The GlcNAc-6-sulfotransferases are a family of Golgi-resident enzymes that modulate glycan function. Two members of this family, GlcNAc6ST-1 and -2, collaborate in the biosynthesis of ligands for the leukocyte adhesion molecule L-selectin. Although their biochemical properties are similar in vitro, the enzymes have distinct glycoprotein substrate preferences in vivo. The sulfotransferases share similar overall architecture with the exception of an extended stem region in GlcNAc6ST-1 that is absent in GlcNAc6ST-2. In this study we probed the importance of the stem region with respect to substrate preference, localization, and oligomerization. Analysis of truncation mutants demonstrated that perturbation of the stem region of GlcNAc6ST-1 affects the cellular substrate preference of the enzyme without altering its retention within the Golgi. A chimeric enzyme comprising the stem region of GlcNAc6ST-1 inserted between the catalytic and transmembrane domains of GlcNAc6ST-2 had the same substrate preference as native GlcNAc6ST-1. In cells, GlcNAc6ST-1 exists as a dimer; two cysteine residues within the stem and transmembrane domain were found to be critical for dimerization. However, disruption of the dimer by mutagenesis did not affect either localization or substrate preference. Collectively, these results indicate that the stem region of GlcNAc6ST-1 influences substrate specificity, independent of its role in dimerization or Golgi retention.

Sulfation of glycans within the secretory pathway is a common modification that modulates their function on the cell surface, often by creating a novel epitope capable of binding a specific receptor (1, 2). For example, sulfation of sialyl Lewis x ((Sia2,3Galβ1,4/Fucα1,3/GlcNAc) αLe(x)-terminated glycans on the 6-position of GlcNAc activates the epitope as a ligand for the leukocyte adhesion molecule L-selectin (3, 4). This receptor-ligand interaction initiates the attachment of circulating leukocytes to endothelial cells that line the blood vessel wall, a requisite first step in the processes of lymphocyte homing and inflammatory leukocyte extravasation (5, 6). Two Golgi-resident GlcNAc-6-sulfotransferases capable of generating sulfated L-selectin ligands have been identified (7, 8). Termed GlcNAc6ST-1 and GlcNAc6ST-2, these enzymes are members of a family of GlcNAc/GalNAc/Gal-6-sulfotransferases that share ~30% overall sequence similarity and act on a variety of glycan types (2, 9). GlcNAc6ST-1 is expressed in most tissues in the mouse, whereas GlcNAc6ST-2 is expressed primarily in specialized blood vessels of the lymph node, where it plays a role in constitutive lymphocyte recirculation (10–12). Like most Golgi-resident enzymes, including other sulfotransferases and glycosyltransferases, GlcNAc6ST-1 and -2 share a similar anatomy (Fig. 1). They are type II proteins with an N-terminal cytosolic tail, a single pass transmembrane domain, a stem region of varying length, and a C-terminal catalytic domain in which the sequence similarity is greatest (Fig. 1).

The substrate preferences of GlcNAc6ST-1 and -2 have been studied in vitro using their truncated soluble catalytic domains and synthetic glycans (13–16). The enzymes behave similarly in these biochemical assays, modifying terminal GlcNAc residues irrespective of the structures of the underlying glycans. However, previous work in our laboratory (17) and others (10, 18) using cell-based systems revealed distinct substrate preferences for GlcNAc6ST-1 and -2. In Chinese hamster ovary (CHO) and COS-7 cells, GlcNAc6ST-1 preferentially sulfates N-linked glycans, whereas GlcNAc6ST-2 prefers to modify O-linked glycans. Furthermore, their distribution within the Golgi compartment was found to differ in Chinese hamster ovary cells (17). GlcNAc6ST-1 resides in the trans-Golgi network, whereas GlcNAc6ST-2 is more broadly distributed, with a strong representation in the earlier parts of the Golgi. A chimera comprising the catalytic domain of GlcNAc6ST-2 fused to the cytosolic tail, transmembrane domain, and stem of GlcNAc6ST-1 possessed the substrate preference and localization characteristic of GlcNAc6ST-1. These observations suggest that the N-terminal region of the enzymes dictates their substrate preference, either indirectly via altered Golgi distribution or by a more direct mechanism.

In this work, we sought to identify the features of the N-terminal region of GlcNAc6ST-1 and -2 that might confer their unique properties. Inspection of their primary sequences reveals an extended stem region in GlcNAc6ST-1 that is absent in GlcNAc6ST-2 (Fig. 1A). We therefore focused on the role of the stem domain in both the localization and substrate preferences of the enzymes. Using a panel of truncation mutants, we determined that perturbations to the stem region affect substrate preference without altering Golgi retention. We also discovered that GlcNAc6ST-1 exists as a disulfide-bond homodimer, mediated primarily by a Cys residue within the stem region, whereas GlcNAc6ST-2 is monomeric. Disruption of GlcNAc6ST-1 dimerization did not
affect substrate preference or Golgi localization. Analysis of a chimera in which the stem of GlcNAc6ST-1 was inserted into the sequence for GlcNAc6ST-2 revealed a direct role for the stem region in governing substrate preference.

EXPERIMENTAL PROCEDURES

Cell Manipulations—HeLa cells were obtained from the ATCC and grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37 °C and 5% CO2. All transfections were carried out in Opti-MEM (Invitrogen) using LipofectAMINE PLUS (Invitrogen) under the manufacturer's standard conditions.

Plasmids and Antibodies—The plasmids containing the human sulfotransferase cDNAs (11) and fucosyltransferase VII (FucTVII) (19) were obtained from D. Steven Rosen and Stefan Hemmerich. The monoclonal antibody G72 (4) was a gift of Dr. Reiji Kannagi. Sheep antisera against TGN46 (20) was purchased from Serotec. MECA-79 (21, 22) and biotinylated goat anti-rat IgM were purchased from BD Pharmingen. Biotinylated goat anti-mouse IgM and streptavidin tricolor were purchased from Caltag. Donkey anti-sheep IgG conjugated to Alexa 647 and rabbit anti-GFP antisera were purchased from Molecular Probes.

Generation of Sulfotransferase-EYFP, CTS-ECPF, and the 2 Stem Chimera—The constructs encoding GlcNAc6ST-1 or -2 fused to enhanced yellow fluorescent protein (EYFP) have been reported previously (17). The gene encoding the CTS domain of GlcNAc6ST-1, corresponding to amino acids 1–114, was amplified by PCR, and then cloned into pcR4Blunt-TOPO vector. The vector fragment was excised from the TOPO vector using restriction sites for enzymes Nhel and KpnI that were included in the PCR primers. The excised sequence was then ligated into EYFP-NI or ECPF-NI (Clontech), which had been digested with Nhel and KpnI.

The -7S variant of GlcNAc6ST-2 was created using a megaprimer PCR method adapted from Skrincosky et al. (23). Using the CTS-ECFP construct as a template, the gene encoding the N-terminal region of GlcNAc6ST-1 (comprising the cytosolic tail, transmembrane domain, and ~12 luminal amino acids) was amplified by PCR with a 3-primer that contained 21 bp of 5′ non-annealing sequence corresponding to the first seven codons of the GlcNAc6ST-1 catalytic domain (DKRQLLV). This megaprimer was gel-purified, and then used in a subsequent round of PCR with a template corresponding to only the catalytic domain of GlcNAc6ST-1. The product of this reaction was inserted into pcR4Blunt TOPO, excised with Nhel and KpnI, and cloned into EYFP-NI.

The -30 GlcNAc6ST-1 construct was generated by digesting the parental GlcNAc6ST-1-EYFP plasmid with SfiI, creating a fragment containing bp 172–803. The remainder of this digestion, comprising bp 1–171 and 990–1458 of GlcNAc6ST-1, was used to amplify DNA corresponding to the tail and transmembrane domain of the enzyme with a 3-primer containing 21 bp of 5′ non-annealing sequence corresponding to His98 to Arg157 (HARDDL) of the same enzyme. The product of this PCR was gel-purified and used in a subsequent PCR with the 172–803 fragment as a template and a 3-primer that included a unique Sfi site in the GlcNAc6ST-1 sequence. This PCR product was cloned into pcR4Blunt TOPO and excised with Nhel and SfiI. The parental GlcNAc6ST-1-EYFP plasmid was digested with the same restriction enzymes, followed by ligation of the TOPO fragment.

The 2-stem-EYFP construct was generated by three rounds of megaprimer PCR. GlcNAc6ST-2-EYFP was used to amplify the sequence corresponding to amino acids 1–39 with a 5′ primer containing 21 bp of 5′ non-annealing sequence, which encoded the first 7 amino acids in the GlcNAc6ST-2 stem (NPDGPLG). This PCR product was used in another PCR employing GlcNAc6ST-1-EYFP as the template and a 5′ primer containing the 5′ non-annealing sequence corresponding to the first 7 amino acids in the catalytic domain of GlcNAc6ST-2 (PERMHVL). After gel purification, this DNA fragment was used as the 3′ primer in a final round of PCR with GlcNAc6ST-2-EYFP as the template. The final PCR product was cloned into pcR4Blunt TOPO, excised with Nhel and KpnI, and ligated into EYFP-NI that had been digested with the same enzymes. All constructs were verified with DNA sequencing.

Generation of the Cys Mutants of GlcNAc6ST-1—The two Cys residues in the CTS-EYFP construct (C12 and C39) were mutated to Ser individually using QuickChange site-directed mutagenesis. The double Cys mutant was constructed by subjecting the C12S mutant to a subsequent round of QuikChange. The full-length variant of GlcNAc6ST-1 with both Cys residues mutated to Ser was generated using megaprimer PCR using the C12S/C39S CTS-EYFP construct as a template.

Generation of Stable Cell Lines—The sulfotransferase-EYFP plasmids were linearized using AseI, then transfected into HeLa cells. After 48 h of transfection, 400 μg/ml geneticin (Invitrogen) was added to the media to select for stable integrants. After 3 weeks of selection, the population of resistant cells was autolysed using a cell sorter, with the fluorescent protein signal used for gating. Individual clones were expanded and used without further subcloning.

Flow Cytometry—Cells (2 × 106) were seeded in a 10-cm dish 24 h prior to transfection. After 24 h of transfection, the cells were lifted with trypsin/EDTA and then replated. After another 24 h, the cells were lifted with 1 mM EDTA in PBS, washed twice with buffer A (0.1% bovine serum albumin and 0.1% sodium azide in PBS), and counted. Flow cytometry analysis was performed on 500,000 cells. All antibodies were used at a concentration of 1 μg/ml cells except for the GT2 hybridoma reagent, which was used at a 1:10 dilution. The primary antibody was applied in 50 μl of buffer A and incubated with the cells on ice for 30 min. The cells were washed twice with buffer A and then incubated in 50 μl of diluted secondary reagent for 30 min. The cells were washed twice and incubated with streptavidin-triclor conjugate. The cells were analyzed using a FACScalibur flow cytometer (BD Biosciences).

Small Molecule Inhibitors of Glycosylation—Cells were plated at ~300,000 cells per well in a 12-well dish. Swainsonine (Sigma) was added to the media at a concentration of 10 μM, whereas a-benzyl GalNAc (Sigma) was used at 4 mM. The cells were grown in the presence of the inhibitors for 2 days and were then processed for flow cytometry as above.

Western Blots—Approximately 6 × 106 cells in a 10-cm dish were washed twice with PBS, then lifted with 0.6 mM EDTA in PBS lacking calcium and magnesium. The cells were washed with PBS and then resuspended in 500 μl of ice-cold lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.2, 300 mM NaCl) containing 1 tablet of Complete protease inhibitors (Roche), supplemented with 1 μM pepstatin. After a 1-h incubation on ice, the lysates were clarified at 20,000 × g for 5 min. The total protein content of each sample was determined using the BCA assay (Pierce) with bovine serum albumin as the standard. An aliquot (9 μg) of each lysate was treated with either denaturing loading buffer (0.1 M DTT added) or the same buffer lacking DTT and then boiled for 8 min. The samples were loaded onto a 5–15% gradient gel, and analyzed by Western blotting. The GFPS were detected with goat-anti-rabbit conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories). The blot was developed using SuperSignal West Pico (Pierce) and Kodak film.

Mannos-β-Gal Antibodies—Cells were seeded on slides mounted with tissue culture wells (LAB-TEK) and allowed to adhere for 2 days. The cells were washed 3 times with PBS, then fixed in 3% paraformaldehyde in PBS. After three washes, the cells were permeabilized with PBS containing 1% bovine serum albumin and 0.1% Triton X-100 (Sigma) for 5 min at room temperature. The cells were blocked in PBS with 1% bovine serum albumin for 20 min, followed by the addition of the first antibody diluted in blocking buffer. After a 1-h incubation on ice, the lysates were clarified at 20,000 × g for 5 min. The total protein content of each sample was determined using the BCA assay (Pierce) with bovine serum albumin as the standard. An aliquot (9 μg) of each lysate was treated with either denaturing loading buffer (0.1 M DTT added) or the same buffer lacking DTT and then boiled for 8 min. The samples were loaded onto a 5–15% gradient gel, and analyzed by Western blotting. The GFPS were detected with goat-anti-rabbit conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories). The blot was developed using SuperSignal West Pico (Pierce) and Kodak film.

RESULTS

Localization and Substrate Preferences of GlcNAc6ST-1-EYFP and Stem Deletion Mutants—Previous studies with glycosyltransferases have implicated their stem regions, in some cases, as determinants of Golgi localization (24–26). Several of these studies exploited mutants in which the stem region was completely or partially deleted. We adopted a similar approach in our investigation of the stem region of GlcNAc6ST-1. Based on sequence alignment of the GlcNAc/GalNAc/Gal-6 sulfotransferase family, we define the stem domain as the 75-residue sequence spanning Asn1-Gly114 (Fig. 1A). Accordingly, the stem region of GlcNAc6ST-2 is defined as the short sequence

Downloaded from http://www.jbc.org/ by guest on July 22, 2018
His^{25}-Pro^{40}. The deletion mutants generated for this study lack residues 40–69 (termed /H1100230) or all 75 residues of the stem (termed /H1100275) (shown in Fig. 1A and schematically in Fig. 1B). For the partial deletion mutant (~30), we chose to eliminate the portion of the stem closest to the transmembrane domain based on previous studies with glycosyltransferases that identified the transmembrane-proximal residues as most important for Golgi localization (25, 26). It should be noted that the region we define as the stem domain does not include /H1101112 residues that are predicted to extend into the Golgi lumen, immediately adjacent to the transmembrane domain. In preliminary studies, we found mutants lacking these additional residues to be misfolded and accumulate in the ER (data not shown).

To study the subcellular localization of the mutants, as well as their cellular substrate preference, we fused EYFP to their C-termini and generated stably transfected HeLa cell lines. Our previous work made extensive use of fluorescent protein-GlcNAc6ST-1 and -2 chimeras (17), and we confirmed in that study that such modifications of the C terminus do not affect either Golgi localization or substrate preference in stably transfected cells. We performed fluorescence colocalization experiments with full-length GlcNAc6ST-1-EYFP and the two deletion mutants (~30 and ~75) along with the known Golgi marker TGN46 (27). As shown in Fig. 2, all three proteins were localized to the Golgi, suggesting that the ~30 and ~75 deletions do not grossly affect cellular distribution.

To determine the preferred substrates of the GlcNAc6ST variants, we utilized antibodies specific for sulfated glycans and glycosylation inhibitors. The antibodies used in this study were monoclonal antibodies G72 and MECA-79; their epitopes are shown in Fig. 3 in the context of a biantennary O-linked glycan that has been identified as a physiological L-selectin ligand (22, 28, 29). The minimal epitope recognized by G72 is GlcNAc-6-sulfated sialyl N-acetyllactosamine (Sia2,3-Galβ1,4(SO3-6)GlcNAc) (4, 18). Its affinity is increased by the addition of a fucose residue to generate 6-sulfo sLe^x (highlighted in green in Fig. 3), which can be accomplished by expression of FucTVII (17). This epitope can be found as a capping structure on either N- or O-linked glycans. MECA-79 recognizes a GlcNAc-6-sulfated core structure elaborated from the Core 1 Gal residue found only on O-linked glycans (Galβ1,4(SO3-6)GlcNAcβ1,3Galβ1,3GalNAc, highlighted in blue in Fig. 3) (22). Biosynthesis of the MECA-79 epitope requires expression of a Core 1 GlcNAc transferase termed Core1-β3GlcNAcT (22). The G72 epitope can be elaborated...
from the MECA-79 epitope on the lower branch in Fig. 3. The same epitope can be generated on the upper branch as well, provided that a Core 2 GlcNAc transferase (Core2GlcNAcT-I) is expressed. HeLa cells express Core2GlcNAcT-I (30), whereas Core1-β3GlcNAcT must be introduced by transfection.

We analyzed HeLa cells stably expressing full-length GlcNAc6ST-1-EYFP, –30 or –75 by flow cytometry using G72 (columns 1 and 2) and MECA-79 (column 3) (Fig. 4A). Cells expressing the full-length enzyme or the –30 variant showed significant immunoreactivity with G72 (column 1), which was increased by transient transfection of FucTVII (column 2). By contrast, the −75 variant did not express detectable G72 epitope in the presence or absence of FucTVII. HeLa cells expressing any of the three GlcNAc6ST-1-EYFP variants showed similar immunoreactivity with MECA-79 (column 3). We confirmed that all cell lines used in this study expressed comparable levels of sulfotransferase-EYFP protein (30 or 35) by Western blot analysis of lysates from these cells. As shown in Fig. 5A, the chimera was capable of generating the G72 epitope, unlike native GlcNAc6ST-2-EYFP. Indeed, the activity of the chimera was similar to that of the enzyme from which its stem was derived, GlcNAc6ST-1. These observations suggest that the stem plays a major role in dictating the spectrum of substrates modified by the sulfotransferase.

**Dimerization of GlcNAc6ST-1-EYFP**—Western blot analyses of GlcNAc6ST-2-EYFP and the –30 and –75 mutants revealed the presence of immunoreactive species with molecular weights corresponding to homodimers (Fig. 6A). Dimerization of glycosyltransferases and associations with other Golgi enzymes have been shown to impact Golgi localization in several systems (33–36), which in turn could affect substrate preference. Therefore, we explored the physiological relevance of GlcNAc6ST-1 dimerization and its impact on Golgi localization and enzyme activity. To determine whether GlcNAc6ST-1-EYFP dimerization was because of disulfide formation, we analyzed the protein by Western blot in the presence and absence of DTT. As shown in Fig. 6B, the protein migrated as a monomer in the presence of DTT and as a dimer in its absence. There are 10 Cys residues in full-length GlcNAc6ST-1 with two residing in the N-terminal region comprising the stem and transmembrane domains (Fig. 1A). These Cys residues are not conserved across the GlcNAc/GalNAc/Gal-6-sulfotransferase family. To explore their contribution to dimerization, we first generated a chimeric protein in which the N-terminal region (cytosolic tail (C), transmembrane (T), and stem (S) domains) of GlcNAc6ST-1 was fused to the cyan fluorescent protein (ECFP). We transiently expressed the protein, termed CTS-ECFP, in HeLa cells that were stably expressing GlcNAc6ST-1-EYFP and confirmed its Golgi localization by fluorescence microscopy. As shown in Fig. 6C, CTS-ECFP localized to the Golgi compartment similarly to GlcNAc6ST-1-EYFP and the Golgi marker TGN46. Lysates from these cells were analyzed by Western blot with anti-GFP, revealing the presence of CTS-ECFP homodimers, GlcNAc6ST-1-EYFP homodimers, and CTS-ECFP/GlcNAc6ST-1-EYFP heterodimers (Fig. 6D). All of the dimers were converted to monomers in the presence of DTT. These results suggest that dimerization is
mediated by one or both Cys residues within the CTS region. Using a similar CTS-EYFP construct, we mutated Cys\textsuperscript{12} and Cys\textsuperscript{39} to Ser, individually and simultaneously, and analyzed their oligomerization state by Western blot. In the absence of reductant, the double mutant migrated as a monomer, C39S migrated in both dimeric and monomeric forms, and C12S migrated primarily as a dimer, similar to the unmutated protein (CTS-EYFP) (Fig. 7A). All variants migrated as monomers in the presence of DTT. These data suggest that Cys\textsuperscript{39} is the principal residue involved in dimerization and that Cys\textsuperscript{12} can also contribute. We explored the importance of dimerization on Golgi localization by fluorescence microscopy of HeLa cells that were transiently expressing the CTS-EYFP variants. As shown in Fig. 7B, there were

![Graph](http://www.jbc.org/Downloaded.png)

FIG. 4. GlcNAc6ST-1 lacking the stem shows reduced sulfation of N-linked glycans but normal sulfation of O-linked glycans. A, column 1: flow cytometry analysis of HeLa cells stably expressing GlcNAc6ST-1-EYFP, the −30 variant, or the −75 variant, labeled with G72 (gray) or an isotype-matched control (clear). Column 2, flow cytometry analysis of the cell from column 1 transiently transfected with FucTVII, then labeled with G72 (gray) or an isotype-matched control (clear). Column 3, flow cytometry analysis of the cells from column 1 transiently transfected with Core1-β3GlcNAcT, then probed with MECA-79 (gray) or an isotype-matched control (clear). B, effects of glycosylation inhibitors on the G72 reactivity of GlcNAc6ST-1-EYFP or −30 expressing HeLa cells. The error bars represent standard deviation of three replicates. MFI, mean fluorescence intensity from flow cytometry analysis.
**Fig. 6.** GlcNAc6ST-1 is a disulfide-bound homodimer. A, Western blot of lysates from parent HeLa cells and HeLa cells stably expressing GlcNAc6ST-1-EYFP, the −30 variant, or the −75 variant, probed with anti-GFP antisera. B, Western blot of lysates from HeLa cells expressing GlcNAc6ST-1-EYFP boiled with or without DTT, probed with anti-GFP antisera. C, fluorescence micrographs of HeLa cells stably expressing GlcNAc6ST-1-EYFP and transiently transfected with the CTS domain of GlcNAc6ST-1 fused to ECFP, and labeled with TGN46 antisera. The top three micrographs show a single section of a deconvolved data set, with the signal from ECFP, EYFP, and anti-TGN46 shown in monochrome. The bottom two images show three color overlays of 4,6-diamidino-2-phenylindole in blue, ECFP in green, and EYFP in red (right). Bar, 3 μm. D, Western blot of lysates from cells transfected as in C treated with or without DTT. The blot was probed with GFP antisera. Red lines indicate the various protein complexes.
no gross differences in Golgi localization among CTS-EYFP and its Cys mutants. Therefore, dimerization does not appear to be a major determinant of Golgi localization.

We performed similar experiments with a C12S/C39S double mutant of full-length GlcNAc6ST-1-EYFP to probe the effect of dimerization on its localization and enzyme activity. The mutant was transiently expressed in HeLa cells and lysates were analyzed by Western blot. As shown in Fig. 8A, the double mutant migrates as a monomer in the presence or absence of reducing agent, confirming that the two Cys residues promote dimerization of the full-length protein. As observed with the CTS-EYFP construct, the double mutant of GlcNAc6ST-1-EYFP localizes to the Golgi compartment similarly to the wild-type protein (Fig. 8B). We probed the cellular activity of the double mutant by flow cytometry analysis of HeLa cells that were cotransfected with FucTVII (Fig. 8C). The G72 immuno-reactivity of these cells was indistinguishable from that of cells expressing wild-type GlcNAc6ST-1-EYFP. Thus, dimerization mediated by the stem domain does not appear to affect either Golgi retention or activity of the sulfotransferase.

DISCUSSION

There are many factors that can influence the cellular substrate preferences of Golgi enzymes, such as Golgi distribution, associations with other proteins, and intrinsic substrate binding activity. Defining the relative contributions of these factors in the assembly of complex glycans, and their molecular basis, is a major challenge in the field of glycobiology. Whereas the catalytic domains of Golgi enzymes play an obvious role in defining the specific reaction between the donor and acceptor residues, the spectrum of glycoconjugates that can be modified in a cellular context may be dictated by other regions of the enzyme. For example, localization determinants can position enzymes in different Golgi subcompartments, where they may encounter a different subset of glycoconjugate substrates (37, 38).

Few studies have addressed the role of Golgi enzyme stem domains in localization or cellular substrate profile. The stem region of a 2,6-sialyltransferase was shown to encode localization, along with the transmembrane domain, but the relationship of localization to substrate preference was not addressed (25, 26, 39). The stem region of GlcNAcTI was shown to be involved in heterodimerization with mannosidase II, an enzyme that acts in the same pathway of N-linked glycan maturation, although disruption of the protein-protein interaction did not compromise Golgi localization (40, 41). Stem domains can also provide sites of proteolysis; there are several reports of secreted catalytic domains of glycosyltransferases (38, 42–45) and sulfotransferases (46, 47) in physiologically relevant systems. It has been speculated that proteolysis within the stem domain and concomitant release of the cata-
lytic domain might be a mechanism of down-regulating cellular activity (38).

In this study, we identified a role for the stem region of GlcNAc6ST-1 in defining the nature of its cellular substrates. A mutant lacking the stem altogether lost the ability to modify GlcNAc residues within2,6-sialyltransferase was addressed by substitution of the stem with a heterologous spacer protein scaffold. Our data suggest that the presence of a stem, in either native GlcNAc6ST-1 or the chimeric GlcNAc6ST-2, confers the ability to sulfate GlcNAc residues on both distal capping structures of N- and O-linked glycans and proximal core structures on O-linked glycans. Conversely, the absence of a stem, in either native GlcNAc6ST-2 or the stem-deletion mutant of GlcNAc6ST-1, restricts activity to GlcNAc residues on both proximal to and distal from their membrane-bound protein scaffold. Our data suggest that the presence of a stem, in either native GlcNAc6ST-1 or the chimeric GlcNAc6ST-2, confers the ability to sulfate GlcNAc residues on both distal capping structures of N- and O-linked glycans and proximal core structures on O-linked glycans. Conversely, the absence of a stem, in either native GlcNAc6ST-2 or the stem-deletion mutant of GlcNAc6ST-1, restricts activity to GlcNAc residues within the core region of O-linked glycans. These data are consistent with the third model, which might be further addressed by substitution of the stem with a heterologous spacer domain of comparable size.

GlcNAc6ST-1 is the first Golgi-resident sulfotransferase reported to exist as a homodimer. This feature is not shared by all members of the GlcNAc/GalNAc/Gal-6-sulfotransferase family, as GlcNAc6ST-2 does not exist as a covalent dimer in cells (data not shown). Consistent with this observation, GlcNAc6ST-2 lacks the two Cys residues that we identified as mediators of GlcNAc6ST-1 dimerization. The functional significance of the GlcNAc6ST-1 dimer remains undefined, but does not appear to be related to substrate preference or localization. By contrast, dimerization of several other Golgi enzymes, such as a rat α2,6-sialyltransferase (36) and a human β1,4Gal transferase (35), is required for proper Golgi localization. It is possible that dimerization of GlcNAc6ST-1 influences properties of
the enzyme that were not monitored in this work. For example, protein stability, residence time in the Golgi compartment, or association with other Golgi enzymes might be affected by dimerization.

Among the family members, the closest relative to GlcNAc6ST-1 with respect to the stem region is GlcNAc6ST-4 (52, 53). This enzyme has an extended stem of similar size, as well as two Cys residues in similar locations. A role for the GlcNAc6ST-4 stem in substrate preference is an intriguing possibility worthy of future study. More generally, the GlcNAc/GalNAc/Gal-6-sulfotransferases provide an appealing experimental system in which to probe the contributions of their various domains to activity in cells. They are a class of related enzymes that appear to use different mechanisms for Golgi localization and substrate selection, while retaining modular domains that can be interchanged. Their catalytic activities seem separable from other functional determinants, and their products are readily detected on cells with specific antibodies. We anticipate that this enzyme family will be useful for probing fundamental mechanisms of glycan assembly and modification in the Golgi compartment.

Acknowledgments—We thank Jackson Egen and Amanda Jamieson for technical advice, Annette Bistrup, Kenji Uchimura, Stefan Hemmerich, and Steven Rosen for helpful discussions, Hector Nolla of the University of California, Berkeley, CRL cell sorting facility, and Steve Ruzin and Denise Schichnes of the University of California, Berkeley, CNR Biological Imaging Facility. We thank Jennifer Kohler and Jennifer Czaplinski for critical reading of this manuscript.

REFERENCES
1. Rosen, S. D. (2004) Annu. Rev. Immunol. 22, 129–156
2. Hemmerich, S., and Rosen, S. D. (2000) Glycobiology 10, 849–856
3. Hemmerich, S., Leffler, H., and Rosen, S. D. (1995) J. Biol. Chem. 270, 12035–12047
4. Mitsuoka, C., and Kannagi, R. (1998) Curr. Opin. Cell Biol. 10, 313–317
5. Hitomi, J., Tynninen, O., Hayry, P., Paavonen, T., and Renkonen, R. (2002) J. Biol. Chem. 277, 531–538
6. Renkonen, J., Tynninen, O., Hayry, P., Paavonen, T., and Renkonen, R. (2002) J. Biol. Chem. 277, 531–538
7. van Zante, A., and Rosen, S. D. (2003) J. Immunol. 169, 22, 1252–1259
8. Honke, K., and Taniguchi, N. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7278–7283
9. Hemmerich, S., Bistrup, A., Singer, M. S., van Zante, A., Lee, J. K., Tsay, D., Peters, M., Carminati, J. L., Brennan, T. J., Carver-Moore, K., Leviten, M., Fuentes, M. E., Ruddle, N. H., and Rosen, S. D. (2001) J. Biol. Chem. 276, 21608–21617
10. Elbein, A. D. (1984) CRC Crit. Rev. Biochem. 16, 21–49
11. McCormick, C., Duncan, G., Goutos, K. T., and Tufaro, F. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 668–673
12. Hemmerich, S., Bistrup, A., Singer, M. S., van Zante, A., Lee, J. K., Tsay, D., Peters, M., Carminati, J. L., Brennan, T. J., Carver-Moore, K., Leviten, M., Fuentes, M. E., Ruddle, N. H., and Rosen, S. D. (2001) J. Biol. Chem. 276, 21608–21617
13. Grunwell, J. R., Rath, V. L., Rasmussen, J., Cabrilo, Z., and Bertozzi, C. R. (2002) J. Biol. Chem. 277, 1406–1416
14. Uchimura, K., El-Fasakhany, F. M., Horii, M., Hemmerich, S., Blink, S. E., Peters, M., Carminati, J. L., Brennan, T. J., Carver-Moore, K., Leviten, M., Fuentes, M. E., Ruddle, N. H., and Rosen, S. D. (2001) J. Biol. Chem. 276, 21608–21617
15. Elbein, A. D. (1984) CRC Crit. Rev. Biochem. 16, 21–49
16. Colley, K. J., Lee, E. U., and Paulson, J. C. (1992) J. Biol. Chem. 267, 7284–7293
17. Prescott, A. R., Lucoq, J. M., James, J., Lister, J. M., and Ponnambalam, S. (1997) Eur. J. Cell Biol. 72, 238–246
18. Hiraoka, N., Kawashima, H., Petryniak, B., Nakayama, J., Mitoma, J., Mart, J. D., Lowe, J. B., and Fukuda, M. (2004) J. Biol. Chem. 279, 3058–3067
19. Mitoma, J., Petryniak, B., Hiraoka, N., Yeh, J. C., Lowe, J. B., and Fukuda, M. (2003) J. Biol. Chem. 278, 9563–9567
20. Ye, J. C., Ong, E., and Fukuda, M. (1999) J. Biol. Chem. 274, 3215–3221
21. Kuan, S. F., Byrd, J. C., Bashbaum, C., and Kim, Y. S. (1989) J. Biol. Chem. 264, 19271–19277
22. Egelman, E. H. (1998) J. Struct. Biol. 120, 140–149
