Biosynthesis of L-Selectin Ligands: Sulfation of Sialyl Lewis x-Related Oligosaccharides by a Family of GlcNAc-6-sulfotransferases[†]

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ABSTRACT: The leukocyte adhesion molecule L-selectin mediates lymphocyte homing to secondary lymphoid organs and to certain sites of inflammation. The cognate ligands for L-selectin possess the unusual sulfated tetrasaccharide epitope 6-sulfo sialyl Lewis x (Siaα2→3Galβ1→4[Fucα1→3][SO_3→6]-GlcNAc). Sulfation of GlcNAc within sialyl Lewis x is a crucial modification for L-selectin binding, and thus, the underlying sulfotransferase may be a key modulator of lymphocyte trafficking. Four recently discovered GlcNAc-6-sulfotransferases are the first candidate contributors to the biosynthesis of 6-sulfo sLex in the context of L-selectin ligands. Here we report the in vitro activity of the four GlcNAc-6-sulfotransferases on a panel of synthetic oligosaccharide substrates that comprise structural motifs derived from sialyl Lewis x. Each enzyme preferred a terminal GlcNAc residue, and was impeded by the addition of a β1,4-linked Gal residue (i.e., terminal LacNAc). Surprisingly, for three of the enzymes, significant activity was observed with sialylated LacNAc, and two of the enzymes were capable of detectable sulfation of GlcNAc in the context of sialyl Lewis x. On the basis of these results, we propose possible pathways for 6-sulfo sialyl Lewis x biosynthesis and suggest that sulfation may be an early committed step.

Carbohydrate sulfation is a pervasive modification of cell surface glycans in virtually every mammalian tissue. The presentation of sulfated epitopes occurs with precise temporal and spatial orchestration during normal and pathogenic processes. Discrete carbohydrate sulfoforms have been shown to mediate highly specific molecular recognition events, including the activation of growth factors and cytokines, the regulation of serum half-life of glycoprotein hormones, and leukocyte−endothelium adhesion at sites of inflammation (*1*−*3*). Thus, the carbohydrate sulfotransferases that adorn cell surface molecules with diverse sulfoforms play a fundamental role in extracellular communication. Accordingly, these enzymes have become the focus of great interest as therapeutic targets. In particular, a family of GlcNAc-6-sulfotransferases has emerged in the past few years whose members have attracted attention for their potential roles in diverse processes. The epitope GlcNAc-6-sulfate is found in both glycosaminoglycans (GAGs)[1] and smaller glycans, including heparin sulfate and keratan sulfate, respiratory mucins of cystic fibrosis patients (*4*), HIV-1 gp120 (*5*), zona pellucida glycoprotein (*6*, *7*), thyroglobulin (*8*), and the PEN5 epitope specific to differentiated natural killer cells (*9*). Thus, GlcNAc-6-sulfate is an element frequently found in a variety of structural contexts and might represent a mechanism for creating unique epitopes from common scaffolds.

A landmark discovery in sulfotransferase biology was the identification of GlcNAc-6-sulfate as an essential component of the L-selectin ligand 6-sulfo sialyl Lewis x (6-sulfo sLex) (*10*−*14*). L-Selectin plays a central role in the immune system by mediating the constitutive recirculation of lymphocytes through secondary lymphoid organs, a process in which lymphocytes exit the bloodstream and migrate into underlying tissue (*15*, *16*). Migration is initiated by transient adhesion of blood-borne leukocytes to specialized endothelium, termed high endothelial venules (HEVs), in the vasculature of lymphoid tissue, and through a similar process at certain sites of inflammation (*17*−*21*). The initial interaction is mediated by L-selectin, an adhesion molecule constitutively expressed by circulating leukocytes, and sulfated glycoprotein ligands presented on the surface of HEVs. High-avidity interaction between L-selectin and its ligands displays a strict requirement for sulfation. In both murine and human lymph node endothelium, these ligands are mucin-like glycoproteins adorned with the unusual sulfated tetrasaccharide 6-sulfo sLex (Siaα2→3Galβ1→4-[Fucα1→3][SO_3→6]GlcNAc) on a Core 2 scaffold (Figure 1) (*10*−*14*). Sulfation of this epitope on the GlcNAc residue confers high-avidity L-selectin binding and is thought to be restricted in the vasculature to sites of sustained lymphocyte recruitment. Collectively, these observations point to a GlcNAc-6-sulfotransferase with a specific role in generating L-selectin ligands that mediate leukocyte recruitment, and significant effort has been directed toward determining its

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[1] Abbreviations: Sia, sialic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; GalNAc, *N*-acetylgalactosamine; HEV, high endothelial venule; sLex, sialyl Lewis x; LacNAc, *N*-acetyllactosamine (Galβ1→4GlcNAc); GAG, glycosaminoglycan.

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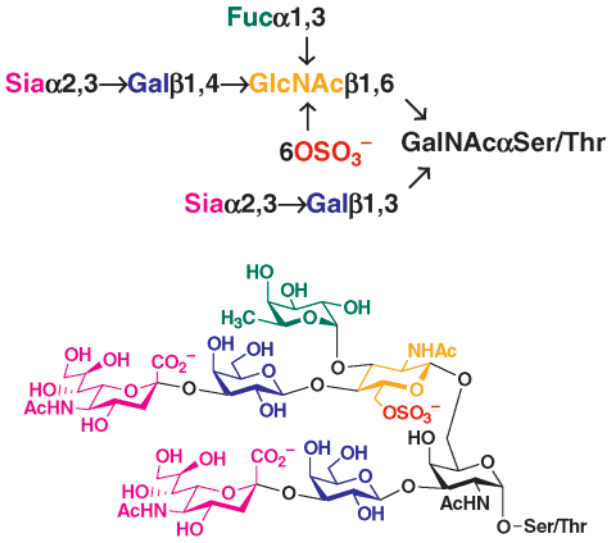


FIGURE 1: 6-Sulfo sialyl Lewis x on a sialylated Core 2 structure. This motif is found on high-affinity L-selectin glycoprotein ligands involved in leukocyte recruitment to secondary lymphoid organs and possibly to sites of chronic inflammation. Monosaccharide structures and abbreviations are color-coded.

identity (*22*). The GlcNAc-6-sulfotransferase may be a key modulator of lymphocyte recruitment to secondary lymphoid organs and sites of chronic inflammation, and therefore, this family of enzymes has become the focus of ardent pursuit as potential targets for anti-inflammatory therapy (*23*, *24*).

In a previous effort to identify a GlcNAc-6-sulfotransferase activity involved in lymphocyte homing, we employed an in vitro biochemical assay to screen a broad range of tissues for sulfotransferase activity (*25*). Using synthetic substrates related to sLex, we characterized a GlcNAc-6-sulfotransferase activity highly restricted to HEVs of lymphoid tissue. The presence an HEV-specific carbohydrate sulfotransferase activity strongly suggested a role in L-selectin ligand biosynthesis, and several groups have since reported the molecular cloning of four human GlcNAc-6-sulfotransferases as candidates for this activity: GlcNAc6ST (*26*) or CHST-2 (*27*), HEC-GlcNAc6ST (*28*) or LSST (*29*), I-GlcNAc6ST (*30*), and C6ST-2 (*31*) or GST5 (*32*). These enzymes are members of a growing family, previously named the Gal/GalNAc/GlcNAc sulfotransferases (GSTs) (*30*), that share 30−50% identity and include the chondroitin (huCSST) and keratan sulfotransferases (Figure 2). The GSTs are distinct from the 15 or so other human carbohydrate sulfotransferases cloned in the past few years which generate heparan sulfate, HNK-1, and other sulfoglycolipids (*2*). Previous characterization of the reported GlcNAc-6-sulfotransferases, including their tissue distribution, is summarized in Table 1.

Two of these enzymes, GlcNAc6ST/CHST-2 and HEC-GlcNAc6ST/LSST, have been investigated in cell-based contexts for their ability to generate L-selectin ligands. The first human clone that was reported, GlcNAc6ST/CHST-2 (*26*, *27*), is robustly expressed across a broad range of tissues and at modest levels in several others, including the HEVs of peripheral and mesenteric lymph nodes. When transfected into a cultured human endothelial cell line, GlcNAc6ST/CHST-2 generates functional L-selectin ligands (*33*). The second GlcNAc-6-sulfotransferase that was identified, HEC-GlcNAc6ST/LSST, has an expression highly restricted to the HEV of peripheral lymph nodes (*28*, *29*, *34*), a compelling attribute that suggests a role essential and specific to that organ. In addition, when expressed in a heterologous host, HEC-GlcNAc6ST/LSST is capable of generating the 6-sulfo sLex epitope recognized by L-selectin (*28*, *34*). These observations underscore the role of HEC-GlcNAc6ST/LSST as a strong candidate for contribution to 6-sulfo sLex biosynthesis in the context of L-selectin ligands. Preliminary in vitro studies of both enzymes using LacNAc-based oligosaccharide substrates indicated a preference for a terminal GlcNAc residue over internal GlcNAc residues, although sialylated and fucosylated structures were not examined (*26*, *29*).

A third enzyme, I-GlcNAc6ST, was identified through an EST database by its homology to the nascent GST family members (*30*). Northern analysis revealed expression restricted to intestinal and colon tissue (*30*), including mesenteric lymph nodes (S. D. Rosen, personal communication). When cotransfected with the L-selectin ligand scaffold protein GlyCAM-1, I-GlcNAc6ST sulfated the pendant O-linked glycans within LacNAc structures, but the products were not reported to support L-selectin recognition (*30*). The most recent GST family member that was reported, C6ST-2/GST5, was initially identified as a chondroitin sulfate sulfotransferase because of its in vitro activity on the 6-hydroxyl of GalNAc within chondroitin sulfate chains (*31*).

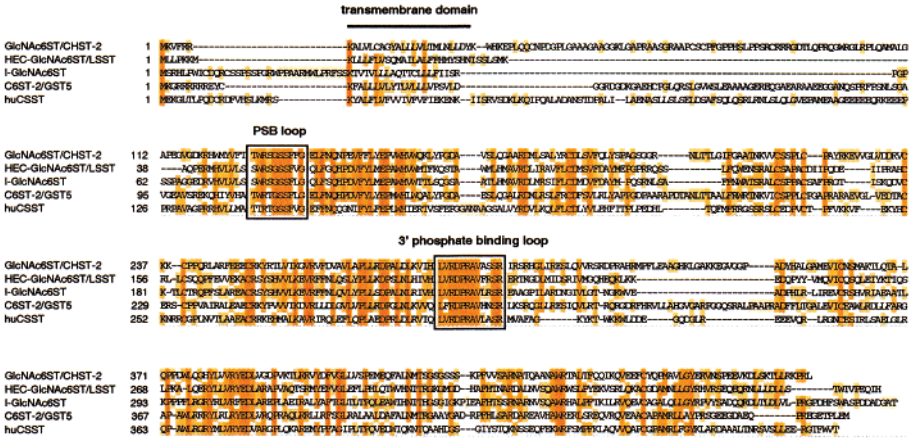


FIGURE 2: Multiple-sequence alignment of the GlcNAc-6-sulfotransferases and human chondroitin sulfotransferase (huCSST). Identified are the putative transmembrane domain and two of the PAPS binding motifs, the 5′-phosphosulfate binding region (PSB loop), and the 3′-phosphate binding region.

Table 1: Reported Characterization of the Family of Four GlcNAc-6-sulfotransferases

name	tissue distribution	capacity to generate L-selectin ligands
GlcNAc6ST/CHST-2	cerebrum, cerebellum, eye, lung, pancreas (*26*)	transcript expression correlates with 6-sulfo sLex presentation during embryogenesis (*42*); generates functional L-selectin ligands in cultured human endothelial cells (*33*) and a heterologous host (*28*)
HEC-GlcNAc6ST/LSST	PLN HEV, spleen (*28*), induced HEV of thyroid neoplasia (*29*)	transcript expression correlates with induction of L-selectin ligands (*29*); directs the synthesis of functional L-selectin ligands in a heterologous host (*28*, *29*, *34*)
I-GlcNAc6ST	small intestine and colon (*30*), mesenteric lymph nodes	not reported
C6ST-2/GST5	liver, heart, spleen (*31*, *32*)	not reported

However, our own studies reveal a robust GlcNAc-6 sulfotransferase activity both in vivo and in vitro and an absence of activity on simple GalNAc glycosides (*32*). The physiological activity of this enzyme remains ambiguous. Detailed investigation of this enzyme's capacity to generate the 6-sulfo sialyl Lewis x epitope has not been reported.

These four GlcNAc-6-sulfotransferases are candidates for generating the critical GlcNAc-6-sulfate motif within the high-avidity L-selectin ligand 6-sulfo sLex. The precise substrate preferences of these enzymes on sLex-related structures would provide insight into the potential of each enzyme to generate GlcNAc-6-sulfate in various contexts, and also would guide interpretation of biological studies related to expression of the GlcNAc-6-sulfate epitope. In addition, the relative order of sulfation and glycosylation steps in the assembly of L-selectin ligands remains a point of controversy, and the preferred oligosaccharide substrate for each enzyme might illuminate the sequence of biosynthetic assembly. In this study, we investigated the activity of the four GlcNAc-6-sulfotransferases on a panel of synthetic oligosaccharide substrates that comprise structural motifs found in the native L-selectin ligands (structures **1**−**4**, Figure 3). All substrates possess the target GlcNAc residue linked to a galactose moiety that mimics the peptide-proximal GalNAc found in the native oligosaccharide. We generated soluble and secreted constructs lacking the putative transmembrane domain of each known GlcNAc-6-sulfotransferase and probed each for activity across the substrate panel. We discovered that each enzyme preferred a terminal GlcNAc residue and was impeded by the addition of a β1,4-linked Gal residue (i.e., terminal LacNAc). Surprisingly, for three of the enzymes, significant activity was observed with sialylated LacNAc, and two of the enzymes displayed detectable activity on sLex.

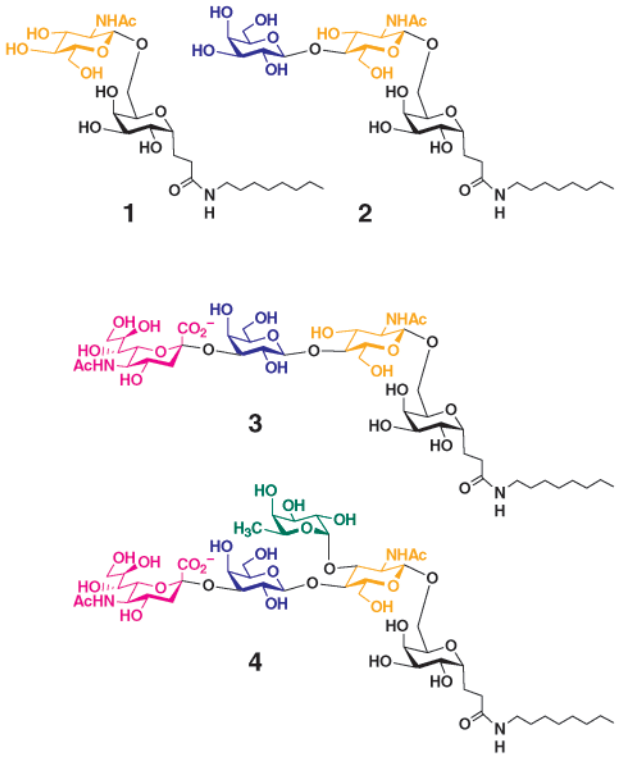


FIGURE 3: Synthetic oligosaccharide substrates **1**−**4**. All substrates preserve the GlcNAcβ1→6 linkage found in the native structure while replacing the native GalNAc with a galactose residue. The monosaccharide residues are color-coded as in Figure 1.

MATERIALS AND METHODS

Materials. Opti-MEM, fetal calf serum (FCS), DMEM, and LipofectAMINE were acquired from Gibco Lifesciences (Grand Island, NY). GDP-β-L-fucose, CMP-β-D-sialic acid, rat recombinant α2,3-sialyltransferase, and human recombinant α1,3-fucosyltransferase V were purchased from CalBiochem (La Jolla, CA). Bovine serum albumin (BSA), calf intestinal alkaline phosphatase, protease inhibitors, inorganic chemicals, and buffers were obtained from Sigma (St. Louis, MO). Organic solvents were purchased from Fischer Scientific (Pittsburgh, PA). Oligonucleotides were custom synthesized, desalted, and lyophilized by Sigma-Genosys (The Woodlands, TX) and used without further purification. All restriction enzymes and T4 DNA ligase were acquired from New England Biolabs (Beverly, MA). PfuTurbo DNA polymerase was obtained from Stratagene (La Jolla, CA). Tissue culture plates were purchased from Falcon (Lincoln, NJ). Centriprep concentrators were obtained from Amicon (Beverly, MA). The BCA Protein Assay Kit was obtained from Pierce (Rockford, IL). Analytical thin-layer chromatography (TLC) plates (HPTLC RP-18 $F_{254}S$) were from EM Science (Gibbstown, NJ). HPLC purifications were carried out on a Rainin Dynamax SD-200 system. The ^{1}H NMR spectra were obtained on a Bruker AMX-500 MHz spectrometer. Chemical shifts are reported in δ values relative to tetramethylsilane, and coupling constants (J) are reported in hertz. Mass spectra were acquired on a Hewlett-Packard Series 1100MSD mass spectrometer. DNA sequencing was performed by the University of California DNA Sequencing Facility. Water used for biochemical procedures was doubly distilled and deionized using a Milli-Q system (Millipore). Protease inhibitor cocktails I and II (PIC-I and PIC-II, respectively) were prepared as 1000-fold concentrated stocks as follows.

PIC-I. To 2.5 mL of an aprotinin solution (5−10 trypsin inhibitory units/mL) were added 3.0 mg of leupeptin, 12.5

mg of PefaBloc SC, 5 mg of antipain, 5 mg of turkey trypsin inhibitor, and 2.5 mg of benzamidine.

PIC-II. Chymostatin (10 mg) and pepstatin (5 mg) were dissolved in 5 mL of DMSO. Procedures for the enzymatic synthesis of [^{35}S]PAPS and the regiochemical analysis of sulfated oligosaccharide products have been previously described (*25*). PVDF membrane was purchased from Applied Biosystems, and quantification of sulfotransferase activity was performed on a Molecular Dynamics Phosphorimager. Oasis reversed-phase 96-well extraction plates were obtained from Waters (Millford, MA).

Plasmid Construction. To facilitate the substrate studies, a soluble and secreted form of each GlcNAc-6-sulfotransferase was generated by subcloning the genes into a modified version of the pSecTag2A vector (Invitrogen). This modified vector, termed pSecHis, was engineered to encode six histidine residues between the N-terminal Igκ secretion signal and the multiple-cloning site. This vector will be described in detail elsewhere (*32*). Each plasmid was constructed by PCR amplification from the full-length gene sequences contained in the pcDNA3.1 vector (obtained from S. D. Rosen, University of California, San Francisco, CA, and S. Hemmerich, Roche Biosciences). PCR primers were designed to amplify an N-terminally truncated gene sequence such that the expression product would lack a transmembrane domain. PCR products were restriction digested, purified by agarose gel electrophoresis, and ligated into pSecHis digested to give compatible ends. The plasmid junctions and inserts were confirmed by sequencing. The primer sequences and restriction sites for each subcloning are given below: GlcNAc6ST/CHST-2, forward *Eco*RI (5′-GCAAGATC-GAATTCGGACTACAAGTGGCACAAGGAGCCG-3′) and reverse *Xho*I (5′-ATGGAGCTTCTCGAGTTAGAGAC-GGGGCTTCCGAAGCAGGG-3′); HEC-GlcNAc6ST/LSST, forward *Bam*HI (5′-GATCGGATCCATGTACAGCCACAA-CATCAGCTCCCTGTCTAT-3′) and reverse *Not*I (5′-GAT-CGCGGCCGCAGCAAAGCCTTCTCAACCCTCTTAGT-GGATT-3′); I-GlcNAc6ST, forward *Eco*RI (5′-CGCGAT-GAATTCAATCTCCCGGCCAGGGCCC-3′) and reverse *Eco*RV (5′-CCGACGCGATATCTCAGTCAGGCGATGC-CC-3′); and C6ST-2/GST5, forward *Bst*XI (5′-CAGCCC-AGTGTGGTGGGACGGCGGCCGCGACGGGGAC-3′) and reverse *Bst*XI (5′-GCTGCCACTGTGCTGGCTACGTG-GCGCCGTCGGCATC-3′).

Cell Culture, Transient Transfection, and Enzyme Isolation. Prior to transfection, COS-7 cells were maintained in DMEM supplemented with 10% FCS at 37 °C in a humidified atmosphere of 5% CO_2 and split 1:8 every 3 days. For generation of recombinant sulfotransferase enzymes, the cells were grown to 60−75% confluence in 150 mm tissue culture plates and transfected with 10 μg of one of the four constructs or the empty pSecHis vector using LipofectAMINE according to the manufacturer's instructions. After transfection, the cells were cultured in Opti-MEM for 4 days and then the conditioned media collected and treated with PIC-I and PIC-II and maintained throughout all subsequent manipulations at 4 °C until they were used in sulfotransferase activity assays. The media were concentrated 100-fold using a Centriprep 30 000 molecular weight cutoff (MWCO), normalized for total protein content using a BCA protein assay, and then stored at 4 °C. Expression products were confirmed by immunoblot using mouse anti-His_6 monoclonal antibody (Clontech). The relative amount of His-tagged enzyme from each expression was determined by densitometry analysis, and enzyme stock solutions were normalized prior to assays with concentrations within 2-fold of one another.

Synthetic Compounds. The synthetic disaccharide **1** and trisaccharide **2** were previously reported (*24*, *25*). Trisaccharide **2** was the starting material for the enzymatic synthesis of compounds **3** and **4**.

*Sialylated Tetrasaccharide **3***. The conditions that were used were derived from those described by Palcic and coworkers (mentioned in ref *25*) and the commercial supplier of the glycosyl transferases (CalBiochem). In a 2 mL microfuge tube, 20 mg of trisaccharide **2** (28 μmol) was incubated with 27.7 mg (42 μmol) of CMP-β-D-sialic acid, 100 milliunits of α2,3-sialyltransferase, and 12 units of calf intestinal alkaline phosphatase in a buffer of 50 mM sodium cacodylate (pH 7.4), 1% BSA, and 30 mM $MnCl_2$ in a final volume of 1.5 mL. The reaction tube was incubated at 37 °C with gentle rocking. After 5 days, an additional 13.5 mg of CMP-β-D-sialic acid and 50 milliunits α2,3-sialyltransferase were added, and incubation was continued for an additional 5 days. At this time, synthesis of the tetrasaccharide **3** was complete by TLC analysis (4:3:2:1 $CHCl_3$/EtOH/MeOH/H_2O mixture, normal phase; 0.25% TFA in a 1:1 CH_3CN:H_2O mixture, reversed phase), and 900 μL of the reaction solution was set aside for later purification.

*Pentasaccharide **4***. To the remainder of the sialylation reaction, above, were added 13.4 mg (21 μmol) of GDP-β-L-fucose and 100 milliunits of α1,3-fucosyltransferase. The reaction mixture was incubated for 5 days at 37 °C, after which an additional 13.4 mg of GDP-β-L-fucose and 100 milliunits of α1,3-fucosyltransferase were added, and the tube incubated an additional 5 days.

Purification of Synthetic Substrates. All compounds were purified from the reaction mixtures without further workup by HPLC on a reversed-phase, semipreparative column using a gradient of water in acetonitrile (from 0 to 35%) with 0.1% TFA to give 12.1 mg (72%) of **3** and 4.0 mg (31%) of **4**.

Spectroscopic Data. Tetrasaccharide **3** (characteristic peaks): ^{1}H NMR (CD_3OD) δ 4.49 (d, 1H, J = 8.0), 3.89 (d, 1H, J = 8.5), 2.83 (dd, 1H, J = 4.0, 12.5), 2.32 (m, 1H), 2.13 (m, 1H), 2.00 (s, 3H), 1.97 (s, 3H), 1.70 (m, 10H), 0.9 (app t, 3H, J = 7.0); MS m/z (ESI$^-$) 1003.3 [(M − H)$^-$].

Pentasaccharide **4** (characteristic peaks): ^{1}H NMR (CD_3OD) δ 5.05 (d, 1H, J = 4.0), 4.51 (d, 1H, J = 8.0), 4.40 (d, 1H, J = 8.5), 2.79 (dd, 2H, J = 3.5, 13.1), 2.00 (s, 3H), 1.97 (s, 3H), 1.49 (m, 4H), 1.48 (m, 10H), 1.32 (m, 3H), 1.16 (d, 3H, J = 6.5), 0.90 (app t, 3H, J = 6.5); ^{13}C NMR δ (anomeric carbons) 104.09, 102.92, 100.49, 99.91; MS m/z (ESI$^-$) 1148.4 [(M − H)$^-$].

Assay for Sulfotransferase Activity. Sulfotransferase assays were performed as previously described (*25*). Briefly, standard assay reactions (25 μL) were carried out in 96-well plates at pH 6.5 in buffer ST [15 mM MOPSO, 1% Triton X-100, 40 mM β-mercaptoethanol, 6 mM $Mg(OAc)_2$, 10 mM NaF, 1 mM ATP, and 13.3% glycerol], 5 μCi of [^{35}S]PAPS, and concentrated conditioned medium containing 10 μg of total protein. Each enzyme was assayed in triplicate for activity on substrates **1**−**4** at concentrations of 0, 0.25, 0.5, and 1.0 mM. Reaction mixtures were incubated for 20 h at 37 °C and then reactions terminated by dilution with 1 mL

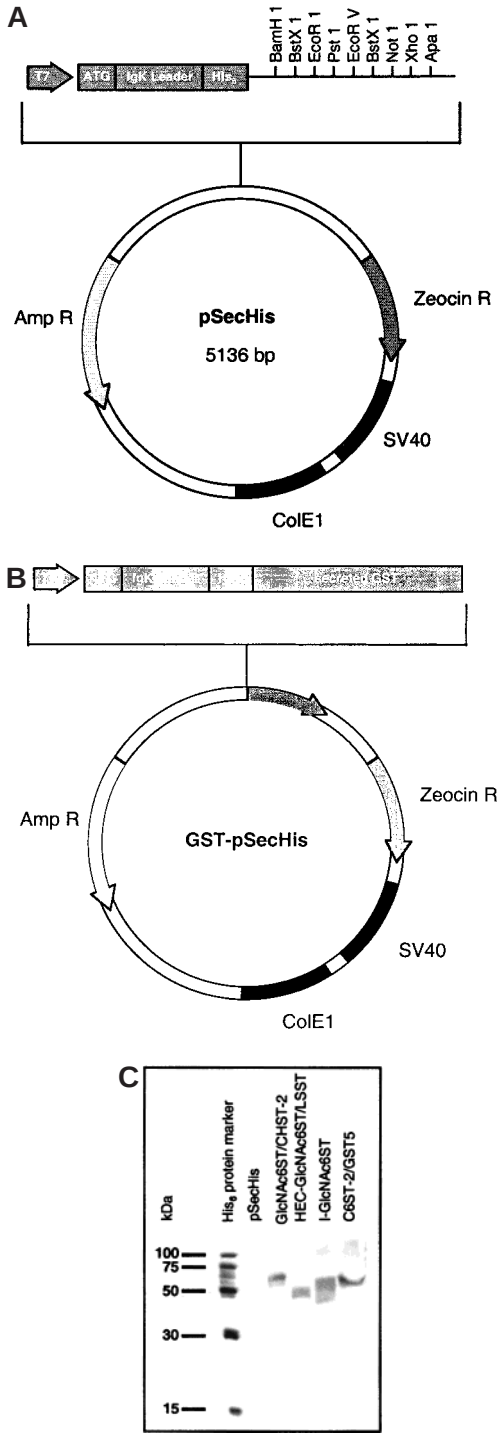


FIGURE 4: Mammalian expression vector pSecHis. The vector features an N-terminal Igκ secretion signal and His_6 sequence (A). An expression construct was generated for each of the enzymes (GlcNAc6ST/CHST-2, HEC-GlcNAc6ST/LSST, I-GlcNAc6ST, and C6ST-2/GST5) (B). COS-7 cells were transfected with each construct or empty vector and cultured in Opti-MEM and the expression products obtained by concentration of the conditioned medium. Western blot analysis of concentrated conditioned medium using anti-His_6 monoclonal antibody is shown for each enzyme (C).

of H_2O followed by loading of the mixtures onto Oasis 96-well reversed-phase extraction plates at a flow rate of 1 mL/min. Each column in the plate was washed with 6 mL of water. The sulfated product was eluted with 300 μL of 70% methanol, and 5 μL of eluant from each reaction was spotted

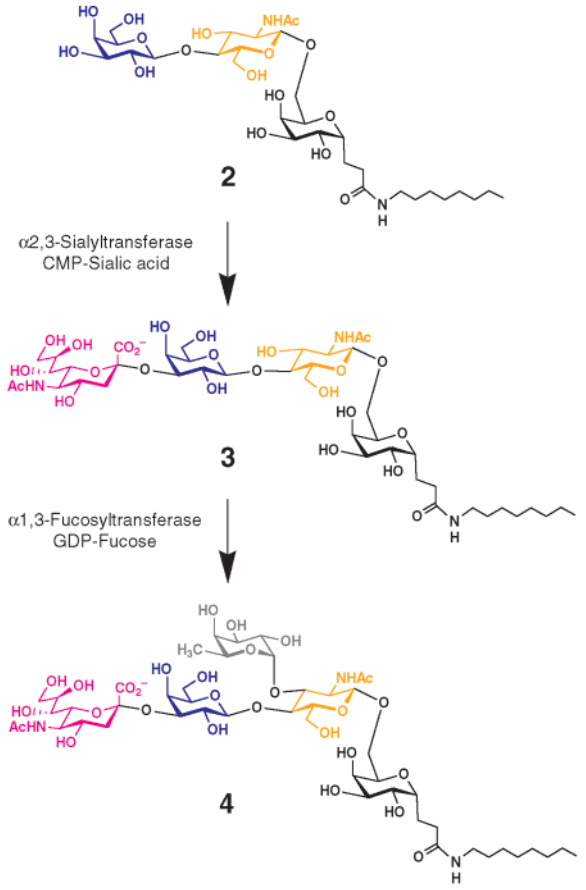


FIGURE 5: Enzymatic synthesis of substrates **3** and **4**. The monosaccharide residues are color-coded as in Figure 1.

onto PVDF membrane and quantified using a Phosphorimager. Data were analyzed using IPLab Gel software.

RESULTS

Construction of Expression Vectors for the GlcNAc-6-sulfotransferases. Primary sequence analysis of the carbohydrate sulfotransferases reveals a type II architecture with a putative catalytic domain residing in the lumen of the Golgi compartment, a single-pass transmembrane domain, and a short cytosolic tail. To facilitate our studies, we generated soluble, secreted constructs for each sulfotransferase in which the transmembrane and cytosolic domains were replaced with an N-terminal His_6 tag. The pSecTag2A expression vector features the Igκ leader secretion signal, and we modified this to incorporate a His_6 sequence between the Igκ signal and the N-terminus of the protein (*32*). This modified vector, termed pSecHis, is shown in Figure 4A. The structure of the expression vector with the subcloned, truncated sulfotransferase gene is shown in Figure 4B.

Sulfotransferase Expression. The enzymes were expressed in the heterologous host COS-7 by individual transfection of each GlcNAc-6-sulfotransferase−pSecHis construct and empty vector pSecHis using LipofectAMINE. The soluble, secreted enzymes were isolated by collecting the conditioned media 4 days post-transfection followed by 100-fold concentration using Centriprep 30 concentrators. We verified expression by immunoblot against the His_6 antigen (Figure 4C). All sulfotransferase stocks were normalized for the relative amount of His_6-tagged protein within 2-fold before being used in a sulfotransferase assay.

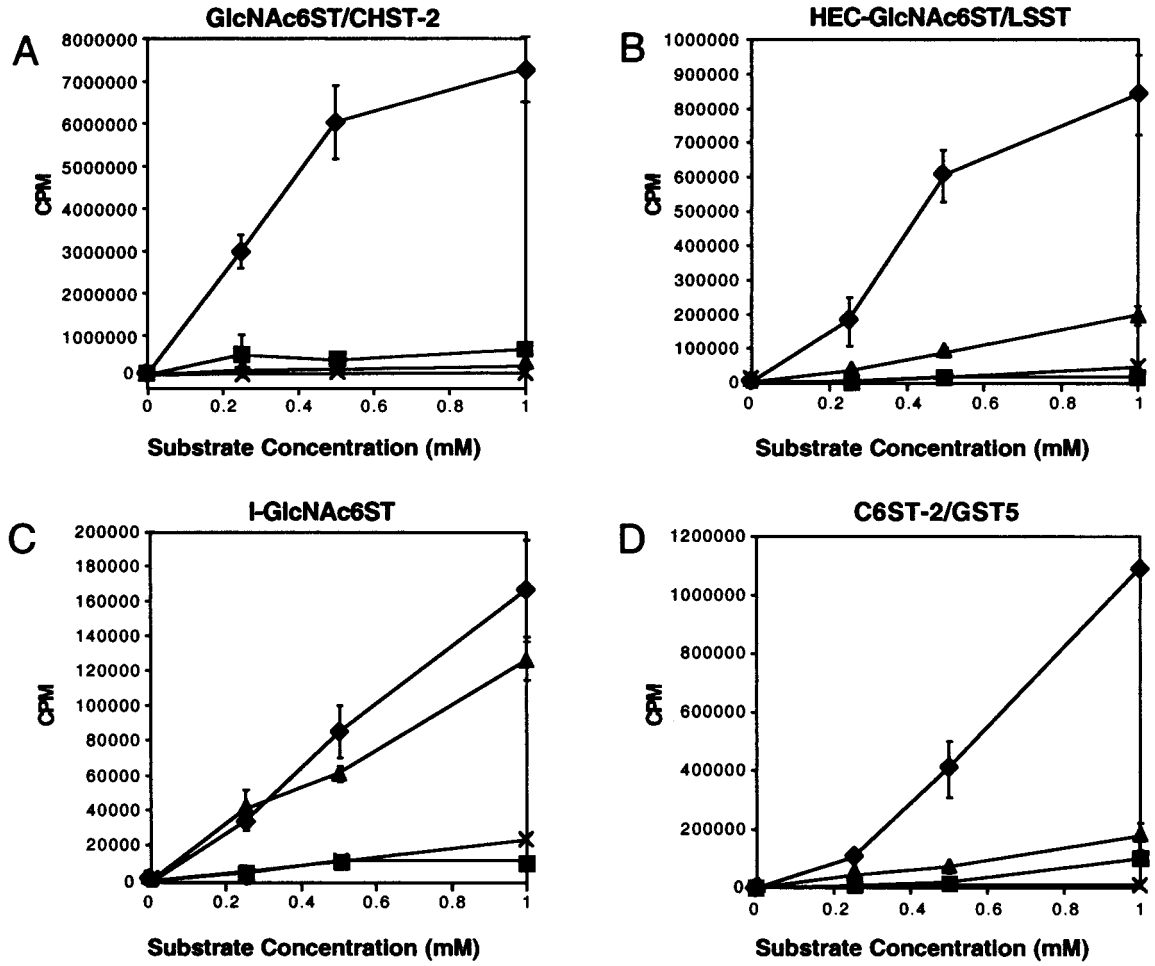


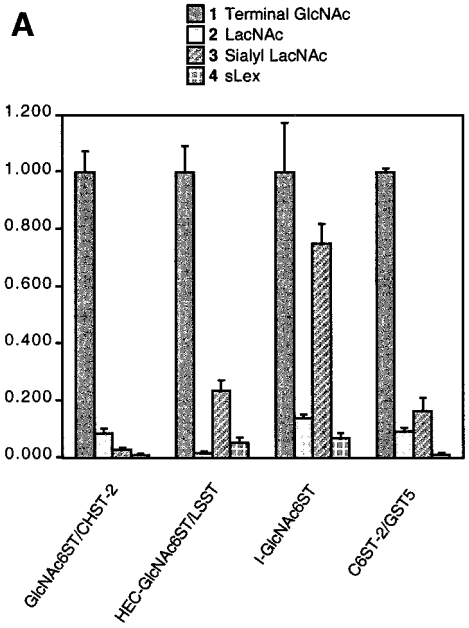
FIGURE 6: Substrate preferences of the four GlcNAc-6-sulfotransferases were determined using synthetic substrates **1** (◆), **2** (■), **3** (▲), and **4** (×). Each enzyme preferred the terminal GlcNAc presented by substrate **1** over the more complex substrates (**2**−**4**): (A) GlcNAc6ST/CHST-2, (B) HEC-GlcNAc6ST/LSST, (C) I-GlcNAc6ST, and (D) C6ST-2/GST5. Error bars represent the standard deviation of the mean for triplicate data points. Where error bars are not apparent, they are smaller than the symbol.

Enzymatic Synthesis of Substrates. The design of the panel of substrates is based upon the modular assembly of the sLex scaffold found in L-selectin ligands. The simplest structure (**1**) serves as the foundation for stepwise enzymatic assembly of substrates **2**−**4**, and possesses the target GlcNAc residue linked to a galactose moiety that mimics the peptide-proximal GalNAc found in the native glycan. The disaccharide substrate **1** was chemically synthesized (*24*, *25*) and the trisaccharide **2** generated in a single enzymatic step from **1** using bovine galactosyltransferase as previously reported (*25*). Tetrasaccharide **3** and pentasaccharide **4** were synthesized enzymatically from **2** using commercially available glycosyltransferases and nucleotide sugar donors (Figure 5).

Sulfotransferase Assays. With the substrates and recombinant enzymes in hand, we carried out [^{35}S]PAPS radiolabel transfer assays (*25*) to assess the substrate preferences of each sulfotransferase. In these assays, the enzymatic transfer of radiolabeled sulfate from [^{35}S]PAPS to the 6-hydroxyl group of GlcNAc within substrate glycolipids is quantified after separation of the products on 96-well reversed-phase extraction plates. For our studies, each enzyme was incubated with each of the substrates (**1**−**4**) at 0−1 mM. The results of these experiments are shown in Figures 6 and 7. For all the GlcNAc-6-sulfotransferases that were probed, the preferred substrate was disaccharide **1**. Activity on trisaccharide **2**, which embodies GlcNAc in the context of LacNAc, was significantly lower for all of the enzymes, between 2 and 14% relative to disaccharide **1**. Curiously, the sialylated tetrasaccharide **3**, a sialyl LacNAc derivative, was a good acceptor for all the sulfotranferases except GlcNAc6ST/CHST-2, and the level of incorporation varied from 3 to 75% relative to **1**. The pentasaccharide **4**, which embodies sLex on a Core 6 scaffold, was a poor acceptor overall. GlcNAc6ST/CHST-2 and CGST-2/GST5 showed no significant activity. However, HEC-GlcNAc6ST/LSST and I-GlcNAc6ST had 5 and 7% activity, respectively, relative to disaccharide **1**.

To confirm that sulfation occurred exclusively on the 6-hydroxyl group of GlcNAc, a regiochemical analysis of each radiolabeled enzyme product was performed as previously described (*25*). Briefly, the sulfotransferase reaction products were subjected to acid hydrolysis by exposure to 100 mM H_2SO_4 at 100 °C for 40 min. High-pH anion exchange chromatography (HPAEC) was first performed on commercially available standards for various sulfated monosaccharides. Elution times of these standards were compared to those of the acid hydrolysates, which were detected by a scintillation detector in parallel with the HPAEC detector. All hydrolysis product elution times coincided with GlcNAc-6-sulfate or LacNAc-6-sulfate, the latter being a product of incomplete hydrolysis.

A



B

Substrate	GlcNAc6ST/CHST-2	HEC-GlcNAc6ST/LSST	I-GlcNAc6ST	C6ST-2/GST5
1 Terminal GlcNAc	1.000	1.000	1.000	1.000
2 LacNAc	0.086	0.018	0.140	0.091
3 SiaLacNAc	0.031	0.234	0.751	0.164
4 sLex	0.010	0.054	0.069	0.011

FIGURE 7: (A) Relative activities of the four enzymes with substrates **1**−**4** (1 mM) normalized for activity relative to disaccharide **1**. (B) Data shown as numerical values. Error bars represent the standard deviation of the mean for triplicate data points.

DISCUSSION

Leukocyte recruitment to secondary lymphoid organs and certain sites of inflammation is initiated by the leukocyte adhesion molecule L-selectin and sulfated carbohydrate ligands on HEV adorned with the unusual sulfated tetrasaccharide epitope 6-sulfo sialyl Lewis x (Figure 1). Given the essential nature of GlcNAc-6-sulfate for L-selectin recognition, the identity and substrate preference of the relevant GlcNAc-6-sulfotransferase(s) represents an important component of understanding lymphocyte homing. Recently, four GlcNAc-6-sulfotransferases were discovered that may participate in L-selectin ligand biosynthesis. Detailed biochemical analysis using chemically defined substrates provides a vehicle for investigating the relative capacity of each enzyme to generate 6-sulfo sLex and related structures, and also to examine scenarios for GlcNAc-6-sulfotransferases in L-selectin ligand biosynthesis. To illuminate potential pathways for the assembly of 6-sulfo sLex by these enzymes and to precisely characterize the substrate profile on sLex-related structures, we synthesized four carbohydrate scaffolds closely related to intermediates in the stepwise construction of sLex. In addition, for each of the reported GlcNAc-6-sulfotransferases, we generated recombinant constructs whose expression products were soluble and secreted.

Compound **1** represents the minimal GlcNAc-containing biosynthetic precursor to sLex. For all of the GlcNAc-6-sulfotransferases, we found that **1** is preferred over those substrates which had been extended by galactosylation, sialylation, and fucosylation, indicating either a preference or requirement for a terminal GlcNAc residue. Compound **2**, which bears the LacNAc motif, was a modest acceptor characterized by only 9, 14, and 9% activity compared to compound **1** by GlcNAc6ST/CHST-2, I-GlcNAcST, and C6ST-2/GST5, respectively (Figure 7). Significant activity was not detectable with HEC-GlcNAc6ST/LSST. Surprisingly, HEC-GlcNAc6ST/LSST and I-GlcNAc6ST exhibited significant activity with sialyl LacNAc substrate **3** relative to compound **1**, 23 and 75%, respectively. C6ST-2/GST5 had modest activity on **3**, 16% compared to compound **1**, and GlcNAc6ST/CHST-2 exhibited poor activity, 3% compared to compound **1**. Compound **4**, which embodies the complete sLex epitope, was a poor acceptor for HEC-GlcNAc6ST/LSST and I-GlcNAc6ST (5 and 7% of sulfate incorporation compared to compound **1**, respectively). GlcNAc6ST/CHST-2 and C6ST-2/GST5 did not have significant activity on **4**. Thus, the GlcNAc-6-sulfotransferases investigated here display restrictive and unique substrate preferences, indicating that the context in which GlcNAc is presented in vivo may determine whether a glycan receives a sulfate ester.

These observations may also may be relevant to the order in which 6-sulfo sLex is biosynthesized, which has been a matter of some controversy. Previous work established that the 6-sulfo sLex epitope on the L-selectin ligands is produced by the successive action of an α2,3-sialyltransferase and an α1,3-fucosyltransferase, most likely FucTVII, on a precursor bearing the Galβ1→4GlcNAc (LacNAc) disaccharide (*35*). Although fucosylation was determined to occur after sialylation and sulfation, the point at which sulfation occurred could not be established. In addition, experiments in which Brefeldin A was used concluded that sulfation is a late modification that occurs in the trans-Golgi network (*36*). However, our studies in conjunction with earlier work indicate that all GlcNAc-6-sulfotransferase activities reported to date prefer a terminal GlcNAc residue (*25*, *26*, *37*). Thus, if these candidate enzymes contribute to L-selectin ligand biosynthesis in accord with their in vitro substrate preference, sulfation of GlcNAc most likely precedes further elaboration by subsequent glycosyltransferases that complete the assembly of 6-sulfo sLex (Figure 8A). Consistent with this proposal is the observation that several human fucosyltransferases recognize GlcNAc-6-sulfated 3′-sialyl LacNAc as a substrate (*35*, *38*, *39*). Moreover, β1,4-galactosyltransferases capable of modifying terminal GlcNAc-6-sulfate have been identified (*37*, *40*), including an activity that prefers GlcNAc-6-sulfate over GlcNAc (*41*). Collectively, these observations suggest that a complement of glycosyltransferases successively installs the galactose, sialic acid, and fucose residues within the trans-Golgi network after GlcNAc sulfation. Additional support for the latter scenario comes from our preliminary observations that three of the GST family members studied here colocalize with known markers of the trans-Golgi network (unpublished). However, the possibility remains that the relevant GlcNAc-6-sulfotransferase resides in an earlier secretory compartment and operates prior to the requisite glycosyltransferases in subsequent Golgi compartments.

Alternatively, the moderate activity on the sialyl LacNAc substrate **3** by three of the GlcNAc-6-sulfotransferases provides for a scenario in which sulfation could occur at a later stage in the biosynthesis (Figure 8B). Sulfation might be a penultimate step, occurring after sialylation and im-

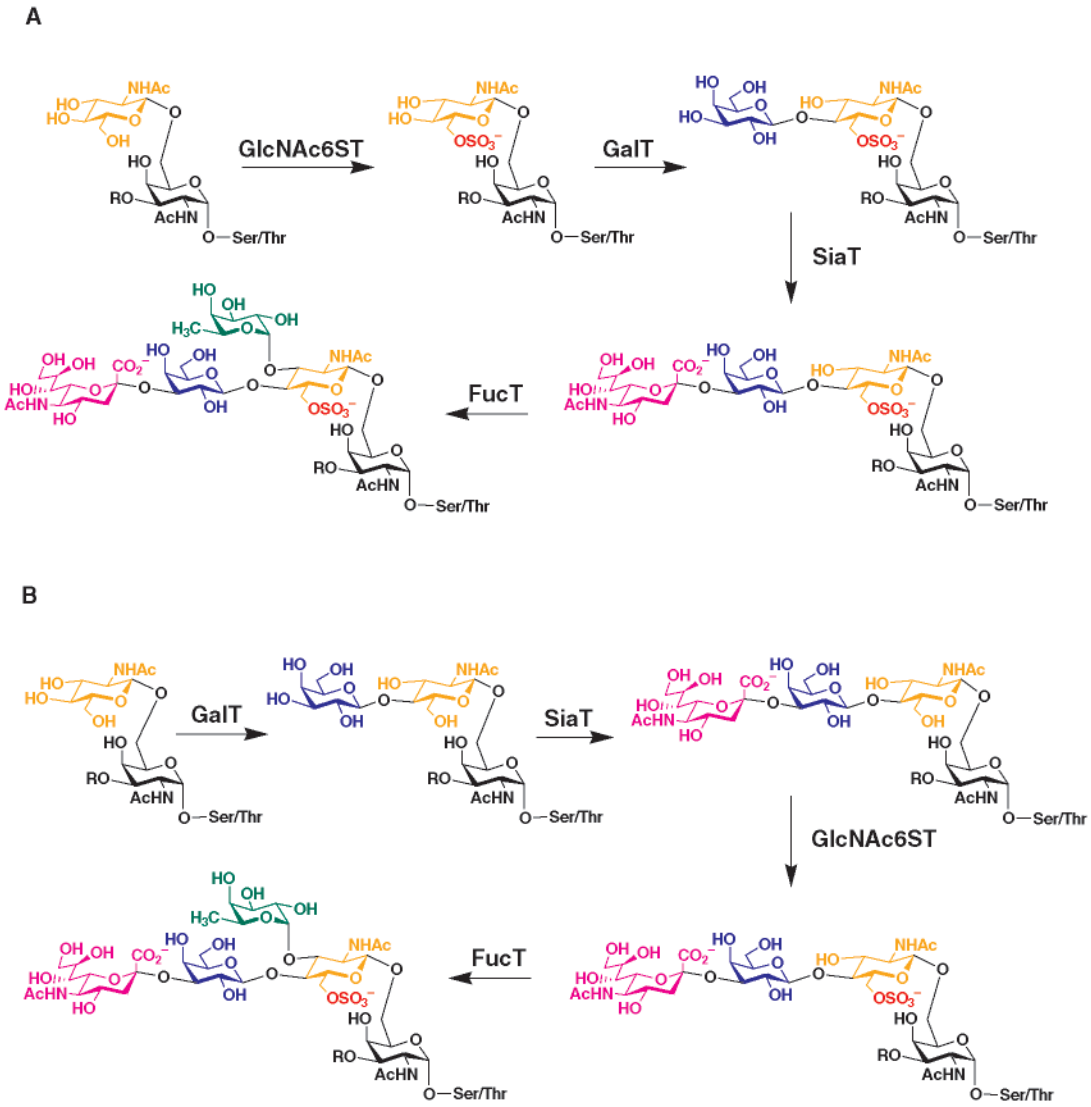


FIGURE 8: Two possible biosynthetic pathways for the assembly of L-selectin ligand glycans. (A) Sulfation may occur on the terminal GlcNAc residue prior to further elaboration of the glycan by glycosyltransferases. (B) The sulfate ester may be installed after galactosylation and sialylation of the GlcNAc residue, but preceding fucosylation. The monosaccharide residues are color-coded as in Figure 1.

mediately preceding a final fucosylation event. Notably, the enzymes with the greatest tolerance for sialylated structures, HEC-GlcNAc6ST/LSST and I-GlcNAc6ST, also have the most restricted expression pattern that includes HEV (Table 1) supporting these two enzymes as plausible candidates for L-selectin ligand construction. Given the low activity of all of the enzymes with sLex substrate **4**, it is unlikely that sulfation is the final modification in the pathway. Consistent with this hypothesis is the presence of unfucosylated, sulfated carbohydrate structures in HEV of mice deficient in FucTVII (*35*).

GlcNAc6ST/CHST-2 has a strikingly restrictive substrate profile. Earlier work reported by Muramatsu and co-workers directly implicated GlcNAc6ST/CHST-2 in the biosynthesis of 6-sulfo sLex, 6-sulfo Lex, and poly 6-sulfo LacNAc (keratan sulfate) across many tissues in a stage-dependent manner during murine embryonic development (*42*). In developmental contexts, these epitopes do not appear to serve as L-selectin ligands, as L-selectin itself was not detected in the same tissues. Thus, the specific biological roles of these GlcNAc-6-sulfated structures remain obscure. Nevertheless, GlcNAc6ST/CHST-2 appears to directly contribute to developmentally regulated structures containing 6-sulfo LacNAc. Coupled with the rigid substrate requirement of a terminal GlcNAc residue revealed by our studies, these observations indicate that participation of GlcNAc6ST/CHST-2 in the biosynthesis of these epitopes must take place at an early step, and perhaps represents a committed step in the biosynthesis of glycans containing GlcNAc-6-sulfate.

Biological information about I-GlcNAc6ST is limited, and its potential contribution to specific GlcNAc-6-sulfate-containing epitopes has not been previously reported. However, the expression of I-GlcNAc6ST is highly restricted to colon and intestinal mucosa. Significantly, the transcript is also present in mesenteric lymph nodes where it could contribute to lymphocyte homing. Our data show that I-GlcNAc6ST accepts GlcNAc either in a terminal position or within 3′-sialyl LacNAc. Thus, this enzyme could contribute to the biogenesis of 6-sulfo sLex or related structures as an early or intermediate step in the glycan assembly (Figure 8A,B) but most likely precedes fucosylation.

C6ST-2/GST5 was initially described as a chondroitin sulfotransferase that sulfated the 6-hydroxyl group of Gal-

NAc (*31*). However, a later report identified this enzyme as a GlcNAc-6-sulfotransferase (*32*). In comparative assays in which *β*-*O*-benzyl GlcNAc, *β*-*O*-benzyl GalNAc, and α-*O*-benzyl GalNAc substrates were used, activity was observed only with the GlcNAc acceptor (*32*). Both groups reported high expression levels of C6ST-2/GST5 in adult spleen and moderate levels in lung, pancreas, ovary, peripheral blood leukocytes, and small intestine. In contrast, the distribution in fetal tissues included abundant expression in heart, lung, skeletal muscle, and spleen. In addition, Hemmerich and coworkers (*32*) demonstrated that this enzyme could generate GlcNAc-6-sulfate and 6-sulfo LacNAc on mucins when expressed by a heterologous host. Our studies reflect a robust GlcNAc-6-sulfotransferase activity on terminal GlcNAc and moderate activity within 3′-sialyl LacNAc, suggesting a possible role in 6-sulfo sLex and related structures. However, the paucity of biological experiments and ambiguity of the in vitro activity of C6ST-2/GST5 make further speculation untenable.

The expression of HEC-GlcNAc6ST/LSST is markedly restricted to the HEV of peripheral lymph node. In addition, its transcript was observed in the HEV of the AKR/J mouse, a well-characterized animal model of thyroid neoplasia in which HEV-like structures which present L-selectin ligands are induced (*29*). HEC-GlcNAc6ST/LSST expression colocalized with L-selectin ligands and FucTVII (*29*), an established contributor to L-selectin ligands at sites of leukocyte trafficking (*35*). When expressed in a heterologous host, HEC-GlcNAc6ST/LSST is capable of generating the 6-sulfo sLex epitope recognized by L-selectin (*28*, *29*, *34*). Altogether, these observations point to HEC-GlcNAc6ST/LSST as the most compelling candidate for contribution to L-selectin ligand expression in the peripheral lymph node HEV. A probable model for 6-sulfo sLex biosynthesis by HEC-GlcNAc6ST/LSST involves sulfation as an early modification in glycan assembly (Figure 8A), although the alternative depicted in Figure 8B cannot be ruled out. It is possible that GlcNAc 6-sulfation is an early committed step in the biosynthesis of 6-sulfo sLex and therefore a key regulatory event in the cascade that leads to leukocyte recruitment.

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