Golgi Localization of Carbohydrate Sulfotransferases Is a Determinant of L-selectin Ligand Biosynthesis*

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Sulfation of endothelial glycoproteins by the sulfotransferase GlcNAc6ST-2 is a regulatory modification that promotes binding of the leukocyte adhesion molecule L-selectin. GlcNAc6ST-2 is a member of a family of related enzymes that act on similar carbohydrate substrates in vitro but discrete glycoproteins in vivo. We demonstrate that GlcNAc6ST-1, -2, and -3 have distinct Golgi distributions, with GlcNAc6ST-1 confined to the trans-Golgi network, GlcNAc6ST-3 confined to the early secretory pathway, and GlcNAc6ST-2 distributed throughout the Golgi. Their localization was correlated with preferred activity on either N-linked or O-linked glycoproteins. A chimera comprising the localization domain of GlcNAc6ST-1 fused to the catalytic domain of GlcNAc6ST-2 was confined to the trans-Golgi network and adopted the substrate preferences of GlcNAc6ST-1. We propose a model in which Golgi enzyme localization and competition orchestrate the biosynthesis of L-selectin ligands.

Cell surface oligosaccharides are major determinants of cell-cell interactions during development, the immune response, and pathogenic processes such as microbial infection and tumor cell metastasis (1–4). Although cell surface glyconjugates are highly complex and diverse in structure, it is now apparent that specific glycans are responsible for mediating discrete molecular recognition events among cells. These unique carbohydrates are a reflection of the biosynthetic machinery within the secretory compartments of the cell. Therewithin, carbohydrate biosynthetic and processing enzymes, organized among the membranes of the endoplasmic reticulum (ER)1 and Golgi cisternae, act in an assembly line to generate functional oligosaccharide structures (5, 6). The organization of these enzymes within subcellular compartments can be a key determinant of cell surface glycosylation patterns and, accordingly, cell-cell interactions.

The leukocyte adhesion molecule L-selectin mediatesthe interaction of lymphocytes with endothelial cells of peripheral lymph node high endothelial venules (HEV), a crucial step during constitutive lymphocyte recirculation through lymph nodes (7). A similar process occurs at sites of chronic inflammation, during which many classes of leukocytes extravasate into the diseased tissue. In both cases, L-selectin binds to unique sulfated and fucosylated carbohydrates (depicted in Fig. 1A and termed “sulfoadhesin” herein) that are presented on O-sulfoglycosylated mucin-like endothelial glycoproteins such as CD34 and GlyCAM-1 (7–12). Elements of the sulfoadhesin structure have been elucidated during the past decade, beginning with the identification of the 6-sulfo-sialyl Lewis x (sLeα) capping group as a major determinant of L-selectin binding (10). This capping group can be displayed on a Core 2 branch (Fig. 1A), initiated by an N-acetylgalactosamine (GlcNAc) residue added by the Core 2 GlcNAc transferase (Core2GlcNAcT-I) (13). In the simplest case, this GlcNAc residue is sulfated at the 6-position and elaborated into the sLeα epitope. However, there exist higher order structures containing multiple N-acetyllactosamine (LacNAc, Galβ1–4GlcNAc) repeats, which may contain additional sulfate groups and/or fucose residues (10, 11).

A second major feature of sulfoadhesin was recently elucidated as the antigen for the MECA-79 antibody (11). This antibody was originally characterized by its specific binding to peripheral lymph node HEV and HEV-like vessels in chronically inflamed tissues and its ability to block L-selectin-mediated leukocyte adhesion (14, 15). Biochemical characterization of MECA-79-reactive mucin-type glycoproteins revealed a novel Core 1 extension (Fig. 1A) initiated by a recently discovered GlcNAc transferase (Core1-β3GlcNAcT) (11). As with the Core 2 branch, this Core 1 GlcNAc residue can be elaborated directly into 6-sulfo-sLeα or extended with LacNAc repeats.

Sulfation of GlcNAc within sulfoadhesin is essential for functional L-selectin binding activity (9, 16). This discovery has directed considerable attention to sulfation as a modulator of chronic inflammation and to its biosynthetic origin. A family of Golgi resident GlcNAc-6-sulfotransferases (GlcNAc6ST-0, -1, -2, -3, -4, and -5) has now been identified, and their endogenous substrates and biological functions are under intense scrutiny (17, 18). GlcNAc6ST-2 is a likely candidate for sulfoadhesin biosynthesis because of its highly restricted expression in HEV (16, 19). GlcNAc6ST-1, by contrast, is expressed in many tissues (including HEV) where it presumably sulfates GlcNAc.

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The abbreviations used in this paper are: ER, endoplasmic reticulum; BFA, brefeldin A; BSA, bovine serum albumin; CGN, cis-Golgi network; CHO, Chinese hamster ovary cell; Core1-β3GlcNAcT, Core 1 GlcNAc transferase; Core2GlcNAcT-I, Core 2 GlcNAc transferase I; DAPI, 4,6-diamidino-2-phenylindole; ECFP, enhanced cyan fluorescent protein; ERGIC, endoplasmic reticulum Golgi intermediate compartment; EFYP, enhanced yellow fluorescent protein; FACS, fluorescence-activated cell sorter; FucoTII, fucosyltransferase VII; GFP, green fluorescent protein; GlcNAc6ST, GlcNAc-6-sulfotransferase; GlcNAcT-I, N-acetylgalactosaminyltransferase I; HEV, high endothelial venule; LacNAc, N-acetylactosamine; mAb, monoclonal antibody; MTOC, microtubule organizing center; PBS, phosphate-buffered saline; sLeα, sialyl Lewis x; TGN, trans-Golgi network.
residues within other glycan structures (20). However, this enzyme can generate 6-sulf-o-LE° oligosaccharides in cultured cells (20). The other GlcNAc6STs are also expressed in tissues lacking sulfoadhesin, such as the intestine (GlcNAc6ST-3) and cornea (GlcNAc6ST-5) (21). Consistent with a role for GlcNAc6ST-2 in sulfoadhesin biosynthesis, mice deficient in this enzyme lack MECA-79 reactivity on the luminal aspect of peripheral lymph node HEV and display significantly reduced lymphocyte homing (22). No other defects are evident. However, some residual MECA-79 reactivity is observed on the abluminal aspect of peripheral lymph node high endothelial cells in GlcNAc6ST-2 knockout mice.

Although GlcNAc 6-sulfate is found in ubiquitous structures such as keratan sulfate and in other N- and O-linked glycoproteins (23–26), its residence within sulfoadhesin is unique to activated endothelium where GlcNAc6ST-2 is expressed (27, 28). Thus, the other GlcNAc6STs, expressed in extralymphoid sites, presumably sulfate GlcNAc residues within different glycoconjugate structures. This substrate specificity, key to understanding how inflammatory leukocyte adhesion is regulated, may be engendered by a combination of factors. For example, each enzyme may have a preferred local context for the target GlcNAc residue, or the underlying polypeptide may contribute to a composite epitope that directs sulfation by an individual enzyme. To address these possibilities, several groups have studied the in vitro activities of recombinant soluble GlcNAc6STs using defined oligosaccharide substrates (29–31). The surprising conclusion from these studies is that all members of the family prefer a terminal GlcNAc residue, adding sulfate before elaboration to higher order, more specific structures. Moreover, the enzymes are relatively insensitive to the underlying glycan structures. Thus, the specificity of GlcNAc6ST-2 for sulfoadhesin, and the other sulfotransferases for their substrates, must be engendered by factors not reflected in these in vitro biochemical studies.

Subcellular and subcompartmental localization play important roles in defining the substrate preference of many enzyme families (32–34). Indeed, localization of glycosyltransferases to particular Golgi cisternae can dictate biosynthetic pathways by providing access to substrates in a prescribed order (35, 36). We considered the possibility that the organization of GlcNAc6STs, specifically GlcNAc6ST-1, -2, and -3, within the Golgi compartment might dictate their biological substrates. Herein we report that these three enzymes have distinct sub-Golgi localization, including a presence in the earliest part of the secretory pathway for GlcNAc6ST-2 and -3. We also show that localization affects their substrate preference and that perturbations to their localization domains can lead to a change in substrate profile. A model is proposed that correlates the distribution of GlcNAc6ST-2 in the Golgi with the process of sulfoadhesin biosynthesis.

EXPERIMENTAL PROCEDURES

Cell Manipulations—CHO-K1 cells were obtained from the ATCC and grown in Ham’s F-12 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 OptiMEM (Invitrogen) using LipofectAMINE PLUS (Invitrogen). All constructs were verified by DNA sequencing.

Fluorescence-activated cell sorting (FACS) analysis was performed on 500,000 cells. All antibodies were used at a concentration of 1 μg/ml.

Small Molecule Inhibitors of Glycosylation—Cells were plated at ~300,000 cells/well in a 12-well dish. Tunicamycin (Sigma) was added to the medium at a concentration of 5 μg/ml, and β-N-GalNAc (Sigma) was used at concentrations varying from 1 to 16 μM.

Isolation of GlyCAM-IgG—GlcNAc6ST-3-EYFP-expressing CHO cells were transfected with GlyCAM-IgG, GlyCAM-IgG and Core2GlNAcT-I, or empty vector. One day after transfection, the cells were washed with PBS and then incubated with human endothelial

Generation of Stable Cell Lines—The sulfotransferase-EYFP fusion plasmids were linearized using AseI, and the GFP fusion was linearized with PvuI. The linearized plasmids were transfected into CHO-K1 cells. After 48 h of transfection, 1 mg/ml G418 (Mediatech) was added to the medium to select for stable integrants. After 2 weeks of selection, the population of resistant cells was autocloned using a cell sorter, with the fluorescent protein signal used for gating. Individual clones were expanded without further subcloning. The open reading frame for GlcNAc6ST-1-ECFP in the GlcNAc6ST-3-EYFP cell line was accomplished by transfection of the plasmid GlcNAc6ST-1-ECFP pIRES puro2. After 48 h of transfection the cells were treated with 5 μg/ml puromycin (Clontech). After 2 weeks of selection, individual foci were selected with cloning rings, expanded to confluence in a 6-well plate, and then cloned by limiting dilution. Cells expressing TGN38 human IgG were selected using the hygromycin resistance gene encoded on the plasmid and were treated with 300 μg/ml hygromycin for 2 weeks. Cells expressing this construct were not cloned.

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 0.05% EDTA and then resuspended. After another 24 h, the cells were lifted with 1 mM EDTA in PBS, washed twice with FACS buffer (0.1% BSA and 0.1% sodium azide in PBS), and then counted. Flow cytometry analysis was performed on 500,000 cells. All antibodies were used at a concentration of 1 μg/ml except for G72 hybridoma supernatant, which was used at a 1/10 dilution. The primary antibody was applied in 50 μl of FACS buffer and incubated with the cells on ice for 30 min. The cells were washed twice with FACS buffer and then incubated in 50 μl of diluted secondary reagent for 30 min. The cells were washed twice and then incubated with streptavidin tricolor conjugate. The cells were analyzed using a FACScalibur flow cytometer (BD Biosciences).

Enzyme Treatments—Approximately 300,000 cells were plated in a 12-well dish 12 h prior to treatment. Heparinase II (Sigma) and keratanase (ICN Biomedicals) were used at 0.1 unit/ml. Cells were washed twice with PBS. Wells that were incubated with heparinase were filled with 200 μl of 20 mM Tris, pH 7.4, containing 4 mM CaCl2, 50 mM NaCl, and 0.1 mg/ml BSA. Keratanase-treated wells were filled with 200 μl of PBS. The cells were incubated at 37 °C for 1 h, lifted with EDTA, and then analyzed as above.

Small Molecule Inhibitors of Glycosylation—Cells were plated at ~300,000 cells/well in a 12-well dish. Tunicamycin (Sigma) was added to the medium at a concentration of 5 μg/ml, and β-N-GalNAc (Sigma) was used at concentrations varying from 1 to 16 μM. The cells were grown in the presence of the inhibitors for 2 days and were then processed for flow cytometry as above.
serum-free medium (Invitrogen) supplemented with 0.25 mCi/ml 35S-labeled sodium Iodide (ICN Biomedicals) for 4 days. The medium was collected, clarified by centrifugation, and then concentrated to 1.5 ml. The concentrate was incubated with 0.5 ml of a slurry of protein A-Sepharose (Zymed Laboratories) (50% by volume) overnight at 4 °C with shaking. The beads were washed with 10 ml of PBS with 0.05% Tween 20. The GlyCAM-IgG chimera was eluted from the beads with 1.5 ml of 100 mM glycine, pH 2.9, then neutralized with 150 μl of 1 M Tris, pH 8. Each sample was lyophilized and resuspended in water. Half of the purified GlyCAM-IgG was analyzed by SDS-PAGE with Coomassie Blue staining to assess the quantity of protein in each lane. The gel was then exposed to a phosphor screen, which was analyzed by phosphorimaging using ImageQuant (Molecular Dynamics).

Western Blots—Approximately 6 × 106 cells in a 10-cm dish were washed three times with ice-cold PBS, then once with cold 10 mM HEPES, pH 7.4. To lyse the cells, 200 μl of the HEPES buffer containing protease inhibitors (Protease Inhibitor Mixture III, CMbiochem) was added to each plate, and the plates were incubated on ice for 10 min. The cells were scraped from the each plate, and the supernatants were transferred to centrifuge tubes and clarified at 2,500 × g for 5 min. The total protein content of each lysate was determined using the BCA assay (Pierce) with BSA as the standard. An aliquot (9 μg) of each lysate was loaded onto a 12% gel and analyzed by Western blot on a nitrocellulose membrane probed with rabbit anti-GFP polyclonal serum (Molecular Probes), followed by goat anti-rabbit conjugated to horseradish peroxidase (Jackson Immunolabs). The blot was developed using SuperSignal West Pico (Pierce) and Kodak film.

Microscopy—Cells were seeded on slides mounted with tissue culture wells (LAB-TEK) and allowed to adhere for 2 days. For experiments involving cycling to the microtubule organizing center (MTOC), brefeldin A (BFA) (Sigma) was added to the cells at a concentration of 10 μg/ml and then incubated at 37 °C for 45 min. The cells were washed three times with PBS, then fixed in 3% paraformaldehyde in PBS. After three washes, the cells were permeabilized with PBS containing 1% BSA and 0.1% Triton X-100 (Sigma) for 5 min at room temperature. The cells were washed three times and then blocked in PBS with 1% BSA for 20 min, followed by the addition of the first antibody diluted in blocking buffer. After a 2-h incubation at room temperature, the cells were washed three times and then incubated with the secondary antibody diluted in blocking buffer for 1 h. After three washes, the cells were mounted using Vectashield with DAPI (Vector Labs). A Leica DMIRE2 inverted microscope equipped with a xenon arc lamp using a 63× lens was employed for imaging. Image stacks containing 50–60 sections spaced 0.3 μm apart were acquired using a Quantix CCD camera (Roper Scientific). An 8900 Sedat quad filter set with single band excitation and emission filters (Chroma) was used, allowing the imaging of DAPI, GFP (or EYFP), Cy3, and Cy5 without any concern for register among the different channels. When ECFP was included, a filter set allowing the acquisition of ECFP and EYFP (Chroma) was used along with the appropriate filter channels of the quad set. Slidebook software (Intelligent Imaging Solutions) was used to control the microscope and the camera. The image stacks were digitally deconvolved using the nearest neighbor algorithm of Slidebook.

Quantitation of Colocalization—Quantitation of colocalization was performed using the software Colocalization 1.2 (Bitplane, Inc., Zurich). Image stacks from individual cells were processed by background subtraction, and individual channels were converted to TIFF format and imported into the software. The pixel intensity values were converted to histograms, and regions of the histograms were chosen which gave cross-correlation values >97%. A binary mask was generated which contained the highly correlated pixels. The plane corresponding to the fluorescence micrograph was displayed. Values for percent colocalization, which denotes the sum of the intensities of colocalized pixels divided by the sum of the intensities of all pixels with nonzero value, were determined using masks giving >97% cross-correlation, according to software protocols. Images of three or four cells from each sample were processed. For comparative purposes, we labeled CHO cells with 1-GFP with the G72 antibody are shown in Fig. 2B). These data demonstrate that GlcNAc6ST-1-GFP is capable of generating 6-sulfosialyl-LacNAc on endogenous glycoconjugate substrates. We next addressed the nature of these sulfated molecules. The major subclasses of glycoconjugates are N-linked and O-linked glycoproteins, proteoglycans, and glycolipids. To determine the expression of G72 antigen among these subclasses, we employed mutant CHO cell lines and chemical inhibitors of glycosylation.

CHO cells display elaborate N-linked glycans that contain LacNAc repeats (46), which could be sulfated by GlcNAc6ST-1-GFP. A mutant CHO cell line known as Lecl lacks N-acetylgalactosaminyltransferase 1 (GlcNAcT-I) activity, which limits the N-linked glycans produced by these cells to immature oligomannosides (39). We generated 10 independent Lecl CHO cell lines stably expressing GlcNAc6ST-1-GFP, and none dis-
played immunoactivity with the G72 antibody (a representative is shown in Fig. 2B). These data suggest that the sulfated epitope generated by GlcNAc6ST-1-GFP in CHO cells is displayed primarily on N-linked glycans.

The lack of mature N-linked glycoproteins in Lec1 CHO cells provided an opportunity to explore O-linked glycoprotein sulfation by GlcNAc6ST-1-GFP in the absence of competing N-linked substrates. CHO cells express minimal or no Core2GlcNAcT-I and therefore cannot extend glycans from the 6-position of the peptide-proximal GalNAc residue (Fig. 1A) (13). The Core1-β3GlcNAcT that extends the lower branch in Fig. 1A (11) and α1,3-fucosyltransferase activity (47) are also absent from CHO cells. Thus, to study sulfation of O-linked glycans related to sulfoadhesin, we added the genes encoding Core2GlcNAcT-I, FucTVII, known to generate 6-sulfo-sLe^a in vivo (48), and the mucin-like membrane protein CD34. CHO cells do express the galactosyltransferases and sialyltransferases necessary to generate sulfo-sLe^a (see below).

As shown in Fig. 2C, Lec1 CHO cells stably expressing GlcNAc6ST-1-GFP and transiently transfected with Core2-GlcNAcT-I, FucTVII, and CD34 show limited reactivity with mAb G72 despite the presence of the O-linked glycan biosynthetic machinery. We confirmed cell surface expression of the underlying O-linked glycoprotein scaffold by flow cytometry analysis using an anti-CD34 mAb and the anti-sLe^a mAb HCEA-452 (not shown). Consistent with a lack of GlcNAc6ST-1-GFP activity on O-linked glycoproteins, expression of the G72 antigen on wild-type CHO cells stably expressing the enzyme was essentially unaltered by the O-linked glycosylation inhibitor α-benzyl galactosamine (α-BnGalNAc) (49) at concentrations up to 16 mM (Fig. 2E). By contrast, tunicamycin, an inhibitor of N-linked glycosylation, completely abrogated G72 epitope expression (Fig. 2E).

It is likely that the G72 epitope on CHO cells expressing GlcNAc6ST-1-GFP forms a capping structure on keratan sulfate oligosaccharides (i.e. poly-LacNAc chains on the N-linked glycans) (46). Consistent with this hypothesis, keratanase treatment of the cells abolished G72 immunoreactivity (Fig. 2E). We tested whether other glycosaminoglycans such as heparan and chondroitin sulfates contribute to G72 immunoactivity. Digestion of CHO cell surfaces with heparinase II had no significant impact on G72 binding (Fig. 2E). Furthermore, stable expression of GlcNAc6ST-1-GFP in the CHO cell mutant pgsA, which lacks glycosaminoglycans because of a defect in xylosyltransferase activity (50), produces the same level of G72 antigen as observed on wild-type CHO cells (Fig. 2D, compare with Fig. 2A). Therefore, heparan sulfate and chondroitin sulfate are not the source of G72 reactivity. Finally, to address the contribution of glycolipids to G72 binding, we subjected wild-type CHO cells stably expressing GlcNAc6ST-1-GFP to exhaustive trypsinization to remove cell surface glycoproteins. As expected, the G72 binding activity was completely abolished, suggesting no contribution from trypsin-resistant glycolipids (not shown). Collectively, these data show that GlcNAc6ST-1-GFP prefers to sulfate GlcNAc residues on the antennae of N-linked glycoproteins. This is consistent with previous studies by Fukuda and co-workers (16), which showed sulfation of N-linked glycans by native GlcNAc6ST-1 in transiently transfected CHO cells.

Substrate Preference of GlcNAc6ST-2-EYFP—We performed a similar series of experiments using CHO cells stably expressing GlcNAc6ST-2 fused at the C terminus to EYFP. By contrast to GlcNAc6ST-1-GFP, CHO cells expressing GlcNAc6ST-2-EYFP did not display detectable G72 antigen on endogenous cell surface glycoproteins (Fig. 3A). Transient transfection with FucTVII, however, did produce a population of CHO cells that bound the G72 mAb (Fig. 3B). This result indicates that GlcNAc6ST-2-EYFP does sulfate N-linked glycoproteins but to a lesser extent than GlcNAc6ST-1-GFP. Detection using G72

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**Fig. 1. Structures of sulfoadhesin and G72 antigen.** A, sulfoadhesin is an O-linked glycan in which the core GalNAc residue is extended at the 6-position by a Core 2 branch and at the 3-position by a galactose residue that is elaborated further with the Core 1 extension. Both the Core 2 and Core 1 extension GlcNAc residues can be elaborated into the sLe^a tetrasaccharide (depicted in green in the upper branch). The sulfation of GlcNAc depicted in red is crucial for L-selectin binding. Both the upper and lower branches can be extended by LacNAc units. Heterogeneity with respect to extent of sulfation and fucosylation and the number of LacNAc repeats has been observed among glycans isolated from HEV-associated glycoproteins. The epitope recognized by the mAb MECA-79 is shown in blue. B, carbohydrate recognized by the mAb G72. Fucose is depicted in parentheses because it increases the affinity of binding but is not absolutely required.
required the presence of fucose residues that increase the affinity of the antibody (28).

The structure of sulfoadhesin combined with genetic validation of GlcNAc6ST-2 as the participating sulfotransferase implies that this enzyme acts on O-linked glycoproteins (16, 22). Indeed, both Rosen and Fukuda and their co-workers (16, 38) have shown that murine GlcNAc6ST-2, when transiently expressed in COS-7 or CHO cells, can sulfate a variety of mucin-like glycoprotein scaffolds. To confirm the substrate specificity of GlcNAc6ST-2, we generated CHO cells stably expressing GlcNAc6ST-3 fused at the C terminus to EYFP. No G72 immunoreactivity was generated on wild-type CHO cells by expression of the enzyme, nor could G72 reactivity be engendered by transfection of the cells with Core2GlcNAcT-I and/or FucTVII. We conclude that GlcNAc6ST-3-EYFP cannot generate sulfated capping structures related to 6-sulfo-Lewis x on either N-linked or O-linked glycoproteins. In its initial characterization, this enzyme was transiently expressed in COS-7 cells, and an activity on the O-linked glycans of the secreted glycoprotein GlyCAM-1 was identified (51). We confirmed this by transient transfection of the GlcNAc6ST-3-EYFP-expressing CHO cells with the gene encoding GlyCAM-1 fused to a human immunoglobulin G heavy chain (GlyCAM-IgG). The secreted molecule was isolated using protein A-Sepharose, and enzyme activity was determined by incorporation of [35S]labeled sulfate into the glycoprotein. As shown in Fig. 3E (lane b), GlyCAM-IgG expressed by the CHO cells in the absence of any other added genes lacks sulfate. However, cotransfection of the CHO cells with Core2GlcNAcT-1 produced GlyCAM-IgG containing sulfate (Fig. 3E, lane c). Thus, it appears that sulfation by GlcNAc6ST-3-EYFP is dependent on the presence of the Core 2 GlcNAc residue, as noted previously for the native enzyme (51). Notably, the COS-7 cells used in the initial characterization of this enzyme express Core2GlcNAcT-1 endogenously. More recently, this requirement for Core2GlcNAcT-1 was demonstrated on several mucin-IgG constructs, suggesting that this result is not dependent on the protein scaffold (37).

Previous work with glycosyltransferases suggests that cellular substrate preference may be altered by dramatic changes in expression level of the enzymes. At saturating levels, highly expressed enzymes may distribute into Golgi subcompartments they would not otherwise occupy, thereby gaining access to novel substrates (52–55). To exclude the possibility that dramatic differences in expression level account for the distinct substrate preferences of GlcNAc6ST-1, -2, and -3 chimeras, we analyzed protein levels in the stable CHO cell lines by Western blot using anti-GFP serum. As shown in Fig. 3F, lanes b–e, all proteins were expressed at levels within 2-fold of each other (quantitation is shown in Fig. 3G). Thus, the substrate preferences observed are unlikely to be the result of gross differences in expression.

Fluorescence Colocalization with Known Golgi Markers—Once the activities of the GlcNAc6ST-1-glucosyltransferase chimeras were established, the Golgi localization of these proteins was determined by colocalization analysis with known Golgi marker proteins. TGN38 (56) fused to an IgG heavy chain served as a marker for the trans-Golgi network (TGN) and was stably expressed in CHO cells simultaneously with the sulfotransferase-fluorescent protein chimeras. We employed BiP as an ER marker, GM130 as a cis-Golgi marker (57), and mannosidase II
Localization and Specificity of GlcNAc-6-sulfotransferases

**Fig. 3. Specificities of the three GlcNAc6STs for N- and O-linked glycans.** In A–D the x axis is the fluorescence intensity of streptavidin tricolor in arbitrary units. A, mAb G72 staining of CHO cells stably expressing GlcNAc6ST-2-EYFP (gray) and parent CHO cells (clear). B, cells in A transiently transfected with the gene encoding FucTVII stained with mAb G72 (gray) or isotype control (clear). C, cells in A transiently transfected with the gene encoding Core1-βGlcNAcT stained with mAb MECA-79 (gray) or isotype control (clear). D, CHO cells stably expressing GlcNAc6ST-1-GFP and transiently transfected with the gene encoding Core1-βGlcNAcT, stained with mAb MECA-79 (gray) or isotype control (clear). E, [35S]sulfate incorporation into GlyCAM-IgG. CHO cells expressing GlcNAc6ST-3-EYFP were transiently transfected with GlyCAM-IgG, and the secreted glycoprotein was purified from the conditioned medium with protein A-Sepharose. [35S]Sulfate incorporation was determined by SDS-PAGE and phosphorimaging analysis. The upper image is the Coomassie-stained gel, and the lower image is the same gel analyzed by phosphorimaging. The molecular mass marker is 63 kDa. Lane a, samples from mock-transfected GlcNAc6ST-3-EYFP-expressing CHO cells; lane b, samples from the same CHO cells transiently transfected with the gene encoding GlyCAM-IgG; lane c, samples from the same CHO cells transiently transfected with genes encoding GlyCAM-IgG and Core2GlcNAcT-1. F, Western blot of GlcNAc6ST-1-GFP (lane b), GlcNAc6ST-2-EYFP (lane c), GlcNAc6ST-3-EYFP (lane d), and the GlcNAc6ST-1/2-EYFP chimera (lane e), from stable CHO cell lines. Lane a is the lysate from parent CHO cells. Lysates with equal protein content were loaded onto a 12% gel. The blot was probed with anti-GFP serum and analyzed by chemiluminescence. The molecular mass marker is 85 kDa.

as a medial-Golgi marker (58); these proteins are endogenous to CHO cells and can be detected with specific mAbs.

GlcNAc6ST-1-GFP lacked significant overlap with GM130 (Fig. 4A, a–d; percent colocalization = 4.2 ± 0.3, Fig. 4B) but strongly colocalized with TGN38-IgG (Fig. 4A, e–h; percent colocalization = 66.2 ± 1.1, Fig. 4B), suggesting a presence in the late Golgi cisternae. The assignment of precise localization within the Golgi compartments can be difficult because of the small distances (approximately 30 nm) that separate neighboring cisternae (59). The fungal metabolite BFA has been used to distinguish between proteins residing within the medial-trans-Golgi cisternae and the TGN. BFA causes the collapse of the contents of the cis, medial, and trans-cisternae back to the ER, whereas proteins residing within the TGN traffic to the MTOC (60, 61). This redistribution allows the straightforward assignment of localization either in the trans-Golgi cisterna or TGN (62). In the presence of BFA, GlcNAc6ST-1-GFP cycled predominantly to a punctate juxtanuclear structure and colocalized with TGN38-IgG (Fig. 4A, i–l; percent colocalization = 63.8 ± 5.6, Fig. 4B). These data demonstrate that GlcNAc6ST-1-GFP resides predominantly in the TGN.

By contrast, GlcNAc6ST-2-EYFP showed a diffuse staining pattern, partially colocalizing with all markers including BiP (ER) (Fig. 5A, a–d; percent colocalization = 35.1 ± 1.8, Fig. 5B), GM130 (cis-Golgi) (Fig. 5A, e–h; percent colocalization = 34.9 ± 1.2, Fig. 5B), mannosidase II (medial-Golgi) (Fig. 5A, i–l; percent colocalization = 24.5 ± 1.6, Fig. 5B), and TGN38-IgG (TGN) (Fig. 5A, m–p; percent colocalization = 42.3 ± 1.9, Fig. 5B). Upon treatment with BFA, GlcNAc6ST-2-EYFP cycled to both the ER and the MTOC, confirming its presence throughout the secretory pathway (Fig. 5A, q–t; percent colocalization = 31.8 ± 2.4, Fig. 5B). Treatment with 10 μg/ml cycloheximide for 3 h at 37 °C, which is sufficient to block ~90% of protein synthesis in CHO cells (63), did not affect the distribution of GlcNAc6ST-2-EYFP (not shown). Thus, the observed fluorescence reflects the true location of the mature protein and is not the result of nascent protein still in the process of trafficking within the Golgi.

GlcNAc6ST-3-EYFP was found very early in the secretory pathway. The most significant colocalization was observed with the ER marker BiP (Fig. 6A, a–d; percent colocaliza-
Although the two molecules were distributed in a similar fashion, the fine structures of their fluorescence signals were distinct. The ER marker BiP was distributed in a reticular structure as is typical of ER-resident proteins (64); however, the sulfotransferase appeared to occupy tubular structures. We considered that GlcNAc6ST-3-EYFP might reside in the ER-Golgi intermediate compartment (ERGIC), but little colocalization with the ERGIC marker ERGIC-53 (65, 66) was observed (Fig. 6A, e–h; GlcNAc6ST-1-GFP colocalizes strongly with the TGN marker TGN38-IgG, i–l, treatment with BFA cycles both TGN38-IgG and GlcNAc6ST-1-GFP to the MTOC, which is a hallmark of TGN-resident proteins. Bar = 3 μm. B, bar graph showing percent colocalization. Error bars represent the standard deviation from images of three or four cells.

As further characterization, we generated CHO cells stably expressing both GlcNAc6ST-1-GFP and GlcNAc6ST-1 fused to the ECFP (GlcNAc6ST-1-ECFP) and evaluated their localization in the presence of BFA. As in previous experiments, GlcNAc6ST-1-ECFP cycled exclusively to the MTOC, whereas GlcNAc6ST-3-EYFP localized to the ER (Fig. 7). Given the dependence of GlcNAc6ST-3-EYFP activity on the presence of Core2GlcNAcT-1, we evaluated the effects of the latter on the localization of the former. No change in localization of GlcNAc6ST-3-EYFP was observed upon coexpression with Core2GlcNAcT-1 (not shown). In conclusion, the three sulfotransferases have very different distributions within the secretory pathway of CHO cells, with GlcNAc6ST-
Effects of Perturbing Golgi Localization on Substrate Preference—Given that the sulfotransferases do not distinguish among terminal GlcNAc substrates in vitro, we considered the possibility that the preferences they display in CHO cells reflect their distinct Golgi distribution. To test this hypothesis, we analyzed the substrate preference of a chimeric sulfotransferase comprising the localization domain of one enzyme fused to the catalytic domain of the other (depicted in Fig. 8A). Precedents for this come from studies of glycosyltransferases, Golgi-resident enzymes with an architecture similar to that of the sulfotransferases. Although there is as
yet no method for predicting Golgi localization based on primary sequence, work with glycosyltransferases has implicated the cytosolic tail, transmembrane domain, and/or stem region inside the Golgi lumen as potential determinants (67, 68). In some cases, localization is determined by a single domain (i.e. the cytosolic tail), whereas in other cases combi-
nations of two or three domains are required for effective Golgi localization. Several groups have shown that the catalytic domains of glycosyltransferases can be replaced with heterologous proteins, such as GFP, without detriment to proper Golgi localization (41). Likewise, the exchange of localization domains between different glycosyltransferases has yielded active proteins whose location within the Golgi depends on the origin of the localization domain, attesting to the modularity of the protein (35, 36, 69, 70).

The cytosolic tail, transmembrane domain, and stem region of GlcNAc6ST-1 were fused to the catalytic domain of GlcNAc6ST-2 bearing C-terminal EYFP, and the substrate preference of the chimera was analyzed in stably transfected CHO cells (the individual domains were defined based on sequence alignment as shown in Fig. 3F, lane e). All of the isolated clones expressed the G72 antigen at a level similar to that of CHO cells stably expressing GlcNAc6ST-1-GFP (a representative is shown in Fig. 9A). We confirmed that the G72 antigen resides on N-linked glycoproteins, as Lec1 CHO cells stably expressing the chimera showed no detectable G72 binding (Fig. 9B). Furthermore, the expression level of the chimera in the CHO cell line was comparable with those of the parent fluorescent sulfotransferases (Fig. 3F, lane e), but the chimeric sulfotransferase colocalized strongly with TGN38-IgG, similarly to GlcNAc6ST-1-GFP (Fig. 9C, e–h; percent colocalization = 51.0 ± 3.8, Fig. 9D). Although the catalytic domain of GlcNAc6ST-2 derives from an enzyme that prefers...
O-linked glycoproteins, this preference shifts to N-linked targets when the catalytic domain is confined similarly to GlcNAc6ST-1.

**DISCUSSION**

The GlcNAc6ST catalytic domains show little difference in substrate preference when probed with oligosaccharides in vitro. For example, the family members require a terminal GlcNAc residue but show limited sensitivity to the context in which that residue occurs. By contrast, analysis of the GlcNAc6ST-2 knockout mouse clearly identifies this enzyme as a participant in sulfoadhesin biosynthesis; other GlcNAc6STs (i.e. GlcNAc6ST-1) resident in the same tissues fail to compensate for its absence. Thus, the in vivo targets of the sulfotransferases are not determined solely by the carbohydrate specificity of the catalytic domain. Our results show that the specific classes of glycoproteins modified by GlcNAc6ST-1-GFP (N-linked) and GlcNAc6ST-2-EYFP and -3-EYFP (O-linked) in CHO cells are influenced by their distribution within the Golgi compartment. Because the sulfotransferases require GlcNAc in a terminal position, they must act prior to elaborating glycosyltransferases in the secretory pathway. In other words, the sulfotransferases must colocalize both temporally and spatially with a substrate presenting a terminal GlcNAc residue in order to sulfate that glycoprotein.

The cellular substrate preferences of GlcNAc6ST-1, -2, and -3 can be understood in the context of the different mechanisms for N-linked and O-linked glycan elaboration. N-Linked glycosylation initiates with the cotranslational transfer of a tetradecasaccharide from a dolichol phosphate precursor to Asn residues on proteins within the ER (Fig. 10A) (71). Subsequent trimming steps throughout the ER and early Golgi compartments reduce this structure to a core oligosaccharide, which is then elaborated with terminal glycan epitopes in the late Golgi compartments. The final trimming step in this process is the removal of two terminal mannose residues by mannosidase II (step 2 in Fig. 10A), known to reside in the medial-Golgi. Thus, extension of terminal glycans can occur only after this point. The sulfated GlcNAc residues generated by GlcNAc6ST-1-GFP are components of the terminal glycans. Concentration of GlcNAc6ST-1-GFP in the TGN would ideally situate this enzyme at a point in the Golgi where its substrate accumulates. GlcNAc6ST-2-EYFP is distributed broadly throughout the Golgi cisternae, with a representation in the TGN. This may explain why GlcNAc6ST-2-EYFP shows weak activity on terminal epitopes of N-linked glycoproteins.
O-Linked glycan biosynthesis, by contrast, occurs in a linear stepwise fashion beginning very early in the Golgi (Fig. 10B). The process is initiated by the polypeptide GalNAc trans- ferases, of which several isoforms have been identified in hu- mans with localization ranging from the ER to the TGN (72– 74). As shown schematically in Fig. 10B (step 4), the Core 1 structure is generated by the addition of a galactose residue to the core GalNAc, and the Core 2 trisaccharide can be formed subsequently by the action of Core2GlcNAcT-I (step 5) which has been localized to the cis/ medial compartment of the Golgi (55). Sulfation of the Core 2 GlcNAc residue must occur at this point (step 6), before further elaboration with poly-LacNAc extensions or the sLeα capping group. GlcNAc6ST-3-EYFP is situated early in the secretory pathway and therefore may only have access to the Core 2 GlcNAc residue. GlcNAc residues installed later on more distal portions of the glycan may be inaccessible to the enzyme. This would explain its inability to generate more distal capping 6-sulfo-sLeα structures that are recognized by the antibodies used in this study. The apparent early localization of GlcNAc6ST-3-EYFP might minimize its cohabitation with Core2GlcNAcT-1, thus ensuring precise and exclusive sulfation of that GlcNAc residue. In stark contrast, the confinement of GlcNAc6ST-2-EYFP to the TGN most likely precludes it from acting on Core 2 GlcNAc residues. Although this is an attractive hypothesis, we cannot rule out that the localization of GlcNAc6ST-3-EYFP has been perturbed by the EYFP fusion or that CHO cells mislocalize the human protein. Still, the activity of the fluorescent protein fusion is consistent with literature reports of native GlcNAc6ST-3 (37).

GlcNAc6ST-2-EYFP has a strong presence in the cis- and medial-Golgi compartments and is therefore situated for efficient sulfation of core GlcNAc residues (step 6) as well as more distal GlcNAc residues within poly-LacNAc repeats (steps 7 and 8, Fig. 10C). The initial biochemical characterization of

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**Fig. 9. Substrate preference and Golgi localization of the GlcNAc6ST-1/2-EYFP chimera.** A, mAb G72 staining of CHO cells stably expressing the GlcNAc6ST-1/2-EYFP chimera (gray) and parent CHO cells (clear). B, mAb G72 staining of Lecl CHO cells stably expressing the GlcNAc6ST-1/2-EYFP chimera (gray) and parent Lecl CHO cells (clear). C, Golgi localization of the GlcNAc6ST-1/2-EYFP chimera in CHO cells. Images are presented as in Figs. 4–6. a–d, the GlcNAc6ST-1/2-EYFP chimera does not colocalize with GM130, e–h, the GlcNAc6ST-1/2-EYFP chimera shows strong colocalization with TGN38-IgG, i–l, treatment with BFA cycles the GlcNAc6ST-1/2-EYFP chimera to the MTOC, where it colocalizes again with TGN38-IgG. D, bar graph showing percent colocalization. Error bars represent the standard deviation from images of three or four cells.
sulfoadhesin from murine lymph nodes revealed that a majority of O-linked glycans were modified with multiple sulfate esters (75). Based on more recent analyses of sulfoadhesin (11), the other sulfated GlcNAc residues most likely include the Core 1 extension residue installed by Core1-fucosylating sialyl-LacNAc on both N-linked and O-linked glycans in CHO cells (69). FucTVII is capable of recognizing the terminal structure for L-selectin (16, 38). It has been proposed that these enzymes have different Golgi distributions. It is interesting to note that GlcNAc6ST-1 has an extended stem region that is not represented in the sequence of GlcNAc6ST-2 and -3 (this stem was included in the GlcNAc6ST-1/2 chimera). How- ever, the precise determinants of their localization are not known and are the subject of future interest.

Several models have been proposed for Golgi localization, including control at the level of transmembrane domain thickness or via associations among clusters of Golgi enzymes (67, 68, 81–84). Given that several pairs of Golgi enzymes have been shown to associate specifically (85–87), including a heparan sulfate epimerase and sulfotransferase that act in tandem in the
Localization and Specificity of GlcNAc-6-sulfotransferases

same pathway (42), the possibility that the GlcNAc6STs also associate with functionally related proteins should be investigated. From a practical standpoint, any specific localization mechanism that is discovered for GlcNAc6ST-2 would provide a new target for the discovery of small molecules with anti-inflammatory properties. More generally, a thorough understanding of carbohydrate biosynthesis cannot be uncoupled from a complete description of Golgi enzyme organization.

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