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Sulfotransferases of Two Specificities Function in the Reconstitution of High Endothelial Cell Ligands for L-selectin

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Abstract. L-selectin, a lectin-like receptor, mediates rolling of lymphocytes on high endothelial venules (HEVs) in secondary lymphoid organs by interacting with HEV ligands. These ligands consist of a complex of sialomucins, candidates for which are glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), CD34, and podocalyxin. The ligands must be sialylated, fucosylated, and sulfated for optimal recognition by L-selectin. Our previous structural characterization of GlyCAM-1 has demonstrated two sulfation modifications, Galα6-sulfate and GlcNAcα6-sulfate in the context of sialyl Lewis x. We now report the cloning of a Galα6-sulfotransferase and a GlcNAcα6-sulfotransferase, which can modify GlyCAM-1 and CD34. The Galα6-sulfotransferase shows a wide tissue distribution. In contrast, the GlcNAcα6-sulfotransferase is highly restricted to HEVs, as revealed by Northern analysis and in situ hybridization. Expression of either enzyme in Chinese hamster ovary cells, along with CD34 and fucosyltransferase VII, results in ligand activity, as detected by binding of an L-selectin/IgM chimera. When coexpressed, the two sulfotransferases synergize to produce strongly enhanced chimera binding.

Key words: sulfotransferase • carbohydrate • L-selectin • high endothelial venule • endothelium

Sulfation of carbohydrates attached to glycoproteins, glycolipids, and proteoglycans confers highly specific functions onto these macromolecules (Nelson et al., 1995; Hooper et al., 1996; Rosenberg et al., 1997). Examples of sulfation-dependent recognition phenomena are seen in diverse biological systems, including blood clotting, cytokine sequestration and receptor binding, control of the circulatory half-life of pituitary hormones, and plant-bacterial symbiosis. Therefore, the sulfotransferases that catalyze the sulfation of carbohydrates have become the focus of increasing research interest.

In recent years, the role of sulfated carbohydrates in leukocyte-endothelial interactions has been established (Kansas, 1996; Rosén and Bertozzi, 1996). During the process of normal lymphocyte recirculation, L-selectin on lymphocytes interacts with a set of sulfated glycoprotein ligands on the endothelial cells of high endothelial venules (HEVs).1 This interaction leads to lymphocyte rolling, which constitutes the first step in the recruitment cascade that culminates in the migration of the lymphocyte into the underlying tissue (Springer, 1994; Butcher and Picker, 1996). The involvement of L-selectin in leukocyte recruitment to certain sites of acute and chronic inflammation has also been demonstrated (Lewinsohn et al., 1987; Ley et al., 1991; Michie et al., 1993). The high endothelial cells (HECs) of HEVs within lymphoid organs and of HEV-like vessels that develop at sites of chronic inflammation have long been known to incorporate large amounts of [35S]sulfate in several species (Andrews et al., 1982; Girard and Springer, 1995a). The functional significance of this

1. Abbreviations used in this paper: BAC, bacterial artificial chromosome; C2GnT, core 2 β1→6 N-acetylgalactosaminyltransferase; C6/KSST, chicken chondroitin/keratan sulfate sulfotransferase; C6ST, human chondroitin-6-sulfotransferase; CM, conditioned medium; EST, expressed sequence tag; FTVII, fucosyltransferase VII; Gal, galactose; GlcNAc, N-acetylglucosamine; GlyCAM-1, glycosylation-dependent cell adhesion molecule 1; HEC, high endothelial cell; HEC-GlcNAc6ST, human chondroitin-6-sulfotransferase (AF131235, AF131236); HEV, high endothelial venule; HPAEC, high pH anion exchange chromatography; HUVEC, human umbilical vein endothelial cell; KSGalβ1,3ST, keratan sulfate Galα6-sulfotransferase; MFI, mean fluorescence intensity; nt, nucleotide(s); PE, phycoerythrin; RT, reverse transcriptase; SLeα, sialyl Lewis x.
extensive sulfation was not understood until the ligands for L-selectin were elucidated.

To date, four discrete H E V-associated glycoprotein ligands for L-selectin have been identified at the molecular level: glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1) (Lasky et al., 1992; CD 34 (B aumhueter et al., 1993; Puri et al., 1995; Shailubhai et al., 1997), mucosal addressin cell adhesion molecule 1 (M A D C M -1) (B erg et al., 1993), and podocalyxin (S assetti et al., 1998). A t least one other component, Sgp200, remains to be cloned (B erg et al., 1991; H emmerich et al., 1994b; H oke et al., 1995). These glycoproteins all contain mucin-like domains and are heavily O-glycosylated (Puri et al., 1995). It is well established that optimal recognition of these ligands by the C-type lectin domain of L-selectin requires sialylation (I mai et al., 1991), fucosylation (M aly et al., 1996), and sulfation (I mai et al., 1993). The protein scaffolds underlying these ligands are also expressed in tissues other than H E Vs, such as mammary gland, non-H E V endothelium, hematopoietic precursors, and kidney podocytes (D owbenko et al., 1993; B aumhueter et al., 1994; K ershaw et al., 1997). H owever, the L-selectin–reactive glycoforms of these proteins are highly restricted to H E Cs (D owbenko et al., 1993; B aumhueter et al., 1994; S assetti et al., 1998).

To understand the posttranslational modifications that underlie recognition of ligands by L-selectin, we previously undertook the analysis of the oligosaccharides of Gi yCA M-1, which was affinity purified with recombinant L-selectin (H emmerich and R osen, 1994; H emmerich et al., 1994a, 1995). We detected two specific sulfated mono-saccharides: N-acetylgalactosamine-6-sulfate (GlcNAc c-6-sulfate) and galactose-6-sulfate (G al-6-sulfate), which were present in equal amounts. These sulfate modifications are found, respectively, within two capping structures based on sialyl Lewis x (sLe x); sialyl 6-sulfo Le x and sialyl 6'-sulfo Le x (see Fig. 1 and Table I). The simplest chains of Gi yCA M-1 consist of sialyl 6'-sulfo Le x and sialyl 6-sulfo Le x as extensions from an internal trisaccharide known as core 2 (see Fig. 1 and Table I). More complex structures with increased chain size and multiple sulfate esters comprise the majority of the chains and remain to be analyzed in detail.

The sulfate dependence for L-selectin binding of H E V ligands was first established in pharmacological experiments with metabolic inhibitors of sulfation, i.e., chlorate and brefeldin A (I mai et al., 1993; H emmerich et al., 1994b; C rommie et al., 1995; Shailubhai et al., 1997). A dditional evidence for the importance of sulfation has emerged from studies with two m Abs, M E C A -79 and G 72. M E C A -79 stains lymph node H E Vs and blocks L-selectin–dependent lymphocyte attachment to H E Vs in vitro and in vivo (S treeter et al., 1988). This antibody also stains H E V-like vessels that develop at sites of chronic inflammation (M ichie et al., 1993; L ee and S arvetnick, 1994; G irrard and Springer, 1995a; O nrust et al., 1996). M E C A -79 reacts with all the L-selectin ligands described above (I mai et al., 1991; B erg et al., 1993; H emmerich et al., 1994b; S assetti et al., 1998). This broad reactivity is thought to reflect its binding to a posttranslational modification that is common to many L-selectin ligands (B erg et al., 1993; H emmerich et al., 1994b). The presence of the M E C A -79 epitope is currently accepted as a predictor of L-selectin ligand activ-
Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics. Total RNA was isolated from HECs, HUVECs, and human tonsillar lymphocytes by lysis and extraction with RNAZol (Bior最高). Total RNA from these tissues was reverse transcribed using oligo (dT) primer (Clontech), which incorporates a long-distance PCR amplification step from first strand cDNA. 1 μg of total HEC RNA was hydrolyzed in a total volume of 10 μl of KlenTaq buffer (Clontech) containing 600 nM primers, 200 μM dNTPs, 0.2 μl KlenTaq Advantage DNA polymerase mix (KlenTaq polymerase; Clontech), and 1.0 μl of twofold serially diluted cDNA as template. Cycling conditions were as follows: 1 min at 94°C; 30 cycles of 30 s at 92°C followed by 1 min 15 s at 68°C; one cycle of 10 min at 68°C. The resulting 1926 nt sequence was confirmed by resequencing in RNA prepared as above was mixed with a modified oligo (dT) primer (Life Technologies, Inc.). PCR reactions were carried out in a total volume of 10 μl of 1× KlenTaq buffer (Clontech) containing 400 nM primers, 200 μM dNTPs, 0.2 μl KlenTaq Advantage DNA polymerase mix (KlenTaq polymerase; Clontech), and 1.0 μl of twofold serially diluted cDNA as template. Cycling conditions were as follows: 1 min at 94°C; 30 cycles of 30 s at 92°C followed by 1 min 15 s at 68°C; one cycle of 10 min at 68°C. The resulting primers were used for: HEC-GicNAc cST; 5′-AAACCTCAAGAAGGAG-GACCAACCCCTACTATGGTAGC-3′ and 5′-GTGGAATTCTGCTCA-GGAGCAGTCCTCAGGAACAAAC-3′, which amplify a 456-bp fragment corresponding to nucleotides (nt) 884-1339 in Fig 3 a for hypoxanthine phosphoribosyltransferase (HPRT), 5′-CCTGTCGATATTACATCAGAAGCT-3′ and 5′-TCCACATCCTGGCTGTTCT-3′. The resulting amplified DNA was electrophoresed and visualized by ethidium bromide.

Preparation of an HEC cDNA Expression Library

A cDNA expression library was prepared from human HECs using the SMART cDNA technology (Clontech), which incorporates a long-distance PCR amplification step of first strand cDNA. 1 μg of total HEC RNA, which had been modified by digestion with SacI followed by religation, was reverse transcribed using oligo (dT) primer (Clontech), which incorporates a universal site for 5′ priming of the PCR reaction and the SMART oligonucleotide (1 μM), which provides a universal site for 5′ priming of the PCR reaction. This mixture was heated at 72°C for 2 min to disrupt RNA secondary structure, and first strand cDNA was synthesized by Moloney murine leukemia virus (MMLV) RT priming of the PCR reaction. This mixture was subjected to 18 cycles of long-distance PCR primed by the universal primers using KlenTaq polymerase (Clontech). The PCR reaction mixture was incubated in the presence of proteinase K for 1 h at 45°C to destroy the KlenTaq polymerase activity, followed by heating in a 3 min at 90°C for 10 min. The double-stranded cDNA was purified by treatment with T4 DNA polymerase at 16°C for 30 min, followed by ligation to EcoRI/BstXI adaptors (Invitrogen) overnight at 16°C in the presence of T4 DNA ligase. A daptor-ligated cDNAs were digested with NotI and then phosphorylated. The double-stranded cDNA was purified by size fractionation, ligated into EcoRI/NotI-digested pCDNA1.1 (Invitrogen), and introduced into E. coli per plate. The library contained 500,000 independent clones with an average insert size of 1.1 kb (about 10% of the total library).

Molecular Cloning of HEC-GlcNAc6ST

HEC-GicNAc cST (human) was cloned from the HEC cDNA library by modification of a pool selection procedure (Kolodkin et al., 1997). In brief, an aliquot (comprising 400,000 colony forming units) of the amplified bacterial stock of the HEC cDNA library was plated onto 200 LB plates and grown for ~18 h at 37°C. Each plate of 2,000 colonies was harvested and plated for an additional 2 h at 37°C, and glycerol stocks were made. PCR analysis was performed, using the HEC-GicNAc cST-specific primers described above, to identify positive pools. 1 of the 9 positive pools was titered and plated onto 40 plates to yield 100 colonies per plate. These pools were expanded and analyzed as in the first round. A single positive subpool was titered and plated onto 20 plates of 10 colonies each. A nalysis of individual colonies by PCR resulted in a singular positive clone, which was sequenced (Koester et al., 1997). To clone the murine HEC-GicNAc cST, 14,241 bp probe (nt 26-267) was amplified from the EST clone (A.A 522184, Research Genetics Inc., Huntsville, AL) and used as a probe for screening a bacterial artificial chromosome (BAC) library from the C57BL/6 mouse (Genome Systems, Inc.). From the single positive clone, DNA was purified and sequenced directly, using primers derived from EST A A 522184 (forward: 5′-TGGGCTCAAGCCTTGCTTACATAAG-3′; reverse: 5′-TTCTAAGAGTTCCGTTCTTTCTGGAC-3′) to the EST sequence from the EST clone, A 522184, and then obtained sequence upstream (1559 nt) and downstream (582 nt). The resulting 1926 nt sequence was confirmed by resequencing in both directions.

Molecular Cloning of KSGal6ST

A human fetal brain library (λ ZAP; Stratagene) was the kind gift of Dr. Marc Tessier-Lavigne at the University of California, San Francisco. A preparation of washed cDNA was used to screen a KSGal6ST cDNA expression library (Clontech, Palo Alto, CA) for positive clones. The positive clones were sequenced using the sequencing protocol of the manufacturer (Clontech). The cDNA encoding the KSGal6ST chimera was constructed by amplifying the entire coding sequence of murine KSGal6ST (KSGal6ST probe transcribed from the IMAGE consortium clone 851801 (EMBL/GenBank/DDBJ accession no. AA522184; Research Genetics, Inc.) which probe transcribed from the IMAGE consortium clone 851801 (EMBL/GenBank/DDBJ accession no. AA522184; Research Genetics, Inc.). Multiple Tissue Northern blots (Clontech) containing poly(A)+ RNA from various human tissues were hybridized at 60°C overnight in Rapid-Hyb (A mer sham Pharmacia Biotech) and then washed twice at room temperature for 15 min in 2x SSC/0.1% SDS followed by 2x min washes at 60°C in 0.1× SSC/0.1% SDS and autoradiography. The Northern blot to establish expression in HECs, poly(A)+ RNA was prepared from 1.5×106 HECs and 2.0×106 HUVECs, respectively. Isolation of the poly(A)+ RNA with oligo(dT) latex beads was performed according to the manufacturer’s protocol (Oligotex Direct kit, QIAEN, Inc.). A approximately 2 μg poly(A)+ RNA was loaded per lane. The RNA was separated by electrophoresis in a 1% de natured agarose-formaldehyde gel and transferred to positively charged nylon filters (Hybond N+). The filters were hybridized and washed as described. Sulfatase activity was then measured using a nucleotide-6 labeled ribo probe transcribed from the IMAGE consortium clone 851801 (EMBL/GenBank/DDBJ accession no. A A 522184; Rearch Genetics, Inc.), which had been modified by digestion with SacI followed by religation. A hybridization, sessions were washed at high stringency, dehydrated, dipped in photographic emulsion NTB2 (Eastman Kodak Co.), stored for 4C for 2-8 wk, developed, and counterstained with hematoxylin and eosin.
Sepharose) was equilibrated in 2 mM pyridine-acetate, pH 5.0, and eluted with a gradient of 2-500 mM pyridine-acetate. The monosulfated oligosaccharides eluted from this column between 50 and 250 mM pyridine-acetate. The eluate was lyophilized, redissolved in 100 μl of water, and 30-μl samples of the resulting solution were subjected directly to HPAEC. HPAEC was performed using a Dionex DX 500 chromato- graphic system and a CarboPac 1 column (Dionex Corp.) essentially as described (Hemmerich et al., 1994a, 1995), with column elution performed isocratic- ally with 150 mM NaOH in 400 mM NaOAc at 1 ml/min. The authentic carbohydrate standards used in this analysis were obtained as described (Hemmerich et al., 1994a, 1995).

**Flow Cytometry**

CHO/FTVII/C2GnT cells were grown to 80% confluency in T75 flasks (6 × 10⁶ cells per flask; Nalge Nunc) and then transfected with plasmids encoding C2GnT (1 μg, pCDNA 1.1), fucosyltransferase VII (FTVII; 1 μg, pCDNA 3.1), human CD34 (2 μg, pRK5), sulfotransferases (at the concentrations indicated for each experiment), and vector plasmid (pCDNA 3.1) to achieve 8 μg total DNA per flask, using Lipofectamine. 48 h after transfection, the cells were harvested in 0.6 mM EDTA in PBS without calcium and magnesium, washed once in assay buffer (0.1% BSA in PBS), and resuspended at 4 × 10⁶/ml in assay buffer. 25 μl of this cell suspension was added to wells of 96-well plates containing 50 μl of L-selectin/gM chimera, or assay buffer. Cells were incubated on ice for 30 min, washed twice in assay buffer, and resuspended in 50 μl assay buffer containing secondary staining reagents. Cells were incubated for another 30 min, washed twice in assay buffer, and then resuspended in 50 μl assay buffer containing primary staining reagents and/or directly conjugated primary mAbs. For the sialyl 6-sulfo Leα or overall sLeα determinations, cells were incubated with G72 mAb or HECA 452 (PharMingen), respectively, diluted in assay buffer, and then reacted with rabbit anti-mouse IgG-FITC (Zymed Laboratories, Inc.) (for G72) or mouse anti-rat IgM-FITC (PharMingen) (for HECA 452). The cells were incubated for 30 min, washed twice, and fixed in 1% paraformaldehyde in PBS, pH 7, for 20 min before transfer into 300 μl of assay buffer. Cells were analyzed on a FACSort™ (Becton Dickinson) flow cytometer using CELLQuest software (Becton Dickinson). To produce the L-selectin/gM chimera (Maly et al., 1996), COS cells (10-cm plates) were transfected with L-selectin/gM cDNA in pCDM8 (8 μg DNA, 50 μl Lipofectamine per plate) and incubated in Opti-MEM for 10 d, at which time the CM was harvested and clarified by centrifugation. The CM was concentrated to half its original volume, titere, and used neat. Biotinylated primary mAbs. For the two-color analysis, we used mouse anti-CD34 phycoerythrin (PE) (QBend10-PE; Coulter Corp.) and the isotype-matched control, mouse IgG1-PE (Caltag Laboratories).

**Results**

**Identification of Two cDNAs Predicting Carbohydrate Sulfotransferases**

Our previous structural analysis of the carbohydrate chains of GlyCAM-1 indicated that the Gal-6-sulfate and GlcNAc-6-sulfate modifications accounted for essentially all of the sulfation of GlyCAM-1 (Hemmerich et al., 1995). The previously cloned chicken chondroitin/kera
tan sulfate sulfotransferase (C6/K SST) has been shown by H abuchi et al. (1997) to catalyze sulfation at C-6 of galactose in SiaO2→3Galα1→4GlcNAc, which is a core structure within the capping groups shown in Fig. 1. We used the cDNA sequence encoding C6/K SST to probe the Na
tional Center for Biotechnology Information (NCBI) dbEST and LifeSeq (Incyte Pharmaceuticals, Inc.) human EST databases for related sequences. When we examined expression of transcripts corresponding to the ESTs by Northern analysis, one (corresponding to LifeSeq clone no. 2620445, derived from a human breast epithelial cell line) was of particular interest, because its tissue distribution was highly restricted (original finding not shown, but result demonstrated in Fig. 2 B). To determine whether this gene was expressed in HECs, a cell type in which L-selectin ligands are elaborated, we carried out a semiquantitative RT-PCR analysis on HEC cDNA with primers derived from this EST. HECs were purified, as described previously, from human tonsils using immunomagnetic separation with MECA-79 as the probe (Girard and Springer, 1995b; Sassetti et al., 1998). The resulting cells had a purity of >99% and represented <1 per 1,000 of the stromal cells in tonsils. In parallel, we prepared cDNA from HUVECs and tonsillar lymphocytes. As shown in Fig. 2 A, the primers specific for the novel gene amplified a fragment of 456 bp from HEC cDNA, but failed to amplify this product from the HUVEC or lymphocyte cDNA. Since we had a relatively small number of HECs available, we used a PCR-based technique (SMART technology; Clontech, Inc.) to produce cDNA from total RNA, from which we prepared a plasmid expression library. Using PCR amplification of the 456-bp fragment to identify positive pools, we isolated a full-length cDNA clone from the library by a pool selection procedure, as described in Materials and Methods. Initially, 200 pools of 2,000 colony-forming units/pool were screened. A second two additional rounds of screening, a single positive clone was obtained. The cDNA corresponding to this clone contains a single open reading frame of 1,158 nucleotides. The cDNA is apparently full-length as indicated by the presence of an upstream stop codon and a Kozak sequence surrounding the first ATG, and a poly(A) tail at the 3' end. This open reading frame predicts a type II transmembrane protein of 386 amino acids with 3 potential sites for N-linked glycosy-
lation. The new cDNA sequence was used to probe the human databases for additional matching ESTs. One EST was identified in the LifeSeq database that mapped to the new gene at the 5' end of its protein coding region. When the clone (no. 2617407, derived from gall bladder) corresponding to this EST was fully sequenced, its sequence completely matched the original cDNA sequence within the coding region. There were two base changes in the 3' untranslated region and divergence in the 5' untranslated region. We present the sequence corresponding to LifeSeq clone no. 2617407 (Fig. 3 A; EMBL/Genbank/DDBJ accession no. AF131235), since the library from which it was cloned was created without a PCR amplification step. The predicted amino acid sequence of this novel gene is 31% identical to C6/KSST (Fukuta et al., 1995), the chicken enzyme used in the original EST search. We have termed this novel gene HEC-specific GlcNAc-6-sulfotransferase (HEC-GlcNAc6ST) on the basis of the characterization described below.

Using the human HEC-GlcNAc6ST cDNA sequence as a probe, we identified a highly related mouse sequence in the NCBI dbEST database (EMBL/Genbank/DDJB accession no. AA522184). A 241-bp probe based on this EST was used for screening of a BAC library from mouse (C57BL/6). The probe was found to hybridize to a single BAC clone within this BAC. The genomic clone within this BAC clone contained an open reading frame of 1167 bp (EMBL/Genbank/DDJB accession no. AF131236), which is 77% identical at the nt level to the coding region of the human HEC-GlcNAc6ST. No introns were detected. The sequence predicts a protein of 388 amino acids (Fig. 3 A), which is 73% identical to that of human HEC-GlcNAc6ST. We term this apparent mouse homologue mHEC-GlcNAc6ST.

The expression of HEC-GlcNAc6ST was confirmed to be highly restricted by further analysis. On a conventional Northern blot (human), transcripts corresponding to this gene were absent from most tissues (Fig. 2 B). Low levels of a 2.4-kb transcript were apparent in lymph node, liver (adult and fetal), and pancreas. A prominent band of the same size was detected at relatively high levels in mRNA from HECs, but was undetectable in HUVEC mRNA (Fig. 2 B). An additional transcript at ~6 kb was expressed in liver and pancreas, and trace levels appeared to be present in lymph node, HECs, and HUVECs (Fig. 2 B). Using an antisense riboprobe based on the original mouse EST, we carried out in situ hybridization on mouse tissues. Strikingly, mHEC-GlcNAc6ST transcripts were detected only in the HEVs of lymph node (Fig. 4). No hybridizing signal was found in other cell types of the lymph node, or in several other organs, including spleen, thymus, liver, skeletal muscle, pancreas, stomach, and kidney (data not shown). A weak signal was detected in gut intestinal epithelium (data not shown). The sense control did not yield signal in any tissue.

Our screening of the human EST databases with the chicken C6/KSST led to the identification of multiple ESTs that mapped to another gene. We cloned a cDNA for this gene by conventional techniques from a fetal brain library as described in Materials and Methods. While this work was in progress, the same cDNA (with only two base changes in untranslated regions) was published independently by Fukuta et al. (1997). The expressed protein was characterized as a keratan sulfate sulfotransferase with Gal-6-sulfotransferase activity (KSGal6ST) (Fukuta et al., 1997). The predicted protein is a type II transmembrane protein of 411 amino acids with 32% identity to HEC-GlcNAc6ST. Fukuta et al. (1997) reported the expression of KSGal6ST in many tissues, including lymph node. We confirmed these results; in addition, we detected its expression in HECs, albeit at apparently low levels, by per-
transfected COS cells with a cDNA encoding a GlyCAM-1 (human) were capable of sulfating an L-selectin ligand, we sought to test whether KSGal6ST and HEC-GlcNAc6ST could contribute to the generation of sialyl 6-sulfo Lex (Table I), we have shown that these enzymes are capable of sulfating GlcNAc6ST and GlcNAc6ST, respectively. The open reading frame is denoted by capital letters, and the predicted amino acid sequences are indicated below the nucleotide sequence. The putative transmembrane domain is underlined, and three potential N-linked glycosylation sites (for each sequence) are indicated by asterisks. (A) Alignment of regions of high conservation among human carbohydrate sulfotransferases. The sequences were aligned using the ClustalW algorithm (Thompson et al., 1994). Black shading indicates identity at that residue among at least three of the sequences; grey shading indicates similarity among at least three of the sequences. (B) Generation of sialyl 6-sulfo Lex with HEC-GlcNAc6ST. To determine whether the HEC-GlcNAc6ST could contribute to the generation of sialyl 6-sulfo Lex (Table I), we carried out transfection experiments with CHO cells, termed CHO/FTVII/C2GnT, which had been stably transfected with a mucin scaffold was not required for the expression of this epitope. Since HEC-GlcNAc6ST and KSGal6ST are both expressed in COS cells, we transiently transfected the HEC-GlcNAc6ST cDNA (but not empty vector) was included in the cotransfection. To establish the regiochemistry of sulfation on radiolabeled GlyCAM-1, we first carried out transfection experiments with CHO cells with HEC-GlcNAc6ST cDNA. The transfected cells were cultured in the presence of [35S]sulfate, and radiolabeled GlyCAM-1/IgG was purified from the CM on a protein A–agarose affinity column and subjected to hydrolysis and Dionex HPLC analysis according to our previously established procedures (Hemmerich et al., 1994a). As shown in Fig. 5 B, transfection with KSGal6ST resulted in sulfated mono- and disaccharides that comigrated with [SO3^-6]Gal and [SO3^-6]GalNAc, confirming this enzyme as a 6-sulfotransferase (Fukuta et al., 1997). In contrast, transfection with HEC-GlcNAc6ST resulted in products that corresponded to [SO3^-6]GalNAc in CHO cells and Galβ1-4[SO3^-6]GalNAc. Thus, this enzyme was established to be a novel GalNAc c-6-sulfotransferase.

**Table I. Nomenclature and Structure of Oligosaccharides**

| Name   | Structure                      |
|--------|--------------------------------|
| sLex   | Siaα2-3Galβ1-4[Fucα1-3]GlcNAc |
| Sialyl 6'-sulfo Leα | Siaα2-3[Siaα6-6]Galβ1-4[Fucα1-3]GlcNAc |
| Sialyl 6-sulfo Leα     | Siaα2-3[Siaα6-6]Galβ1-4[Fucα1-3]Siaα6-6GlcNAc |
| Sialyl 6',6-disulfo Leα | Siaα2-3[Siaα6-6]Galβ1-4[Fucα1-3][SO3^-6]GlcNAc |
| Core 2    | Galβ1-3GlcNAcβ1-6Galβ1-3[SO3^-6]GlcNAc |
| 6',6-disulfo lactose | [SO3^-6]Galβ1-4[SO3^-6]Glc |

**Figure 3.** Molecular features of human and mouse HEC-GlcNAc6ST. (A) cDNA sequence for human HEC-GlcNAc6ST and predicted protein sequences of human (h) and mouse (m) HEC-GlcNAc6STs (EMBL/DDBJ accession nos. AF131325 and AF131236, respectively). The open reading frame is denoted by capital letters, and the predicted amino acid sequences are indicated below the nucleotide sequence. The putative transmembrane domain is underlined, and three potential N-linked glycosylation sites (for each sequence) are indicated by asterisks. (B) Alignment of regions of high conservation among human carbohydrate sulfotransferases. The sequences were aligned using the ClustalW algorithm (Thompson et al., 1994). Black shading indicates identity at that residue among at least three of the sequences; grey shading indicates similarity among at least three of the sequences. (B) Generation of sialyl 6-sulfo Lex with HEC-GlcNAc6ST. To determine whether the HEC-GlcNAc6ST could contribute to the generation of sialyl 6-sulfo Lex (Table I), we took advantage of the G72 mAb, which recognizes this structure in a sulfate-dependent manner (Mitsuoka et al., 1998). We carried out transfection experiments with CHO cells, termed CHO/FTVII/C2GnT, which had been stably transfected with (a) fucosyltransferase VII (FTVII) to provide a core structure for O-linked glycans (Fig. 6), whereas transfection with KSGal6ST did not yield staining (not shown). Similar results were obtained with G152 (Mitsuoka et al., 1998), a related mAb (data not shown). Inclusion of a CD34 cDNA in the transfection did not significantly enhance staining by G72, indicating that an exogenous mucin scaffold was not required for the expression of this epitope.
in HECs and are capable of sulfating GlyCAM-1/IgG in transfected cells, they are candidates to participate in the biosynthesis of L-selectin ligands. Our previous structural analysis indicated that Gal-6-sulfate and GlcNAc-6-sulfate are present equally in native GlyCAM-1 oligosaccharides. However, as reviewed above, the relative contribution of the two sulfate esters to L-selectin binding affinity has been a matter of considerable uncertainty. To test whether these enzymes can contribute to the generation of ligand activity, we carried out further flow cytometry experiments with an L-selectin/IgM chimera as a probe. The CHO/FTVII/C2GnT cells were transiently transfected with cDNAs for the two sulfotransferases (singly or in combination) plus a CD34 cDNA. As shown in Fig. 7 A, no binding of the chimera was detected in the absence of the sulfotransferases. Transfection with either sulfotransferase yielded positive staining, both in terms of the proportion of positive cells and their mean fluorescence intensity (MFI). Strikingly, the combination of KSGal6ST and HEC-GlcNAc6ST cDNAs produced strongly enhanced binding of L-selectin/IgM, which greatly exceeded the sum of the signals from the individual sulfotransferases (Fig. 7 A). This apparent synergistic effect was evident over a considerable range of cDNA concentrations in the transfection mixtures (Tables II and III). The binding of the L-selectin/IgM chimera induced by the sulfotransferases was confirmed to be specific as indicated by its calcium dependence (data not shown) and complete inhibition binding by a function-blocking anti-L-selectin mAb (MEL-14; Fig. 7 B). The effects of the sulfotransferases on the L-selectin/IgM chimera were not due to differences in transfection efficiency, as the CD34 expression levels were essentially constant, irrespective of the combinations of sulfotransferase cDNAs in the transfection mixtures (Table II). Further...

Figure 4. In situ hybridization to detect HEC-GlcNAc6ST transcripts in mouse lymph node. Sections of C56BL/6 mouse lymph node were hybridized with 35S-labeled sense or antisense riboprobes based on the clone corresponding to the mouse homologue of HEC-GlcNAc6ST. Dark field micrographs of the sections are shown. Signal is seen as bright dots. (A) Hybridization with antisense probe, whole lymph node shown. The only source of signal are HEVs, seen as distinctive high-walled vessels in the cortex of the node. (B) Hybridization with sense probe of section adjacent to that in A. (C) Higher magnification view of area indicated by arrow in A. Two large HEVs are evident.

Figure 5. Sulfation of GlyCAM-1/IgG by sulfotransferases. COS cells were transfected with combinations of plasmids encoding GlyCAM-1/IgG, KSGal6ST, and HEC-GlcNAc6ST, as indicated. Transfected cells were cultured in the presence of [35S]sulfate, and recombinant GlyCAM-1/IgG was isolated from the CM. 1% of the captured material was analyzed by SDs-PAGE, and the remainder was subjected to hydrolysis and compositional analysis. (A) Autoradiograph (top) and Coomassie Blue staining (bottom) of SDS gel. Densitometric quantification of the Coomassie Blue-stained bands showed that each lane, except the control lane without GlyCAM-1/IgG plasmid, contained approximately equal amounts of GlyCAM-1/IgG. (B) Sulfated carbohydrates produced in GlyCAM-1 by transfection with HEC-GlcNAc6ST (△) or KSGal6ST (●) were analyzed by Dionex chromatography after acid hydrolysis. The following standards are indicated: 1, GlcNAc c-3SO3−; 2, [35S]SO42−; 3, Galβ1→4SO3−→6]GlcNAc c; 4, [SO3−→6]Galβ1→4GlcNAc c; 5, Galβ3; 6, Galβ3SO3−; 7, GlcNAc c-6SO3−; 8, Galβ3SO3−.
thence, to control for the possibility that the sulfotransferases might cause changes in essential glycosylation parameters that could affect L-selectin binding, we stained the transfected cells with the HECA 452 mAb. This antibody recognizes sLex-related structures and is widely used as a reporter for glycosylation modifications (sialylation and fucosylation) pertinent to selectin ligands (Wagers et al., 1997; Tu et al., 1999). Because this antibody reacts equally well with sulfated (at Gal-6, GlcNAc-6, or both) and nonsulfated sLex structures (Mitsuoka et al., 1998), it was of particular utility for detecting the overall presence of sLex on the cell surface of the transfectants. As shown in Table III, the expression of the HECA 452 epitope was not significantly altered (<25% variation) by transfection with the sulfotransferase cDNAs in any combination. The final issue we investigated was the contribution of CD34 to the expression of L-selectin ligand activity. In striking contrast to the results with G72, the binding of L-selectin/IgM was dependent on the presence of the CD34 protein scaffold, as there was a nearly complete loss of staining when the CD34 cDNA was omitted from the transfection (Fig. 7 C). Interestingly, among the CD34-positive population, only those cells expressing the highest levels stained with the L-selectin/IgM chimera (Fig. 7 A).

Table II. L-selectin/IgM and CD34 Staining of CHO Cells Transfected with Combinations of Sulfotransferase cDNAs

| cDNA transfected | MFI  
|------------------|--------
| KSGal6ST         | HEC-GlcNAc6ST |
| L-sel/IgM staining | CD34 staining |
| μg               |         |
| 0                | 0       |
| 1.0              | 364     |
| 1.5              | 391     |
| 0                | 1.0     |
| 1.0              | 1.5     |
| 0.5              | 0.5     |
| 1.0              | 0.5     |
| 0.5              | 1.0     |
| 0                | 830     |
| 1.0              | 703     |

CHO/FTVII/C2GnT cells were cotransfected with plasmids encoding CD34 (2 μg) and each sulfotransferase singly or in combination in the indicated amounts. Data are expressed as the mean fluorescence intensity (MFI) of L-selectin/IgM staining in the L-sel-IgM/CD34+ population with background signal (from transfectants with CD34 cDNA alone, value 139) subtracted. Also shown is the MFI for staining with a CD34 mAb (Qbend-10) for the population that was positive for CD34, as defined by staining with the class-matched control antibody.

Discussion

Sulfation plays a central role in the interactions of L-selectin and P-selectin with their physiological ligands (Rosen...
and Bertozzi, 1996). PSL L-1, a leukocyte ligand for both P-selectin and L-selectin (McEver and Cummings, 1997), possesses a cluster of sulfated tyrosine residues, which facilitate binding of both selectins (Pouyani and Seed, 1995; Sako et al., 1995; Wilkins et al., 1995; Snapp et al., 1998). As reviewed above, L-selectin recognition of its cognate HEV ligands requires sulfation, as well as fucosylation and sialylation, for optimal binding. For the molecularly defined HEV ligands, sulfation is on sugar moieties rather than tyrosine residues (Rosen and Bertozzi, 1996). Our objective has been the molecular identification of the sulfotransferases that participate in the biosynthesis of these endothelial ligands. The specificities of the desired sulfotransferases were dictated by the analysis of the oligosaccharides of L-selectin–reactive GlyCAM-1, which revealed the presence of Gal-6-sulfate and GlcNAc-c-6-sulfate in the context of Sl(Le)\(^3\) (sialyl 6-sulfo Le\(^3\)) and sialyl 6-sulfo Le\(^3\), respectively). Similarly, the functional glycoforms of CD 34 also contain Gal-6-sulfate and GlcNAc-c-6-sulfate in approximately equal representations (Hemmerich and Rosen, unpublished observations). Histochemical evidence with carbohydrate-directed mAb bs shows that sialyl 6-sulfo Le\(^3\) is prominently displayed on the HEVs of human peripheral lymph node (Mitsuoka et al., 1998), further implicating a GlcNAc-c-6-sulfotransferase activity in the biosynthesis of L-selectin ligands. Bowman et al. (1998) recently characterized such an activity from porcine lymph node, which was highly enriched in HECs.

To identify the relevant sulfotransferases at the molecular level, we probed human EST databases for homologues of the chicken C6/KST, a bona fide Gal-6-sulfotransferase. Here we report the cloning of two carbohydrate sulfotransferases. One is indeed a Gal-6-sulfotransferase, which was independently discovered by Fukuta et al. (1997) and named KSGal6ST. The second enzyme, which we have termed HEC-GlcNAc6ST, is a novel GlcNAc-c-6-sulfotransferase. These two sulfotransferases, together with the chicken C6/KST (Fukuta et al., 1995), and the recently reported human chondroitin-6-sulfotransferase (C6ST, specificity for C-6 of GalNAc) (Fukuta et al., 1998) and GlcNAc-c-6-sulfotransferase (GlcNAc-c6ST) (Uchimura et al., 1998a,b) constitute a family of highly conserved enzymes. Overall, amino acid identities within the family range from 27 to 42%. These enzymes are type II transmembrane proteins with short cytoplasmic tails, features that are typical of glycosyltransferases and carbohydrate sulfotransferases.

Within this new family of carbohydrate sulfotransferases, there are three regions of amino acid sequence in which amino acid identity ranges from 45 to 54% and similarity from 80 to 90% (Fig. 3 C). Regions one and two contain elements that conform to the recently described consensus binding motifs for the high energy sulfate donor, 3'-phosphoadenosine 5'-phosphosulfate. These elements are found in all sulfotransferases characterized to date (Kakuta et al., 1998). In addition, regions one and three contain two stretches of sequence of 11 amino acids each (corresponding to amino acids 124-134 and 328-339, respectively, in the HEC-GlcNAc c6ST sequence) that are highly conserved (>90% similarity). It is possible that these two elements contribute to a binding pocket that interacts with the 6-hydroxyl group of an appropriate oligosaccharide acceptor (Gal, GalNAc, or GlcNAc) to bring it into apposition with the donor phosphosulfate group.

Our characterization of the KSGal6ST and HEC-GlcNAc c6ST supports their involvement in the synthesis of L-selectin ligands. KSGal6ST has a wide tissue distribution which includes HECSs. HEC-GlcNAc c6ST shows a highly restricted, although not absolute, localization to HECs. Second, the sulfotransferases catalyze the two specific sulfation modifications on a recombinant L-selectin ligand that have been established to occur on native ligands. In the case of HEC-GlcNAc c6ST, we have used the G72 mAb to show that transfection with the cDNA leads to 6-sulfation of Sl(Le)\(^3\) on the cell surface of CHO/FTVII/C2GnT cells. This finding establishes that recombinant HEC-GlcNAc c6ST can be used to generate a highly specific sulfated structure that is present on lymph node HEVs (Mitsuoka et al., 1998).

Using a flow cytometry assay based on the binding of an L-selectin/IgM chimera to transfected CHO/FTVII/C2GnT cells, we showed that each enzyme imparts L-selectin binding. Control experiments established that the effects of the sulfotransferases were not due to indirect effects on transfection efficiencies or to global changes in glycosylation parameters of the cells. In contrast to the generation of the G72 epitope, L-selectin/IgM binding required cotransfection of the cells with a CD 34 cDNA. These results indicate that a specific protein scaffold, not present endogenously in CHO cells, is needed for optimal ligand activity, although at least one of the relevant sulfated carbohydrate epitopes (sialyl 6-sulfo Le\(^3\)) can be formed without the provision of such a scaffold. This finding is analogous to the situation with P-selectin, in which Sl(Le)\(^3\) determinants can be formed by transfection of COS cells with an appropriate fucosyltransferase cDNA, but ligand activity requires the inclusion of PSL L-1 cDNA (Sako et al., 1993).

A s noted above, a shared feature of the molecularly defined HEV-associated ligands for L-selectin is the presence of a mucin region (Puri et al., 1995). This feature provides the potential for multivalent presentation of carbohydrate recognition determinants, which is thought to be important for enhancing the avidity of L-selectin interactions (Nicholson et al., 1998). Thus, the CD 34 contribution to ligand activity seen in the present study may be primarily due to its mucin character. The ability of other

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### Table III. L-selectin/IgM and HECA 452 Staining of CHO Cells Transfected with Combinations of Sulfotransferase cDNAs

| cDNA transfected         | MFI |
|--------------------------|-----|
| KSGal6ST                 |     |
| HEC-GlcNAc6ST            |     |
| L-sel/IgM staining       |     |
| HECA staining            |     |

| µg | 0 | 0.5 | 1.0 |
|----|---|-----|-----|
| 0  | 665 | 561 | 496 |
| 1.0| 197 | 697 | 561 |
| 0  | 60  | 636 | 561 |

CHO/FTVII/C2GnT cells were cotransfected with plasmids encoding CD34 (2 µg) and each sulfotransferase singly or in combination in the indicated amounts. Data are expressed as the MFI of L-selectin/IgM staining in the L-sel-IgM/CD34 population with background signal (from transfectants with CD34 cDNA alone, value 182) subtracted. Also shown is the MFI for staining with the HECA 452 mAb for the entire population. The MFI for staining with a control antibody was <10.
involved in the biosynthesis of the sialyl 6-sulfo Lex6ion pattern, HEC-GlcNAc6ST is likely to be specifically
We are currently addressing these issues using a parallel
these parameters are the key determinants of the dynamic
mine the impact of the two sulfation modifications on the
(Warnock et al., 1998). Future experiments must deter-
lymphocytes on HEVs under conditions of blood flow
will be required to resolve these important structural ques-
tions.
L-selectin normally functions in tethering and rolling of
lymphocytes on HEVs under conditions of blood flow (War-
nock et al., 1998). Future experiments must deter-
mine the impact of the two sulfation modifications on
the kinetic parameters of L-selectin binding to ligands,
as these parameters are the key determinants of the dynamic
interactions of lymphocytes with HEVs (AIon et al., 1997).
We are currently addressing these issues using a parallel
plate flow chamber to recapitulate physiological flow dyn-
amics within blood vessels (Lawrence et al., 1995).
On the basis of its activities and highly restricted expres-
sion pattern, HEC-GlcNAc6ST is likely to be specifically
involved in the biosynthesis of the sialyl 6-sulfo Le6 epitope and related structures within L-selectin ligands.
Ultimately, it may be necessary to inactivate the gene in
mice by gene knockout to define the biological roles of
this enzyme. Experiments of this type were required to
demonstrate the critical role for FTVII in the generation
of selectin ligands, including the HEV ligands for L-selec-
tin (Maly et al., 1996). We cannot rule out the possible
involvement of another recently cloned GlcNAc6-sulfotransferase identified in mice and humans (Uchimura et al.,
1998a,b) in ligand biosynthesis. A revealed by in situ hy-
bridization, transcripts encoding the latter enzyme were
present in HECs of mouse lymph nodes, although the
overall tissue expression pattern was extremely broad
in both species, suggesting other functions as well. In this
regard, it should be noted that 6-sulfated GlcNAc residues
are present in the GAG chains of keratan sulfate and a
variety of glycoproteins, including the N-linked oligosaccha-
rides of thyroglobulin (Kamerling et al., 1988), gp120 of
HIV (Shilatifard et al., 1993), and porcine zona pellucida
(Noguchi and Nakano, 1992). Furthermore, the GlcNAc-
c-6-sulfate motif in the context of the core-2 structure has
been identified in respiratory mucins from cystic fibrosis
patients (Lo-Guidice et al., 1994), and a GlcNAc-c-6-sul-
photransferase activity, which can act on mucins, has been
detected in human respiratory mucosa (Degroote et al.,
1997).
With respect to the sialyl 6-sulfo Le6 epitope, our data
on the acceptor specificity and expression pattern of
KSGal6ST are consistent with a possible role for this en-
zyme in the generation of the epitope within HECs. How-
ever, this enzyme (Fukuta et al., 1997), like the GlcNAc-
c-6-sulfotransferase described by U chimura and colleagues
(1998a,b), is broadly distributed, being expressed in all of
the major organs. In analogy with HEC-GlcNAc6ST de-
scribed in the present study, one might predict the exis-
tence of an HEC-specific, or more highly restricted, Gal-
6-sulfotransferase that sulfates L-selectin ligands in vivo.
L-selectin-dependent leukocyte trafficking into lym-
phoid organs and inflammatory sites is likely to be subject
to complex regulation, which may be affected at the level
of L-selectin ligand biosynthesis or by posttranslational modification. Given the dramatic sulfate dependency of the interaction
between L-selectin and its ligands, and the high [35S] sul-
late incorporation seen in HEVs and HEV-like vessels
(Girard and Springer, 1995a), it is tempting to speculate
that regulation of the expression or activity of one or both
of the relevant sulfotransferases represents critical control
points for this process. It is further plausible that the
differential expression of the sulfotransferases may con-
tribute to the apparent variation in the posttranslational modi-
fications of L-selectin ligands in different lymphoid organs
(Berg et al., 1998; Mitsuoka et al., 1998). The expression of
KSGal6ST and HEC-GlcNAc6ST, as well as other candi-
date sulfotransferases, should also be examined in HEV-
like vessels that are induced at sites of chronic inflamma-
tion (Michie et al., 1993; Girard and Springer, 1995a;
Orrust et al., 1996). Endothelial ligands for L-selectin are
also inducible on flat-walled vessels in vivo, although the
biochemical nature of these ligands is poorly understood
(Lev and Teder, 1995). In vitro models of endothelial ac-
vitiation suggest that sulfation is required for the activity of
these ligands (Giuffre et al., 1997; Zakrzewicz et al., 1997;
Tu et al., 1999). Endothelial sulfotransferases that are
implicated in inflammatory leukocyte trafficking would
clearly assume importance as potential targets for antiin-
flammatory therapeutics.

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