Effects of N-glycosylation on the activity and localization of GlcNAc-6-sulfotransferase 1

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N-Acetylgalactosamine-6-sulfotransferase-1 (GlcNAc6ST-1) is a Golgi-resident glycoprotein that is responsible for sulfation of the N-linked glycans on endothelial cells. Here, we report the sites at which GlcNAc6ST-1 is modified with N-linked glycans and the effects that each glycan has on enzyme activity, specificity, and localization. We determined that glycans are added at three of four potential N-linked glycosylation sites: N196, N410, and N428. The N428 glycan is required for the production of sulfated cell surface glycans: cells expressing a mutant enzyme lacking this glycan were unable to sulfate the sialyl Lewis X tetrasaccharide or a putative extended core 1 O-linked glycan. The N196 and N410 glycans differentially affect sulfation of two different substrates: cells that express an enzyme lacking the N410 glycan are able to sulfate the sialyl Lewis X substrate, but produce reduced levels of a sulfated peripheral lymph node addressin epitope and cells that express an enzyme lacking the N196 glycan are able to produce a sulfated peripheral lymph node addressin epitope, but are impaired in their ability to sulfate sialyl Lewis X. The glycans’ effects on enzyme activity may be mediated, in part, by changes in enzyme localization. While most mutants that lacked glycans localized normally within the Golgi, the N428A mutant and a mutant lacking all glycans were also found to localize ectopically. Altered trafficking of mutants may be associated with the mechanisms by which misglycosylated enzyme is degraded.

Keywords: enzyme specificity/N-linked glycosylation/sulfation

Introduction

Cell surface sulfation is a critical signal that regulates the recruitment of white blood cells (Hemmerich and Rosen 2000). This recruitment process is essential for normal lymphocyte trafficking, but can also contribute to the symptoms of chronic inflammation (Uchimura and Rosen 2006; Yang et al. 2006). Sulfated glycoproteins are displayed on endothelial cells found lining the blood vessels in the peripheral lymph nodes and at sites of chronic inflammation (Rosen 2004). The requisite sulfated structures are produced by Golgi-resident enzymes that add sulfate to carbohydrates (or tyrosines) in glycoproteins destined for cell surface display (Grunwell and Bertozzi 2002; Sperandio 2006). For example, recruitment of leukocytes to sites of inflammation is mediated, in part, by a binding interaction between L-selectin (CD62L) on leukocytes and sulfated glycans on the endothelium. Monoclonal antibodies have served as key tools in efforts to determine the structures of these sulfated glycans. For example, the addition of sulfate at the 6-position of N-acetylgalactosamine (GlcNAc) in the sialyl Lewis x (sLe X) tetrasaccharide creates 6-sulfosLe X, recognized by the G72 antibody (Figure 1A) (Hemmerich et al. 1995), while the MECA-79 antibody recognizes that sulfated GlcNAc is found on an extended core 1 branch of O-linked glycans (Figure 1B) (Yeh et al. 2001). Since Golgi-resident sulfotransferases are key regulators of the inflammatory response, further study of the factors that control the activity and specificity of these enzymes is required to evaluate the sulfotransferases’ suitability as targets for pharmaceutical intervention (Hemmerich et al. 2004).

The GlcNAc-Gal-GalNAc-6-O-sulfotransferases comprise a family of seven enzymes that transfer sulfate from adenosine 3’-phosphate 5’-phosphosulfate (PAPS) to the 6-position of a GlcNAc, galactose (Gal), or N-acetylgalactosamine (GalNAc) residue (Grunwell and Bertozzi 2002; Uchimura and Rosen 2006). These enzymes share a common topology: they are type II transmembrane proteins with a short cytoplasmic tail, a hydrophobic single-pass transmembrane spanning domain, an intervening stem region, and a large globular catalytic domain that resides in the Golgi lumen (Figure 1C).

The physiological roles of GlcNAc-6-sulfotransferase-1 (GlcNAc6ST-1) and other GlcNAc-6-sulfotransferases have been examined in mice (Hemmerich et al. 2001; van Zante et al. 2003; Uchimura et al. 2004, 2005; Kawashima et al. 2005). Animals that lack both GlcNAc6ST-1 and GlcNAc-6-sulfotransferase-2 (GlcNAc6ST-2) are unable to produce 6-selectin ligands (including 6-sulfosLe X); therefore, they exhibit severely impaired lymphocyte homing to peripheral lymph nodes and Peyer’s patches (Kawashima et al. 2005; Uchimura et al. 2005). Biochemically, GlcNAc6ST-1 has been demonstrated to be responsible for sulfation of the 6-position of nonreducing GlcNAc, thereby producing 6-sulfosLe X, the G72 epitope (Uchimura et al. 1998). This epitope is found on the distal end of both N-linked and O-linked glycans. In addition to forming the G72 epitope, GlcNAc6ST-1 is able to sulfate the extended core 1 branch of O-linked glycans, producing the epitope recognized by the MECA-79 antibody (Berg et al. 1998; Yeh et al. 2001; de Graffenried and Bertozzi 2003) and a related epitope recognized by the JG-1 antibody (Berg et al. 1998).

Golgi-resident proteins are often glycosylated and all GlcNAc-Gal-GalNAc-6-O-sulfotransferases display the consensus sequence for N-linked glycosylation, although little information is available concerning the occupancy of the putative glycosylation sites (Grunwell et al. 2002). In particular,
N-linked glycosylation is essential to the activity, specificity, and trafficking of many Golgi-resident glycosyltransferases and sulfotransferases (Fast et al. 1993; Haraguchi et al. 1995; Nagai et al. 1997; Martina et al. 1998; Baboval et al. 2000; Christensen et al. 2000; Muhlenhoff et al. 2001; Eckhardt et al. 2002; Kato et al. 2005; Yusa et al. 2005, 2006). For example, the Golgi-resident polysialyltransferase ST8Sia2 requires the attachment of N-glycans at specific sites to ensure activity in vitro and in cells (Muhlenhoff et al. 2001). In addition, N-linked oligosaccharides have been shown to be essential for the production of active chondroitin 4-sulfotransferase-1 (C4ST-1) and chondroitin 6-sulfotransferase-1 (C6ST-1) (Yusa et al. 2005, 2006).

Previous work suggested that GlcNAc6ST-1 is glycosylated (de Graffenried and Bertozzi 2004). Inspired by this information and by reports that glycosylation affects enzyme activity and specificity (Muhlenhoff et al. 2001; Yusa et al. 2005, 2006), we designed experiments to identify the N-glycosylation sites on GlcNAc6ST-1 and to determine what, if any, effect these glycans have on the activity and localization of GlcNAc6ST-1. In this context, the role of glycosylation in the regulation of C6ST-1 activity is of particular interest because C6ST-1 exhibits sequence homology with GlcNAc6ST-1. Notably, putative N-linked glycosylation sites in the two genes, N428 in the GlcNAc6ST-1 and N413 in the C6ST-1, align, suggesting that they could play related roles (Figure 1C). In C6ST-1, the glycosylation state of N413 regulates the specificity of the enzyme and is required for sulfation of keratan sulfate (Yusa et al. 2006). We speculated that the putative N428 glycosylation site in GlcNAc6ST-1 might have some import for its activity or specificity. An understanding of which glycosylation events are crucial for the formation of active enzyme will facilitate future structural and biochemical studies of sulfotransferase family members (Grunwell et al. 2002).

### Fig. 1. GlcNAc6ST-1’s sulfated products and putative N-linked glycosylation sites.

(A) Structure of the G72 epitope produced by GlcNAc6ST-1. The G72 antibody recognizes the sLeX tetrasaccharide, sulfated on the 6-position of GlcNAc (sulfation shown in red). (B) Structure of the MECA-79 epitope. The MECA-79 epitope defines peripheral node addressin (PNAd) and is sulfated on the 6-position of GlcNAc (sulfation shown in red). (C) Sequence alignment of human GlcNAc6ST-1 and murine C6ST-1 amino acid sequences. Alignment was performed using the ClustalW program implemented with its default parameters. Potential N-linked glycosylation sites are indicated in red. The approximate boundaries of the cytoplasmic tails, transmembrane domains, stem regions, and catalytic domains are indicated above the sequences.

<table>
<thead>
<tr>
<th>Amino Acid Sequence</th>
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<th>Mouse C6ST-1</th>
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<td>mC6ST-1</td>
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with wild-type GlcNAc6ST-1-EYFP was subjected to digestion. To test this hypothesis, cell lysate from HeLa cells transfected with GlcNAc6ST-1-EYFP or one of its mutants were separated by SDS–PAGE and analyzed by Western blot, as described. For the N428A mutant, 24 μg of cellular protein was loaded on the gel; for wild-type protein and other mutants, 8 μg of total cellular protein was used. As a control, 6.4 μg of cellular protein from cells transfected with wild-type GlcNAc6ST-1-EYFP was treated with PNGaseF (lane 1). Molecular weights are indicated to the right of the blot.

**Results**

GlcNAc6ST-1 has three sites of N-linked glycosylation

All experiments were conducted using an expression plasmid encoding GlcNAc6ST-1 with enhanced yellow fluorescent protein (EYFP) fused to its C-terminus. This fusion protein has been employed previously to study the activity and specificity of GlcNAc6ST-1 in Chinese hamster ovary (CHO) and HeLa cells (de Graffenried and Bertozzi 2003, 2004; de Graffenried et al. 2004). Previous Western blot analysis of the GlcNAc6ST-1-EYFP chimera resulted in several immunoreactive bands, suggesting that this enzyme is subject to post-translational modification (de Graffenried and Bertozzi 2003, 2004).

We examined the sequence of GlcNAc6ST-1 and identified four asparagines – N105, N196, N410, and N428 – that conform to the consensus sequence (Asn-Xaa-Ser/Thr) for N-glycosylation (Bause 1983). In addition, we used ClustalW (Thompson et al. 1994) to align the amino acid sequence of GlcNAc6ST-1 with that of another sulfotransferase, murine C6ST-1, which has six potential sites of N-linked glycosylation (Figure 1C) (Yusa et al. 2006). We discovered that N428 in GlcNAc6ST-1 aligns with N413 in C6ST-1 the glycan at this position has been shown to control the specificity of C6ST-1 for chondroitin sulfate versus keratan sulfate substrates (Yusa et al. 2006).

We conducted site-directed mutagenesis to convert each potential glycosylation site in the GlcNAc6ST-1-EYFP chimera from asparagine to alanine, thereby precluding the possibility of glycosylation. The four mutant genes were termed N105A, N196A, N410A, and N428A. We also prepared a double mutant in which both N410 and N428 are replaced by alanine (N410A:428A), and a quadruple mutant (NallA) in which all four asparagines are substituted with alanine. We expected that the bands in the N410A and N428A lanes would be completely devoid of N-linked glycans.

We cultured HeLa cells and transiently transfected the cells with EYFP-tagged GlcNAc6ST-1 or one of its mutants. After 2 days, cells were lysed and the contents were analyzed using an antibody that recognizes EYFP. Western blotting of GlcNAc6ST-1-EYFP revealed that wild-type GlcNAc6ST-1 migrates as multiple species with differing molecular weights (Figure 2). We hypothesized that the slower migrating bands corresponded to protein bearing one or more N-linked glycans. To test this hypothesis, cell lysate from HeLa cells transfected with wild-type GlcNAc6ST-1-EYFP was subjected to digestion with peptide N-glycosidase F (PNGase F), an enzyme that removes N-linked glycans. PNGase F treatment afforded the protein that migrated with faster mobility, although the presence of multiple bands indicated that deglycosylation may not have proceeded to completion. The lowest molecular band was observed near 80 kDa, consistent with GlcNAc6ST-1-EYFP’s calculated molecular weight of 84 kDa. Furthermore, the NallA mutant, expected to lack N-linked glycans, migrated similarly to the lowest band in the PNGase F–treated lane. These results suggest that wild-type GlcNAc6ST-1 bears multiple N-linked glycans.

The N105A mutant and the wild-type protein migrate identically in the Western blot, leading us to conclude that N105 is not a site for N-linked glycosylation. In contrast, immunoblotting of the N196A, N410A, and N428A mutants produced multiple bands, all of which appeared to have a lower molecular weight than a fully glycosylated wild-type protein. Therefore, we concluded that N196, N410, and N428 were sites of N-linked glycosylation. The existence of the N196 glycan was confirmed by examining the N410:428A double mutant, which migrated slower than the quadruple mutant or deglycosylated enzyme. We also noted that the bands in the N410A and N428A lanes had similar mobility, suggesting that the glycans attached to N410 and N428 were similar in size. Together, these results point to the presence of at least three N-linked glycans attached to residues N196, N410, and N428 of GlcNAc6ST-1-EYFP.

The N428 glycan is required for the production of sulfated sialyl Lewis X

Next, we tested whether cells expressing glycan-deficient forms of GlcNAc6ST-1-EYFP were able to produce 6-sulfo-sLeX. In these experiments, we used flow cytometry to measure cell surface 6-sulfo-sLeX levels. HeLa cells were transiently transfected with plasmid DNA coding for GlcNAc6ST-1-EYFP or one of its mutants, along with a plasmid encoding fucosyltransferase 7 (FUT7), an enzyme required for the synthesis of sLeX in HeLa cells. In these and subsequent flow cytometry experiments, we identified transfected cells on the basis of their EYFP fluorescence and analyzed glycan expression on EYFP-positive cells. Upon repetition of this experiment, we found that the transfection efficiency varied, but did not affect the relative activity of wild-type GlcNAc6ST-1-EYFP and its mutants. Within a single experiment, all samples exhibited transfection efficiencies within 10% of one another.

Cell surfaces were probed using the G72 antibody, which recognizes 6-sulfo-sLeX, and G72 antibody binding was quantified by flow cytometry (Figure 3). As demonstrated previously, HeLa cells expressing wild-type GlcNAc6ST-1-EYFP fusion protein produced high levels of cell surface 6-sulfo-sLeX (de Graffenried and Bertozzi 2004). Similarly, cells expressing the GlcNAc6ST-1-EYFP mutants N105A and N410A exhibited high levels of G72 antibody binding, indicating that they were able to generate 6-sulfo-sLeX at the same levels as wild-type enzyme. In contrast, the N196A mutant demonstrated a significantly lower level of G72 antibody binding, suggesting that this mutant produces lower levels of 6-sulfo-sLeX or that it makes a different epitope that has lower affinity for the G72 antibody. Finally, cells transfected with the N428A, N410:428A, or NallA mutants exhibited G72 antibody binding similar to the control cells, which were transfected with GlcNAc6ST-1-EYFP.
Fig. 3. Cellular production of G72 epitope by GlcNAc6ST-1-EYFP and its mutants. Flow cytometry was performed on HeLa cells transiently transfected with DNA encoding wild-type GlcNAc6ST-1-EYFP, one of its N-glycosylation mutants, or a GlcNAc6ST-1-EYFP mutant lacking the catalytic domain (no Cat), along with DNA encoding FUT7. Cells were stained with the G72 antibody to detect the presence of 6-sulfo-sLeX on the cell surface. Each sample contained 20,000 live cells and analysis was performed in triplicate. The data shown represent cells expressing the EYFP fusion protein, as determined by fluorescence on the FL-1 channel of the flow cytometer. (A) The N105A and N410A mutants exhibit similar levels of activity as wild-type protein. (B) The N196A mutant exhibits partial activity. (C) The N428A, N410:428A, and NallA mutants are inactive and behave similarly to the “No cat” control. (D) Flow cytometry data from panels A–C shown in histogram form.

The N410 and N428 glycans are required for the production of the JG-1 epitope

We found that GlcNAc6ST-1 is also able to produce a peripheral lymph node addressin (PNAd) ligand recognized by the JG-1 antibody. The JG-1 epitope has not been clearly delineated, but may be related to MECA-79’s antigen, 6-sulfo- \( \text{N} \)-acyetyllactosamine (Figure 1B) (Yeh et al. 2001) since these two antibodies partially block one another (Berg et al. 1998). Unlike MECA-79, JG-1 binding requires sialic acid (Berg et al. 1998). To determine how GlcNAc6ST-1’s glycosylation state affects JG-1 epitope production, HeLa cells were transiently transfected with GlcNAc6ST-1-EYFP or one of its mutants, along with \( \beta3 \text{GlcNAcT-3} \), the GlcNAc-transferase that is required for extension of the core 1 branch in HeLa cells (Yeh et al. 2001). Cell surfaces were probed with the JG-1 antibody and binding was quantified by flow cytometry (Figure 4). HeLa cells transfected with \( \beta3 \text{GlcNAcT-3} \) and GlcNAc6ST-1-EYFP that lacks a catalytic domain do not bind the JG-1 antibody, but cells transfected with wild-type GlcNAc6ST-1-EYFP and \( \beta3 \text{GlcNAcT-3} \) produced significant amounts of the JG-1 antigen, consistent with the MECA-79 antigen production observed previously for this enzyme (de Graffenried and Bertozzi 2003). The N105A and N196A mutants were also active for JG-1 antigen production. The N428A, N410:428A, and NallA mutants were unable to produce the JG-1 antigen and the N410A mutant also exhibited a pronounced decrease in JG-1 antigen production. These data confirm that the N428 glycan is required for GlcNAc6ST-1 activity in HeLa cells and indicate that the N410 glycan also plays a role in the production of the JG-1 epitope.

The N428 glycan is required for enzyme activity, but the N196 and N410 glycans are not

Next, we wished to determine whether the observed inability of GlcNAc6ST-1-EYFP mutants to produce the G72 and JG-1 epitopes was due to decreased catalytic activity of the mutant sulfotransferase. To test the activity of the mutant sulfotransferases, we conducted an in vitro sulfation experiment (Figure 5). We transfected HeLa cells with plasmids encoding wild-type or mutant GlcNAc6ST-1-EYFP and prepared lysates from the cells. The cell lysates were incubated with \( \beta \)-benzyl GlcNAc and \( 35 \text{S-PAPS} \) to test the sulfotransferase’s ability to transfer sulfate from PAPS to terminal GlcNAc. The reaction products were separated by thin-layer chromatography (TLC), and the radioactive species were visualized with a phosphorimager. As expected, lysates containing wild-type GlcNAc6ST-1-EYFP were able to sulfate \( \beta \)-benzyl GlcNAc, as evidenced by the
Fig. 4. Cellular production of the JG-1 epitope by GlcNAc6ST-1-EYFP and its mutants. Flow cytometry was performed on HeLa cells transiently transfected with DNA encoding wild-type GlcNAc6ST-1-EYFP, one of its N-glycosylation mutants, or a GlcNAc6ST-1-EYFP mutant lacking the catalytic domain (no Cat), along with DNA encoding β3GlcNAcT-3. Cells were stained with the JG-1 antibody to detect the presence of PNAd on the cell surface. Each sample contained 20,000 live cells and analysis was performed in triplicate. The data shown represent cells expressing the EYFP fusion protein, as determined by fluorescence on the FL-1 channel of the flow cytometer. (A) The N105A and N196A mutants exhibit similar levels of activity to wild-type protein. (B) The N410A, N428A, N410:428A, and NallA mutants are inactive and behave similarly to the “No cat” control. (C) Flow cytometry data from panels A and B shown in histogram form.

Fig. 5. In vitro sulfation activity of GlcNAc6ST-1-EYFP and its mutants. Cells were transiently transfected with pcDNA3.1 (mock), GlcNAc6ST-1-EYFP (wild-type), or one of its mutants. Cells lysates were incubated with 35S-PAPS, the sulfate donor, and an artificial sulfate acceptor, β-hexyl GlcNAc. Heat-killed samples of both mock and wild type were prepared (mock HK and wild-type HK) as negative controls, and recombinant GlcNAc6ST-1 catalytic domain served as a positive control (rGlcNAc6ST-1). Reactions mixtures were separated by HPTLC and visualized by phosphorimaging. A new 35S-containing species interpreted to be 35SO4-GlcNAc appears in the samples incubated with rGlcNAc6ST-1 or with lysates from wild-type GlcNAc6ST-1-EYFP, N105A, N196A, and N410A mutants. Lysates from N428A-, N410:428A-, and NallA-transfected cells are severely impaired in their ability to make this product. Another 35S-containing species (unidentified product) is produced by all cell lysates including the mock-transfected lysate and likely reflects some endogenous enzymatic activity present in HeLa cells. Species marked by an asterisk are present in the 35S-PAPS preparation and are believed to be 35S-PAPS degradation products such as 35S-adenosine 5′-phosphosulfate (35S-APS) or 35SO4~2~.

appearance of a new radioactive product. This new product had the same mobility as the product of recombinant GlcNAc6ST-1 catalytic domain (purchased from R&D Systems). Similarly, the N105A, N196A, and N410A mutants produced sulfated GlcNAc at levels comparable to the wild-type enzyme. All mutants containing the N428A mutation (N428A, N410:428A, and NallA) exhibited severely impaired enzyme activity: sulfated product was observed only upon lengthy exposure of the TLC plate to the phosphorimaging screen. These results indicate that the N428 glycan is required for the production of fully active GlcNAc6ST-1-EYFP.

The N428 glycan influences enzyme localization
Our analysis of cell surface antigens revealed that GlcNAc6ST-1-EYFP mutants lacking the N196, N410, or N428 glycan were impaired in their ability to produce one or both sulfated epitopes, yet only the N428 glycan was demonstrated to be essential for in vitro activity. One possible explanation for the observed decreases in sulfated cell surface glycans is that enzymes lacking the N196 and N410 glycans are mislocalized to compartments where they were unable to access their substrates. To test this hypothesis, we conducted fluorescence microscopy of GlcNAc6ST-1-EYFP and its mutants to determine their subcellular localization (Figure 6). Wild-type GlcNAc6ST-1-EYFP exhibits a perinuclear, punctate fluorescence pattern, reminiscent of Golgi localization. Previous work demonstrated that this localization pattern corresponded to the trans-Golgi network (TGN) (de Graffenried and Bertozzi 2003). To confirm the TGN localization, cells were probed with an antibody against the endogenous TGN protein, GCC185. These experiments revealed that wild-type enzyme colocalizes with GCC185. Likewise, the N105A, N196A, N410A, and N410:428A mutants also colocalized well with GCC185 and were not observed in other parts of the secretory pathway (supplementary Figure S2). In contrast, while the N428A and NallA mutants did colocalize with
GlcNAc6ST-1, detectable amounts of these enzymes were found in regions outside of the Golgi. The data do not readily account for the activity changes observed for the N196A, N410A, and N410:428A mutants, but do indicate that the inactive N428A and NallA mutants are at least partially mislocalized.

The N428A and NallA mutants exhibit an unusual ectopic localization

We wondered whether the ectopically localized N428A and NallA mutants resided in the endoplasmic reticulum (ER) since misfolded Golgi-resident proteins often accumulate in this organelle. Cells expressing these mutants were immunostained for calreticulin, an ER resident protein. We observed no significant colocalization of N428A or NallA with the ER marker, indicating that the altered localization of these mutants was not a result of their slowed trafficking through the secretory pathway (Figure 7).

Our Western blot results (Figure 2) indicated that the N428A mutant was expressed at a significantly lower level than the wild-type protein. The addition of either proteosomal or lysosomal inhibitors resulted in increased quantities of the N428 mutant (data not shown), so we tested whether the ectopically localized mutants colocalized with markers of the early endosome (EEA1), late endosome (mannose-6-phosphate receptor), or the lysosome (LAMP-1) (Figure 7). The N428A mutant demonstrated minimal colocalization with the mannose-6-phosphate receptor and no colocalization with EEA1 or LAMP-1, while

![Fig. 6. Colocalization of GlcNAc6ST-1-EYFP and its mutants with the trans-Golgi network marker GCC185. Confocal immunofluorescence microscopy was performed with HeLa cells transiently transfected with empty vector (pcDNA3.1, Mock), wild-type GlcNAc6ST-1-EYFP or its N-glycosylation mutants. The fluorescence emanating from EYFP is shown in column 1. Immunostaining for GCC185 is shown in column 2. Column 3 represents the overlay of the images: EYFP is shown in green, GCC185 is shown in red, and the nuclear stain (DAPI) is in blue. Colocalization between the EYFP fusion protein and GCC185 appears yellow in the merged image.](https://academic.oup.com/glycob/article-abstract/19/10/1068/1987735)

![Fig. 7. The NallA and N428A mutants do not localize appreciably to the ER, endosome, or lysosome. Confocal immunofluorescence microscopy was performed with HeLa cells transiently transfected with wild-type GlcNAc6ST-1-EYFP (A), the NallA mutant (B), or the N428A mutant (C). In each panel, immunostaining for marker proteins calreticulin (ER marker), EEA1 (early endosomal marker), mannose-6-phosphate receptor (late endosomal marker), and LAMP-1 (lysosomal marker) is shown in column 1. The fluorescence emanating from EYFP is shown in column 2. Column 3 represents overlay of the images: EYFP is shown in green, marker proteins are shown in red, and the nuclear stain (DAPI) is in blue. Colocalization between the EYFP fusion protein and organelle markers appears yellow in the merged image.](https://academic.oup.com/glycob/article-abstract/19/10/1068/1987735)
the NallA mutant showed at most a minor amount of colocalization with each of the above markers. Wild-type GlcNAc6ST-1-EYFP exhibited essentially no colocalization with any of these markers. These results indicate that the mislocalized N428A and NallA mutant enzymes are localized primarily to locations other than the endosome or lysosome.

Discussion
GlcNAc6ST-1 has three N-linked glycans
After identifying four potential N-glycosylation sites in GlcNAc6ST-1, we confirmed that at least three of those sites are occupied with glycans when the enzyme is produced in HeLa cells. Although we did not conduct a comprehensive study of double mutants, our results suggest that N-glycosylation at the different sites occurs independently, that is, the addition of a glycan at one site is not required for the addition of glycans at other sites. We also noted the presence of the incompletely glycosylated protein for wild-type GlcNAc6ST-1 and all of its mutants (Figure 2). We did not determine the structures of the three attached N-linked glycans, but our Western blot data suggest that they may not be identical: the N196A mutant displayed different mobility than either N410A or N428A GlcNAc6ST-1-EYFP.

The N428 glycan is critical for enzyme activity
Our most striking observation is that the N428 glycan is required for the production of cell surface sulfated glycans. We prepared three mutants that lack the N428 glycan: N428A, N410:428A, and NallA. All three of these mutants are unable to produce cell surface G72 and JG-1 epitopes. We considered several possible explanations for this loss of activity: mislocalization, misfolding, and the requirement of specific glycans for interactions with substrates. Our fluorescence microscopy data demonstrated that the N428A and NallA mutants show some ectopic localization, but that most of the protein localized normally, to the TGN. Surprisingly, the N410:428A mutant showed a localization pattern indistinguishable from wild-type protein. In addition, all mutants lacking the N428 glycan exhibited severely impaired activity in an in vitro assay. These data indicate that mislocalization is not the primary explanation for this loss of activity. Rather, GlcNAc6ST-1 that lacks the N428 glycan may be misfolded or unable to interact with the substrate.

Interplay between N410 and N428 glycans
We considered the possibility that misfolding of GlcNAc6ST-1 mutants might be the cause of lost enzyme activity. In our Western blot analysis of the mutant proteins (Figure 2), we observed substantially higher expression levels of the wild-type enzyme, as compared to mutant enzymes. In particular, levels of the N428A mutant were so low that additional lysate was required to visualize it on the same blot. Although the N428A mutant appears to be destabilized, the double mutant N410:428A was present at levels similar to wild-type and also appears to localize normally. The differential behavior of the N428A mutant and the N410:428A mutant suggests a role for the N410 glycan in quality control. Mutants that lack the N410 glycan may be subject to less stringent examination prior to their exit from the ER.

N-Glycans may influence substrate specificity
Other factors may also play a role in the lack of activity observed for mutants lacking glycans. We found that the N410A mutant produces the G72 epitope at levels similar to wild-type protein, but is severely impaired in JG-1 epitope production. Conversely, the N196A mutant produces decreased amounts of the G72 epitope as compared with wild-type protein but produces levels of the JG-1 epitope similar to those produced by wild-type protein. These observations cannot be explained simply by lower expression levels of the underglycosylated enzymes since the two mutants show the decreased production of different epitopes. One possible explanation is that one or both of the N196 and N410 glycans influence the specificity of GlcNAc6ST-1, either by participating in direct interactions with specific substrates, interacting with other Golgi-resident enzymes, or by subtly altering the localization of the enzyme. In vitro enzyme assays conducted with the defined glycan acceptor, such as sLeα and the extended core 1 branch, may prove useful in distinguishing among these possibilities. In addition, it would be of great interest to conduct the cellular activity experiments with the MECA-79 antibody since the molecular knowledge of that antibody’s antigen (Yeh et al. 2001) would allow one to make more substantive conclusions regarding the substrate specificity of GlcNAc6ST-1 mutants. Unfortunately, when we tried to conduct these experiments, we observed nonspecific binding of a rat IgM isotype control, which prevented us from accurately measuring MECA-79 binding. Nonetheless, our observations concerning the role of the N196 and N410 glycans in GlcNAc6ST-1 specificity are reminiscent of the role that the N413 glycan of C6ST plays in controlling substrate specificity of that enzyme (Yusa et al. 2006).

Fate of misglycosylated proteins
While performing localization studies of the N-glycosylation mutants of GlcNAc6ST-1, we noted that two of the mutants, N428A and NallA, exhibited some distribution to sites outside of the TGN (Figure 6). We also observed a difference in enzyme expression levels when comparing the N428A mutant to wild-type enzyme (Figure 2), suggesting that this incompletely glycosylated protein might be subject to accelerated degradation. Given these results, we examined whether the ectopically localized N428A and NallA mutants were found at endosomal and/or lysosomal sites, but observed extremely minimal colocalization with endosomal and lysosomal markers. In fact, most of the ectopically localized mutant enzyme did not colocalize with any marker that we examined. Although the exact location of these mutants remains unresolved, we note that they exhibit a punctate pattern and are not homogeneously distributed throughout the cytoplasm. An intriguingly similar localization pattern has been observed for the human neutral α-mannosidase that is responsible for cytoplasmic catabolism of soluble N-glycans (KuoKKanen et al. 2007). This mannosidase also exhibited a punctate staining pattern and did not colocalize with organelle markers, including the ER, lysosome/late endosome, and autophagosome. We speculate that incompletely glycosylated GlcNAc6ST-1 could be a substrate for this mannosidase or other associated catabolic enzymes and might colocalize to similar structures.
Material and methods

Materials

Mouse anti-G72 supernatant was a kind gift of Reiji Kannagi. Biotin-conjugated goat anti-mouse antibody and streptavidin tricolor conjugate were purchased from Caltag/Invitrogen, Carlsbad, CA. Biotin-conjugated HECA-452 and biotin-conjugated goat anti-rat Ig polyclonal antibody were purchased from BD Biosciences, San Jose, CA. The expression plasmids coding for GlcNAc6ST-1-EYFP and β3GlcNAcT-3 were gifts from Carolyn Bertozzi (de Graffenried and Bertozzi 2003, 2004) and the pcDNA3.1(-)FUT7 expression plasmid was obtained from Steven Rosen (UCSF). Goat anti-rabbit horseradish peroxidase conjugate, rabbit anti-GFP antibody, and AlexaFluor 647-conjugated goat anti-rabbit and goat anti-mouse antibodies were purchased from Molecular Probes (Invitrogen). Rabbit anti-GCC185 was a gift from Suzanne Pfeffer. 35S-PAPS was prepared by the Pfeffer lab, following a previously published protocol (Itin et al. 1997). The mouse anti-LAMP-1 ascites fluid developed by August and Hildreth and rat anti-PNAa (JG-1) supernatant developed by Butcher were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Mouse anti-EEA1 and anti-Man-6-phosphate receptor (Man6PR) were purchased from Abcam, Cambridge, MA. PNGase F was obtained from New England Biolabs (Ipswich, MA). 7.5% and 5% Tris-HCl ReadyGels were purchased from Bio-Rad Laboratories, Hercules, CA. β-Benzyl-GlcNAc was purchased from Toronto Research Chemicals, Toronto, ON, Canada. Recombinant GlcNAc6ST-1 catalytic domain was obtained from R&D Systems, Minneapolis, MN. Merck silica gel 60 high-performance thin-layer chromatography (HPTLC) plates (20 × 10 cm) were purchased from Fisher Scientific, Pittsburgh, PA.

Mutagenesis of GlcNAc6ST-1

Site-directed mutagenesis performed according to the Quikchange (Stratagene) method was used to introduce asparagine to alanine mutations in an expression plasmid encoding the GlcNAc6ST-1 EYFP fusion protein. We used the following primers:

- N105A 5′ primer GCCCCTCCAGCGCCTGACCTGGG, N105A 3′ primer CCCCCAGTGCCACGCCCTGGAGGGGC
- N196A 5′ primer CAGCGGGGGGCGCGCCCTACCA CGCTG, N196A 3′ primer CAGCGTGTTGAGGCCGCG CCCCCCGCTG
- N410A 5′ primer CAGTTTCGCCTGGCCATGACCAGTG, N410A 3′ primer CCACCTGTCATGGCCAGGGCAACTG

Mutated genes were sequenced by MCLab (South San Francisco) to verify the mutations and to confirm that no spurious changes were introduced into the GlcNAc6ST-1-EYFP gene.

Cell culture

HeLa cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (v/v), 100 units/mL penicillin, and 50 μg/mL streptomycin. Cells were passaged by 10-fold dilution every 3 days. They were grown at 37°C in an atmosphere containing 5% CO2.

Western blotting

Two days before transfection, 5 × 105 cells were plated in 6 cm tissue culture dishes. Cells were transiently transfected with plasmid DNA encoding the GlcNAc6ST-1 EYFP fusion protein or mutant protein using Lipofectamine 2000 (Invitrogen) according to manufacturer’s protocol. Cells were lysed 2 days later using 0.5 mL lysis buffer per sample (50 mM Tris-HCl, adjusted to pH 7.4, 150 mM NaCl, 0.1% SDS) with protease inhibitor cocktail (antipain, turkey trypsin inhibitor, leupeptin, benzamidine) added. The PNGase F-treated sample was prepared by removing an 80 μL aliquot of lystate from cells transfected with wild-type GlcNAc6ST-1 and incubating at 37°C overnight with 500 units of PNGase F. Total protein concentration was measured by the DC Protein Assay (BioRad, Hercules, CA). Each lane contained 8 μg protein, except the N428A sample, which contained 24 μg of protein. Samples were mixed with equal volumes of SDS loading buffer containing DTT and boiled for 10 min. Samples were applied to 7.5% Tris-HCl SDS-PAGE Ready Gels (Bio-Rad) and separated by electrophoresis. The proteins in the gel were transferred to a nitrocellulose membrane, which was subsequently blocked overnight in 5% nonfat dry milk in phosphate-buffered saline, pH 7.0 (PBS) with 0.1% Tween-20 (PBST). The membrane was incubated for 2 h with rabbit anti-GFP diluted 1:2500 in PBST containing 5% nonfat dry milk and then washed three times with PBS and once with PBST. The membrane was then incubated with an HRP-conjugated goat anti-rabbit antibody diluted 1:10,000 in PBST containing 5% nonfat dry milk, and then washed three times with PBST and once with PBS. The blot was developed using SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL) and was exposed to film.

Flow cytometry

Flow cytometry assays were carried out on a FACSScan analyzer (Becton-Dickinson Instruments). Two days prior to transfection, 5 × 105 cells were plated in 6 cm tissue culture dishes. For experiments using the G72 antibody, cells were transiently transfected with DNA encoding GlcNAc6ST-1 or one of its mutants as well as DNA encoding the fucosyltransferase FUT7. The two plasmids were used in equal amounts. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. One day post-transfection, six aliquots each containing 2.5 × 105 cells from each transfection were plated in 12-well tissue culture plates. The following day, cells were washed twice with PBS and removed from the tissue culture plates using 1 mM EDTA in PBS. The cells were transferred to V-bottom 96-well plates for staining. Three aliquots of each sample were washed three times with the FACS buffer (0.1% bovine serum albumin (BSA) in PBS), and then incubated with 50 μL of a 1:50 dilution of G72 supernatant in the blocking buffer. These aliquots were washed with the FACS buffer and then incubated 30 min with the secondary antibody, biotin-conjugated goat anti-mouse diluted 1:50 in the FACS buffer. The remaining three aliquots were washed three times with the FACS buffer, and then incubated for 1 h with 50 μL of a 1:50 dilution of biotinylated HECA-452. All samples were washed with the FACS buffer and then incubated for 30 min with...
streptavidin tricolor conjugate diluted 1:100 in the FACS buffer. Samples were washed and resuspended in the 200 μL FACS buffer. For each sample, fluorescence was measured for FL-1 (measuring EYFP expression) to identify the cells expressing GlcNAc6ST-1-EYFP fusions proteins. The FL-1-positive cells were further examined on the FL-3 channel (measuring tricolor staining) to determine G72 binding. We analyzed 20,000 cells per sample.

To analyze sulfation of the core 1 extension, cells were transiently transfected with DNA encoding GlcNAc6ST-1 or one of its mutants as well as a plasmid encoding β3GlcNAcT-3 (de Graffenried and Bertozzi 2004). The two plasmids were used in equal amounts. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. One day post-transfection, three aliquots each containing 2.5 × 10^5 cells from each transfection were plated in 12-well tissue culture plates. The following day, cells were washed twice with PBS and removed from the tissue culture plates using 1 mM EDTA in PBS. The cells were transferred to V-bottom 96-well plates for staining. The aliquots of each sample were washed three times with the FACS buffer, and then incubated with 50 μL of a 1:20 dilution of JG-1 supernatant in the blocking buffer. Cells were washed with the FACS buffer and then incubated for 30 min with the secondary antibody, biotin-conjugated goat anti-rat Ig diluted 1:50 in the FACS buffer. All samples were washed with the FACS buffer and then incubated for 30 min with streptavidin tricolor conjugate diluted 1:100 in the FACS buffer. For each sample, fluorescence was measured for FL-1 (measuring EYFP expression) to identify the cells expressing GlcNAc6ST-1-EYFP fusions proteins. The FL-1-positive cells were further examined on the FL-3 channel (measuring tricolor staining) to determine JG-1 binding. We analyzed 20,000 cells per sample.

Sulfation activity assay
HeLa cells were transiently transfected as above with either wild-type or mutant GlcNAc6ST-1 DNA, or with pcDNA3.1 (mock) using Lipofectamine 2000 according to manufacturer’s instructions. Two days post-transfection, cells were trypsinized, washed twice with DPBS, pelleted, and lysed by homogenization in 100 μL of 2× reaction buffer (100 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 20 mM NaF, 10 mM Mg(OAc)2, 2 mM ATP, pH 7.0). Insoluble matter was removed by centrifugation, and the supernatant was collected and stored on ice until use. Thirty microliters of lysates from cells transfected with pcDNA 3.1 (mock) and with DNA encoding wild-type GlcNAc6ST-1 was heated to 95°C for 10 min to heat kill the samples for use as negative controls. A reaction containing 0.381 μg of GlcNAc6ST-1 catalytic domain (purchased from R&D Systems) was used as a positive control. Reactions were performed in a final volume of 20 μL containing final concentrations of 0.1% Triton X-100, 10% glycerol, 50 mM HEPES, 10 mM NaF, 5 mM Mg(OAc)2, 1 mM ATP, 25 μCi 35S-PAPS, and 1 mM β-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) and 2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) and were incubated for 20 min with 3.7% formaldehyde in DPBS. The cells were washed again and were permeabilized for 5 min with 0.1% Triton X-100 and 1% BSA fraction V in DPBS. The cells were then blocked for 20 min with 1% BSA fraction V in DPBS (blocking buffer) and then incubated with 1:500 rabbit anti-GCC185 in the blocking buffer for 2 h. The cells were then washed three times with DPBS and then blocked for 20 min with the blocking buffer. They were then incubated with 1:500 goat anti-rabbit Alexa Fluor 647 in the blocking buffer for 1 h. The cells were washed with DPBS. Vectashield with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) was added to the slides, and they were sealed with coverslips. Staining with other antibodies was performed similarly. Concentrations used were 1:500 goat anti-mouse Alexa Fluor 647, 1:250 mouse anti-LAMP1, 1:100 mouse anti-EEA1, 1:250 rabbit anti-calreticulin, and 1:250 mouse anti-Man6PR. Cells were imaged using a Leica SP2 AOBS confocal microscope at the Stanford Imaging Facility.

Supplementary Data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest statement
None declared.