% of the STcys SA23 were found as insoluble oligomers at pH 6.3. In contrast, only 28% of the STcys Δtail SA23 was found as an insoluble oligomer at pH 6.3. This was very similar to the extent of oligomerization of the STtyr (35%) at this pH. These results suggested that the cytosolic tail and transmembrane regions independently mediate events necessary for oligomerization and that oligomerization is likely to be a secondary event that enhances the localization process.

**Alteration of the cytosolic tail and transmembrane region of the STtyr increases the rate of its Golgi exit only slightly.** As shown above, when we alter the STcys cytoplasmic tail and transmembrane region, the STcys Δtail SA23 protein is now cleaved and secreted with the same kinetics as the STtyr and fails to oligomerize to the extent that is observed for the unaltered STcys protein. If indeed the cytosolic tail and transmembrane region are the only two localization signals in the protein, then a STtyr Δtail SA23 protein should be more rapidly cleaved and secreted than the unaltered STtyr protein. To evaluate this possibility we generated HeLa cells stably expressing both the STtyr and STtyr Δtail SA23 and compared the amount of enzyme exiting the Golgi between 2 and 6 h of chase. We did the same comparison in transiently expressing cells. In both cases, we found that there was a slight increase in the rate of exit of the STtyr Δtail SA23 relative to the unaltered STtyr (data not shown). The amount of the stably expressed STtyr found intracellularly did not vary between 2 and 6 h of chase, while the amount of the stably expressed STtyr Δtail SA23 in the cell (Golgi) decreased by 12% during this time period. Notably, the transiently expressed enzymes were secreted to a greater extent than the stably expressed enzymes with a decrease of cellular (Golgi) enzyme of 53% for the STtyr and 66% for the STtyr Δtail SA23 between 2 and 6 h of chase. Overall, altering the tail and transmembrane region of the STtyr led to a 12-13% increase in its secretion between 2 and 6 h of chase. In addition, the differences in the rates of cleavage and secretion of the transiently and stably expressed enzymes suggested that the higher level of expression in the transiently expressing cells was likely saturating the existing localization mechanism(s). In sum, altering the cytosolic tail and transmembrane region of the STtyr only slightly enhanced the Golgi exit of the protein and suggested the possibility that other interactions, likely mediated via the unaltered luminal sequences, might be slowing exit from the Golgi.

**DISCUSSION**

In this work we have shown that the ST6Gal I cytosolic tail and transmembrane region function as independent Golgi localization signals which may mediate the same or different Golgi localization mechanisms. Deletion of the five core amino acids of the cytosolic tail (leaving MKKK), or replacement of the transmembrane region with a longer region from a plasma membrane protein, did not substantially impact STcys localization,
processing or oligomerization. In contrast, making these changes simultaneously in the Δtail SA23 mutant converted the STcys into a protein that exhibited enhanced proteolytic processing and secretion and decreased pH-dependent oligomer formation. Surprisingly, a Δtail SA23 mutant form of the STtyr isoform exhibited only a 12-13% increase in the rate of its Golgi exit relative to the unaltered enzyme, suggesting that additional interactions mediated by lumenal sequences may slow the movement of enzymes through the Golgi. Because the cytosolic tail and transmembrane sequences of the two isoforms are identical, and the STtyr only weakly forms oligomers, we believe that it is unlikely that these regions directly mediate oligomerization. Instead, it is more likely that they mediate the concentration of the enzymes in the Golgi and that this allows the STcys, but not the STtyr, to form stable oligomers. We predict that these oligomers enhance retention of the STcys in the cell by promoting retrograde transport into earlier regions of the Golgi and even the ER, explaining the combined ER-Golgi localization pattern previously observed for this ST6Gal I isoform (8).

It has been appreciated for many years that the amino terminal sequences of glycosylation enzymes including their cytosolic tails, transmembrane regions and stem sequences, play a role in their Golgi localization (6,18,30). Early work showed that the transmembrane regions of the Δ1, 4 galactosyltransferase (Gal-T1), ST6Gal I, N-acetylglucosaminyltransferase I (GnT-I) were necessary for Golgi localization (13,15,41,44,46-50). Work done by Munro (14,41) and Masibay *et al.* (13) demonstrated the importance of transmembrane region length for the Golgi localization of a ST6Gal I chimeric protein and the intact Gal-T1 protein, respectively. These observations led to the proposal of the bilayer thickness model of Golgi protein retention (12,51). However, a comparison of Golgi protein transmembrane region length demonstrates that enzymes with shorter transmembrane regions are many times physically and functionally found later in the Golgi relative to enzymes with longer transmembrane regions (for a discussion see 52). With this in mind, as well as our inability to disrupt the Golgi localization and trafficking of either full length ST6Gal I isoform by increasing the length of the transmembrane region (9,15), we suggest that a membrane partitioning mechanism is only one of several mechanisms acting together to mediate the Golgi localization of glycosylation enzymes.

Early work by Gleeson's laboratory elegantly showed that all regions of the GnTI, including the luminal sequences, play roles in its Golgi retention (31). Other work by this group showed that the GnTI and GnTII form salt resistant oligomers that correlate with their Golgi localization, and that luminal sequences, but not the cytosolic tails or transmembrane regions, are important for oligomer formation (20,47,53). Previous work from our laboratory (9) and the results presented in this work demonstrate that STcys oligomerization correlates with its stable retention in the Golgi, and suggest that the conformation of the STcys catalytic domain is critical for oligomer formation. Interestingly, other changes made in the catalytic domain can recapitulate the effect of having a Cys residue at position 123 (9). For example, the presence of a Ser residue at this position or the deletion of one of the two N-glycosylation sites in the STtyr protein generates proteins that oligomerize and are stably retained in the Golgi like the STcys (9).

Luminal sequences of glycosylation enzymes have not only been implicated in the oligomerization of the GnTI and GnTII and ST6Gal I STcys isoform, but also for a “kin recognition” interaction between the Δ-mannosidase II and GnTI (21,32). The original kin recognition hypothesis proposed by Nilsson and Warren (17) suggested that glycosylation enzymes in the same Golgi cisternae may form hetero-oligomers. The interaction of enzymes in the same glycosylation pathway is an attractive idea from the standpoint of efficient protein glycosylation and substrate channeling. The work of Giraudo *et al.* (54,55) and Pinhal *et al.* (56) provides additional support for such interactions between Golgi enzymes involved in glycolipid and heparan sulfate biosynthetic pathways. More recently, Sprong *et al.* (57) have demonstrated that the UDP-Gal transporter that resides in the Golgi of most cells is partially
relocated to the ER when co-expressed with the ER localized galactosyltransferase that catalyzes the synthesis of galactosylceramide. It is possible that lumenal interactions with other enzymes, the CMP-sialic acid transporter, and/or glycoprotein substrates are slowing the exit of the ST6Gal I isoforms from the Golgi, and these kinds of interactions might explain why the STtyr Δtail SA23 and STcys Δtail SA23 proteins are not more rapidly exiting this compartment.

In this work, we provide evidence that the cytoplasmic tail of the ST6Gal I is involved in its Golgi localization. Burke et al. (31) showed that the cytosolic tail of the GnTI plays a minor role in Golgi localization, while more recent work by Sandrin and colleagues (34,35) suggests a more major role for the cytosolic tails of both the Δ1, 2-fucosyltransferase and Δ1, 3-galactosyltransferase. Their work suggests that the cytosolic tail of Δ1, 2-fucosyltransferase, and particularly the presence of an hydroxylated amino acid in this region, mediates the intra-Golgi localization of the enzyme, while its transmembrane region mediates general trans Golgi network retention (35). While our data supports a role for the ST6Gal I cytosolic tail in Golgi localization, we have been unable to demonstrate that the Thr residue in this region is critical for its function.

We envision at least three mechanisms that work simultaneously to maintain the localization of glycosylation enzymes in the Golgi. The amino terminal cytoplasmic tail sequences of Golgi enzymes are likely to mediate their intra-Golgi localization by interacting with cytosolic coat proteins or Golgi matrix proteins, the former interactions being required for continuous retrograde transport as predicted by the cisternal maturation model, and the latter being required for more stable retention as predicted by the vesicular transport model. The transmembrane regions of glycosylation enzymes could be involved in membrane partitioning mechanisms that restrict movement of the enzymes beyond the Golgi and/or promote enzyme incorporation into retrograde transport vesicles. While transmembrane regions could also mediate protein-protein interactions for some enzymes, the luminal sequences of the ST6Gal I have been found to be primarily responsible for oligomerization. We propose that this oligomerization, in the case of the ST6Gal I isoforms, is the result of initial concentration of the enzymes mediated by other sequences. Based on the differences in their steady state localization, we predict that the oligomeric STcys isoform is trafficked differently by the cell than the monomeric/dimeric STtyr protein, and that it more readily is transported in a retrograde fashion into early regions of the Golgi and to the ER. Whether enzyme’s cytosolic sequences mediate interactions with coat proteins leading to incorporation into retrograde transport carriers, or interactions with matrix proteins leading to retention, is still not clear. In addition, we do not fully understand the factors controlling enzyme oligomerization and whether this process is reversible. These, and other questions are currently being addressed by our laboratory.

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FOOTNOTES

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1 Abbreviations used are: ST6Gal I (Δ2, 6-sialyltransferase); STcys and STtyr, isoforms of the ST6Gal I; endoplasmic reticulum, ER; DMEM, Dulbecco’s modified Eagle’s medium; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline; Gal-T1, Δ1, 4-galactosyltransferase; GnTI and GnTII, N-acetylgalcosaminyltransferase I and II; LacCer, lactosylceramide.

2 F. H. Fenteany and K. J. Colley, unpublished results.

FIGURE LEGENDS

Fig. 1. Schematic representation of ST6GalI isoforms and mutants. The full-length ST6Gal I isoforms and the three mutants of the STcys isoform are depicted. The STtyr and STcys isoforms differ by a single amino acid at position 123 in their catalytic domains (7). The construction of the STcys tail and STcys SA23 mutants were previously described (9,37). CT, cytosolic tail; TM, transmembrane region.

Fig. 2. Localization of the ST6Gal I isoforms and mutant proteins. COS-1 cells transiently expressing STtyr, STcys, and the STcys mutants were analyzed by indirect immunofluorescence microscopy using an anti-ST6Gal I antibody. Cells were fixed and permeabilized with −20°C methanol prior to staining to visualize internal structures. Stained cells were visualized and photographed Leica DMRB microscope.
equipped with a Hamamatsu CCD camera driven by the OpenLab imaging program. Calibration bar = 10 µm.

**Fig. 3.** Deletion of the core amino acids of the cytoplasmic tail and lengthening the transmembrane region of the STcys attenuates its stable localization in the Golgi. COS-1 cells transiently expressing STcys, STtyr and the tail and transmembrane region mutants were metabolically labeled for 1 h with 35S-Express protein labeling mix and chased for 6 h with unlabeled media. Proteins were immunoprecipitated from cell lysates and media samples using an anti-ST6Gal I antibody and analyzed under reducing conditions by SDS-PAGE and fluorography. Molecular mass markers: 51 kDa ovalbumin, 34 kDa carbonic anhydrase. For each protein, the percentage of material remaining in the cells and secreted into the medium was determined using the NIH Image program (Percentage of Total).

**Fig. 4.** Comparison of rates of cleavage and secretion of STtyr, STcys, and STcys Δtail SA23. COS-1 cells transiently expressing STtyr, STcys, and their Δtail SA23 mutants were metabolically labeled for 1 h with 35S-Express protein labeling mix and chased for 0, 2, 6, and 12 h. *Left,* Proteins were immunoprecipitated from cell lysates and medium samples using an anti-ST6Gal I antibody and analyzed by SDS-PAGE and fluorography. Molecular mass markers: 51 kDa ovalbumin, 34 kDa carbonic anhydrase. *Right,* The bands were quantified using the NIH Image program and the percentage of protein remaining in the cell was plotted as a function of chase time using Igor Pro, version 4.0.

**Fig. 5.** Evaluation of the pH-dependent insoluble oligomer formation of the STtyr, STcys, and STcys mutant proteins. STtyr, STcys and the STcys cytosolic tail and transmembrane region mutants were transiently expressed in COS-1 cells. Golgi-enriched membranes were isolated from these cells (40). 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. (39). Membrane fractions were analyzed by SDS-PAGE and immunoblotted using the affinity purified rabbit anti-rat ST6Gal I antibody and HRP-conjugated goat anti-rabbit IgG.
Figure 1
Figure 3

|                | Cell Lysates | Medium Fractions |
|----------------|--------------|------------------|
| Percentage of Total | 63 83 84 86 62 37 17 16 14 38 |
Figure 4
Multiple signals are required for α2, 6-sialyltransferase (ST6Gal I) oligomerization and localization

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