Specific Sequences in the Signal Anchor of the \(\beta\)-Galactoside \(\alpha\)-2,6-Sialyltransferase Are Not Essential for Golgi Localization

MEMBRANE FLANKING SEQUENCES MAY SPECIFY GOLGI RETENTION*

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Refka Y. Dahdah and Karen J. Colley

From the Department of Biochemistry, University of Illinois at Chicago, College of Medicine, Chicago, Illinois 60612

The \(\beta\)-galactoside \(\alpha\)-2,6-sialyltransferase is a trans Golgi/trans Golgi network glycosyltransferase which adds sialic acid residues to Asn-linked oligosaccharides of glycoproteins. Previous results suggested that the sialyltransferase stem and signal anchor including flanking sequences may be two independent Golgi retention regions. However, other experiments demonstrated that the sequence of the signal anchor itself was not important. To investigate whether the sialyltransferase signal anchor was necessary and sufficient for Golgi retention, several mutant and chimeric proteins were expressed and localized in Cos-1 and Chinese hamster ovary cells. We found that the signal anchor and flanking sequences were able to retain the sialyltransferase catalytic domain in the Golgi. However, efficient Golgi retention was still observed when the signal anchor was altered or entirely replaced in either the presence or absence of most of the luminal stem region. Chimeric proteins consisting of the sialyltransferase cytoplasmic tail and signal anchor fused to the extracellular domains of two different cell surface proteins demonstrated poor Golgi retention. A significant increase in the Golgi retention of one of these chimeras was observed when two lysines were placed next to the signal anchor on the luminal side. Taken together these results suggest that the sialyltransferase signal anchor is not necessary or sufficient for Golgi retention, rather, appropriate spaced cytoplasmic and luminal flanking sequences are the important elements of the sialyltransferase Golgi retention region.

Central to the study of protein sorting in the eukaryotic secretory pathway is a precise understanding of the signals and mechanisms which allow the specific localization of soluble and transmembrane proteins. Following their synthesis on endoplasmic reticulum (ER)\(^1\) membrane-associated ribosomes, nascent secretory proteins are cotranslationally translocated into the lumen of the ER where they are core glycosylated on Asn residues and correctly folded and oligomerized (reviewed in Refs. 1-4). The transport of secretory proteins from the ER, through the Golgi complex, and to the plasma membrane is thought to occur in a bulk flow fashion, with nascent proteins moving in vesicular carriers with the bulk flow of lipid until they are retained in a specific compartment (1). Retention or targeting signals have been identified for a variety of ER proteins (2, 4-7), Golgi proteins (reviewed in Refs. 8 and 9), and lysosomal proteins (reviewed in Refs. 10-12). Pelham and colleagues (2, 4) have elucidated the retention/retrieval signal and mechanism for soluble ER proteins, while other groups (5-7) have identified specific signals for the retention of ER transmembrane proteins. Several researchers have found that Golgi glycosyltransferases and Golgi-Localized viral proteins require sequences in and around their membrane spanning regions for Golgi retention (reviewed in Refs. 8 and 9). Soluble lysosomal enzymes are targeted to lysosomes by receptors which recognize mannose 6-phosphate recognition markers found on the Asn-linked oligosaccharides of these proteins (10, 11), while membrane associated lysosomal proteins have targeting signals within their cytoplasmic tails (12).

The Golgi complex is central to the sorting and modification of proteins in the secretory pathway. Morphologically the Golgi complex is composed of a series of cisternae (cis, medial, trans, and trans Golgi network (TGN) which have been functionally defined by the specific localization of glycosidases and glycosyltransferases that sequentially process the Asn-linked oligosaccharides of secretory proteins as they pass through these compartments (13, 14). Recently, Mellman and Simons (15) have suggested viewing the Golgi complex as a three-compartment structure consisting of the cis Golgi network, the medial Golgi, and the TGN. The cis Golgi network, consisting of the cis Golgi cisternae and the transitional elements between the EE and cis Golgi, is involved in the traffic from ER to Golgi and also in the recycling of proteins and lipids from this region back to the ER. The medial Golgi is thought to function as a glycosylation compartment and contain the majority of the enzymes involved in these processes. Presumably, the sequential, and at times overlapping, organization of Golgi glycosyltransferases would be maintained in this medial compartment (13, 16). The final Golgi compartment, the TGN, is known to play the central role in the sorting of plasma membrane, lysosomal, and regulated secretory proteins (13, 17). Recent results suggest that retention signals and mechanisms employed by proteins exclusively localized in the TGN could be different from those in the other Golgi compartments (18, 19).

While the terminal Golgi glycosyltransferases have similar type II domain structures, they possess no obvious sequence homology which would suggest a common Golgi retention signal (9, 14). Despite this limitation, several investigators have identified sequences in Golgi glycosyltransferases and other...
Sialyltransferase Golgi Localization Sequences

Golgi proteins which are important for their localization (reviewed in Refs. 8 and 9). Machamer and Rose (20) demonstrated that the first membrane spanning region of the infectious bronchitis virus (IBV) E1 glycoprotein is necessary for this protein's cis Golgi retention. Later work demonstrated that uncharged polar residues within the transmembrane α-helix are important components of this retention signal (21). Construction and expression of soluble forms of the β-galactoside α-2,6-sialyltransferase (ST) (22) and the β-1,4-galactosyltransferase (23), demonstrated that their Golgi retention signals reside in their amino-terminal cytoplasmic tail, signal anchor, and/or luminal stem regions. Further analysis demonstrated that the sequences in and around the signal anchor regions of the β-1,4-galactosyltransferase (23–26), N-acetylgalcosaminyltransferase I (27), and ST (28–30) are required for the Golgi retention of these proteins. Evidence from our laboratory (29) and that of Munro (28) suggest that the Golgi retention signal of the ST may be somewhat more complicated than that of other glycosyltransferases. Unlike other Golgi glycosyltransferases, changes in the sequences of the signal anchor of the ST do not result in the surface expression of the protein. In general, other experiments have demonstrated that other ST sequences, such as those which flank the signal anchor and within the luminal stem region, may participate in Golgi retention or constitute independent retention signals (28, 29).

To determine the relative importance of the signal anchor region in the Golgi localization of the ST, we constructed a series of mutant and chimeric proteins to determine what sequences of the ST are necessary and sufficient for Golgi localization. Our results suggest that the precise sequences of the ST signal anchor region are not necessary for Golgi retention since they can be altered or completely replaced with no change in Golgi retention. Since the signal anchor region and flanking sequences are able to retain the ST catalytic domain in the Golgi, it is likely that correctly spaced membrane flanking sequences constitute the ST Golgi retention signal. Localization of chimeric proteins demonstrated that the ST cytoplasmic tail and signal anchor region alone are not sufficient for Golgi retention. More efficient Golgi retention required 2 lysines residues in the luminal sequences flanking the signal anchor region. These results suggest that the signal anchor region of the ST is not necessary or sufficient for Golgi retention and that it can be replaced by any transmembrane region which allows correct spacing and folding of sequences flanking the membrane.

EXPERIMENTAL PROCEDURES

Materials

Blue script (bs®) and Escherichia coli strain XL-1 Blue were purchased from Stratagene, LaJolla, CA. Restriction enzymes were obtained from Pharmacia LIK Biotechnology Inc. and from Life Technologies, Inc. The pGEM-2 and pGEM-7 vectors were kindly provided by Dr. Susan Ross and Robert Cost, University of Illinois, Chicago, IL. Sequencing DNA sequencing kit was purchased from Pharmacia. Oligonucleotides were synthesized by GENOSYS, The Woodlanda, TX, and by Oligos, ETC., Wilsonville, OR. 35S-Express label was purchased from DuPont NEN. Qiagen columns for DNA purification were obtained from Boehringer Mannheim. 

Methods

In Vitro Oligonucleotide-directed Mutagenesis

In vitro oligonucleotide-directed mutagenesis was performed as previously described (29) according to the method of Zoller and Smith (31). For the ΔTAS mutant and the ΔSSA mutants, single-stranded DNA was made from ΔST-ΔST cDNA (29) subcloned into the bs®(phagemid) expression vector (Pharmacia) for expression in Cos-1 cells. The following oligonucleotides were used in the construction of the ΔTAS mutant and the ΔSSA mutants: ΔTAS, CTGCAATCTTATAGAAGAAGGTTA-CCGAC; ΔSSA1, GAGAAAAAGATCATCACCAGGGAGTCCGGTTGTCTCTTTTCT; ΔSSA2, GTTTCTGAGGTCTCTTGTCTCGCAG; ΔSSA3, CTTCATTGCTGGTCTTGTGGAGTCTGGTGT; SSA4, CTTTTCTTCGTGCAATGTGCCTGATCATGAGAGAGGAGCC. 

Construction of Signal Anchor Replacements and Chimeric Proteins

Polymerase chain reaction (PCR) using Vent polymerase (New England Biolabs) was performed according to manufacturer's instructions using ST, NA, and TR coding sequences in either bs® or pSVL as templates. The PCR fragments encoding the specific sequences of the chimeric proteins were gel-purified using low melting point agarose (Life Technologies, Inc.). and were then ligated into pSVL for expression in Cos-1 cells. The vector possessing the recombinant vaccinia virus, Specific restriction enzyme sites were incorporated into the oligonucleotides used in the PCR reactions and allowed the ligation of DNA fragments into these expression vectors and with other DNA fragments. The sequence of each mutant construct was verified by DNA sequencing using Sequenase enzyme (U.S. Biomedical Corp.).

Signal Anchor Replacements—Initially, SA29 containing the entire NA signal anchor region was constructed using these oligonucleotides: primer 1, 5'TATACAGGCTCTAAGGAG; primer 2, 5'GAATTCCTGCTCATCGTAGACTCTG; primer 3, GGTTTTAAAAAGGGACGGAGGTAATC; primer 4, CCCGGCCCCCATTTAATCTCCAGAA; primer 5, GGGAGGATTAAATACCGTCGTCATCT; primer 6, AAGCTTAGTACCATACCATTTGAGATATT. Primers 1 and 2 were used to synthesize the ST cytoplasmic tail, primers 3 and 4 were used to synthesize the ST stem and a portion of the catalytic domain, primers 5 and 6 were used to synthesize the signal anchor region of the NA. After sequential construction of the SA29 construct into bs®, the remaining portion of the ST catalytic domain was added by cleavage of BS-bs® and the partial SA29 construct with SacI (within the stem region) and SacI (polylinker of bs®) and insertion of this region of the wild type ST into the partial SA29 construct. Both SA23 and SA17 were constructed using SA29 as a template and simply truncating the signal anchor region which was ligated to the stem and catalytic domain of the ST. The T7 primer (primer 1) and SA23 primer (GATTGAAGTACTATTGTTTCAATG) were used to synthesize the ST tail and 33 amino acids of the stem signal anchor. The T7 primer (primer 1) and SA17 primer (GATTGACTTTTTCGGTTGCTAT) were used to synthesize the ST tail and 17 amino acids of the NA signal anchor, and the T3 primer and primer 3 (shown above) were used to synthesize the ST stem and catalytic domains for both constructs. Initially the SA23 and SA17 tail and signal anchor fragments (cut with XhoI and SacI) were subcloned into the XhoI and SacI sites of the pSVL vector. The SacI/XhoI ligation regenerates the SA1 site which was used with the HindIII site in the pGS7 polylinker to subclone the Drl (also compatible with SacI ends). HindIII ST stem and catalytic domain fragment to complete both constructs. Both SA23 and SA17 constructs were subcloned into the XhoI and SacI sites of pSVL for expression in Cos-1 cells.

ST/NA Chimeric Proteins—The ST/NA chimeras were generated by fusing the ST tail and signal anchor (generated from the ST-bs® plasmid}
Immunofluorescence Microscopy was not added. Pulse-chase analysis of expressed proteins proceeded as described previously (29) except Protein A-Sepharose was used for ST immunoprecipitations (with rabbit polyclonal antibody) and Protein G-Sepharose was used for NA (with goat polyclonal antibody) and TR (with mouse monoclonal IgG1 antibody). Immunoprecipitated proteins were eluted from Sepharose beads by boiling for 5 min in 1 ml of 1% SDS, 5% Tris-HCl, pH 7.5, 0.5 mg/ml bromphenol blue) with 10% β-mercaptoethanol.

Cell Surface Biotinylation

Cos-1 cells were transfected and labeled as described above, culture dishes were then placed on ice and washed with cold PBS. Cells were then biotinylated as previously described (29). Three milliliters of cold PBS containing 1mg/ml sulfosuccinimidyl (biotinamidyl) hexacontane was added to the cells, and the incubation was continued for 30 min with gentle rocking at 4 °C. Cells were washed four times with 5 ml of PBS containing 50 mM lysine to block any unreacted reagent. Cells were lysed and proteins immunoprecipitated with appropriate antibodies and immobilized secondary reagents as described above. The proteins were eluted from Protein G or Protein A-Sepharose beads by boiling for 5 min in 100 μl of 0.2 M Tris-HCl, pH 6.8, 1.0% SDS, 0.5 mM EDTA. Pellets were washed further in 100 μl of lysine buffer (10% SDS, 4% Triton X-100, 3% nonidet NP-40). Both the elution and wash were combined and 25 μl was reserved as "total." The remaining 225 μl were rotated for 1 h with 40 μl of a 50% suspension of streptavidin-agarose (Pierce Chemical Co.) at 4 °C. Complexes were pelleted, washed, and eluted as described above in preparation for SDS-polyacrylamide gel electrophoresis. In the CHO-recombinant vaccinia expression system, we and others have found that the level of wild type NA found on the cell surface is very low; however, we attempted to quantitate these results by densitometry scanning to try to compare the levels of the NA, STNA, and STKKNA at the cell surface relative to the amount of total protein expressed in each case.

RESULTS

Immunofluorescence Microscopy

Cos-1 and CHO cells expressing wild type, mutant, and chimeric proteins were processed for immunofluorescence microscopy as previously described (29). Following fixation, permeabilization (if required), and blocking steps, cells were incubated for 45 min with a 1:100 dilution of rabbit affinity-purified antibodies raised against the rat liver ST, rabbit polyclonal antibodies raised against the WSN NA (N1), or a monoclonal antibody (19B11) in blocking buffer. Following four PBS washes, primary antibody conjugates to fluorescein isothiocyanate were then incubated with the cells at a 1:100 dilution in blocking buffer for 45 min. Again, cells were washed four times with PBS prior to mounting on glass slides using 20 μl of mounting media (15% (v/v) Vinal 206 polyvinyl alcohol (Serva, 55% (v/v) Vinal 85 in PBS). Slides were visualized and photographed using a Nikon Axioshot microscope equipped with epifluorescence illumination and a 60X oil immersion Plan Apochromat objective.

Sialyltransferase Signal Anchor Region and Flanking Sequences Are Able to Retain the Catalytic Domain in the Golgi Apparatus—Previous experiments which localized mutant proteins consisting of either the ST stem and catalytic domain (sc-ST) or the ST tail, signal anchor (plus KRGSD in stem) and catalytic domain (ΔSTem-ST) to the Golgi apparatus (29), were consistent with the possibility that the ST possessed two independent Golgi retention signals, one in the signal anchor region and another in the stem region. The existence of two independent retention signals in the ST coding sequence may have made it impossible for us to observe the effects of altering the signal anchor sequence in the intact protein (29). To begin to test the hypothesis that the ST does have two retention signals and to analyze each separately, we constructed a series of mutants by altering the signal anchor sequence in the intact protein (29). In the case of the ΔSTe signal protein, we maintained the MKKK bordering the signal anchor on the cytoplasmic side of the membrane and KRGSD bordering the signal anchor on the luminal side of the membrane.
A. Sialyltransferase Mutants

ST  MIEHNLKKSFLIIFVLPPAVICWVKKSSR-----STEM---CATALYTIC DOMAIN
ΔΔΔΔ MIEHNLKKSFLIIFVLPPAVICWVKKSSR-----STEM---CATALYTIC DOMAIN
ASSA1 MIEHNLKKSFLIIFVLPPAVICWVKKSSR-----STEM---CATALYTIC DOMAIN
ASSA2 MIEHNLKKSFLIIFVLPPAVICWVKKSSR-----STEM---CATALYTIC DOMAIN
ASSA3 MIEHNLKKSFLIIFVLPPAVICWVKKSSR-----STEM---CATALYTIC DOMAIN
ASSA4 MIEHNLKKSFLIIFVLPPAVICWVKKSSR-----STEM---CATALYTIC DOMAIN

B. Sialyltransferase Signal Anchor Replacements

SA29 MIEHNLKKSFLIIFVLPPAVICWVKKSSR-----STEM---CATALYTIC DOMAIN
SA23 MIEHNLKKSFLIIFVLPPAVICWVKKSSR-----STEM---CATALYTIC DOMAIN
SA17 MIEHNLKKSFLIIFVLPPAVICWVKKSSR-----STEM---CATALYTIC DOMAIN

C. Sialyltransferase Chimeric Proteins

STTR MIEHNLKKSFLIIFVLPPAVICWVﺳرF----STEM---CATALYTIC DOMAIN
STNA MIEHNLKKSFLIIFVLPPAVICWV----STEM---CATALYTIC DOMAIN
STNA MIEHNLKKSFLIIFVLPPAVICWV----STEM---CATALYTIC DOMAIN
STNA MIEHNLKKSFLIIFVLPPAVICWV----STEM---CATALYTIC DOMAIN

Fig. 1. Construction of mutant ST proteins and chimeric proteins. A, construction of ΔΔΔΔ and ASSA mutants. In vivo oligonucleotide-directed mutagenesis was performed as described under "Methods," and the altered DNA was purified and sequenced to confirm the mutations. The ΔΔΔΔ construct contained MKKK on the cytoplasmic side of the ST signal anchor and stem amino acids, KKGS, were left on the luminal side of the signal anchor. The ASSA mutants were constructed by replacing 4–5 amino acids of the signal anchor of ST with amino acids from the signal anchor of the plasma membrane protein, influenza NA. B, construction of ST signal anchor replacement mutants. PCR techniques were used to synthesize DNA fragments encoding the ST tail and stem-catalytic domain and stretches of the NA transmembrane domain corresponding to 17, 23, and 29 amino acids. DNA fragments were ligated together using the restriction enzyme sites encoding the ST tail and stem-catalytic domain and stretches of the NA transmembrane domain. The ASSA mutants were all contained the full nine-amino acid cytoplasmic tail, but only the stem sequences, KKGS, bordering the signal anchor on the luminal side of the membrane.

When expressed in Cos-1 cells, metabolically labeled with [35S]Express protein label, and immunoprecipitated with anti-ST antibodies, the ΔΔΔΔ and the four ASSA mutant proteins migrated on SDS-polyacrylamide gels with the approximate molecular mass of the ΔStem-ST protein (~43–44 kDa) (Fig. 2). Localization of the ΔΔΔΔ protein in Cos-1 cells by indirect immunofluorescence microscopy demonstrated that it is localized in the Golgi complex just like the wild type ST enzyme (Fig. 3). These experiments demonstrated that the signal anchor region of the ST, MKKK flanking this region on the cytoplasmic side of the membrane, and KKGS flanking the signal anchor region on the luminal side of the membrane, are able to retain the ST catalytic domain in the Golgi complex.

Sequence of the Sialyltransferase Signal Anchor Region Is Not Critical for Golgi Retention Even in the Absence of Stem Sequences—To disrupt a possible Golgi retention signal in the signal anchor region of the protein in the absence of a second potential retention signal in the stem region, we constructed the ASSA mutants (Fig. 1A). In these mutants, the signal anchor region of the ΔStem-ST was altered by the replacement of 4–5 amino acids along its entire length using amino acids from the signal anchor region of the type II plasma membrane protein, influenza NA. Analysis of the localization of these mutants by indirect immunofluorescence microscopy also demonstrated that these proteins are localized in the Golgi complex like the wild type ST (Fig. 3). These results demonstrated that even in the absence of the stem region, the sequence of the ST signal anchor region is not critical for Golgi retention.

Replacement of the Sialyltransferase Signal Anchor Region with Other Signal Anchor Sequences of Different Lengths Does

Fig. 2. Expression of ATAS and ASSA mutants in Cos-1 cells and immunoprecipitation of expressed proteins. Cos-1 cells were transfected with the mutant cDNAs, subcloned into pSVL expression vector, using the lipofectin method and allowed to express for 16 h. Cells were pulse labeled with [35S]Express protein label in methionine-free DMEM and then were chased for 6 h in DMEM containing 10% fetal calf serum. Cells were lysed and mutant ST proteins were immunoprecipitated from the cell lysates. The expression and molecular mass of the mutant ST proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. All proteins were expressed at comparable levels and had similar half-lives. 14C-Methylated molecular mass marker indicated is ovalbumin (46 kDa).

Fig. 3. Localization of ΔΔΔΔ and ASSA mutants in the Golgi apparatus of the Cos-1 cells by indirect immunofluorescence microscopy. Cos-1 cells were grown on coverslips and transfected with the mutant cDNA using the lipofectin method. After 16 h of expression, cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. The ST proteins were detected by incubation with affinity-purified rabbit anti-ST antibodies followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG second antibody. Cells were visualized using a Nikon Axiphot fluorescence microscope. No cell surface immunofluorescence was detected for any of these mutant proteins. Magnification, × 750.
Not Interfere with Golgi Retention—To determine whether that signal anchor region of the ST was necessary for Golgi localization and to investigate the impact of the length of the signal anchor region on Golgi localization, we constructed a series of replacements of the ST signal anchor region using PCR techniques (Fig. 1B). In these mutant proteins, we replaced the ST signal anchor region with the entire signal anchor region of the plasma membrane protein, influenza NA (29 amino acids), in the SA29 protein, or the first 23 and 17 amino acids in this region in the SA23 and SA17 proteins, respectively. Following construction of these altered coding sequences, the mutant proteins were transiently expressed in Cos-1 cells and analyzed by metabolic labeling of the expressing cells, immunoprecipitation of the expressed proteins, analysis of these immunoprecipitated proteins by SDS polyacrylamide gel electrophoresis, and fluorography. The SA29 and SA23 proteins were immunoprecipitated by the anti-ST antibody and migrated with molecular mass predicted based on an increase in length of 12 and 6 amino acids, respectively (Fig. 4). Analysis of the localization of both the SA29 and SA23 by indirect immunofluorescence microscopy demonstrated that they are expressed in the Golgi complex like the wild type ST (Fig. 5). Overexpression of these mutant ST proteins in Cos-1 cells did not lead to expression at the cell surface (data not shown). In contrast, the SA17 protein appeared to express very poorly and was difficult to detect following immunoprecipitation (Fig. 4). Not surprisingly, indirect immunofluorescence microscopy demonstrated that this mutant ST protein is weakly expressed in the ER (Fig. 5). It is possible that the first 17 amino acids of the NA signal anchor region is not sufficient to span the membrane and this results in a grossly misfolded protein which does not leave the ER and is either poorly recognized by anti-ST antibodies and/or rapidly degraded. Similar changes in the intact NA protein demonstrated that 23 amino acids of the signal anchor region are adequate to span the membrane, whereas 17 amino acids of the signal anchor are not. These results strongly suggest that the signal anchor region of the ST can be replaced by a signal anchor region which adequately spans the membrane no matter what its length in amino acids.

Sialyltransferase Cytoplasmic Tail and Signal Anchor Region Are Not Sufficient for Golgi Retention of Chimeric Proteins—To determine whether the ST signal anchor domain and flanking sequences are sufficient for the localization of a non-Golgi protein in the Golgi, we constructed two chimeric proteins by fusing the ST cytoplasmic tail and signal anchor to either the extracellular domain of the TR (32) (STTR), or the extracellular stalk and head group of the influenza NA (STNA) (Fig. 1C). The above results suggested that the signal anchor region of the ST is not necessary for Golgi localization and that the sequences flanking this region in the ST (KKK- - - - - - KKGSD) are the primary determinants in the Golgi localization signal. If this is the case, then these two chimeric proteins would be predicted to be inefficiently or partially localized in the Golgi apparatus.

The STTR and wild type TR in the pSVL expression vector were transiently expressed and localized in Cos-1 cells, while the STNA and wild type NA in either the pGEM-2 or bs′ vector were transiently expressed in CHO cells using the recombinant vaccinia virus expressing the T7 polymerase (33). The STTR and STNA proteins and the wild type TR and NA proteins were immunoprecipitated from lysates of metabolically labeled cells using the appropriate anti-TR or anti-NA antibodies and immunoprecipitated proteins analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The STTR and STNA proteins were efficiently expressed and folded in Cos-1 or CHO cells, as demonstrated by the immunoprecipitation of radiolabeled proteins of the appropriate molecular mass from these cells (Fig. 6).

Indirect immunofluorescence microscopy of permeabilized (internal) and unpermeabilized (surface) cells expressing either the TR, NA, or STTR and STNA chimeric proteins was used to determine whether the ST tail and signal anchor were suffi-

![Fig. 4. Expression of the ST and signal anchor replacement mutants, SA29, SA23, and SA17 in Cos-1 cells and immunoprecipitation of expressed proteins. ST, SA29, SA23, and SA17 cDNAs in the pSVL expression vector were transfected into COS-1 cells using the lipofectin method. Cells were pulse-labeled with 35S-Express protein label in methionine-free DMEM and chased for 6 h in DMEM containing 10% fetal calf serum. ST, SA29, SA23, and SA17 proteins were immunoprecipitated from cell lysates using affinity-purified anti-ST antiserum and Protein A-Sepharose. Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The ST was more highly expressed than the SA29 and SA23 proteins; however, no significant differences in these protein's half-lives were observed over several experiments. 14C-Methylated molecular mass marker shown is ovalbumin (46 kDa). Contaminating actin (~44 kDa) is indicated by *.]
cient to localize these non-Golgi proteins in the Golgi apparatus (Figs. 7 and 8). Both the wild type TR and NA proteins are observed in the cell surface (Figs. 7 and 8, NA and TR). In addition, the high level of protein expression results in a significant proportion of these proteins being observed intracellularly. Like their wild type counterparts, the STTR and STNA chimeric proteins, are also localized intracellularly and at the surface of the expressing cells (Figs. 7 and 8, STNA and STTR).

The significant surface expression of these chimeric proteins, particularly the STTR chimera, demonstrated that the ST cytoplasmic tail and signal anchor regions alone are not sufficient to localize these chimeric proteins in the Golgi (Figs. 7 and 8).

The level of surface expression of these chimeras appeared to be related to the level of their expression in the cells. This is particularly well illustrated in the immunofluorescence micrographs of the STTR chimera (Fig. 8), in which two cells expressing different levels of this protein are presented. The cell expressing lower protein levels demonstrates less observable surface staining, while the cell expressing higher levels of protein demonstrates significant surface staining. These results contrast with what has been observed in the overexpression of the wild type ST. As has been previously demonstrated by our laboratory (29) and others (28), the overexpressed ST does not move to the cell surface, but appears to backup along the cisternae of the Golgi and into the ER. It is clear from these results that chimeric proteins possessing only the ST cytoplasmic tail and signal anchor region are poorly retained in Golgi apparatus and are lacking sequences and/or characteristics required for efficient, nonsaturable Golgi retention.

**Luminal Lysine Residues Significantly Improve Golgi Retention of Sialyltransferase-Neuraminidase Chimeric Proteins**—To reconstitute the complete retention signal containing the luminal flanking sequences of the stem region, in addition to those in the cytoplasmic tail and a spacer signal anchor region, we constructed the STKKNA chimeric protein which differed from the STNA chimeric protein in possessing KKSTQ rather than HMIQ flanking the ST signal anchor region on the luminal side of the membrane. Following expression in CHO cells using the recombinant vaccinia-T7 polymerase expression system and transfection with the STKKNA construct in pGEM-2, we analyzed the localization of this chimera by indirect immunofluorescence microscopy using anti-NA antibodies. From these experiments, it is clear that the STKKNA protein is much more efficiently retained in the Golgi and demonstrates little to no surface expression even when highly expressed in CHO cells (Fig. 7).

To demonstrate the difference in surface expression biochemically, we biotinylated cell surface proteins of metabolically labeled and chased CHO cells expressing NA, STNA, or STKKNA in order to compare intracellular and surface forms of each protein (Fig. 9). Quantitation of the protein bands by densitometry scanning showed that the proportion of total STNA protein at the CHO cell surface significantly higher than the proportion of total STKKNA protein at the cell surface. If we calculate the percentage of total protein which is found at the cell surface for NA, STNA, and STKKNA, and we arbitrarily set the surface percentage for NA at 100%, we calculate that surface levels of STNA and STKKNA are 71% and 20% of the surface NA level, respectively. These results suggest that the stalk and head regions of NA are not efficiently retained in the Golgi apparatus by the tail and signal anchor regions of the ST unless two stem lysine residues are present in the chimeric protein.

The processing of these proteins' Asn-linked oligosaccharides also suggested that the STKKNA protein was retained more efficiently in the Golgi than the STNA protein (Figs. 6 and 9). The wild type ST in rat liver and expressed in Cos-1 cells possesses a high proportion of Asn-linked carbohydrate structures which lack terminal processing (29). In contrast, the secreted form of the ST catalytic domain (sp-ST (29)) expressed in either Cos-1 cells (22) or CHO cells possesses more completely processed Asn-linked oligosaccharides. These results suggest that the wild type ST protein which is retained in the Golgi apparatus is unable to be completely terminal glycosylated, while the soluble form of the protein may be more accessible to glycosyltransferases and therefore more completely processed.
We observe the same phenomena with the incompletely retained STNA protein and the more completely retained STKKNA protein. As demonstrated by the differences in migration on SDS-polyacrylamide gels (Fig. 6) and the increased sensitivity of the STKKNA to endo-β-N-acetylgalcosaminidase H digestion (data not shown), the oligosaccharides of the STKKNA, which is efficiently retained in the Golgi, are less well processed than those of the STNA protein, which is observed at the cell surface and migrates more slowly on SDS-polyacrylamide gels (Figs. 6 and 9).

**DISCUSSION**

In this study we provide evidence that the sequences of the ST signal anchor are not necessary or sufficient for the Golgi retention of the wild type ST or chimeric proteins. Replacement of all or some of the signal anchor in the presence or absence of most of the stem region results in no change in ST Golgi retention (SA29 and SA23 (Fig. 5) and ΔSSA mutants (Fig. 3)). These results, and the Golgi localization of the ΔTAS protein, suggest that the sequences flanking the signal anchor region may be the most critical portion of the ST Golgi retention signal and that the signal anchor region plays the role of a spacer between these two portions of the retention signal. Localization of chimeric proteins, consisting of the ST cytoplasmic tail and signal anchor fused to either the TR or NA extracellular domains, demonstrates that the tail and signal anchor are not sufficient for efficient Golgi retention of these plasma membrane proteins (STNA and STTR, Figs. 7 and 8). More efficient retention of a STNA chimera is achieved only after two lysines are placed adjacent to the signal anchor on the luminal side of the membrane (STKKNA, Fig. 7). These data suggest that these flanking lysines may have reconstituted a ST retention signal which consists of cytoplasmic KKK, a spacer transmembrane region and luminal KK or KKGSD.

Immunofluorescence microscopy is a good method for obtaining general information on the localization of proteins in cells. However, this technique does not allow a precise cisternal localization of proteins in the Golgi complex (39). It is therefore possible that the Golgi-localized mutant and chimeric proteins analyzed in this work are localized in different Golgi cisternae than the wild type protein, and that changes within, or replacement of, the signal anchor region may result in slightly altered localization.

**Fig. 7. Localization of NA, STNA, and STKKNA chimeric proteins on the CHO cell surface.** CHO cells were grown on coverslips and infected with the recombinant vaccinia virus expressing the T7 polymerase at a multiplicity of infection of 5. These cells were then transfected with the cDNA encoding the chimeric proteins using lipofectin method. After expression for 16 h, cells were fixed with 3% paraformaldehyde followed by permeabilization with 0.1% Triton X-100 if internal staining was desired. The proteins were detected by incubation with rabbit anti-NA antibodies followed by incubation with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG second antibody. Cells were visualized using a Nikon Axiphot fluorescence microscope. Magnification, ×750.

**Fig. 8. Localization of the STTR and TR proteins on the COS-1 cell surface.** COS-1 cells were grown on coverslips and transfected with the STTR or TR cDNAs in the pSVL expression vector using the lipofectin transfection method. After 24–36 h of expression, the COS-1 cells were fixed and permeabilized as described under “Methods.” The STTR and TR proteins were detected with anti-TR antibodies and fluorescein isothiocyanate-conjugated goat anti-mouse IgG second antibody. Cells were visualized using a Nikon Axiphot fluorescence microscope. Magnification, ×750.

**Fig. 9. Quantitation of cell surface expression of NA, STNA, and STKKNA by cell surface biotinylation of expressing CHO cells.** Cells were infected with the recombinant vaccinia virus expressing the T7 polymerase, transfected using the lipofectin method, then pulsed labeled for 2 h with 35S-Express protein label in methionine-free DMEM and chased for 6 h in DMEM containing 10% fetal calf serum. Cells were then incubated with sulfo succinimidyl (biotinamidyl) hexo nate for 30 min at 4 °C, washed extensively with 50 mM lysine in PBS, and then lysed, and the expressed proteins were immunoprecipitated with goat anti-NA antibodies. Following several washes of the Protein G-Sepharose beads and elution of the immunoprecipitated proteins, 1/10 of the eluate was reserved as the “total” and 9/10 (“surface”) was incubated with streptavidin-agarose for 1 h at 4 °C. The complexes were pelleted, washed, and eluted by boiling in sample buffer. Proteins in each fraction were analyzed on SDS-polyacrylamide gels and visualized by fluorography. 14C-Methylated molecular mass markers shown are ovalbumin (46 kDa) and bovine serum albumin (69 kDa).
cisternal localization. Because of the unusual dual localization of the ST in the trans Golgi and TGN in the rat hepatocyte (40), its differential localization in the Golgi in intestinal absorptive and goblet cells (41), and the potential for more than one independent ST Golgi retention signal (29), it will be ultimately important to determine the precise localization of the ST mutant and chimeric proteins using immunoelectron microscopy.

The transmembrane regions of many Golgi proteins appear to be completely sufficient for Golgi retention, while other proteins require additional sequences flanking these transmembrane regions for efficient Golgi retention (reviewed in Refs. 8 and 9). The first transmembrane region of the IV E1 glycoprotein is necessary and sufficient for Golgi localization, and specific uncharged polar residues in this region are required for the retention of a chimeric protein containing this transmembrane region (20, 21). The β-1,4-galactosyltransferase, which is localized in the trans cisternae of the Golgi, also possesses a Golgi retention signal which appears to be entirely contained within its signal anchor region (23-26). Golgi retention signals of the N-acetylglucosaminyltransferase I and the ST appear to require sequences flanking their transmembrane regions for Golgi retention. Tang et al. (27) demonstrated that the cytoplasmic tail and signal anchor of the medial Golgi protein, N-acetylglucosaminyltransferase I, are not sufficient for Golgi retention of a dipetidyl peptidase IV reporter protein, and that complete Golgi retention is achieved only when 12 amino acids from the N-acetylglucosaminyltransferase I stem region are included. Similar requirements have been demonstrated for the ST by our laboratory (29) and that of Munro (28) (this work).

For protein sequences to be considered retention signals they must be shown to be both necessary and sufficient. Replacement of the ST signal anchor with either 29 or 23 amino acids of the influenza NA signal anchor does not change the Golgi localization of the ST. Since the influenza NA is a plasma membrane protein, no Golgi retention signals should be present in its sequences, and therefore this experiment demonstrates that the ST signal anchor is not necessary for its retention in the Golgi. This being the case, what does constitute the ST Golgi retention signal(s)? Previous results suggested that the stem region might contain a separate retention signal (28, 29), and results in this work suggest that the sequences flanking the signal anchor region may constitute a separate Golgi retention signal (ASSA mutants). Munro (28) replaced the ST signal anchor region with the 23-amino acid transmembrane region of dipetidyl peptidase IV and found that the resulting chimeric protein exhibited cell surface staining. He also demonstrated that 17 leucines plus the ST stem were sufficient for Golgi localization of a chimeric protein containing a lysozyme carboxyl-terminal domain. However, the presence of 23 leucines plus the ST stem resulted in the cell surface expression of a similar chimera. We suggest that these chimeras may not allow for the correct presentation of the ST sequences which flank the membrane on the luminal side. This would lead to a masking of the retention signal and the appearance of these chimeras at the cell surface. In our SA29 and SA23 proteins, it seems that the two lengths of the NA transmembrane region fold so that they are able to correctly present the ST luminal (and perhaps cytoplasmic) flanking sequences in a way that they can achieve retention in the Golgi.

To determine whether the ST signal anchor region is sufficient for Golgi retention, we and others (28, 30) have constructed a series of chimeric proteins containing this region. Both our STNA and STTR chimeras which possess the ST cytoplasmic tail and signal anchor region fused to the extracellular domains of NA and TR, are poorly retained in the Golgi. Golgi retention is significantly increased when two luminal lysine residues flanking the ST signal anchor region are included in the STNA chimera (STKKNA), suggesting that we have (partially) reconstituted a Golgi retention signal which consists of lysine residues flanking a spacer transmembrane region. In order to achieve complete Golgi retention, we attempted to construct chimeras which included the entire ST stem region, however these chimeras were misfolded and rapidly degraded. Consistent with our results, Munro (28) demonstrated that the signal anchor of the ST did not completely retain a chimeric protein in the Golgi. In contrast, Wong et al. (30) observed no cell surface expression of a chimera in which the 17-amino acid ST signal anchor region replaced the transmembrane region of the plasma membrane protein, dipetidyl peptidase IV. It was suggested that the cell surface expression that Munro observed with his chimeras, possessing only the ST signal anchor region, was a result of the aberrantly high level of expression in Munro’s transient expression system (30). Previous results have demonstrated that the wild type ST protein is never expressed on the cell surface of Cos-1 cells or CHO cells even when overexpressed in a transient expression system (28, 29) (this work). In contrast, the cell surface levels of the STNA and STTR chimeras are increased with increased expression (Fig. 8). These observations suggest that the ST cytoplasmic tail and signal anchor are not adequate for the efficient Golgi retention. Addition of two luminal flanking lysines significantly improves Golgi retention, however some of the STKKNA chimeras is still observed at the cell surface (Fig. 7). Interestingly, the STTR protein naturally possesses a lysine flanking the ST signal anchor region and yet this protein is very poorly retained in the Golgi (Fig. 8). The inefficient retention of the chimeric proteins could reflect both the lack of complete retention signals and the presence of carboxyl-terminal regions which either cause the improper folding of the amino-terminal retention regions and/or may not accommodate the ST Golgi retention mechanism.

It is likely that certain characteristics of the stem and catalytic domain of the ST perfectly accommodate the Golgi retention mechanism and allow for absolute retention. Variations in Golgi retention efficiency of chimeras may result from how well the non-Golgi reporter proteins can accommodate the Golgi retention mechanism. The ability to contact ST or other resident Golgi proteins may be important, especially if the Golgi retention mechanism involves oligomerization. With this in mind we chose the NA as a reporter protein. NA has basically the same type II domain structure as the ST and it also forms disulfide-bonded dimers which ultimately associate into tetramers (42, 43). The contacts which exist in these NA oligomers are known to occur between NA transmembrane, stalk and head regions (42, 43). We reasoned that if the Golgi retention mechanism involved interactions in carboxyl-terminal regions, then the NA stalk and head groups would most likely be able to make these contacts and ultimately be retained in the Golgi, when fused with the correct ST retention sequences. This worked to a large degree in the STKKNA protein, however this chimera was still not completely retained in the Golgi like the wild type ST. Until a mechanism for Golgi retention is elucidated, it will be difficult to know what features in these carboxyl-terminal regions are important for retention.

Based on the requirements for ST Golgi retention one can speculate on the type(s) of Golgi retention mechanisms which may be likely for this glycosyltransferase and potentially other Golgi proteins (see Ref. 8 for review). One potential retention mechanism is a protein or lipid receptor-mediated retention mechanism. Specific association with membranes of a defined lipid composition is a logical model since the sequences re-
quired for the Golgi retention of glycosyltransferases and other Golgi proteins are found in and around their membrane spanning regions. A gradient of cholesterol is known to exist across the Golgi cisternae, and similarly, particular lipids are thought to be enriched in either the cis or trans face of this organelle, with the cis cisternae having a lipid composition most like the ER and the trans cisternae having a lipid composition most like the plasma membrane (44–47). Lipid gradients could be the basis for the gradient of glycosyltransferases and glycosidases observed across the Golgi, and gradual alterations in these lipid gradients would also accommodate the variations in glycosyltransferase and glycosidase localization observed in different cell types (16, 41, 48). Several examples of cell type dependent variation in glycosyltransferase localization have now been reported. Roth et al. (41) demonstrated that the ST and the blood group A α1,3-N-acetylglactosaminyltransferase A are broadly localized in the medial and trans regions of intestinal absorptive cell, while being restricted to a trans region in adjacent goblet cells. Nilsson et al. (46) demonstrated an unusual overlapping localization of the β-1,4-galactosyl transferase I and β-1,4-galactosyltransferase in the trans Golgi of HeLa cells. More recently, Velasco et al. (48) demonstrated a cell type dependent distribution of the α-mannosidase I in the medial/trans Golgi in several different cell types. These results were particularly surprising since a cis Golgi localization has been always assumed for the α-mannosidase I in the traditional ordered compartmentation of enzymes in the Asn-linked glycosylation pathway. Although ER, Golgi, and plasma membrane lipid compositions are available for rat liver and kidney cells, further support for a lipid-Golgi protein interaction model must await the precise analysis of Golgi cisternae lipids in a variety of cell types.

A second potential mechanism is that of self-aggregation or hetero-oligomerization in the specific microenvironments of the Golgi cisternae. Possibly the glycosyltransferases or other Golgi proteins are induced to form self-aggregates or oligomers with other resident Golgi proteins because of the specific lipid composition, pH levels, and/or calcium concentrations in their resident Golgi cisternae. One potential problem with this mechanism is that at a trans time only a small fraction of the ST and β-1,4-galactosyltransferase are known to exist in rat Golgi membranes as demonstrated by the tarantol inactivation experiments of Fleischer et al. (49). However, others have reported larger detergent-insoluble complexes containing Golgi proteins (50).

It is clear that the ST does not bypass the Golgi when overexpressed, but rather appears to backup along the Golgi cisterne and into the ER (28, 29). This observation suggests that the ST Golgi localization mechanism may not exclusively involve a receptor-mediated process because it is not saturable. We can contrast this observation with the behavior of a group of mammalian and yeast proteins which contain Golgi localization signals in their cytoplasmic tails and upon overexpression are either found at the cell surface (TGN38) (18) or in the yeast vacuole (Kex2p) (19). This group of Golgi proteins may interact with clathrin and/or clathrin-associated proteins via tyrosine-based signals in their cytoplasmic tails (51). The ambiguous nature of the glycosyltransferase Golgi retention signal(s) also draws into question an exclusively receptor-mediated retention mechanism. One possibility is that a combination of events occurs to achieve efficient Golgi localization. Resident Golgi proteins could first associate with specific lipid compositions via sequences in and around their membrane-spanning regions, then due to either the microenvironment of the particular Golgi cisternae or perhaps a certain critical protein concentration, self-aggregate or interact with other resident Golgi proteins or lipids resulting in the formation of stable proteolipid complexes and their retention. It seems probable that the sequences required for the initial retention and the formation of resident Golgi complexes could be identical or overlapping so that different Golgi proteins would appear to have slightly different requirements for Golgi retention. The Golgi protein sequences spanning and flanking the membrane are most likely to make contacts with lipid head groups and fatty acid chains, while both these regions and luminal stem sequences would be involved in any further protein-protein contacts. The results presented in this paper suggest that sequences flanking the ST signal anchor region may actually make membrane contacts while the signal anchor sequences themselves may function to appropriately space these sequences across the membrane and ultimately act with the stem region in complex formation.

The current challenge is now to uncover evidence for specific Golgi retention mechanisms and to further characterize the regions of the ST and other glycosyltransferases and Golgi proteins which participate in these mechanisms. Electron microscopic and biochemical analysis of wild type, mutant, and chimeric proteins should provide insight into these problems and ultimately lead to a greater understanding of the structure and function of the Golgi complex.

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