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# A Novel, High Endothelial Venule–Specific Sulfotransferase Expresses 6-Sulfo Sialyl Lewis$^x$, an L-Selectin Ligand Displayed by CD34

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## Summary

**L-selectin mediates lymphocyte homing by facilitating lymphocyte adhesion to unique carbohydrate ligands, sulfated sialyl Lewis$^x$, which are expressed on high endothelial venules (HEV) in secondary lymphoid organs. The nature of the sulfotransferase(s) that contribute to sulfation of such L-selectin counterreceptors has been uncertain. We herein describe a novel L-selectin ligand sulfotransferase, termed LSST, that directs the synthesis of the 6-sulfo sialyl Lewis$^x$ on L-selectin counterreceptors CD34, GlyCAM-1, and MAdCAM-1. LSST is predominantly expressed in HEV and exhibits striking catalytic preference for core 2–branched mucin-type *O*-glycans as found in natural L-selectin counterreceptors. LSST enhances L-selectin–mediated adhesion under shear compared to non-sulfated controls. LSST therefore corresponds to an HEV-specific sulfotransferase that contributes to the biosynthesis of L-selectin ligands required for lymphocyte homing.**

## Introduction

Detection of foreign antigens by the immune system, and subsequent processes that neutralize these molecules, are critically dependent upon lymphocyte recirculation through lymph nodes and Peyer's patches. Lymphocyte recirculation processes are directed by secondary lymphoid organ-specific expression of molecules displayed by specialized postcapillary venules, called high endothelial venules (HEV) (Butcher and Picker, 1996). The luminal surface of the specialized endothelium characteristic of postcapillary venules in peripheral and mesenteric lymph nodes displays counterreceptors for the leukocyte adhesion molecule L-selectin. These counterreceptors capture circulating lymphocytes via L-selectin–dependent adhesive interactions that lead, in turn, to lymphocyte tethering and rolling, chemokine-dependent activation, integrin-mediated firm arrest, and transmigration. Antibody blockade experiments and analysis of L-selectin–deficient mice (Arbones et al., 1994) demonstrate an absolute requirement for L-selectin and its HEV-borne ligands in the recruitment of lymphocytes to lymph nodes (reviewed in Springer, 1994; Butcher and Picker, 1996).

L-selectin and its ligands are also implicated in lymphocyte recruitment in certain chronic inflammatory states. In this context, inducible L-selectin ligand activity is observed on microvascular venular endothelium in rheumatoid arthritis, lymphocytic thyroiditis, and inflammatory bowel disease (Michie et al., 1993; Salmi et al., 1994), and in association with the insulitis characteristic of the nonobese diabetic mouse and the rejection of heart transplants of rodents (Hänninen et al., 1993; Tarunen et al., 1995). Inducible L-selectin ligand activity and an associated HEV-like microvasculature are also observed in the thymic hyperplasia characteristic of the AKR mouse and may contribute to the pathogenesis of this preneoplastic disorder (Michie et al., 1995).

L-selectin contains an $NH_2$-terminal carbohydrate-binding domain characterized by its dependence on $Ca^{2+}$ for activity. HEV-borne L-selectin counterreceptors include the glycoproteins GlyCAM-1 (Lasky et al., 1992), CD34 (Baumhueter et al., 1993), MAdCAM-1 (Berg et al., 1993), podocalyxin-like protein (Sassetti et al., 1998), and $Sgp^{200}$ (Hemmerich et al., 1994a). The functionality of these L-selectin counterreceptors is determined by their decoration with specific sialylated sulfated oligosaccharides (Imai et al., 1993). In particular, sulfated isomers of the sialyl Lewis$^x$ (sLe$^x$) tetrasaccharides (NeuNAcα2→3Galβ1→4(Fucα1→3)GlcNAc), as found on mucin-type core 2–branched *O*-glycans displayed by GlyCAM-1 and other glycoprotein counterreceptors for L-selectin (Figure 1), have been implicated as terminal glycans essential for high-affinity binding by L-selectin (Hemmerich et al., 1994b; Tsuboi et al., 1996). While indirect evidence supports a functional role for the 6-sulfo sLe$^x$ isomer (Mitsuoka et al., 1998), direct evidence for this hypothesis has not been available, and the chemical nature of the physiologically relevant sulfated structures necessary for L-selectin ligand activity has remained unclear.

The formation of these core 2 branches is dependent on core 2 β1,6-*N*-acetylglucosaminyltransferase (C2GnT) (Bierhuizen and Fukuda, 1992). It is assumed that those core 2–branched oligosaccharides are modified further by a sulfotransferase, α2,3-sialyltransferase, and α1,3-fucosyltransferase VII (Fuc-TVII) to form the sulfated

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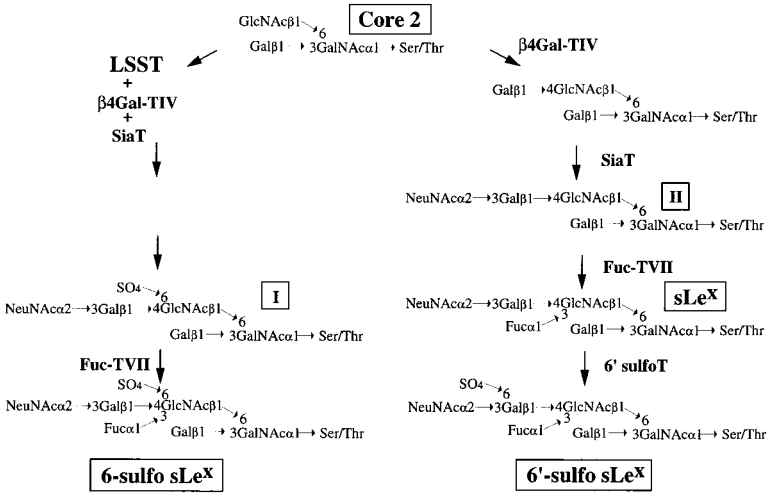


Figure 1. Structure and Biosynthesis of L-Selectin Ligand Oligosaccharides

Core 2 *O*-glycans are synthesized by C2GnT. Core 2 *O*-glycans may be sulfated by 6-sulfo T (or LSST), galactosylated, and sialylated to form 6-sulfo sialyl *N*-acetyllactosamines in core 2 *O*-glycans (I). Compound I is then fucosylated by Fuc-TVII, forming 6-sulfo sLe$^x$. The order of sulfation, galactosylation, and sialylation is not known when LSST is involved in the synthesis. β1,4-galactosyltransferase-IV (β4GalT-IV) is involved in core 2 galactosylation (Ujita et al., 1998). In a different pathway, core 2 *O*-glycans are first galactosylated and sialylated to form sialyl *N*-acetyllactosamine in core 2 *O*-glycans (compound II). This compound is fucosylated by Fuc-TVII, forming sLe$^x$. sLe$^x$ is then sulfated by 6′-sulfotransferase to form 6′-sulfo sLe$^x$ (Maly et al., 1996). SiaT, α2,3-sialyltransferase.

sLe$^x$ (Figure 1) (Hemmerich et al., 1995). Analysis of mice deficient in Fuc-TVII demonstrates an essential role for this enzyme in L-selectin ligand synthesis (Maly et al., 1996). The acceptor substrate specificity of Fuc-TVII implies that 6-sulfo sLe$^x$ can be formed by initial addition of sulfate on core 2–branched oligosaccharides followed by sequential addition of galactose, sialic acid, and fucose (Figure 1) (Maly et al., 1996; Bowman et al., 1998). By contrast, 6′-sulfo sialyl *N*-acetyllactosamine, NeuNAcα2→3(6-sulfo)Galβ1→4GlcNAc, is not a favorable acceptor for Fuc-TVII, and sulfation of the terminal galactose residue in this structure is inferred to occur after α1,3-fucosylation by Fuc-TVII (Maly et al., 1996). Considered together, these observations imply that one or more HEV-specific sulfotransferases play critical roles in the formation of L-selectin ligands, and, in turn, control lymphocyte homing and lymphocyte recruitment in the context of inflammation-induced expression of vascular ligands for L-selectin. Candidate sulfotransferases relevant to L-selectin ligand synthesis include a recently cloned GlcNAc-6-*O*-sulfotransferase, GlcNAc6ST (Uchimura et al., 1998). While this enzyme adds sulfate to the C-6 position of *N*-acetylglucosamine, which may be further modified to form 6-sulfo sLe$^x$, a physiological role for this enzyme in L-selectin ligand synthesis has not been demonstrated.

We herein report the molecular cloning and characterization of a murine cDNA encoding a sulfotransferase that directs formation of 6-sulfo sLe$^x$ on mucin-type *O*-glycans. This enzyme, termed L-selectin ligand sulfotransferase (LSST), displays an HEV-specific expression pattern, is inducible in the context of inducible venular L-selectin ligand activity, and exhibits a remarkable preference for core 2 glycan-type precursors displayed by the mucin-type L-selectin counterreceptors. Core 2-type 6-sulfo sLe$^x$ structures formed by LSST mediate L-selectin–dependent cell adhesion under shear exceeding adhesion mediated by counterreceptors bearing only nonsulfated core 2-type sLe$^x$ ligands. These observations strongly suggest that LSST plays a critical role in lymphocyte homing and in lymphocyte trafficking during chronic inflammation.

## Results

### Isolation of Novel cDNA Encoding L-Selectin Ligand Sulfotransferase

Previous work demonstrated that chondroitin sulfate 6-sulfotransferase (C6ST) (Fukuta et al., 1995) and keratan sulfate Gal-6-sulfotransferase (KSST) (Fukuta et al., 1997) are involved in the biosynthesis of proteoglycans and can add sulfate with low efficiency to sialyl *N*-acetyllactosamine to form 6′-sulfo sialyl *N*-acetyllactosamine, NeuNAcα2→3(6-sulfo)Galβ1→4GlcNAc, but not to sLe$^x$ or 6-sulfo sLe$^x$ (Habuchi et al., 1997; Torii et al., 1998). These observations and the presence of weak but discernible similarity in catalytic domains of different sulfotransferases (Kakuta et al., 1998; Ong et al., 1998) suggested the possibility that other sulfotransferases may be identified by their similarity to C6ST and KSST, including those capable of participating in the formation of sulfated forms of sLe$^x$. Indeed, a search of the EST database for cDNAs related to C6ST and KSST identified one cDNA (EST AA522184), whose transcript was detected in HEV but not in many other tissues. The full-length cDNA encodes an open reading frame of 1167 base pairs and predicts a protein of 388 amino acid residues (44,635 Da) (Figure 2). This protein, termed LSST, is predicted to exhibit a type II membrane topology representative of virtually all known mammalian glycosyltransferases (Schachter, 1994). The amino acid sequence of LSST shares 35.6%, 35.4%, and 32.3% amino acid sequence identity with mouse GlcNAc6ST, human KSST, and chicken C6ST, respectively.

### The Expression of LSST Is Restricted to High Endothelial Venules

Northern blot analysis demonstrates that LSST transcripts are expressed at extremely low levels in most adult tissues, in contrast to the rather wider expression pattern observed for GlcNAc6ST (Figures 3A and 3B; Uchimura et al., 1998). Evidence for HEV-specific expression of LSST was obtained (Figure 3C) using an RT-PCR analysis of a cell preparation enriched for HEV cells by a MECA-79–based immunoaffinity enrichment

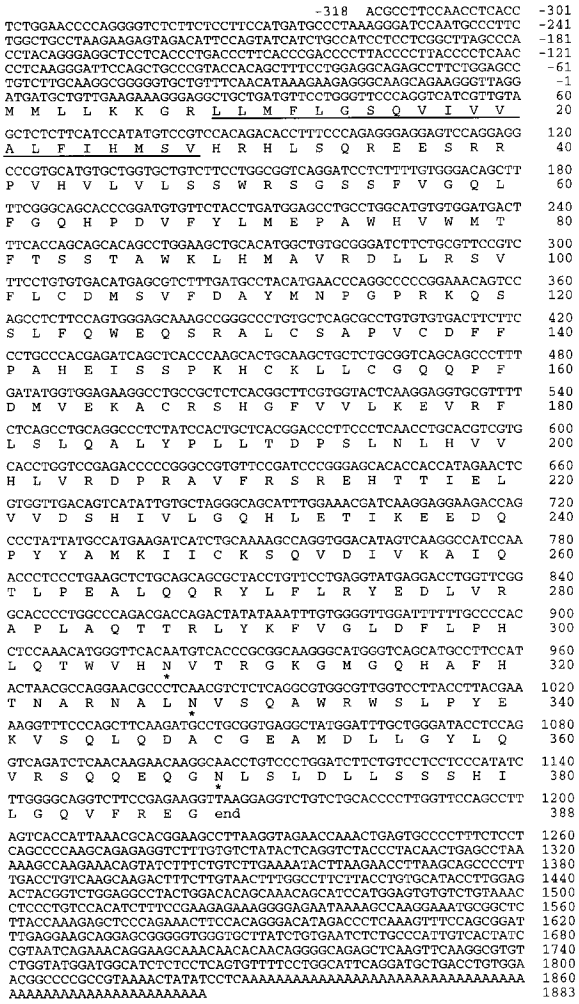


Figure 2. Nucleotide and Translated Amino Acid Sequences of LSST

The signal/membrane-anchoring domain is underlined. Potential *N*-glycosylation sites are marked with asterisks. The initiation codon is numbered as nucleotides 1–3 (GenBank accession number AF109155). Although there are two methionines in a potential translation initiation site, the codon for the first methionine conforms better with consensus sequence for translation initiation (Kozak, 1991). The original EST clone (AA522184) encodes nucleotides 931–1883.

procedure (see Experimental Procedures). By contrast, the same procedure yielded a very faint signal for GlcNAc6ST (Figure 3C). In situ hybridization procedures disclose expression of LSST transcripts in HEV (Figure 3F), as confirmed by colocalized expression with the MECA-79 epitope (Figure 3D), a sulfated antigen characteristic of HEV (Streeter et al., 1988; Hemmerich et al., 1994a). HEV-specific expression of the GlcNAc6ST locus was also observed (Figure 3H), though the relatively stronger hybridization signal of the LSST probe in comparison to the GlcNAc6ST-specific signal implies that expression of the LSST sulfotransferase locus predominates in HEV.

### Coordinate Expression of LSST, Fuc-TVII, and L-Selectin Ligands by High Endothelial Venules Induced in Thymic Hyperplasia

In contrast to the constitutive expression of L-selectin ligand-positive HEV in secondary lymphoid organs, conversion of flat-walled vascular endothelium to an HEV-like morphology is observed in association with some inflammatory and preneoplastic conditions (Butcher and Picker, 1996). A well-characterized murine example is found in the AKR/J mouse; this strain suffers from a retrovirus-associated thymic hyperplasia characterized by increased numbers of small medullary blood vessels with HEV morphology and L-selectin ligand expression (Michie et al., 1995). In situ hybridization analyses demonstrate that the LSST locus is transcribed by the endothelial cells that comprise the HEV of the hyperplastic AKR/J thymus detected by an L-selectin-IgM chimera and MECA-79 antibody (Figure 4). These transcripts colocalize in adjacent sections with transcripts for Fuc-TVII also implicated in L-selectin ligand expression (Maly et al., 1996). Colocalized HEV-specific expression of the GlcNAc6ST locus is also observed at apparently lower levels than transcripts for the former two enzymes (Figure 4). These observations imply that LSST contributes to inducible L-selectin ligand expression that accompanies vascular transformation to the HEV morphology.

### Sulfation of Mucin-Type *O*-Linked Oligosaccharides in CD34 by LSST

The substrate specificity of LSST was examined using a series of in vitro and in vivo sulfation assays. In vitro, LSST did not transfer $^{35}$S-sulfate from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to keratan sulfate or chondroitin sulfate or glycan acceptor substrates such as Galβ1→4GlcNAcβ1→6Manα1→6Manβ1→octyl, GlcNAcβ1→3Galβ1→4GlcNAcβ1→6Manα1→6Manβ1→octyl, and GlcNAcβ1→6(Galβ1→3)GalNAcα→*p*-nitrophenol. These observations implied a possible requirement for specific contributions to sulfotransferase substrate activity by protein or additional glycan components.

A possible requirement for mucin-type *O*-glycan-modified glycoproteins was explored by coexpressing LSST with candidate glycoproteins in cultured cell lines. Initial experiments determined if LSST can catalyze the addition of sulfate moieties to mucin-type L-selectin counterreceptors. COS-1 cells were used in these experiments since this transfection host constitutively expresses C2GnT (Bierhuizen and Fukuda, 1992; Bierhuizen et al., 1994). Cells were transfected with an LSST expression vector together with cDNAs encoding a CD34·IgG chimera, a GlyCAM-1·IgG chimera, or a MAdCAM-1·IgG chimera. Analysis of the chimeric proteins rescued from the labeled transfectants disclosed a robust, LSST-dependent increment in $^{35}$S-sulfate incorporation into GlyCAM-1·IgG, CD34·IgG, and MAdCAM-1·IgG (Figure 5A). *N*-glycanase digestion of the radiolabeled CD34·IgG chimera yielded a decrement in apparent molecular mass, which is consistent with its modification by *N*-glycans (Figure 5B). Nonetheless, the LSST-dependent sulfation of this chimera was essentially resistant to *N*-glycanase digestion, implying that

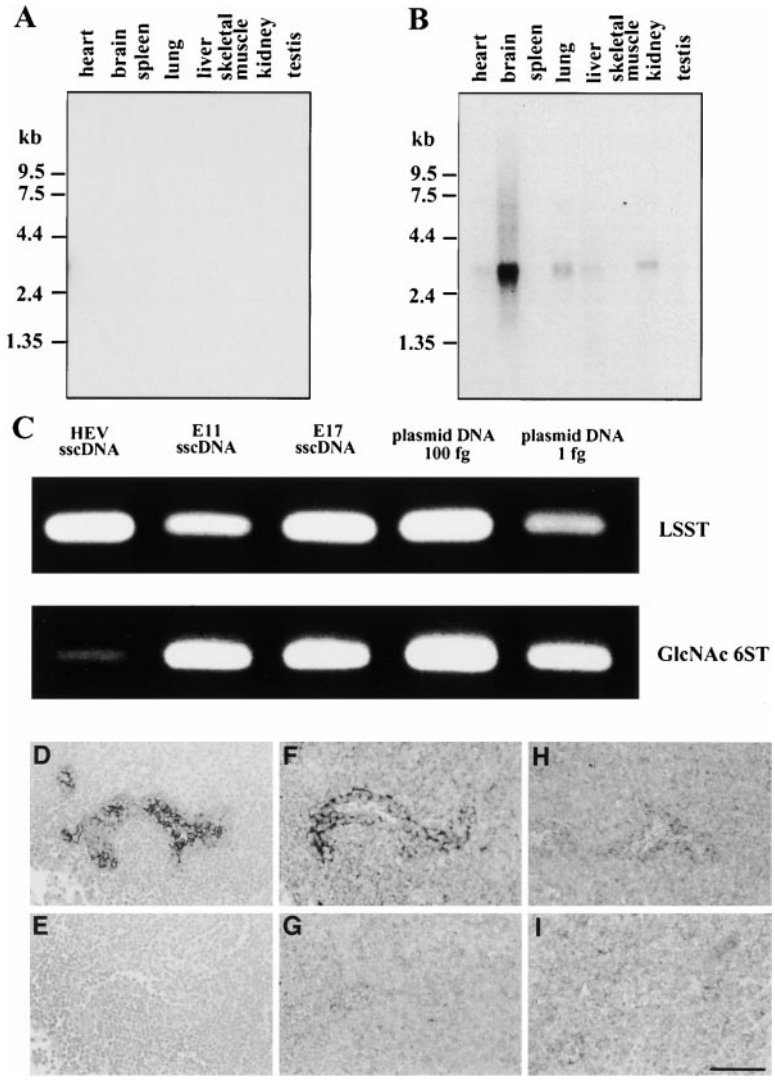


Figure 3. Expression of the Transcripts for LSST and GlcNAc6ST

(A and B) Northern blot analysis of LSST (A) and GlcNAc6ST (B) transcripts using the same mouse multiple tissue blots. (C) Polymerase chain reaction was carried out using templates of the single strand cDNAs constructed from MECA-79–positive cells (HEV), mouse embryo pc day 11 and 17 (E11 and E17), 100 fg and 1 fg of plasmid DNA harboring the full-length LSST and mouse GlcNAc6ST, respectively. The products were separated by 2% agarose gel electrophoresis. (D–I) Expression of LSST and GlcNAc6ST transcripts and MECA-79 in HEV. In situ hybridization of a mouse peripheral lymph node section with anti-sense probes for LSST (F) or GlcNAc6ST (H) and sense probes for LSST (G) or Glc NAc6ST (I). HEV were stained with MECA-79 antibody (D) or negative control antibody (E).

LSST preferentially catalyzes $^{35}$S-sulfate incorporation into *O*-glycans (Figure 5B).

To further examine the acceptor specificity of LSST, sulfate incorporation studies were completed using the GlyCAM-1·IgG (modified almost exclusively by *O*-glycans; Lasky et al., 1992) or a chimera derived from NCAM (modified exclusively by *N*-glycans; Angata et al., 1998). Robust LSST-dependent sulfation of the GlyCAM-1·IgG chimera was again observed, and the sulfate label was resistant to *N*-glycanase digestion (Figure 5B). Furthermore, virtually no LSST-dependent sulfation was observed on the NCAM·IgG chimera (Figure 5B). Considered together, these observations indicate that LSST directs sulfation of *O*-glycans on CD34 and GlyCAM-1.

To determine if core 2 branching on CD34-associated *O*-glycans is essential for sulfation by LSST, the CD34·IgG chimera was expressed in two cell lines. These included CHO cells (deficient in expression of C2GnT and the corresponding core 2–branched *O*-glycans; Bierhuizen and Fukuda, 1992) and Lec1 mutant CHO cells, which are deficient in expression of complex type *N*-glycans (defective *N*-acetylglucosaminyltransferase I; Stanley et al., 1975) as well as C2GnT-deficient.

In the absence of C2GnT, LSST directs a small increment in $^{35}$S-sulfate incorporation into the CD34·IgG chimera, relative to mock-transfected cells (Figure 5C; compare lanes 1 and 2). Most of this $^{35}$S-sulfate incorporation, whether endogenous or LSST dependent, was apparently associated with *N*-glycans, since C2GnT-independent sulfate incorporation was not observed when the Lec1 mutant CHO cells were used as a transfection host (Figure 5C; compare lanes 5 and 6). By contrast, coexpression of LSST with C2GnT yielded a striking increment in CD34·IgG-associated $^{35}$S-sulfate incorporation (Figure 5C, compare lanes 2 and 4 and lanes 6 and 8).

Small amounts of $^{35}$S-sulfate associated with the CD34·IgG chimera are observed in the absence of LSST reconstitution in CHO cells (Figure 5C; lane 1) and in COS-1 cells (Figure 5B; lanes 1, 2, 9 and 10), implying that CHO and COS-1 cells maintain a low level of expression of an endogenous sulfotransferase that modifies the chimera. Considered together, these observations indicate that substrate recognition by LSST is characterized by a strong preference for core 2-type *O*-glycans.

In the fourth set of experiments, GlcNAc6ST, KSST,

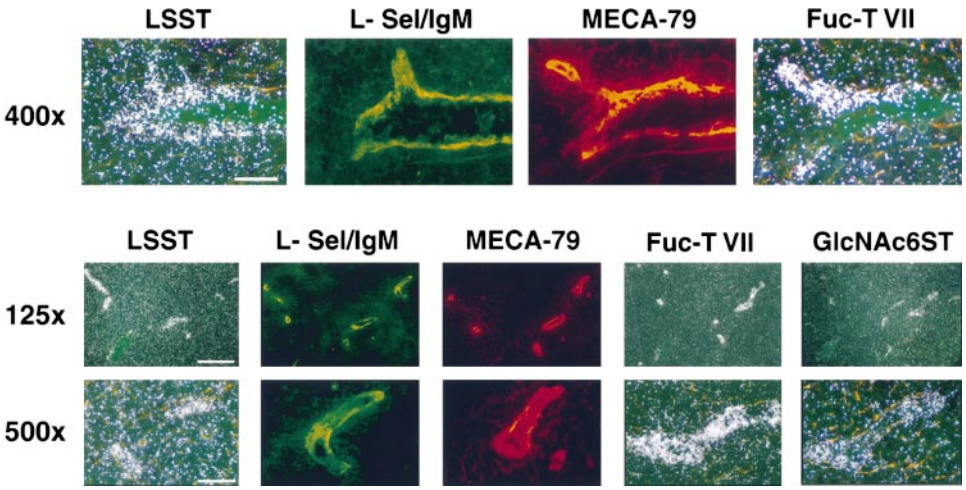


Figure 4. Colocalized Expression of LSST, Fuc-TVII, and L-Selectin Ligands in the HEV-like Vessels of the Hyperplastic AKR/J Thymus

Serial 10 μm thick frozen sections were prepared from the hyperplastic thymus from a 6-month-old AKR/J mouse. Some adjacent sections were subjected to in situ hybridization procedures that detect transcripts corresponding to mouse LSST, Fuc-TVII, and GlcNAc6ST (G6ST), and were photographed using darkfield microscopy (Experimental Procedures). Other adjacent sections were subjected to immunofluorescence staining procedures using the MECA-79 monoclonal antibody or an L-selectin-IgM chimera. Final magnifications of each row of panels are indicated at left. The length of the white bars in the left-most panel of each row corresponds to a length of 50 μm (in the rows displayed at 400× and 500× magnifications) or 200 μm (in the row displayed at 125× magnification).

or C6ST was expressed in CHO cells with or without C2GnT. The results show that GlcNAc6ST and KSST add a sulfate group to CD34·IgG regardless of whether C2GnT was present or not (Figure 5D, lanes 3, 5, 8, and 10) while C6ST transferred a minimal amount of sulfate to CD34·IgG (Figure 5D, lanes 4 and 9). These results indicate that these three sulfotransferases have no clear dependence on core 2-type *O*-glycans for their activity, in contrast to LSST.

### LSST Transfers a Sulfate Group to the 6 Position of *N*-Acetylglucosamine Residue in sLe$^x$ Oligosaccharide

Structural analyses of CD34·IgG-associated sulfated glycans were completed to define the chemical nature of the sulfate modification directed by LSST or by GlcNAc6ST.

The majority of the $^{35}$S-labeled, CD34·IgG-derived glycopeptides generated in LSST-, C2GnT-, and Fuc-TVII-expressing cells eluted near the void volume after Sephadex G-50 gel filtration (Figure 6A, I). These large molecular weight glycopeptides were inferred to correspond to peptide sequences containing multiply-clustered *O*-glycans since pronase cannot cleave peptides containing clustered *O*-glycans (Carlsson et al., 1986). In fact, a nearly identical elution profile was obtained after *N*-glycanase treatment, with the small amount of released *N*-glycans (18% of the total radioactivity) eluting between fractions 39 and 52 (data not shown). Moreover, alkaline borohydride treatment of these high molecular weight glycopeptides released *O*-glycans (Figure 6C).

The two LSST-sulfated *O*-glycans (Figure 6C, IA and IB) were individually applied to a column of QAE-Sephadex. IA contained more acidic compounds than IB (Figure 6D). Those containing two and three anionic charges derived from IA and IB were combined and treated with α2,3-specific neuraminidase. Since the products still contained $^{35}$S-sulfate and the single remaining anionic charge (Figure 6E, III), these results indicate that the two peaks in panel D correspond to molecules modified by one and two α2,3-linked sialic acid residues, respectively.

Bio-Gel P-4 gel filtration analysis indicates that the sulfated oligosaccharide obtained after desialylation of III elutes at a position (solid line; Figure 6F), somewhat earlier than a pentasaccharide standard Galβ1→4(Fucα1→3)GlcNAcβ1→6(Galβ1→3)GalNAcOH (Figure 6F). Digestion of these sulfated glycans with α1,3/4-fucosidase and β-galactosidase yields a peak (IV) that retains the sulfate label and elutes slightly earlier than GlcNAcβ1→6(Galβ1→3)GalNAcOH (Figure 6F). Further digestion of IV with β-hexosaminidase A released an $^{35}$S-labeled fraction (V) that eluted at the same elution position as 6-sulfo GlcNAc and Galβ1→3GalNAcOH (core 1 structure) (Figure 6G; see arrow 1). Repeated digestion of the remaining radioactivity in Figure 6G produced 6-sulfo GlcNAc and the core 1 structure. Finally, HPLC analysis demonstrated that the sulfated *N*-acetylglucosamine is 6-sulfo GlcNAc (Figure 6H).

To determine the core structure, a compound corresponding to peak III was prepared in the absence of Fuc-TVII and desulfated. HPLC analyses establish that the core structure of the oligosaccharide is the core 2–branched oligosaccharide Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcα→Ser/Thr (Figure 6I).

Considered together, these results indicate that LSST, when coexpressed with C2GnT and Fuc-TVII, can direct the synthesis of CD34-associated oligosaccharides with the di-sialylated, sulfated structure [NeuNAcα2→3Galβ1→4(6-sulfo)(Fucα1→3)GlcNAcβ1→6](NeuNAcα2→3Galβ1→3)GalNAcα→Ser/Thr.

By contrast, most of the CD34·IgG-derived glycopeptides generated in GlcNAc6ST-, C2GnT-, and Fuc-TVII-expressing cells correspond to *N*-glycans (Figure 6B),

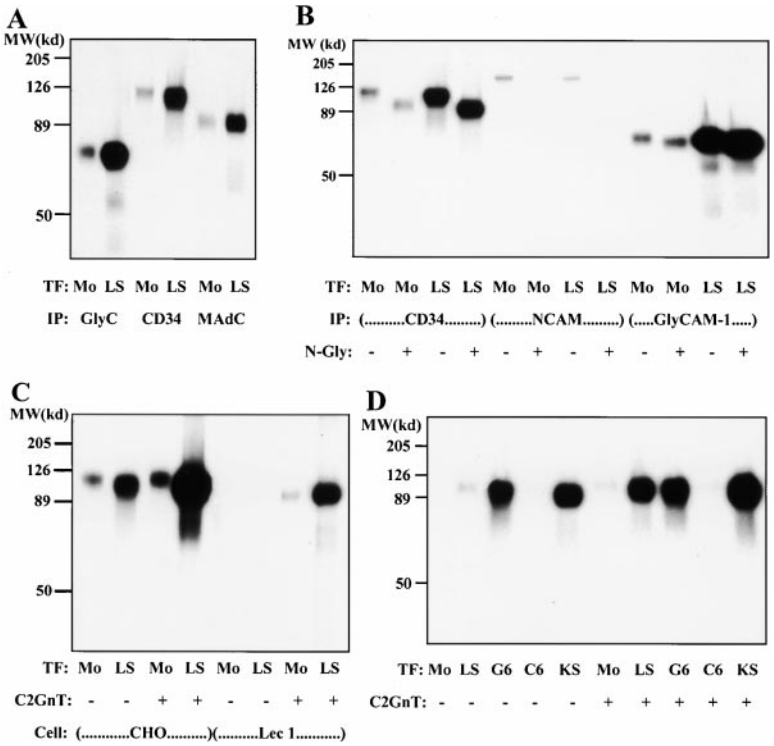


Figure 5. Incorporation of $^{35}$S-Sulfate to CD34, GlyCAM-1, and MAdCAM-1 Chimeric Proteins by LSST

(A and B) COS-1 cells were transfected with pcDNA3.1-LSST (LS) or pcDNA3.1 (Mo) together with pcDM8-CD34·IgG (CD34) (A and B), pcDNA3-GlyCAM-1·IgG (GlyC) (A and B), pcDNA3-MAdCAM-1·IgG (MAdC) (A), or pcDM8-NCAM·IgG (B). In (B), the samples were analyzed before (-) and after (+) *N*-glycanase treatment.

(C and D) CHO (C and D) and Lec1 (C) cells were transfected with pcDM8-CD34·IgG and pcDNA3.1-LSST (LS), pcDNA3-GlcNAc6ST (G6), pcDNA3.1-C6ST (C6), or pcDNA3.1-KSST (KS) or pcDNA3.1 (Mo). The cells were cotransfected with (+) or without (-) pcDNAI-C2GnT. Metabolic labeling with sodium [$^{35}$S] sulfate was performed for 2 days beginning 1 day after transfection. Chimeric proteins released into the medium were purified and separated by 7.5% acrylamide gel electrophoresis in SDS and subjected to fluorography. Western blot analyses using an anti-human IgG antibody were used to ensure that the same amount of each protein was analyzed in each experiment. TF, transfection; IP, immunoprecipitation; N-Gly, N-glycanase.

and 81% of the total radioactivity was released after *N*-glycanase treatment (data not shown). These observations confirm that GlcNAc6ST contributes minimally to the synthesis of sulfated L-selectin counterreceptors corresponding to mucin-type *O*-glycans.

### LSST-Dependent Sulfation Enhances Shear-Dependent L-Selectin Ligand Activity

To further define a role for LSST in L-selectin ligand biosynthesis, a parallel flow chamber system was used to measure the contribution of 6-sulfo sLe$^x$ to L-selectin ligand activity under shear forces that mimic vascular flow. CHO cells expressing CD34, C2GnT, and Fuc-TVII were transiently transfected with pcDNA3.1-LSST or pcDNA3.1 and then subjected to an analysis that determines rolling velocity on an immobilized L-selectin-IgG substrate. After mock transfection, a predominance of cells is observed in the velocity classes exceeding 20 microns per second at two different physiologically relevant shear forces (Figures 7A–7D). By contrast, transfection with pcDNA3.1-LSST consistently yields an increased population of cells rolling at velocities below 20 microns per second (Figures 7A and 7B). These experiments indicate that LSST-dependent sulfation of the sLe$^x$-type *O*-linked glycans expressed by these cells yields L-selectin ligands with shear-dependent affinities exceeding those conferred by the nonsulfated sLe$^x$ structures on the parental cell line.

In contrast, the population of cells transfected with the GlcNAc6ST expression vector yields a velocity class profile similar to mock-tranfected cells (Figures 7C and 7D). These observations demonstrate that an increment in L-selectin ligand activity is not conferred by Glc NAc6ST-dependent sulfation, shown to occur largely on *N*-glycans, and imply that sulfation of core 2-type *O*-glycans is a prerequisite for enhanced L-selectin ligand activity. Considered together with the structural analyses, these observations further indicate that mono- and/or di-sialyated forms of the core 2-type structure [NeuNAcα2→3Galβ1→4(6-sulfo)(Fucα1→3)GlcNAcβ1→6](NeuNAcα2→3Galβ1→3)GalNAcα→Ser/Thr, expressed by CD34, provide a contribution to shear-dependent L-selectin ligand activity that supersedes the contribution made by the corresponding nonsulfated form of this *O*-glycan.

## Discussion

Structural analyses of the oligosaccharides associated with GlyCAM-1 identify sulfate modifications corresponding to *N*-acetylglucosamine-6-sulfate and galactose-6-sulfate, within the context of the core 2–branched 6-sulfo sLe$^x$ and 6′-sulfo sLe$^x$ molecules (Figure 1), and possibly a disulfated analog (Hemmerich et al., 1994b, 1995). In vitro studies that have attempted to address the relative abilities of such structures to contribute to L-selectin ligand activity are conflicting (Sanders et al., 1996; Tsuboi et al., 1996; Galustian et al., 1997; Mitsuoka et al., 1998). The cell adhesion studies we report here provide direct evidence that L-selectin-dependent cell adhesion under shear is facilitated by a core 2-based 6-sulfo sLe$^x$ epitope displayed by the mucin-type glycoprotein CD34. Our observations imply that sulfation contributes to, but is not absolutely required for, L-selectin ligand activity, since cells expressing nonsulfated forms of this counterreceptor were observed to engage in rolling-type L-selectin-dependent adhesive interactions, consistent with previous reports (Foxall et al., 1992). A better understanding of the physiological contribution

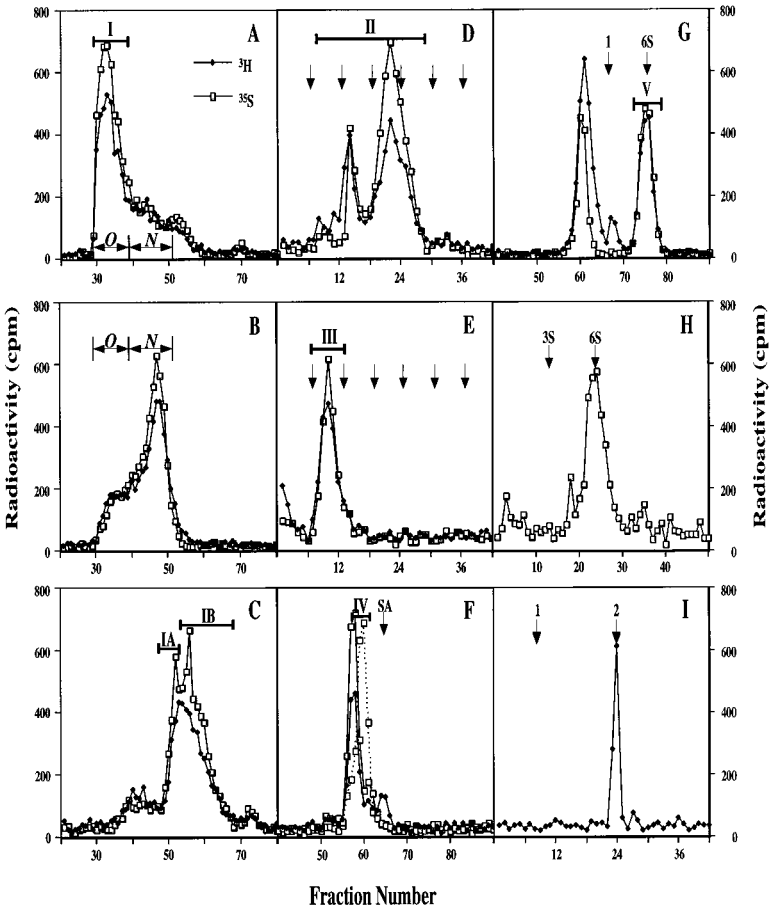


Figure 6. Analysis of Sulfated sLe$^x$ *O*-Linked Oligosaccharides Attached to CD34·IgG

CHO cells were transiently cotransfected with vectors encoding CD34·IgG, C2GnT, Fuc-TVII, and LSST (A and C to I) or GlcNAc6ST (B) and metabolically labeled with [$^3$H]glucosamine (closed diamond) and [$^{35}$S]sulfate (open square). (A, B, and C) Sephadex G-50 gel filtration of glycopeptides prepared from CD34·IgG of CHO transfected with LSST (A) or GlcNAc6ST (B) and alkaline borohydride treated sample I shown in (A) (C). Elution positions for glycopeptides containing *O*- and *N*-glycans are denoted. (D and E) QAE-Sephadex column chromatography of IA (D) and α2,3-sialidase treated sample of II (E). The arrows indicate the changes in increasing NaCl concentration (70 mM, 120 mM, 140 mM, 170 mM, 250 mM, and 1 M NaCl from the left). (F) Bio-Gel P-4 gel filtration of peak III before (solid line) and after α1,3/4-fucosidase and jack bean β-galactosidase treatment (dotted line). (G) Bio-Gel P-4 gel filtration of peak IV in (F) after β-hexosaminidase A treatment. (H) HPLC separation of peak V in (G). (I) HPLC separation of *O*-glycans after desialylation and desulfation of peak III except fucose is absent. The elution positions of Galβ1→3GalNAcOH (1), Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH (2), 6-sulfo *N*-acetylglucosamine (6S), 3-sulfo *N*-acetylglucosamine (3S), and sialic acid (SA) are shown in (F–I). In (F), Galβ1→4(Fucα1→3)GlcNAcβ1→6(Galβ1→3)GalNAcOH and GlcNAcβ1→6(Galβ1→3)GalNAcOH eluted at fractions 59 and 64, respectively.

made by sulfation to L-selectin-dependent lymphocyte trafficking will await the generation and analysis of mice that are deficient in sulfation of HEV-borne L-selectin counterreceptors.

Molecular cloning studies reported here identify LSST as a sulfotransferase that forms 6-sulfo sLe$^x$ in core 2–branched oligosaccharides attached to CD34. LSST thus differs from most of the other glycosyltransferases that can utilize low molecular oligosaccharides. Structural analyses disclosed that LSST directs the formation of the 6-*O*-sulfated form of the sLe$^x$ determinant, a major capping group displayed by HEV-borne GlyCAM-1 and other L-selectin counterreceptors, in cell lines in which a CD34-based L-selectin ligand synthetic pathway has been reconstituted. These properties contrast sharply with those of three other known glycan-type sulfotransferases, C6ST, GlcNAc6ST, and KSST. Our results demonstrate that C6ST does not contribute to sulfation of CD34-associated glycans, whereas GlcNAc6ST contributes to sulfation of CD34-associated *N*-glycans but much less to CD34-associated *O*-glycans. These biochemical observations and the cell adhesion assays completed with GlcNAc6ST imply that neither sulfotransferase participates in a core 2–dependent L-selectin ligand biosynthetic pathway. Although KSST can contribute to CD34-dependent sulfation, this occurs in a core 2–independent manner, and is most likely not involved in the synthesis of 6′-sulfo sLe$^x$ (Torii et al., 1998).

Our studies indicate that CHO cells transfected with LSST or mouse GlcNAc6ST are not recognized by MECA-79 antibody (data not shown). Kimura et al. (1999), on the other hand, demonstrated that the transfection of the ECV304 endothelial cell line with a human GlcNAc6ST resulted in positive staining of MECA-79 antigen. GlcNAc6ST and Fuc-TVII were also detected in human umbilical vein endothelial cells, which do not express MECA-79 epitope. These results indicate that one or more sulfotransferases other than GlcNAc6ST or LSST contribute to expression of MECA-79 epitope.

An HEV-specific expression pattern was observed for LSST in peripheral lymph nodes and in the hyperplastic thymus of the AKR/J mouse, a circumstance associated with conversion of flat-walled, L-selectin ligand-negative endothelium to the high endothelial venular endothelium. The α1,3-fucosyltransferase, Fuc-TVII, exhibits a similarly restricted expression pattern. These observations imply that the HEV phenotype is associated with coordinated regulation of a series of enzymes that dictate the glycosylation phenotype conferring L-selectin counterreceptor activity. It will be of interest to determine if similar coordinated regulation processes are involved in other circumstances, like chronic inflammation, development, and in association with peripheral node deafferentiation, where it is possible to observe an interconversion between a flat-walled, L-selectin ligand-deficient vasculature and L-selectin ligand-positive HEV.

In mucin-type *O*-glycans found on blood leukocytes,

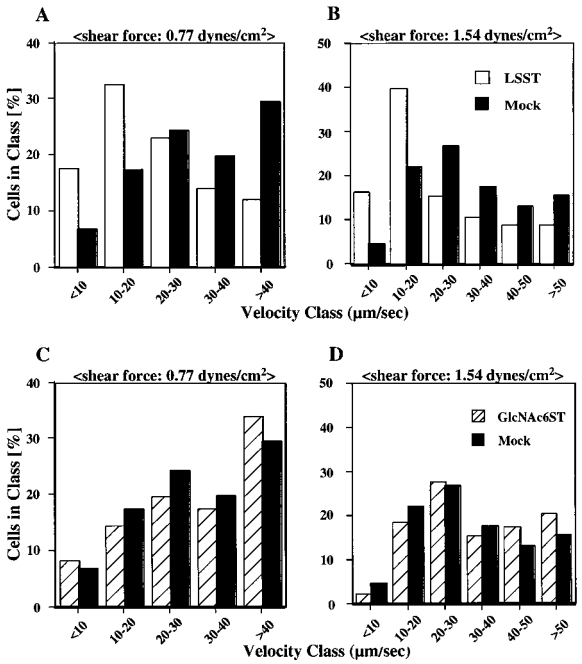


Figure 7. LSST-Dependent Decrements in L-Selectin-Mediated Rolling Adhesion Velocities

CHO cells that stably express CD34, Fuc-TVII, and C2GnT-leukocyte type were transiently transfected with pcDNA3.1-LSST (LSST, [A] and [B]), pcDNA3-GlcNAc6ST (GlcNAc6ST, [C] and [D]), or the insertless vector pcDNA3.1 (Mock). The cells were subsequently harvested and were infused into a parallel plate flow chamber coated with an L-selectin·IgG chimera at a site density of 2690 ± 153 (SD) molecules per square micron. Rolling velocities were measured at shear forces that engage L-selectin-dependent rolling adhesions (0.77 dynes/cm$^2$, [A] and [C]; 1.54 dynes/cm$^2$, [B] and [D]). The histograms display the fraction of all rolling cells that were observed to roll at the indicated velocities. A similar difference between the LSST transfectants and control cells was observed in three independent experiments, and representative results are shown in the figure. No cells were observed to roll in the presence of 1 mM EDTA (data not shown).

core 2–type branched glycans are critical for the elaboration of functional P-selectin counterreceptors decorated with sLe$^x$ moiety (Fukuda et al., 1986; Maemura and Fukuda, 1992; Wilkins et al., 1996). Mucin-type core 2 *O*-glycans have also been implicated in L-selectin counterreceptor activity. Indeed, the absence of core 2-type glycans in mice deficient in C2GnT is associated with deficient expression of neutrophil-borne counterreceptors for L-selectin (Ellies et al., 1998). By contrast, the same studies demonstrate that C2GnT deficiency yields only a moderate decrease in L-selectin–dependent lymphocyte homing efficiency. This observation implies that L-selectin ligands distinct from those containing core 2 *O*-glycans may contribute to L-selectin binding (Ellies et al., 1998) and is consistent with others' experiments that identify *O*-glycoprotease–resistant L-selectin counterreceptor activity on HEV (Clark et al., 1998). However, the apparent absence of a requirement for C2GnT in the peripheral node L-selectin counterreceptor synthetic pathway may be accounted for by our recent discovery of a novel C2GnT, termed C2GnT-mucin type. This enzyme maintains a structure and expression pattern that is distinct from those of C2GnT, now termed C2GnT-leukocyte type (Yeh et al., 1999). These analyses disclose that C2GnT-mucin type is apparently also involved in the biosynthesis of core 2–branched oligosaccharides in HEV and can compensate for the deficiency of C2GnT-leukocyte type in the C2GnT null mice.

In conclusion, our observations define LSST as a novel, HEV-specific sulfotransferase that synthesizes the CD34-associated *O*-glycan–linked 6-sulfo sLe$^x$ capping group implicated in L-selectin counterreceptor activity. Shear-dependent cell adhesion assays disclose that LSST-dependent sulfation events enhance L-selectin–dependent adhesion activity, relative to control cells that display nonsulfated forms of the *O*-glycan–associated sLe$^x$ capping group. These observations, and the HEV-specific expression pattern of this sulfotransferase locus, imply that this enzyme provides a primary contribution to the synthesis of HEV-borne L-selectin ligands, to lymphocyte homing to secondary lymphoid organs, and to lymphocyte trafficking associated with transformation of flat-walled vasculature into HEV-like vessels.

## Experimental Procedures

### Isolation of cDNA Encoding LSST

The coding sequences of chicken C6ST (amino acid residues 114–151, 169–214, 221–290, 337–370, and 405–454, which are highly homologous to human KSST) were used as probes to search dbEST using the tblstx program. Two query genes (AA103962 and AA522184) were found to have 30% and 31.9% identity with the coding regions of C6ST. Preliminary Northern blot analyses indicated that one (AA103962; later identified as GlcNAc6ST; Uchimura et al., 1998) is widely expressed in various mouse adult tissues. RT-PCR of mouse embryo poly(A)$^+$RNA using primers based on the sequence of the EST clone AA 522184, followed by application of 5′-RACE, yielded an LSST cDNA that was cloned into pcDNA3.1, resulting in pcDNA3.1-LSST.

### Synthesis of Sulfotransferase cDNAs from MECA-79–Positive Cells

MECA-79 positive cells, high endothelial venule cells, were isolated from mouse peripheral lymph nodes and mesenteric lymph nodes using biotin-conjugated MECA-79 as described (Girard and Springer, 1995). Total RNA (120 ng) isolated from MECA-79–positive cells, or poly(A)$^+$ RNA derived from mouse embryo at postcoital (pc) days 11 and 17, was reverse transcribed by using SuperScript II reverse transcriptase (GIBCO-BRL) with 1.5 pmoles of NotI-oligo(dT)$_{18}$ as a primer in 10 μl of reaction mixture.

One tenth of RT mixture was used as template for PCR. One fifth of each PCR reaction product was separated by 2% agarose gel electrophoresis. Oligonucleotide primer pairs were used for specific amplification of mouse LSST (nucleotides 956–976 and 1208–1189 for 5′- and 3′-primers) and mouse GlcNAc6ST (nucleotides 688–704 and 1083–1066 for 5′- and 3′-primers).

### In Situ Hybridization

In situ hybridization and immunohistochemical procedures were completed as described previously (Smith et al., 1996; Angata et al., 1997). $^{35}$S-labeled or digoxigenin-labeled sense and anti-sense riboprobes were prepared by in vitro transcription from plasmids containing segments of the mouse LSST cDNA (nucleotides 238–426) or the mouse GlcNAc6ST cDNA (nucleotides 1663–1818).

### Northern Blot Analysis

Northern blots of multiple mouse tissues (Clontech) were hybridized with $^{32}$P-labeled cDNA inserts of pcDNA3.1-LSST or mouse GlcNAc6ST cDNA as described previously (Angata et al., 1997).

### Assay of Sulfotransferase Activity In Vitro

The cell lysates from COS-1 cells transfected transiently with pcDNA3.1-LSST was prepared and assayed using various acceptors

as described (Fukuta et al., 1995, 1997; Bowman et al., 1998; Ujita et al., 1998).

**Transient Transfection and Metabolic Cell Labeling**

The cDNA encoding amino acid residues 1–284 of mouse CD34 (Suda et al., 1992) was ligated to a DNA segment encoding the human $IgG_1$ hinge plus constant region (Aruffo et al., 1990) and subcloned into pcDM8, resulting in pcDM8-CD34·IgG. Similar approaches linked mouse GlyCAM-1 (amino acids 1–127) and mouse MAdCAM-1 (amino acids 1–375) to these human $IgG_1$ segments in pcDM8-GlyCAM-1·IgG and pcDM8-MAdCAM-1·IgG. Cells were transiently cotransfected with pcDM8-CD34·IgG, pcDNA3.1-LSST, pcDNAI-C2GnT, and pcDM8-FucTVII using lipofectamine PLUS (GIBCO-BRL). In some of the experiments, pcDM8-FucTVII or pcDNAI-C2GnT was omitted, or pcDNA3.1-LSST was replaced with an expression vector containing cDNAs encoding mouse C6ST and human KSST (pcDNA3.1), or mouse GlcNAc6ST (pcDNA3).

Twenty-four hours after transfection, the medium was replaced with sulfate-free medium S-MEM (GIBCO-BRL) containing 10% dialyzed fetal bovine serum, 0.1 mM MEM nonessential amino acid solutions, supplemented with sodium[$^{35}$S]sulfate (100 μCi/ml). After an additional 48 hr of culture, CD34·IgG in the culture medium was purified by proteinA-Sepharose as described previously (Tsuboi and Fukuda, 1997; Angata et al., 1998). NCAM·IgG (Angata et al., 1998), GlyCAM-1·IgG, and MAdCAM-1·IgG were similarly expressed and purified.

**Structural Analysis of Oligosaccharides Attached to CD34·IgG**

CD34·IgG was isolated from CHO cells as described above except that the transfected cells were metabolically labeled with [$^3$H]glucosamine (20 μCi/ml) together with sodium[$^{35}$S]sulfate in the sulfate-free medium. Purified CD34·IgG was digested with pronase and subjected to Sephadex G-50 gel filtration (Carlsson et al., 1986). Glycans eluting near the void volume (mucin-type *O*-glycans) were subjected to alkaline borohydride treatment (Bierhuizen et al., 1994), and released *O*-glycans were recovered after Sephadex G-50 gel filtration.

Sialylated sulfated oligosaccharides were digested by α2,3-specific neuraminidase (NANaseI, GlyKo) and then digested with *Streptomyces* species α1,3/4-fucosidase and jack bean β-galactosidase (Sigma) (Lee et al., 1990; Hemmerich et al., 1995). The resultant oligosaccharides were then digested by human placental β-hexosaminidase A (Sigma), which can cleave 6-*O*-sulfated and nonsulfated GlcNAc (Kytzia and Sandhoff, 1985). In parallel, the oligosaccharide was subjected to solvolysis to remove sulfate (Nagasawa et al., 1979). QAE-Sephadex A-25 column chromatography was carried out in 10 mM pyridine-acetate buffer (pH 5.5) and stepwisely eluted with the increasing concentrations of NaCl. By using standard oligosaccharides, we found that monosialosyl, disialosyl, and trisialosyl oligosaccharides elute with 70 mM, 120 mM, and 140 mM NaCl, while 6-sulfo Gal (and 6-sulfo $GlcNH_2$) and 2,3-disulfo $GlcNH_2$ elute with 70 mM and 500 mM NaCl, respectively. Bio-Gel P-4 gel filtration was carried out in 0.1 M ammonium acetate buffer (pH 6.7). HPLC (Bierhuizen and Fukuda, 1992) was used to identify core 2 oligosaccharide, Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH.

Sulfated GlcNAc was identified using an $NH_2$-bonded HPLC column (Asahipak $NH_2$P50-4E, 4.6 × 250 mm). The column was eluted for 10 min with a linear gradient from the solvent A (64% acetonitrile, 36% $H_2O$) to an 85:15 mixture of solvent A and solvent B (64% acetonitrile, 36% 69 mM $NaH_2PO_4/H_2O$ [pH 4.2]) followed by a 40 min isochratic elution with the same mixture.

**Measurement of CHO Cell Rolling Adhesion**

Shear force–dependent cell adhesion was analyzed using a modification of an in vitro rolling adhesion procedure (Lawrence and Springer, 1991). Soluble murine L-selectin IgG chimeric molecules (Maly et al., 1996) were coated onto polystyrene dishes (Ellies et al., 1998). The L-selectin-IgG-coated dishes were then used as the bottom plate of a parallel plate flow chamber (GlycoTech). CHO cells stably expressing C2GnT, Fuc-TVII, and CD34 (Tsuboi et al., 1996) were transiently transfected with pcDNA3.1-LSST or with pcDNA3-GlcNAc6ST. Sixty-two hours after the transfection, the transfected CHO cells were dissociated and resuspended at 1 × $10^6$ cells/ml as described previously (Sawada et al., 1994). Cells were initially introduced into the flow chamber at a wall shear stress of 5 dynes/cm$^2$ for 15 s, followed by termination of flow to allow the cells to adhere under static conditions. Flow was then reinitiated at the shear forces indicated in Figure 7. Image analysis was performed as described (Ellis et al., 1998), and rolling velocities for individual cells (between 120 and 200 rolling cell events per experiment) were determined.

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