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Molecular cloning and characterization of three carbohydrate sulfotransferases: Their possible relevance to the generation of L-selectin ligands

by

Annette Bistrup

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Microbiology

in the

GRADUATE DIVISION

of the

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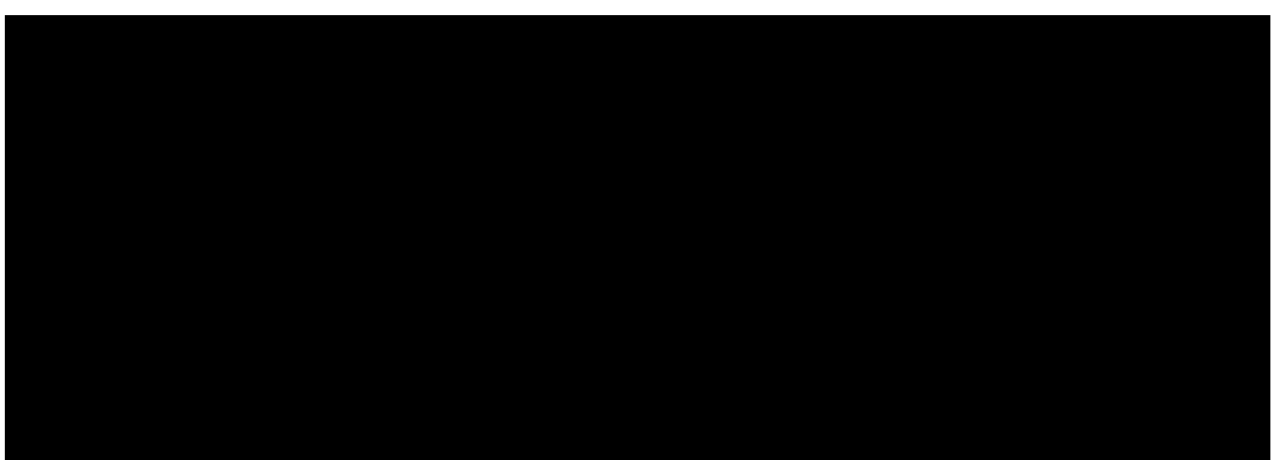

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Molecular cloning and characterization of three carbohydrate sulfotransferases: Their possible relevance to the generation of L-selectin ligands

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by

Annette Bistrup

This treatise is dedicated to my mother,

Inge Margrethe Glahn Bistrup,

and my father,

Fritz Willem Bistrup,

whose unwavering support and infinite love

have sustained me through the many years

and over the many miles of our separation.

Jeg takker jer for jeres kærlighed og tro på mig

fra det dybeste sted I mit hjerte.

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breadth of knowledge in the field of immunology was an inspiration to me, as was her work ethic and approach to problem solving. I am fortunate to have been the recipient of her wisdom and her friendship. I thank Chris Sassetti for always being willing to spare time, even to interrupt his own work, whether to help with a technical problem, discuss a theoretical one, or just to talk. I hope I have learned from his competency and his charitable view of the world, and I have truly enjoyed his company over the years. I am indebted to Kirsten Tangemann for collaborating with me in the characterization of the sulfotransferases and for her moral support during various phases of the project. I have been the privileged to recipient of both Kirsten's and Siegfried Voessner's great hospitality and treasure our many joint musical, bicycling and culinary adventures. I thank Richard Bruehl, Russell Klein, Jin Kyu Lee, Rebecca Newton, Diana Palmeri and Mieke van Zante for their kindness and patience during these past few months. I was fortunate to succeed in recruiting Eugene Belov to work with me during the past year. His assistance with many experiments during the last phase of this project was invaluable, and I thank him in particular for always approaching his work with a seemingly boundless positive attitude. I thank Arceli Joves for her many kindnesses to me, and for being an efficient and resourceful lab caretaker.

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her infectious enthusiasm for their biology have enriched my learning in this somewhat unwieldy area of science. The advice I received from Jason Cyster helped me maintain the perspective of my work as immunology and helped me prioritize experiments to undertake. I greatly appreciate his taking time to discuss aspects of my research with me, as well as his input with respect to publication strategies. Hakon Leffler was extremely helpful in the early stages of my project, discussing both carbohydrate biology and cloning strategies with me, and providing moral support. I thank him for remaining on my committee despite the inconvenience associated with being overseas.

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I have been privileged to enjoy the indulgences of friends, whose forgiveness of my neglect is very much appreciated. First and foremost, I would like to thank Karoline Scheffler for her extraordinary attention to my well being, particularly during these past few months. She has nourished my body with delicious home cooked meals and my spirit with her company. Pam Axelson and Chris Chidsey, and their children Eliza and Gus, provided me with a home for four and a half years and considered me as a family member. I am grateful to them for their love and support, especially during the difficult first year of graduate school. I thank Cynthia Williams for her hospitality and support,

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Molecular cloning and characterization of three carbohydrate sulfotransferases: Their possible relevance to the generation of L-selectin ligands

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ABSTRACT

L-selectin, a lectin-like receptor, mediates rolling of lymphocytes on high endothelial venules (HEV) in secondary lymphoid organs by interacting with HEV-ligands. The HEV-ligands consist of mucin-like glycoproteins bearing O-linked carbohydrates. Candidate ligands are GlyCAM-1, CD34 and podocalyxin. Sialylation, fucosylation and sulfation of the ligands are required for optimal recognition by L-selectin. Structural analysis of the oligosaccharides of GlyCAM-1 revealed the presence of N-acetylglucosamine-6-sulfate and galactose-6-sulfate, in the context of sialyl Lewis x (sialyl 6-sulfo Le^x and sialyl 6'-sulfo Le^x, respectively). Sialyl 6-sulfo Le^x is present on HEV in human lymphoid tissues. This thesis describes the molecular cloning and characterization of three carbohydrate sulfotransferases that may be relevant to the generation of L-selectin ligands in vivo.

Three distinct genes were identified as candidate sulfotransferases in the human expressed sequence tag databases by their homology with the cDNA sequence for a

chicken galactose-6-O-sulfotransferase. The cDNAs corresponding to each of these genes were isolated by screening cDNA libraries utilizing conventional plaque-lifting techniques and PCR. Northern blot and in situ hybridization analysis indicated that two of these genes are broadly expressed, whereas the third is highly restricted to HEV.

The cDNAs encoding each of the genes were expressed in COS cells and extracts of the cells were tested for their ability to sulfate oligosaccharide acceptors in vitro. The sulfated acceptors were subjected to acid hydrolysis and the regiospecificity of sulfation was determined by high-pH anion-exchange chromatography. This analysis established that two of the cDNAs encode N-acetylglucosamine-6-O-sulfotransferases and one encodes a galactose-6-O-sulfotransferase. Coexpression of the sulfotransferase cDNAs with cDNAs encoding GlyCAM-1 or CD34 in COS cells showed that the sulfotransferases are capable of sulfating L-selectin ligands.

Flow cytometry analysis of CHO cells transfected with cDNAs encoding CD34, fucosyltransferase-VII and the sulfotransferase cDNAs indicated that 1) the two N-acetylglucosamine-6-O-sulfotransferases can confer expression of the sialyl 6-sulfo Le^x epitope; 2) either of the three sulfotransferases can contribute to the generation of L-selectin ligand activity; 3) the galactose-6-sulfate and the N-acetylglucosamine-6-sulfate esters apparently synergize in enhancing L-selectin binding. Analysis in a parallel-plate flow chamber indicated that recombinant GlyCAM-1, when sulfated by either of the three enzymes, supports enhanced rolling of lymphocytes.

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LIST OF ABBREVIATIONS

aa:	amino acid
APS:	adenosine phosphosulfate
ATP:	adenosine triphosphate
bp:	base pairs
BFA:	Brefeldin A
C4S:	chondroitin 4-sulfate
C6/KSST:	chondroitin 6-/keratan sulfate sulfotransferase
C6S:	chondroitin 6-sulfate
C6ST:	chondroitin 6-sulfate sulfotransferase
CF:	cystic fibrosis
CHST2:	carbohydrate sulfotransferase 2
CPM:	counts per minute
CS:	chondroitin sulfate
db EST:	database for ESTs
ECM:	extracellular matrix
EGF:	epidermal growth factor
ELISA:	enzyme-linked immunosorbent assay
ER:	endoplasmic reticulum
ESL-1:	E-selectin ligand-1
EST:	xpressed sequence tag
EstST:	estrogen sulfotransferase
FT:	fucosyltransferase
Fuc:	fucose
GAG:	glycosaminoglycan
Gal:	galactose
GalCer:	galactosyl ceramide
gl:	glycolipid
GlcN:	glucosamine
GlcNAc:	N-acetylglucosamine
GluA:	glucuronic acid
GlyCAM-1:	lycosylation-dependent cell adhesion molecule-1
gp:	glycoprotein
GPIbα:	glycoprotein Ibα
GST:	glycosyl transferase
GTP:	guanosine triphosphate

LIST OF ABBREVIATIONS (continued)

HA:	hyaluronic acid
HEC:	high endothelial cells
HEC-GlcNAc6ST:	HEC-specific GlcNAc-6-O-sulfotransferase
HEV:	high endothelial venule
HIV:	human immunodeficiency virus
HPAEC:	high pH anion-exchange chromatography
HPLC:	high-pressure liquid chromatography
HS:	heparan sulfate
HuGlcNAc6ST:	human GlcNAc-6-O-sulfotransferase
HUVEC:	human umbilical vein endothelial cells
kb:	kilobases
KS:	keratan sulfate
KSGal6ST:	keratan sulfate Gal-6-O-sulfotransferase
KSST:	keratan sulfate sulfotransferase
LADII:	leukocyte adhesion deficiency II
Le^x:	Lewis x
LH:	luteinizing hormone
LPS:	lipopolysaccharide
mAb:	monoclonal antibody
MadCAM-1:	mucosal addressin cell adhesion molecule-1
MFI:	mean fluorescence intensity
NCAM-1:	neural cell adhesion molecule-1
NCBI:	National Center for Biotechnology Information
nt:	nucleotides
ORF:	open reading frame
PAPS:	3'-phosphoadenosine 5'-phosphosulfate
PBL:	peripheral blood leukocyte
PNAd:	peripheral node addressin
PSGL-1:	P-selectin glycoprotein ligand-1
RT:	reverse transcriptase
RT-PCR:	reverse transcription-polymerase chain reaction
sgp:	sulfated glycoprotein
Sia:	sialic acid
sLe^x:	sialyl Lewis x
SO_4:	sulfate
SO_3:	sulfonyl
TGN:	trans Golgi network
TNFα:	tumor necrosis factor α

Chapter One

Introduction

The ability of the body to both maintain immune surveillance and mount an effective immune response requires the efficient and selective trafficking of leukocytes from the blood vascular compartment into lymphoid organs and inflammatory sites. The continuous normal circulation of lymphocytes from the blood into lymphoid tissues and back to the blood via the lymphatics ensures that these cells (mostly B and T cells) gain access, and therefore exposure, to the full range of foreign antigens encountered by the organism (Picker and Butcher, 1992; Springer, 1994; Yednock and Rosen, 1989). Leukocytes including neutrophils, monocytes and lymphocytes can also be recruited into sites of acute or chronic inflammation, (Faveeuw et al., 1994; Freemont, 1987; Girard and Springer, 1995b). Leukocyte emigration from the vasculature has been shown to occur in a series of successive but overlapping steps, the end result of which is the translocation of the leukocyte across the endothelial cell layer lining the blood vessel (reviewed in Springer, 1994). Rapidly flowing leukocytes initially make transient contacts (tethering) with the endothelium, which may lead to rolling. These interactions, which occur within the span of seconds and under conditions of great shear stress (Bargatze and Butcher, 1993), are mediated by the selectin family of adhesion molecules (Lasky, 1995; McEver, 1994; Rosen and Bertozzi, 1994; Tedder et al., 1995). Tethering and rolling are prerequisites for the subsequent chemokine-induced activation-dependent, integrin-mediated arrest and firm adherence of the leukocyte on the endothelium

(Springer, 1994). The firmly adherent leukocyte then traverses the endothelial cell layer and basement membrane into the underlying tissue (Anderson et al., 1976). The sequential engagement of different leukocyte receptors with distinct and sometimes tissue specific endothelial ligands provides a mechanism for achieving great specificity in recruitment.

The selectins are critically involved in the earliest steps of leukocyte-endothelial, leukocyte-platelet and leukocyte-leukocyte interactions (reviewed Bevilacqua and Nelson, 1993; Kansas, 1996; Lasky, 1995; McEver, 1994; Rosen and Bertozzi, 1994). There are presently three known selectins: L-selectin, P-selectin and E-selectin. L-selectin is expressed constitutively on all classes of leukocytes, except for a subset of memory T cells. The expression of P- and E-selectin is induced on endothelial cells in response to inflammatory stimuli such as TNFα and LPS. P-selectin expression can also be induced on platelets. The organization of a number shared domain motifs is unique within this family: each selectin contains an amino-terminal lectin domain, followed by an epidermal growth factor (EGF)-like domain and a variable number of short consensus repeats with homology to repeats found in complement regulatory proteins. The selectins are Type I transmembrane proteins with short cytoplasmic tails (Fig. 1.1). The highest degree of amino acid sequence conservation is found within the lectin domains, which are homologous to the lectin domains of other Ca^{++}-dependent (C-type) mammalian lectins (Drickamer, 1993). Indeed, adhesive interactions mediated by the selectins occur through the recognition by their lectin domains of specific carbohydrate structures presented by a discrete set of glycoprotein counter-receptors on the opposing cells. There is some

The Selectin Family of Adhesion Molecules

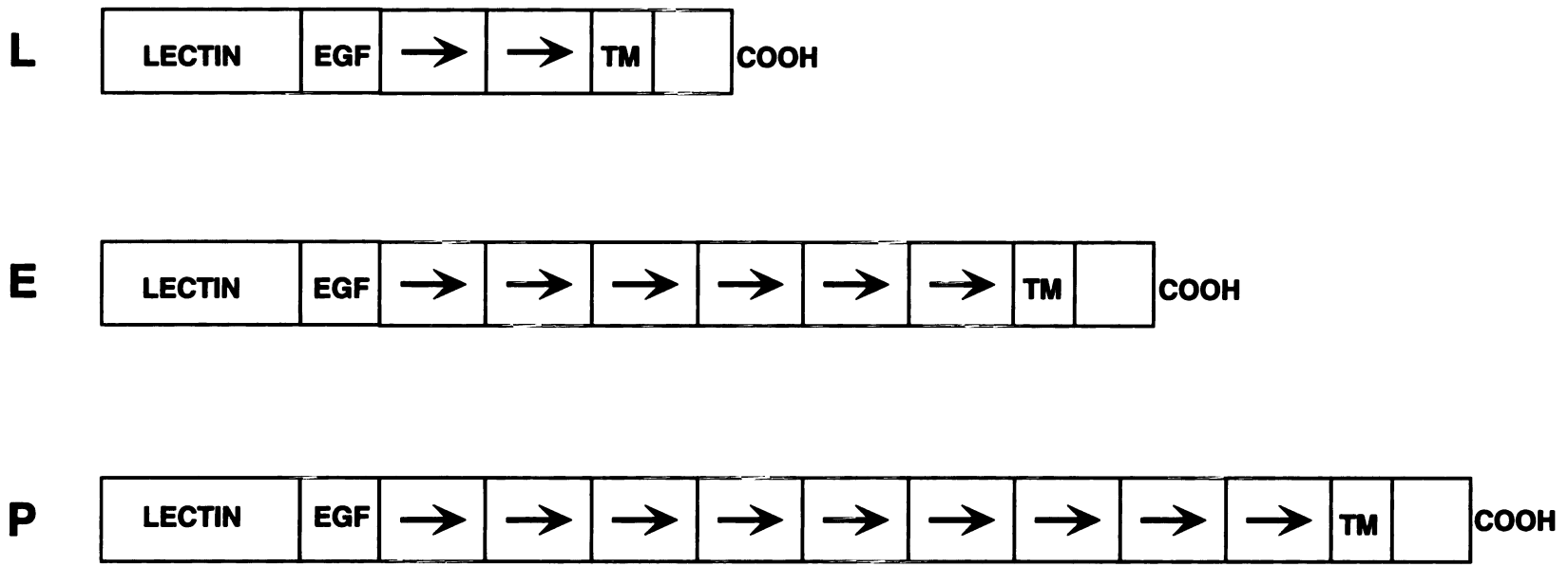


Figure 1.1 The selectin family of adesion molecules

The three members of the family are shown: L-selectin (L), P-selectin (P) and E-selectin (E). Each contains an amino-terminal lectin domain, an EGF-domain and a variable number of short consensus repeats (indicated by arrow) with homology to repeats found in complement regulatory proteins.

evidence that the EGF-like domains may also contribute to ligand binding specificity (Kansas et al., 1994; Kansas et al., 1991; Siegelman et al., 1990).

L-selectin was initially characterized based on its role in the tissue specific homing of lymphocytes to secondary lymphoid organs (Gallatin et al., 1983). An estimated 1.4 x 10^4 lymphocytes per second enter a single lymph node from the blood (Cahill et al., 1976). The specialized structures through which such massive recruitment occurs are postcapillary venules called high endothelial venules (HEV) (reviewed in Girard and Springer, 1995b). HEV are found in all secondary lymphoid organs including lymph nodes, tonsils, Peyer's patches and appendix. The spleen notably does not contain HEV, despite the fact that it supports lymphocyte recirculation far exceeding the combined activity of all the lymph nodes (Kraal, 1992; Pabst, 1988). Lymphocyte entry into the spleen parenchyma is thought to occur via blood sinusoids in the marginal zone (Kraal, 1992; Pabst, 1988). Morphologically, the endothelial cells of HEV are characterized by their "plump" appearance (high endothelial cells, HEC) and their attachment to each other by intermittent, non-occluding junctional complexes (Anderson and Shaw, 1993; Anderson et al., 1976). Large numbers of lymphocytes were first observed to be present within the walls of HEV in 1899 (Schumacher, 1899). HEV are metabolically much more active than flat endothelium and secrete a prominent glycocalyx. It has long been known that HEC incorporate large amounts of ^{35}S-sulfate (^{35}S-SO_4) (Andrews et al., 1980; Girard and Springer, 1995b). HEV-like vessels are present in chronically inflamed tissues from a variety of human chronic inflammatory diseases (Freemont, 1988) and in transgenic and non-transgenic animal models of chronic inflammation (Faveeuw et al.,

1994; Hänninen et al., 1993; Higuchi et al., 1992; Lee and Sarvetnick, 1994; Onrust et al., 1996; Wogensen et al., 1993). Sulfate incorporation has been directly demonstrated (Higuchi et al., 1992) or inferred from the induction of a sulfate-dependent epitope (MECA-79, see below) in several of these (Faveeuw et al., 1994; Hänninen et al., 1993; Lee and Sarvetnick, 1994; Onrust et al., 1996; Wogensen et al., 1993). Acquisition of the HEV phenotype may thus be a key factor in amplifying leukocyte trafficking across vascular endothelium.

HEV-derived ligands for L-selectin consist of a set of glycoproteins that contain mucin-like domains and are heavily O-glycosylated (Puri et al., 1995; Rosen and Bertozzi, 1994). These ligands have been defined biochemically based on their reactivity with an L-selectin/IgG chimera (Imai et al., 1991). Four discrete HEV-associated ligands have been identified in murine lymph node and Peyer's patch. CD34 is a transmembrane sialomucin that is widely expressed on vascular endothelium (Baumhueter et al., 1993). Glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) is a secreted mucin-like glycoprotein produced predominantly in HEV (Brustein et al., 1992; Kikuta and Rosen, 1994). Mucosal addressin cell adhesion molecule-1 (MadCAM-1) is a transmembrane protein which serves as the principal ligand for the leukocyte integrin $\alpha 4\beta 7$ in HEV of Peyer's patch and mesenteric lymph node (Berg et al., 1993; Berlin et al., 1993). The mucin-like domain of MadCAM-1 can function as a ligand for L-selectin in Peyer's patch (Bargatze et al., 1995; Berg et al., 1993). At least one other component, a 200-kd sulfated glycoprotein, sgp 200, remains to be cloned (Berg et al., 1991; Hemmerich et al., 1994b; Hoke et al., 1995). In humans, the HEV-associated ligands for L-selectin are less

well characterized but include a set of 4 discrete protein bands migrating at 65, 105, 160 and 200 kd on SDS-PAGE (Berg et al., 1991). They are all sialomucins (Berg et al., 1991). CD34 constitutes the majority of the 105-kd band and approximately 30% of the total protein in the set (Puri et al., 1995). Recently, podocalyxin was demonstrated to be an L-selectin ligand, constituting at least 75% of the 160 kd component (Sassetti et al., 1998). CD34 and podocalyxin are both type I transmembrane proteins possessing an extensive amino-terminal mucin domain.

An important property of all the above molecules is that their interactions with L-selectin require the appropriate posttranslational modifications. Sialylation (Imai et al., 1991), fucosylation (Maly et al., 1996) and sulfation (Hemmerich et al., 1994b; Imai et al., 1993) are all required for optimal recognition by L-selectin. Polypeptides corresponding to GlyCAM-1, CD34 and podocalyxin are present in tissues such as non-HEV endothelium, mammary gland and kidney podocytes, but the L-selectin reactive glycoforms of these proteins are restricted to HEV (Berg et al., 1993; Dowbenko et al., 1993; Puri et al., 1995; Sassetti et al., 1998).

A variety of evidence indicates the existence of non-HEV endothelial ligands for L-selectin (Giuffrè et al., 1997; Jung et al., 1998; Kansas, 1996; Tu et al., 1999; Zakrzewicz et al., 1997), none of which have yet been identified at the molecular level. CD34 has been ruled out in human umbilical vein (HUVEC) and human cardiac microvascular endothelial cells (Tu et al., 1999; Zakrzewicz et al., 1997). P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes was recently shown to be able to serve as

a ligand for L-selectin (Guyer et al., 1996; Spertini et al., 1996; Walcheck et al., 1996). As with the HEV-derived ligands, L-selectin recognition appears to require the ligands to possess the appropriate posttranslational modifications (Tu et al., 1999).

Several possible functions have so far been determined for the HEV-associated ligands recognized by L-selectin. The biochemically defined set of human ligands described above can support L-selectin mediated tethering and rolling of lymphocytes when coated down as a substratum in a rolling chamber (Puri et al., 1995). Moreover, purified CD34, GlyCAM-1, podocalyxin and MadCAM-1 were each capable of serving as rolling substrates in similar experiments (Berg et al., 1993; Puri et al., 1995; Puri et al., 1997; Sassetti et al., 1998; Dwir et al., 1997). A recently ascribed function for HEV ligands is as a counter-receptor for P-selectin on activated platelets (Diacovo et al., 1996). This interaction would allow for the platelet-mediated recruitment of L-selectin negative lymphocytes, such as memory T cells, to lymphoid organs. Finally, GlyCAM-1 has recently been shown to be able to deliver a signal through L-selectin on lymphocytes that causes the activation of both β1 and β2 integrin function (Giblin et al., 1997; Hwang et al., 1996). As the modulation of integrin function did not occur in the memory T lymphocyte subset, one proposed consequence of this signaling function is the preferential recruitment of naive T cells into the lymph node.

Ligands for P- and E-selectin, while not as well characterized as L-selectin ligands, have also been shown to depend on the presentation of carbohydrate epitopes to the selectin. The major ligand for P-selectin is PSGL-1, which is widely expressed on blood

leukocytes (Moore et al., 1992; Sako et al., 1993). PSGL-1 is a disulfide-linked homodimer of two identical 120 kd polypeptide chains that contain mucin-like domains rich in serine and threonine residues (Sako et al., 1993). E-selectin ligands are expressed on neutrophils, monocytes, natural killer cells, eosinophils and a subset of memory T cells (Kansas, 1996). These ligands consist of specific oligosaccharide structures covalently associated with glycoproteins and glycolipids (Kansas, 1996; Varki, 1994). Recently, a widely expressed 150-kd murine glycoprotein was shown to be a ligand for E-selectin (Levinovitz et al., 1993). Molecular cloning of this protein, termed E-selectin ligand-1 (ESL-1), showed it to be highly homologous (94% amino acid identity) to a chicken fibroblast growth factor receptor (Steegmaler et al., 1995). While not a mucin-like molecule, ESL-1 does contain five potential glycosylation sites, at least some of which appear to be glycosylated in vivo. In addition, PSGL-1 can serve as a ligand for E-selectin (Sako et al., 1993). As is the case with the L-selectin/ligand interactions, only the proper glycoforms of these proteins constitute ligands for P- and E-selectin.

Accumulated evidence indicates that a variety of simple and complex carbohydrate structures, variously containing sialic acid, fucose and sulfate, are capable of binding to the selectins (reviewed in Varki, 1994), but the biological relevance of these specificities is still unknown. Significantly, L- and P-selectin both bind sulfated structures such as sulfatide and heparin, whereas E-selectin does not. A common recognition motif for all three of the selectins is the naturally occurring tetrasaccharide sialyl Lewis x (sLe^x; Siaα2$\rightarrow$3Galβ1$\rightarrow$4[Fucα1$\rightarrow$3]GlcNAc) (Fig. 1.2), which binds all three of the selectins weakly (K_Ds in the millimolar range) (Varki, 1994). Sialylation and fucosylation of the

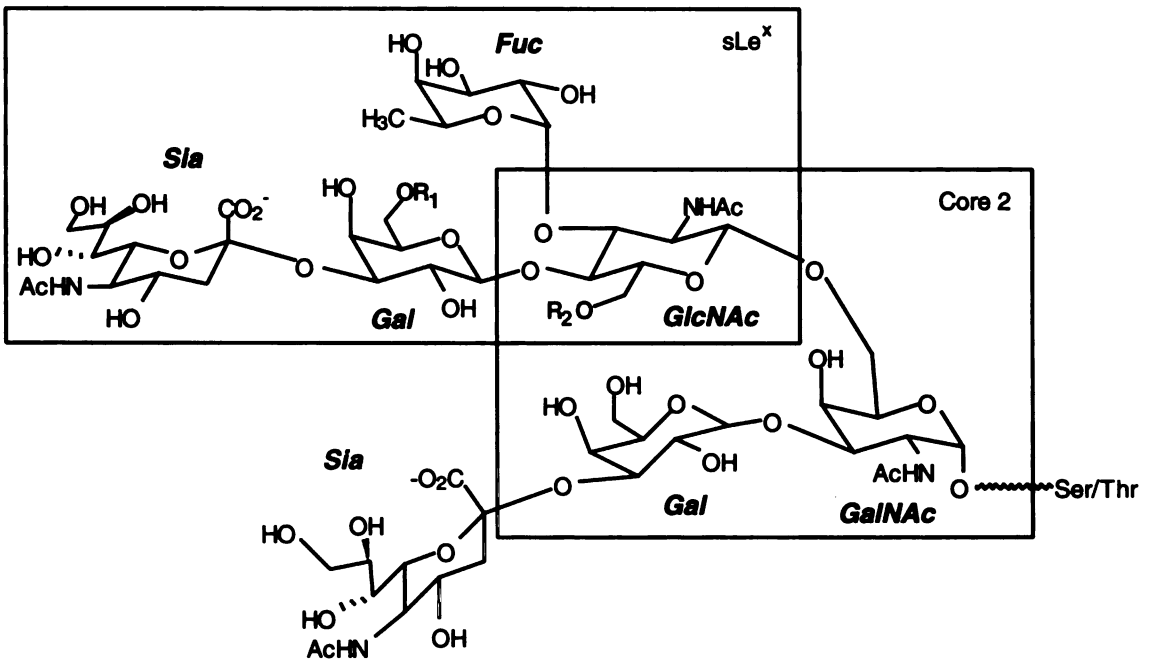
Figure 1.2

The simplest O-glycans of GlyCAM-1, and sialyl Lewis x

(A) Structures of the simplest O-glycans of GlyCAM-1 are shown. These structures were inferred from metabolic labeling studies. The heptasaccharide comprises sialyl Lewis x (sLe^x, indicated by a box and shown in (B)). Sulfate esters were found at C-6 of Gal to form sialyl 6'-sulfo Le^x (structure 1) and at C-6 of GlcNAc to form sialyl 6-sulfo Le^x (structure 2). The presence of sialyl 6'6-disulfo Le^x is suspected. Structures of the more complex O-glycans of GlyCAM-1 remain to be determined.

(B) Structure of sialyl Lewis x is shown. This tetrasaccharide is contained within the O-glycans of GlyCAM-1 shown in (A) and has been shown to bind all three of the selectins.

A

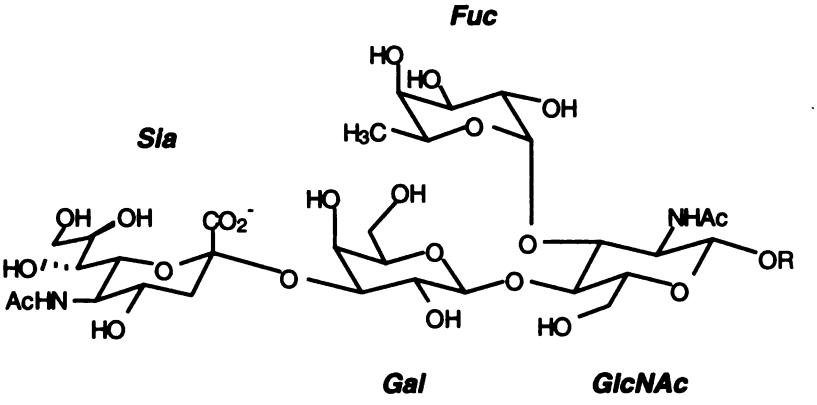


R_1= SO_3; R_2= H : sialyl 6'-sulfo Le^X (structure 1)

R_2= SO_3; R_1= H : sialyl 6-sulfo Le^X (structure 2)

R_1= R_2 = SO_3 : sialyl 6',6-disulfo Le^X

B



sialyl Lewis x, (sLe^X)

core disaccharide (N-acetyllactosamine) (Fig. 1.2) are required for interactions with either selectin (L-, P- or E-selectin) (Feizi, 1993) (See Table 1.1 for nomenclature and structures of several oligosaccharides). The requirement for sialylation is reflected in the sialic acid-dependence of L-selectin interactions with HEV-ligands (Imai et al., 1992; Rosen et al., 1989; Rosen et al., 1985; True et al., 1990). Similarly, fucose was implicated in early metabolic studies as a constituent sugar on functional L-selectin ligands (Imai et al., 1993; Imai et al., 1991).

Table 1.1. Nomenclature and Structure of Oligosaccharides

Name	Structure
sLe^x (sialyl Lewis x)	Siaα2→3Galβ1→4[Fucα1→3]GlcNAc
sialyl 6'-sulfo Le^x	Siaα2→3[SO_3→6]Galβ1→4[Fucα1→3]GlcNAc
sialyl 6-sulfo Le^x	Siaα2→3Galβ1→4[Fucα1→3][SO_3→6]GlcNAc
sialyl 6',6-disulfo Le^x	Siaα2→3[SO_3→6]Galβ1→4[Fucα1→3][SO_3→6]GlcNAc
3'-sulfo Le^x (Glc)	[SO_3→3]Galβ1→4[Fucα1→3]Glc
3'6'-disulfo Le^x(Glc)	[SO_3→3][SO_3→6]Galβ1→4[Fucα1→3]Glc
3'6-disulfo Le^x(Glc)	[SO_3→3]Galβ1→4[Fucα1→3][SO_3→6]Glc
6'-sulfo lactose	[SO_3→6]Galβ1→4Glc
6-sulfo lactose	Galβ1→4[SO_3→6]Glc
6'6-disulfo lactose	[SO_3→6]Galβ1→4[SO_3→6]Glc
Disaccharide acceptor	GlcNAcβ1→6Galα-R
Trisaccharide acceptor	Galβ1→4 GlcNAcβ1→6Galα-R
core 2	Galβ1→3[GlcNAcβ1→6]GalNAc
R	*C*-CH_2-CH_2-$CONH_2$-$(CH_2)_7$-CH_3

The demonstration that mice genetically deficient in fucosyltransferase (FT) VII lack functional HEV-ligands for L-selectin confirmed this hypothesis (Maly et al., 1996). The importance of fucose within functional selectin ligands is further illustrated by the human

genetic disease leukocyte adhesion deficiency II (LADII) (Etzioni et al., 1992; Price et al., 1994; Von Andrian et al., 1993). Neutrophil recruitment into sites of inflammation is severely compromised in LADII patients who suffer recurrent infections and neutrophilia. A global defect in fucose metabolism causes a complete absence of sLe^x structures, and hence selectin ligands, in these patients (Karsan et al., 1998). Using intravital microscopy, von Andrian et al. (Von Andrian et al., 1993) showed that LADII neutrophils roll poorly on exteriorized rabbit venules under shear flow but are fully capable of sticking to the same venules under static conditions, indicating that their integrin mediated adhesion mechanisms are intact.

Another approach to the identification of ligands for L-selectin was taken by Streeter et al. (Streeter et al., 1988a; Streeter et al., 1988b) who prepared a panel of monoclonal antibodies directed against mouse lymph node endothelial cells. One of these, MECA-79, stained lymph node HEV specifically and blocked lymphocyte attachment to HEV *in vitro* as well as lymphocyte homing to peripheral lymph node (Streeter et al., 1988b). MECA-79 recognizes the same set of glycoproteins as L-selectin, in both mouse lymph node and human tonsil (Berg et al., 1991; Hemmerich et al., 1994b; Imai et al., 1991; Sassetti et al., 1998), and these have collectively been termed peripheral node addressin (PNAd) (Streeter et al., 1988a; Streeter et al., 1988b). MECA-79 also recognizes a subset of MadCAM-1 (Berg et al., 1993). Indeed, MECA-79 stains HEV in secondary lymphoid organs in many species, including mouse, human, sheep and pig (Mackay et al., 1992; Michie et al., 1993; Shailubhai et al., 1997; Streeter et al., 1988b). This broad reactivity presumably reflects its binding to a posttranslational modification that is common to many L-selectin ligands (Berg et al., 1991; Hemmerich et al., 1994b). The

MECA-79 epitope is also found on HEV-like vessels that develop at sites of chronic inflammation (Binns et al., 1992; Faveeuw et al., 1994; Hänninen et al., 1993; Lee and Sarvetnick, 1994; Mackay et al., 1992; Michie et al., 1993; Onrust et al., 1996; Salmi et al., 1994; Shailubhai et al., 1997). The presence of the MECA-79 epitope is currently accepted as a predictor of L-selectin ligand activity (Girard and Springer, 1995b; Michie et al., 1993; Onrust et al., 1996), although MECA-79 negative ligands for L-selectin have been identified (Clark et al., 1998; Fuhlbrigge et al., 1996). Although the precise structure of the MECA-79 epitope has not been elucidated, it is known that it does not require fucose or sialic acid (Hemmerich et al., 1994b; Maly et al., 1996; Shailubhai et al., 1997), in sharp contrast to the L-selectin ligands, which, as stated above, must be sialylated (Imai et al., 1991) and fucosylated (Maly et al., 1996) for optimal recognition by L-selectin. The MECA-79 epitope is, however, dependent on sulfation (Hemmerich et al., 1994b; Imai et al., 1993; Shailubhai et al., 1997).

Early characterization of HEV relied on light-microscopic and ultrastructural techniques, as well as metabolic labeling studies. As mentioned above, a striking finding was the distinctive and substantial incorporation of ^{35}S-sulfate by HEV in normal lymph node (Ager, 1987; Andrews et al., 1980; Ise et al., 1988) and by HEV-like vessels at sites of chronic inflammation (Freemont, 1987). By labeling murine lymph nodes in organ culture with ^{35}S-sulfate, Imai et al. (Imai et al., 1991) were the first to demonstrate a direct interaction between L-selectin and sulfated, HEV-derived glycoproteins (GlyCAM-1 and CD34). This interaction has since also been demonstrated for murine sgp 200 (Hemmerich et al., 1994b) and for HEV-ligands in porcine lymph node

(Shailubhai et al., 1997). The sulfate dependence of the interaction was established through the use of metabolic inhibitors of sulfation, namely, chlorate and Brefeldin A (BFA). Chlorate is an inhibitor of ATP sulfurylase, the first enzyme in the synthesis of 3'-phosphoadenosine 5'-phosphosulfate, which is the high energy sulfate donor utilized by mammalian cells in all sulfation reactions (Baeuerle and Huttner, 1986; Klaassen and Boles, 1997). Without affecting protein synthesis, chlorate causes a nearly complete inhibition of sulfation of both protein tyrosine and carbohydrate residues. When isolated from lymph nodes cultured in the presence of chlorate, the murine ligands GlyCAM-1, CD34 and sgp 200, as well as the porcine ligands described above, are unable to bind L-selectin (Hemmerich et al., 1994b; Imai et al., 1993; Shailubhai et al., 1997). In support of these findings, undersulfated GlyCAM-1 from murine lymph nodes cultured in the presence of BFA was completely unreactive with L-selectin (Crommie and Rosen, 1995) (see Chapter Two for a more detailed discussion of the results obtained by Crommie and Rosen with BFA).

Interestingly, in view of the ability of P-selectin to bind sulfated carbohydrate structures (see above), the P-selectin/PSGL-1 interaction has been shown to be require sulfation of PSGL-1 (Pouyani and Seed, 1995; Sako et al., 1993; Wilkins et al., 1995). This is also the case for the L-selectin/PSGL-1 interaction (Spertini et al., 1996). Sulfation of PSGL-1 occurs exclusively at a cluster of three tyrosine residues located in the N-terminal region of PSGL-1 (Spertini et al., 1996). GlyCAM-1, CD34 and podocalyxin all lack tyrosine sulfation motifs in their primary amino acid sequence (Kershaw et al., 1997; Lasky et al., 1992; Simmons et al., 1992).

In an effort to reconcile the known requirements for L-selectin binding, i.e., sialylation, fucosylation and sulfation, with actual structures present on biological ligands, Rosen and colleagues undertook a series of studies aimed at elucidating the nature of the carbohydrate structures present on GlyCAM-1 (Hemmerich et al., 1994a; Hemmerich et al., 1995; Hemmerich and Rosen, 1994; Imai and Rosen, 1993; Imai et al., 1991). The molecule does not contain N-linked sugars, as there was no incorporation of mannose and furthermore, treatment with N-glycanase did not affect the mobility or intensity of radiolabeled GlyCAM-1 on SDS-PAGE (Hemmerich et al., 1994a; Imai et al., 1991). The O-linked sugar chains exhibited considerable size heterogeneity and were shown to contain sialic acid, fucose and sulfate. Anion-exchange chromatography indicated that sulfate groups contribute the majority of the overall charge on the chains (Imai and Rosen, 1993). It is estimated that more than half of the O-linked chains carry sulfate and that of these, 20% are monosulfated, 40% are disulfated and the remainder tri- and tetrasulfated (Hemmerich et al., 1994a; Hemmerich et al., 1995; Imai and Rosen, 1993). Sulfated monosaccharide constituents of GlyCAM-1 were identified as Galactose-6-sulfate (Gal-6-sulfate) and N-acetylglucosamine-6-sulfate (GlcNAc-6-sulfate), both in the context of N-acetyllactosamine (Galβ1→4GlcNAc, Fig. 1.2) (Hemmerich et al., 1994a). Gal-6-sulfate and GlcNAc-6-sulfate were present in approximately equal amounts. No evidence was found for Gal-3-sulfate, despite the fact that L-selectin is known to bind various Gal-3-sulfated structures, e.g., 3'-sulfo Le^x, 3'-sulfo Le^a and sulfatide (Feizi, 1993). Similar results were obtained with murine CD34 (Hemmerich and Rosen, unpublished results).

Further analysis of entire O-linked glycans, released by β-elimination, predicted the simplest structure to consist of 7 sugar units assembled around the core 2 structure (Galβ1→3[GlcNAcβ1→6]GalNAc) (Hemmerich and Rosen, 1994), as shown in Fig. 1.2.A. Significantly, two major capping groups were identified as sulfated versions of sLe^x: sialyl 6'-sulfo Le^x, in which the sulfate ester is at C-6 of Gal, and sialyl 6-sulfo Le^x, in which C-6 of GlcNAc carries the sulfate ester (Hemmerich et al., 1995; Hemmerich and Rosen, 1994). The possibility of a disulfated capping structure (i.e., sialyl 6',6-disulfo Le^x, Fig. 1.2.A) has not been excluded and indeed is likely in view of the demonstrated preponderance of disulfated chains. More complex O-glycans, all based on the core 2 structure, account for the majority of the chains. These are also likely to carry multiple sulfate esters.

Given the approximately equal representation of the two sulfate esters in GlyCAM-1, the question of their respective contribution to L-selectin binding affinity has been of interest for some time. To date, the analysis has been limited to direct binding measurements and competition studies employing sulfated sLe^x structures or analogues thereof. The data are equivocal, as evidence has been presented for enhanced binding (as compared to sLe^x) from the Gal-6-sulfate modification (Koenig et al., 1997; Tsuboi et al., 1996), the GlcNAc-6-sulfate modification (Galustian et al., 1997; Saunders et al., 1996; Scudder et al., 1994) and from both (Yoshino et al., 1997). The inhibition studies of Scudder et al. (1994) and Koenig et al. (1997) showed the inhibitory potency of sulfated sLe^x structures to be enhanced by 2.4 - 4 fold, relative to sLe^x. Significantly, Bertozzi et al. (Bertozzi et

al., 1995) found that several sulfated variants of lactose (Galβ1→4Glc) were equal to or more potent than sLe^x in inhibiting the binding of L-selectin to GlyCAM-1 in an ELISA. For example, 6',6-disulfo lactose (Table 1.1) competed 2-fold better than sLe^x, demonstrating that the relevant sulfate modifications by themselves, in the absence of any contribution from sialic acid or fucose, can confer a significant degree of binding to L-selectin.

Direct evidence for the presence of sialyl 6-sulfo Le^x on HEV has come from Kannagi and colleagues (Mitsuoka et al., 1998a), who examined a panel of mAbs directed against variants of the sLe^x structure. One of these mAbs, G72, reacts with synthetic sialyl 6-sulfo Le^x in a sialic acid- and fucose-dependent manner. G72 stains HEV in human peripheral lymph node intensely and blocks the binding of a human L-selectin/IgG chimera to HEV in vitro.

The inhibition studies described above reported IC_{50} values for sulfated sLe^x and related structures in the μM to low mM range (Koenig et al., 1997; Saunders et al., 1996; Scudder et al., 1994), consistent with data on the binding strength between the selectins and many naturally occurring oligosaccharides (reviewed in Varki, 1994). The interaction between monomeric L-selectin and GlyCAM-1 was shown by Nicholson et al., employing the BIAcore instrument, to occur with a K_D of 100 μM (Nicholson et al., 1998). Considering that IC_{50} values are predicted to overestimate true K_Ds (Hulme and Birdsall, 1992), the implication is that the essential recognition determinants for L-selectin on GlyCAM-1 and the other HEV-ligands are embodied by the sulfated capping

groups. However, under equilibrium conditions, it is known that GlyCAM-1 can bind L-selectin at the cell surface with nanomolar affinity (Giblin et al., 1997; Hwang et al., 1996). Although the basis for this discrepancy in binding strength has not been ascertained, a likely contributing factor is multivalency. A characteristic of many lectins is their oligomeric structure which allows for multiple individual lectin domains to interact with the cognate carbohydrate determinant, thereby dramatically amplifying the intrinsically low-affinity interaction (Drickamer, 1995; Lee and Lee, 1995). Under this view, oligomeric L-selectin in the membrane would bind to sulfated capping groups arrayed along the polypeptide backbone of its HEV ligands. In support of this model Nicholson et al. (Nicholson et al., 1998) found that the affinity of L-selectin immobilized onto the BIAcore chip at high density achieved nanomolar affinity for soluble GlyCAM-1. Furthermore, native L-selectin is tetrameric in detergent solution, as determined by both size-exclusion chromatography and chemical cross-linking (Crommie, 1994). An interesting implication of this model is that the modest enhancement in binding ability conferred by sulfation of individual sLe^x moieties (approximately 4 fold relative to sLe^x, see above) would be magnified to yield a large increase in affinity for the overall interaction (Kiessling and Pohl, 1996; Lee and Lee, 1995; Mortell et al., 1996). This would explain the dramatic sulfate dependency of the interaction of physiological ligands with L-selectin (Hemmerich et al., 1994b; Imai et al., 1993; Shailubhai et al., 1997).

Summary

Taken together, the experiments reviewed in the previous section provide compelling evidence for the contribution by sulfate esters on L-selectin ligands to the interaction between these ligands and L-selectin. Early experiments established that the cell type that produces the ligands and supports the massive recruitment of lymphocytes into lymph node, the HEC, incorporates large amounts of sulfate. Subsequently, it was shown that this metabolic feature, as well as some morphological features of HEC, could be acquired by endothelial cells in chronically inflamed tissues, which also recruit substantial numbers of leukocytes. Rosen and colleagues were the first to establish that the interactions between lymphocytes and HEV involved a carbohydrate-binding molecule on the lymphocytes. An explanation for this observation was obtained upon the cloning of L-selectin, which (together with the two endothelial selectins, P- and E-selectin) was shown to possess a C-type lectin domain. HEV ligands for L-selectin have been shown to be mucin-like proteins bearing abundant O-linked carbohydrates and requiring three specific posttranslational modifications for their interactions with L-selectin: Sialylation, fucosylation and sulfation. The ability of sLe^x and related molecules to inhibit the L-selectin/ligand interaction strongly implicated this structure as part of the binding motif for L-selectin. The sulfate dependence of the L-selectin/ligand interaction was demonstrated by the dramatic finding that GlyCAM-1 isolated from lymph nodes incubated in the presence of chlorate completely lost its ability to bind L-selectin. These observations were rationalized when the structural analysis of the O-

linked oligosaccharides attached to GlyCAM-1 revealed the presence of two sulfated sLe^x variants as major capping groups: sialyl 6-sulfo Le^x and sialyl 6'-sulfo Le^x. Further support for the relevance of these structures to interactions in vivo has been provided by the demonstration that sialyl 6-sulfo Le^x is present on HEV and that antibodies directed against this epitope inhibit the binding of L-selectin to HEV. Taken together, these results strongly implicate sulfated structures including sialyl 6-sulfo Le^x as important recognition motifs for L-selectin on HEV.

Accordingly, we were interested in molecularly identifying and characterizing the sulfotransferases responsible for generating sialyl 6-sulfo Le^x and sialyl 6'-sulfo Le^x on L-selectin ligands. The following chapters will describe our efforts towards reaching this goal. Chapter Two will describe our initial work to develop *in vitro* assays for measuring the two relevant sulfotransferase activities. We used these assays to demonstrate a Gal-6-O-sulfotransferase activity in murine lymph node and, in collaboration with the laboratories of Dr. Carolyn Bertozzi at University of California (UC) at Berkeley and Dr. Stefan Hemmerich of Roche Bioscience, a GlcNAc-6-O-sulfotransferase activity in porcine lymph node. The latter activity was significantly enriched in HEC. Included in this discussion will be a review of other Gal-6- and GlcNAc-6-O-sulfotransferase activities described in the literature and their possible relevance to the lymph node activities. Chapter Three will describe the approach we took to isolating cDNA clones encoding three different sulfotransferases, of which two are GlcNAc-6-O-sulfotransferases and one is a Gal-6-O-sulfotransferase. These proteins constitute a novel family of highly related enzymes consisting as of this writing of five members. And

finally, Chapter Four incorporates our characterization of these enzymes and the demonstration that they can generate epitopes known to be present in HEV as well as L-selectin ligand activity on recombinant L-selectin ligands.

The following sections provide a review of the role of sulfation in other biological systems and a brief introduction to those carbohydrate sulfotransferases or sulfotransferase activities that have so far been described in the literature.

Sulfation as a biological phenomenon

The presence of a covalently attached sulfonyl group (SO_3^-) affects the physicochemical properties and/or biological effects of small and large molecules (reviewed Bowman and Bertozzi, 1999; Klaassen and Boles, 1997). All eukaryotic cells are capable of carrying out sulfation (or more precisely: sulfonation) reactions, the substrates for which include a wide variety of compounds. Two general classes of sulfotransferases exist: The cytosolic sulfotransferases, whose substrates include small, usually hydrophobic, molecules such as steroids, phenols and neurotransmitters (Bowman and Bertozzi, 1999; Falany, 1997), and the Golgi-localized, membrane-bound sulfotransferases which modify protein tyrosine residues and carbohydrates (Bowman and Bertozzi, 1999; Niehrs et al., 1994). The activities of the cytosolic sulfotransferases primarily cause the inactivation and elimination of endogenous metabolites (catabolites) and xenobiotics. In contrast, the

set of biological activities conferred onto their respective substrates by the Golgi-resident sulfotransferases is extremely diverse.

The eukaryotic sulfotransferases catalyze the transfer of a sulfate (or, more precisely, sulfonyl, SO_3^-) group from an activated donor onto the hydroxyl (or amino) group of the acceptor molecule. The activated donor is invariably 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (Klaassen and Boles, 1997). PAPS is synthesized in the cytosol from ATP and SO_4^{2-} by the sequential action of ATP sulfurylase, which generates adenosine 5'-phosphosulfate (APS), and APS kinase. These two activities have been termed PAPS synthetase and are found in mammals within a single, bifunctional protein, the cDNA for which was recently cloned in humans (Girard et al., 1998). The translocation of PAPS from the cytosol into the lumen of the Golgi is mediated by a specific PAPS translocase (Mandon et al., 1994; Ozeran et al., 1996). As will be discussed further in Chapter Three, available evidence suggests that transfer of the sulfate group occurs by an in-line mechanism similar to the one described for the transfer of phosphoryl groups by many kinases (Matte et al., 1998).

Protein tyrosine sulfation

Although protein tyrosine phosphorylation is perhaps more commonly acknowledged as a modulator of molecular (and hence cellular) activity, protein tyrosine O-sulfation is in fact the most common post-translational modification known for this residue (Niehrs et al., 1994). All the proteins so far identified that carry tyrosine sulfate are secretory or

plasma membrane-localized (Niehrs et al., 1994). Although the role of tyrosine sulfation is incompletely understood, the promotion of protein-protein interactions has been established in several systems. A number of tyrosine-sulfated proteins have been identified that are relevant to events occurring in the vasculature, including complement factor C4 (Hortin et al., 1989), coagulation factors V and VIII (Hortin, 1990; Leyte et al., 1991; Pittman et al., 1992), platelet glycoprotein Ibα (GP Ibα) (Dong et al., 1994; Marchese et al., 1995) and PSGL-1 (Spertini et al., 1996; Wilkins et al., 1995). The functional requirement for tyrosine sulfate has been established for several of these. For example, optimal interaction between von Willebrand factor and its ligands factor VIII, GP Ibα and α-thrombin, requires tyrosine sulfation of these ligands (Dong et al., 1994; Leyte et al., 1991; Marchese et al., 1995; Pittman et al., 1992). Similarly, as discussed in a previous section, tyrosine sulfation of PSGL-1 is required for recognition by both L- and P-selectin (Pouyani and Seed, 1995; Sako et al., 1995; Wilkins et al., 1995).

Sulfation of carbohydrates

Like protein tyrosine sulfation, sulfation of carbohydrates constitutes a mechanism for imparting highly specific activities to the macromolecular structures to which the sugars are attached (reviewed in Bowman and Bertozzi, 1999; Hooper et al., 1996). These glycoconjugates represent an extremely diverse set of substrates for sulfation, a fact reflected in the wide variety of activities in which they participate. Glycosylation is the most common post-translational modification of transmembrane and secretory proteins,

and is a feature of many membrane lipids as well. A well established role for extracellular carbohydrate is in the maintenance of the structural integrity of the extracellular matrix (ECM). However, the structures of cell surface-associated glycoconjugates vary dynamically with differentiation state and lineage, suggesting that information transfer is another important aspect of their function. Indeed, the potential for structural diversity inherent to oligosaccharides makes them the ideal vehicle for intercellular communication: There are a great number of different modular units (monosaccharides), which can be assembled in a stereospecific manner into branched oligomers. Furthermore, individual monosaccharide constituents can be covalently modified by acetylation, methylation and phosporylation, in addition to sulfation (Hooper et al., 1996). An additional element of structural diversity derived from sulfate esters on carbohydrate is their propensity to occur in clusters, thus allowing for the presentation of a ligand structure that is composed of discontiguous elements with a particular spatial configuration (Bowman and Bertozzi, 1999; Lindahl et al., 1998; Pittman et al., 1992; Varki, 1994).

Examples of recognition phenomena dependent on carbohydrate sulfation are seen in diverse biological systems including cell adhesion, blood clotting, cytokine sequestration and receptor binding, regulation of enzymatic activity, demyelination, control of the circulatory half-life of pituitary hormones and plant-bacterial symbiosis (reviewed in Bowman and Bertozzi, 1999). Additionally, recent genetic evidence has implicated sulfated carbohydrates in the control of dorsal-ventral patterning in Drosophila (Sen et al., 1998).

Sulfated glycosaminoglycans

The most ubiquitous class of molecules on which sulfated carbohydrates occur are the proteoglycans, which comprise a family of proteins of widely varying size (10-400,000 kDa) located at the cell surface and in the ECM (Lander, 1998). The proteoglycans are defined by a common type of posttranslational modification, the glycosaminoglycan (GAG). GAGs are long, unbranched repeats of heterogeneous disaccharide units attached to the proteoglycan at serine residues. In addition to their well established effect on the biophysical properties of proteoglycans, evidence is emerging that GAGs mediate many of the functions of these molecules as well. The structural diversity of GAGs derives from variations on a limited number of post-polymerization modifications: N-acetylation of glucosamine (GlcN), epimerization and sulfation. To date, four different GAGs associated with proteoglycans have been described: Heparin/heparan sulfate, chondroitin, dermatan, keratan. These differ chiefly in their constituent disaccharide repeat units, and in the extent to which they are sulfated. A fifth GAG, hyaluronic acid (HA), is neither sulfated nor part of a proteoglycan. Sulfation of GAG disaccharide units is most commonly in the form of O-sulfation, although N-sulfation occurs in GlcN of heparin/heparan sulfate.

Heparan sulfate (HS) is synthesized by nearly all cell types in the body and participates in a variety of processes, including cell adhesion (to other cells and to the ECM),

immobilization and activation of diffusible molecules such as growth factors and cytokines, and infection by bacteria and viruses (reviewed in Bowman and Bertozzi, 1999; Salmivirta et al., 1996). The core disaccharide repeat for HS is $(GluA\beta1\rightarrow4GlcNAc\alpha1\rightarrow4)_n$, which can be O-sulfated at the 3 and 6 positions of GlcN and at the 2 position of GluA, in addition to N-sulfation. Heparin differs from HS primarily in its source (mast cells only) and greater degree of sulfation. Specific functions of HS and heparin have been shown to require particular sulfated forms of these molecules (Rosenberg et al., 1997). For example, the protease inhibitor antithrombin, which inactivates several serine proteases of the blood coagulation cascade, binds tightly to defined sulfated structures within HS, and this complex dramatically enhances the inhibitory activity of antihrombin. It was recently shown that this high affinity binding event is critically dependent on the presence of GlcN-3-O-sulfate on HS (Liu et al., 1996), and the 3-O-sulfotransferase activity was shown to be a rate limiting step in generating this structure (Liu et al., 1996). The 3-O-sulfotransferase has been molecularly cloned, as have the 2-O- and 6-O- and three different N-sulfotransferases (Table 3.1, Chapter Three).

Chondroitin sulfate (CS) is abundant in cartilage and brain and participates in both the maintenance of ECM and in specific receptor-ligand interactions (Lander, 1998). Sulfation of the CS disaccharide repeat unit $(GluA\beta1\rightarrow3GalNAc\beta1\rightarrow4)_n$ occurs most commonly on the 4- or 6- position of GalNAc. The ratio of 6- to 4-sulfation varies during development and with the differentiation state of the source cells, and both chondroitin 6-sulfate (C6S) and chondroitin 4-sulfate (C4S) are capable of mediating

specific cellular responses. In the brain, for example, C6S inhibits neurite outgrowth whereas C4S stimulates it (Dou and Levine, 1995). Similarly, C4S, but not C6S, is a cell surface receptor for *Plasmodium falciparum*-infected erythrocytes (Chaiyaroj et al., 1996). cDNAs encoding C6S sulfotransferases have been cloned in chicken, mouse and human (Table 3.1, Chapter Three) and a C4S sulfotransferase activity was recently purified from rat chondrocytes (Yamauchi et al., 1999).

Another principal component of cartilage is keratan sulfate (KS) which is also abundant in cornea and brain (Lander, 1998). The disaccharide repeat of KS is $(Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow3)_n$, which can bear sulfate at the 6-position of both Gal and GlcNAc. Although specific roles for the different sulfated forms of KS have not been established, they are suspected because of the regulated expression of KS during development and in disease (Edward et al., 1990; Nakazawa et al., 1995). A human cDNA clone encoding a KS Gal-6-O-sulfotransferase was recently reported (Fukuta et al., 1997) (Table 3.1, Chapter Three), which also can generate GalNAc6-sulfate on CS (Mazany et al., 1998). This substrate profile mirrors that found in a cloned chicken sulfotransferase, which was characterized as a KS/CS Gal-6-O-sulfotranfserase (Fukuta et al., 1997), as which will be discussed further in Chapter Three.

Sulfated glycoproteins and glycolipids

The sulfated mucin-like ligands for L-selectin described in the first part of this chapter represent an example of another type of carbohydrate structure that undergoes sulfation: Oligosaccharides attached to glycoproteins (gp) or glycolipids (gl). Other examples of such sulfated macromolecules include respiratory and colonic sulfated mucins, sulfated gl such as sulfatide and the bacterial nodulation factor Nod, and sulfated gp such as luteinizing hormone (LH), human immunodeficiency virus (HIV) gp120 and porcine zona pellucida (reviewed in Bowman and Bertozzi, 1999; Hooper et al., 1996). In many cases, sulfation of the oligosaccharide converts a common structure into a unique recognition motif for specific binding by a receptor, thereby significantly affecting the biological activity of the associated macromolecule. As discussed above, this principle clearly applies to the interaction between L-selectin and its ligands.

The acquisition of specificity for a particular receptor upon sulfation has been well documented for the gp LH (Hooper et al., 1996). LH exhibits a highly pulsatile presence in the bloodstream, a feature that is required for its biological activity. The regulation of this fluctuation is at least partly achieved at the level of clearance, which is mediated by a receptor expressed on hepatic endothelium and Kupffer cells. Unlike most gp hormones, which terminate in Siaα2/3→6Galβ1→4GlcNAc, LH carries N-linked oligosaccharides terminating in [SO_3→4]GalNAcβ1→4GlcNAc, and it is this unique terminal structure which is recognized by the hepatic receptor. The corresponding GalNAc-4-O-

sulfotransferase has been purified and extensively characterized (Hooper et al., 1995; Skelton et al., 1991) but has so far not been molecularly cloned.

A sulfated oligosaccharide epitope recognized by the mAb HNK-1, which was raised against human natural killer cells, has in recent years been shown to be carried by a variety of cell-surface gl and gp involved in cell adhesion (reviewed in Schachner et al., 1995). These include the neural cell adhesion molecule-1 (NCAM-1), myelin-associated gp, L1, P0, tenascin and some integrins. Recognition of these molecules by HNK-1 is completely sulfate dependent (Ilyas et al., 1990). Structural analysis of both the glycolipid and glycoprotein forms of the epitope revealed it to terminate in $[SO_3 \rightarrow 3]GluA\beta 1 \rightarrow 3Gal\beta 1 \rightarrow 4GlcNAc$. Indeed, many of the demonstrated functions of the sulfated carbohydrate antigen have been shown to require the presence of the sulfate ester. For example, laminin binding and neural cell migration mediated by the HNK-1 epitope require the GluA moiety to be sulfated. The HNK-1 antigen is expressed predominantly in the nervous system (central and peripheral) in a spatially and developmentally regulated manner (Chou et al., 1991; Holley and Yu, 1987; Low et al., 1994; Prasadaro et al., 1990; Wernecke et al., 1985). This antigen is recognized in a sulfate dependent manner by autoantibodies in human peripheral demyelinative neuropathy (Ariga et al., 1987; Chou et al., 1986), and it is suspected to play a role in pathology (Schachner et al., 1995). cDNAs encoding a sulfotransferase that directs the synthesis of the HNK-1 epitope were recently obtained in rat (Bakker et al., 1997)and human (Ong et al., 1998) (Table 3.1, Chapter Three). The expressed sulfotransferase transferred sulfate to GluA in gl and in the gp NCAM-1 (Ong et al., 1998), suggesting

that one sulfotransferase may be capable of modifying both types of macromolecules in vivo.

Sulfoglycolipids (sulfatides) are abundant in the brain (primarily associated with myelin), spermatozoa, kidney and small intestine (reviewed in Vos et al., 1994) and have been implicated in many physiological functions through their specific interactions with molecules such as von Willebrand factor, human growth factor, laminin and thrombospondin. The major mammalian species of sulfoglycolipids is $[SO_3 \rightarrow 3]$galactosylceramide (GalCer). The human GalCer 3-O-sulfotransferase was recently cloned (Honke et al., 1997) (Table 3.1, Chapter Three). It is expressed at high levels in human renal cancer cell lines (Honke et al., 1998), but the significance of this observation has not been established.

The utilization of sulfated carbohydrates to provoke particular cellular responses is conserved in a fascinating biological system of information exchange across species. Nitrogen-fixing bacteria of the genus *Rhizobium* engage in a symbiotic relationship with leguminous plants such as pea, alfalfa and vetch by inducing the formation of plant nodules through which the bacterium invades the plant tissue (reviewed in Denarie and Cullimore, 1993; Long and Staskawicz, 1993). Many of these bacteria have a very narrow host range, which is determined by so-called nodulation (Nod) factors synthesized and secreted by the bacteria. For example, the exclusive nodulation of alfalfa by *R. meliloti* is initiated by a specific sulfated lipo-oligosaccharide consisting of $COC_{15:2}GlcN\beta1 \rightarrow 4(GlcNAc)_2$-$[SO_3 \rightarrow 6]GlcNAc$. Indeed, mutant *R. meliloti* which

synthesize only non-sulfated forms of the same lipo-oligosaccharide acquire a new host (vetch) while simultaneously losing the ability to symbiose with alfalfa. The GlcNAc-6-O-sulfotransferase that sulfates the reducing-terminal GlcNAc residue has been extensively characterized (Ehrhardt et al., 1995) and is the product of the nodH gene located in the nod gene cluster of *R. meliloti* (Roche et al., 1991) (Table 3.1, Chapter Three).

In addition to the specific purified sulfotransferase activities or clones described above, a number of other sulfotransferase activities have been described but not yet fully characterized. These include Gal-3-O-sulfotransferase activities (distinct from the GalCer 3-O-sulfotransferase) in human bronchial mucosa (Lo-Guidice et al., 1995), rat colonic mucosa (Kuhns et al., 1995) and human colorectal cancer cells (Vavasseur et al., 1994) The activity of the latter sulfotransferase appeared to be decreasing with the state of malignant transformation, reflecting the decreased mucin sulfation observed in ulcerative colitis and colon and breast cancer cells (Brockhausen et al., 1995; Corfield et al., 1992; Raouf et al., 1992; Yamori et al., 1987). Sulfotransferase activities that modify C-6 of Gal or GlcNAc have been described and will be discussed further in Chapter Two.

Sulfated carbohydrates on CD43 and CD44

Several gp expressed on immunologically relevant cells have recently been shown to bear sulfated carbohydrates. One is CD43, the major sialoglycoprotein on T lymphocytes,

thymocytes and neutrophils (Cyster et al., 1990; Killeen et al., 1987; Pallant et al., 1989). CD43 is also expressed on activated (but not resting) B cells and other leukocytes (Brown et al., 1981; Nathan et al., 1993; Shelley et al., 1990). CD43 is heavily substituted with sialylated, O-linked oligosaccharides (Carlsson and Fukuda, 1986; Fukuda et al., 1986; Remold-O'Donnell et al., 1984; Remold-O'Donnell et al., 1987), the structure of which varies among cell types (Carlsson et al., 1986) and with T cell activation (Carlsson et al., 1986). The large amount of negative charge produced at the cell-surface by CD43 is thought to help prevent non-specific adhesion among leukocytes; however, proadhesive activities of CD43 have also been described (de Smet et al., 1993; McEvoy et al., 1997; Sanchez-Mateos et al., 1995; Sperling et al., 1995). The glycosylation pattern of CD43 has been shown to be altered in T lymphocytes from individuals with leukemia (Saitoh et al., 1991) and the immunodeficiency Wiscott-Aldrich syndrome (Higgins et al., 1991; Piller et al., 1991), as well those infected with (HIV) (Giordanengo et al., 1995a). Furthermore, autoantibodies to this altered glycoform are detectable in HIV-infected individuals (Giordanengo et al., 1995a). Wilson et al. were the first to report sulfation of CD43, in the murine T lymphoma line RDM-4 (Wilson and Rider, 1992). The sulfate was almost exclusively associated with O-linked carbohydrates, and there was no evidence for tyrosine sulfation. Subsequent reports from Lefebvre et al. demonstrated sulfation of human CD43 from the HIV-infected T cell line CEM (Lefebvre et al., 1994) and its possible role in homotypic aggregation of cells (Giordanengo et al., 1995b). (These workers also reported the immunoprecipitation of sulfate-labeled CD45; however, no further characterization of this molecule was presented, and it is therefore difficult to assess the significance of this finding (Giordanengo et al., 1995b)) . The exact structures

in CD43 to which the sulfate esters are attached have not been elucidated, nor has there been a description of a sulfotransferase activity capable of modifying this molecule. Clearly, given that alterations in CD43 glycosylation are implicated in several pathogenic processes, the possibility of regulated changes in sulfation patterns raises interest in the identification of the associated sulfotransferase(-s).

Finally, a provocative recent report from Maiti et al. suggested a role for carbohydrate sulfation in the interaction between CD44 on activated leukocytes and endothelium (Maiti et al., 1998). CD44 is a widely expressed and tightly regulated cell adhesion molecule implicated in a variety of processes including cell motility, growth control, tumor metastasis and lymphocyte activation (Lesley et al., 1993). Several alternatively spliced forms of CD44 exist, all of which contain mucin-like regions that are heavily substituted with O-linked carbohydrate, as well as sites for N-linked glycosylation. The cell surface expression of CD44 on leukocytes is increased in response to inflammatory stimuli, and its activity is regulated by several mechanisms, including clustering, phosphorylation and alterations in glycosylation. The principal ECM ligand for CD44 on leukocytes is HA. The interaction between CD44 and HA requires activation of the leukocytes by antigen or cytokines (Kincade et al., 1997; Lesley et al., 1993). The CD44-mediated binding of activated memory/effector T-cells to HA has been implicated in their rolling and extravasation at sites of inflammation (Camp et al., 1993; Clark et al., 1996; DeGrendele et al., 1996; DeGrendele et al., 1997). Maiti et al. (Maiti et al., 1998) studied the effects of TNF-α treatment on the adhesive state of CD44 expressed by cells of the human leukemic line SR91. TNF-α is known to promote leukocyte adhesion at sites of

inflammation (Bevilacqua, 1993). As expected, TNF-α caused increased cell surface expression of CD44 (and ICAM-1) and induction of HA binding. Furthermore, TNF-α treated SR91 cells adhered to the murine endothelial cell line SVEC4-10 in a CD44- and HA-dependent manner. The novel and remarkable finding was that the induced CD44-mediated binding of SR91 cells to HA and SVEC4-10 required sulfation of the CD44 molecule, as chlorate treatment of the SR91 cells completely abrogated the binding. Indeed, sulfation of CD44 was significantly increased upon TNF-α treatment. The sulfate is apparently associated with carbohydrate, as there was no evidence for sulfotyrosine by amino acid analysis. The question of the degree to which the sulfate is associated with O-linked carbohydrate has not been resolved; however, preliminary evidence favors significant sulfation on O-linked and none on N-linked sugars (P. Johnson, personal communication).

These results imply that induction of CD44 sulfation on leukocytes is one potential mechanism for regulating their adhesion to endothelium. They therefore represent a significant advance in our understanding the mechanisms of leukocyte-endothelial interactions and the role of sulfation in promoting these interactions. First, when considered together with the sulfate dependence of the interactions between the selectins and their ligands, this represents a dramatic validation of the concept of sulfated carbohydrates as a mediators of specific information transfer between cells participating in the maintenance of immunity. Second, these experiments provide a novel context in which to consider the regulation of cell adhesion by induction of enzymatic activities that produce posttranslational modifications. As reviewed above, evidence in support of this

notion has been obtained for the selectin/ligand interactions with respect to both the fucosylation and the sulfation of the ligands. Fucosylation of P-selectin ligands has been shown to be required to attain optimal leukocyte recruitment to a site of acute inflammation (Maly et al., 1996) and this activity was shown in that particular model to be largely dependent on a single enzyme, FTVII (Maly et al., 1996). Furthermore, the activation of human peripheral blood T cells induces the coordinate expression of sLe^x-like structures, E-selectin binding and FTVII activity (Knibbs et al., 1996). With respect to sulfation, there is ample evidence of the induction of sulfated ligands for L-selectin at sites of chronic inflammation (reviewed above). Several recent reports (Tu et al., 1999; Zakrzewicz et al., 1997) showed the induction of L-selectin ligand activity on TNF-α activated human endothelium that was at least partially inhibited by chlorate treatment (Tu et al., 1999; Zakrzewicz et al., 1997). The direct demonstration by Maiti et al. that sulfation of CD44 was induced with TNF-α treatment provides a strong impetus for further study of the mechanisms of such induction. Finally, the identity and function of the sulfotransferase(-s) responsible for sulfating CD44 carbohydrates are obviously of great interest. The elucidation of its/their relatedness to the sulfotransferases that sulfate L-selectin ligands is a fascinating area for future study.

Summary

In summary, sulfation of macromolecules is a mechanism used by cells to impart highly specific information to other cells and their environment. Sulfated carbohydrates are particularly well suited for this function, as the combinatorial biosynthesis of these

molecules provides an exponential capacity for structural diversity. The versatility of information transfer by sulfated carbohydrates is illustrated by the conservation of this mechanism from bacterial to mammals. The recent reports that sulfation of two immunologically relevant molecules, distinct from selectin ligands, affects their function provides further impetus to the identification of sulfotransferases that modify such molecules, including the ligands for L-selectin.

Chapter Two

Detection of a Galactose-6-O-sulfotransferase Activity in Murine Lymph Node by an *in vitro* Sulfotransferase Assay

Chapter One provided a review of the essential contribution of sulfation in defining L-selectin binding activity within HEV-associated ligands. Our structural analysis of GlyCAM-1 and CD34 had indicated that the sulfate groups are introduced to an equal extent at the hydroxyl groups of C-6 of Gal and C-6 of GlcNAc within the capping sLe^x groups. Our major objective was to identify and characterize the sulfotransferases that catalyze these modifications. For this purpose, we needed specific and sensitive sulfotransferase assays for the relevant activities.

Initially, we focused our attention on the Gal-6-O-sulfotransferase activity. With respect to sulfation at C-6 of Gal, there have been several reports of N-acetyllactosamine (Siaα2→3Galβ1→4GlcNAc), structures in which Gal is substituted with sulfate on C-6 (and Sia on C-3), however, these were not shown to be in the context of sLe^x (Brown et al., 1994a; Brown et al., 1994b; Pfeiffer et al., 1992). Recently, a Gal-6-O-sulfotransferase activity capable of modifying Siaα2→3Galβ1→4GlcNAc was reported to be present in rat spleen, testes, brain and lymph node (Spiro and Bhoyroo, 1998). This activity required the Gal to be modified with α2→3 linked Sia, implying that sialylation must precede Gal-6-sulfation. Interestingly, the Gal-6-O-sulfotransferase had no activity

towards the acceptor sLe^x (Siaα2→3Galβ1→4[Fucα1→3]GlcNAc), implying that fucosylation occurs after 6'-sulfation in the generation of sialyl 6'-sulfo sLe^x. This result is in apparent disagreement with those of Jain (Jain et al., 1994) and Chandrasekaran et al. (Chandrasekaran et al., 1996) who reported that neither 6'-sulfo N-acetyllactosamine ([SO_3→6]Galβ1→4GlcNAc) nor sialyl 6'-sulfo N-acetyllactosamine (Siaα2→3[SO_3→6]Galβ1→4GlcNAc) could serve as acceptors for several FTs, including the cloned FTs III, IV or V. Significantly, FTVII, which has been shown to be critical for the generation of L-selectin ligands in vivo (Maly et al., 1996), was also unable to synthesize sialyl 6'-sulfo Lex from sialyl 6'-sulfo LacNAc. It is therefore unlikely that the spleen Gal-6-O-sulfotransferase characterized by Spiro et al. (Spiro and Bhoyroo, 1998) is the activity responsible for sulfating L-selectin ligands in vivo.

To develop at Gal-6-O-sulfotransferase assay, we followed the approach taken by the Baenziger laboratory in their study of the pituitary GalNAc4-O-sulfotransferase (Hooper et al., 1995; Skelton et al., 1991), in which they utilized an oligosaccharide mimic of the natural structure as an acceptor. The principle of this assay is the detection of a transfer of ^{35}S-SO_4 from ^{35}S-PAPS, the universal sulfate donor (Farooqui, 1980), to an acceptor structure. Due to synthetic considerations, we initially focused on the simple disaccharide lactose (Galβ1→4Glc) which conserves the essential elements of the core disaccharide of GlyCAM-1 (Galβ1→4GlcNAc, see Fig 1.2 Chapter 1). We synthesized a hydrophobic glycoside of lactose by first reacting it with allyl amine to form β-allylamino lactose, which was then reacted with pelargonyl chloride to generate the lipid-conjugated lactose derivative (Fig. 2.1).

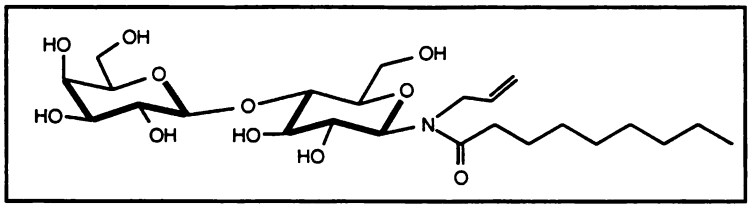


Figure 2.1. Lipid-conjugated lactose as a sulfotransferase acceptor.

The hydrophobic lipid (pelargonyl) group allows for the use of a reversed-phase C-18 column for separation of the sulfated glycoside from components in the reaction mixture. As a source of sulfotransferase activity, we initially used a Triton X-100 extract of microsomes prepared from mouse lymph nodes. The membrane extract was mixed with ^{35}S-PAPS, ATP, NaF, Mg^{++}, glycerol, protease inhibitors and the lipid-conjugated lactose acceptor. After incubation for 1 hr at 37°C, the sulfated product was isolated by passage of the reaction mixture over a C-18 column, followed by washing in aqueous buffer and elution with methanol (Skelton et al., 1991). Sulfate was incorporated into the acceptor in a time-dependent (Fig. 2.2.A), extract-dependent (Fig. 2.2.B), and acceptor-dependent manner (Figure 2.4)

In order to determine where the sulfate modification occurred, purified ^{35}S-labeled acceptor was subjected to acid hydrolysis. The hydrolysate was passed over a C-18 column as above and the flow through, containing a mixture of non-lipid-associated mono- and oligosaccharides, was collected and lyophilized. The lyophilized material was

Figure 2.2

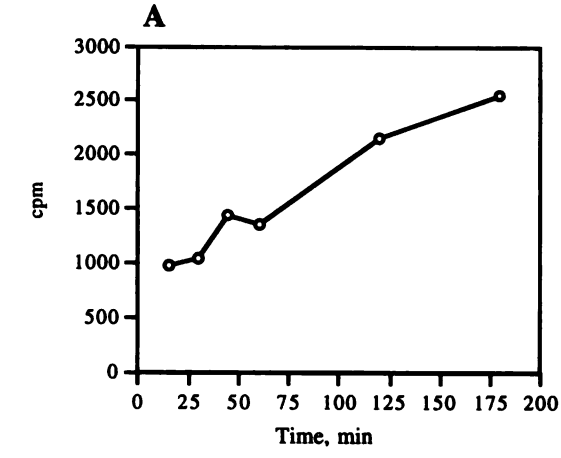
Lymph node sulfotransferase activities towards the lipid-conjugated lactose acceptor and analysis of the regiochemistry of sulfation on lactose by these activities

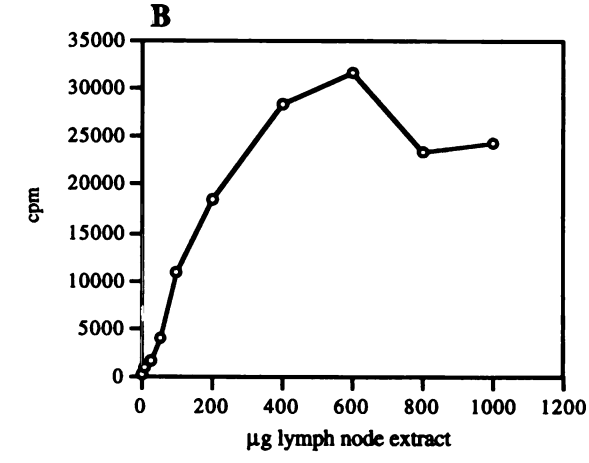
Lymph node extracts were incubated in the presence of ^{35}S-PAPS and lipid-conjugated lactose. Incorporation of sulfate was measured as a function of time and concentration of extract. The sulfated acceptor was subjected to hydrolysis and analysis by high pH anion-exchange chromatography (HPAEC).

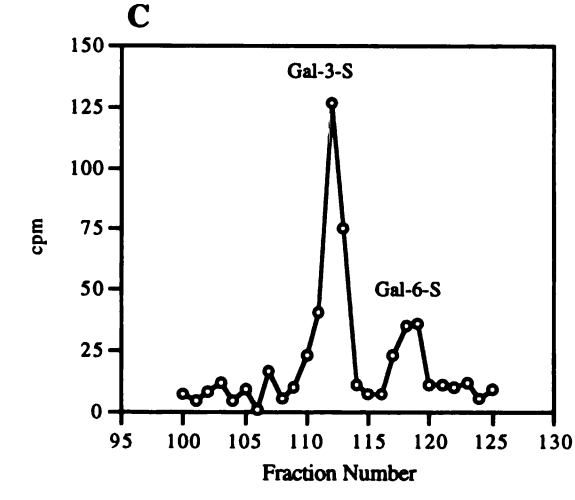
(A) Time dependence of the transfer of sulfate. Incorporation of sulfate into lactose increased linearly for up to 3 hours. Concentration of lipid-conjugated lactose was 10mM. Data points are representative of two separate experiments.

(B) Transfer of sulfate as a function of the concentration of lymph node extract. Incorporation of sulfate into lactose was measured for one hour. Concentration of lipid-conjugated lactose was 10mM. Incorporation of sulfate into lactose increased linearly with the amount of lymph node extract below concentrations of 500 μg/ml. Data points are representative of two separate experiments.

(C) Sulfated monosaccharides produced by lymph node sulfotransferase activities acting on the lipid-conjugated lactose acceptor. The isolated sulfated acceptor was subjected to acid hydrolysis followed by anion exchange chromatography and then HPAEC (Dionex). The retention times (in terms of the fraction number) of authentic sulfated monosaccharide standards by HPAEC are shown.


A
3000
2500
2000
1500
1000
500
0
cpm
0 25 50 75 100 125 150 175 200
Time, min


B
35000
30000
25000
20000
15000
10000
5000
0
cpm
0 200 400 600 800 1000 1200
µg lymph node extract


C
150
125
100
75
50
25
0
cpm
Gal-3-S
Gal-6-S
95 100 105 110 115 120 125 130
Fraction Number

applied to an anion-exchange column to obtain monosulfated saccharides. These were then applied to a high pH anion-exchange column (HPAEC, Dionex). The specific site of sulfate incorporation was determined by comparing the elution profile of the sulfated monosaccharides from the acceptor with those of known sulfated monosaccharides (Hemmerich et al., 1994a; Hemmerich et al., 1995). This analysis revealed major peaks corresponding to Gal-3-sulfate and Gal-6-sulfate (Fig. 2.2.C). In order to eliminate the Gal 3-O-sulfotransferase activity , which was not of interest (since this modification is not detected in GlyCAM-1 or CD34), we synthesized lipid conjugates in which C-6 of Gal was substituted with either sialic acid (3'-sialyllactose) (Fig. 2.3) or acetate (3'-OAc-lactose, not shown).

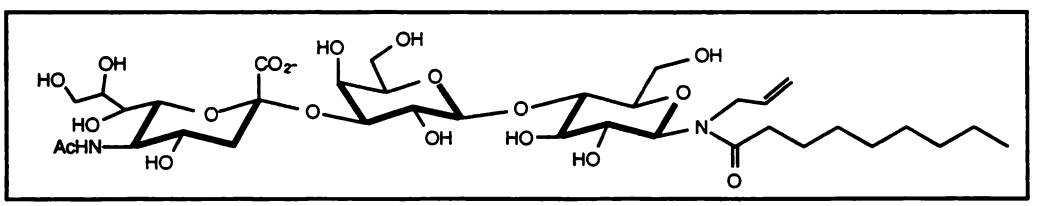


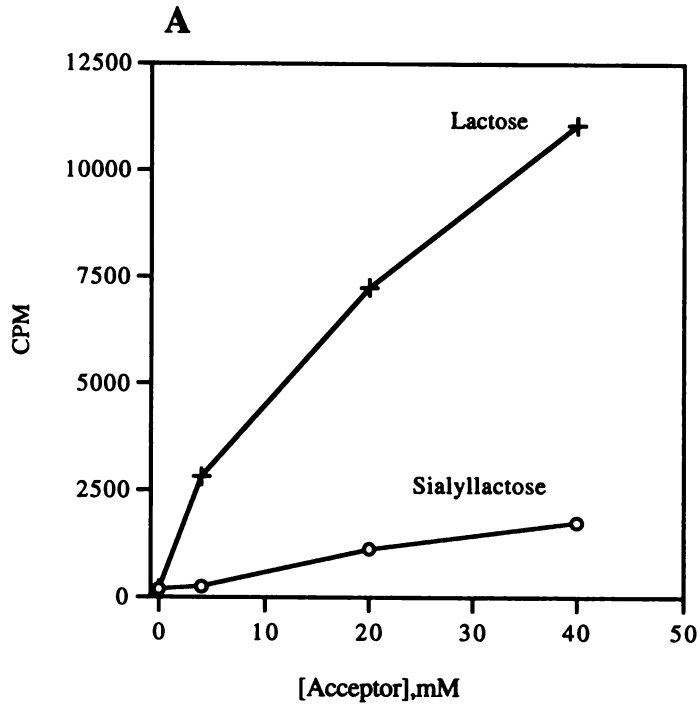
Figure 2.3. Lipid-conjugated 3'-sialyllactose as an acceptor for Gal-6-O-sulfotransferase.

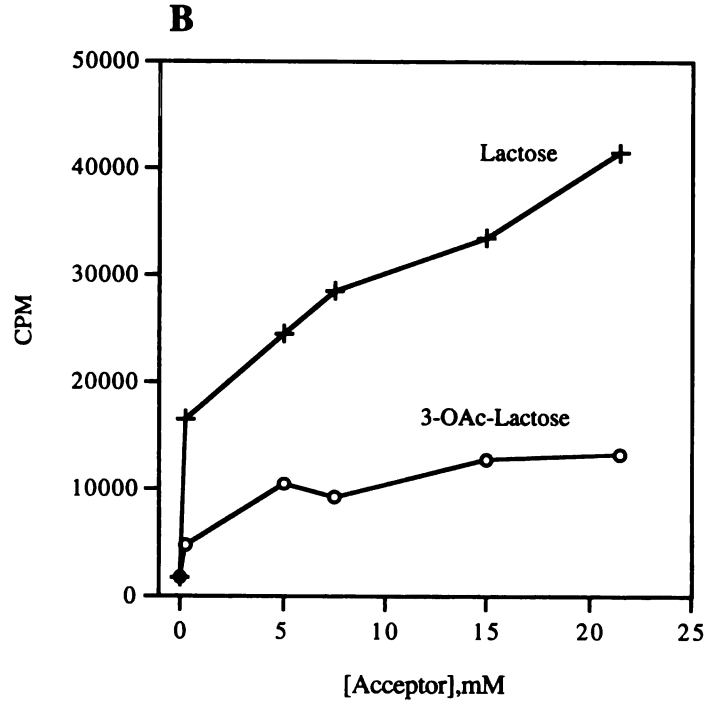
When analyzed in parallel with the lactose-lipid acceptor, either of the 3'-modified lactoses incorporated significantly fewer counts per minute (CPM) than lactose, consistent with the elimination of 3'-sulfation (Fig. 2.4.A and 2.4.B). In addition, incorporation of sulfate into the 3'-modified lactoses approached saturation with increasing concentrations of acceptor (range of 5-25mM concentrations), in contrast to the results obtained with lactose (Fig 2.4).

Figure 2.4

Lymph node sulfotransferase activities towards the lipid-conjugated acceptors lactose, 3'-sialyllactose and 3'-O-acetyllactose

Lymph node extracts were incubated in the presence of ^{35}S-PAPS and the lipid-conjugated acceptors at the indicated concentrations for one hour. The presence of sialic acid (A) or an acetate group (B) at the 3'-position of lactose significantly reduced the level of sulfate incorporation into the acceptor. The two graphs reflect data from different experiments. The data points for each graph represent a single experiment.


A
12500
10000
7500
5000
2500
0
CPM
Lactose
Sialyllactose
0
10
20
30
40
50
[Acceptor],mM


B
50000
40000
30000
20000
10000
0
CPM
Lactose
3-OAc-Lactose
0
5
10
15
20
25
[Acceptor],mM

As discussed in Chapter One, GlcNAc-6-sulfate was found in equal amounts to Gal-6-sulfate within GlyCAM-1 oligosaccharides (Hemmerich et al., 1994a). Furthermore, sialyl 6-sulfo Le^x was found to be present on HEV of human lymph nodes, and an antibody directed against his epitope inhibited the binding of an L-selectin/IgG chimera to HEV *in vitro* (Mitsuoka et al., 1998b). We were therefore also interested in the GlcNAc-6-O-sulfotransferase activity responsible for generating these structures in vivo. GlcNAc-6-sulfated structures have been identified in a number of cell-surface associated proteins, including keratan sulfate GAGs (Brown et al., 1994a), thyroglobulin (Kamerling et al., 1988), HIV gp120 (Shilatifard et al., 1993) and porcine zona pellucida (Noguchi and Nakano, 1992). The context within which the GlcNAc-6-sulfate is found varies among all of these proteins, and different sulfotransferases are therefore inferred to contribute to their biosynthesis.

A rat liver Golgi GlcNAc-6-O-sulfotransferase activity was recently described that modified the structure GlcNAcβ1→6Man, which is found within many types of N-linked oligosaccharides (Spiro et al., 1996). This enzyme required a terminal GlcNAc for its activity and could not sulfate Galβ1→4GlcNAc. Conversely, the same rat liver Golgi preparation contained a β1→4-galactosyltransferase activity that was capable of modifying [SO_3→6]GlcNAcβ1→6Man (Spiro et al., 1996). Other β1→4galactosyltransferases have been described which are capable of modifying terminal GlcNAc-6-sulfate (Degroote et al., 1997; Seko et al., 1998), in some cases with greater affinity for this structure than for unsulfated GlcNAc (Seko et al., 1998). Taken

together, these data imply that the biosynthesis of GlcNAc-6-sulfated N-linked oligosaccharides requires sulfation to occur before β1→4 galactosylation.

Other than L-selectin ligands, the occurrence of GlcNAc-6-sulfate within the context of core 2 oligosaccharides (i.e., GlcNAcβ1→6[Galβ1→3]GalNAc) has been described in two instances to date. Mucins from a highly metastatic human colon carcinoma cell line were shown to contain the motif [SO_3→6]GlcNAc[Galβ1→3]GalNAc (Capon et al., 1997), implying the existence of a corresponding GlcNAc-6-O-sulfotransferase. In addition, among the carbohydrate chains in respiratory mucins from patients with cystic fibrosis (CF) is a structure nearly identical to structure 2 in Fig 1.2 of Chapter one (differing only in not being sialylated on Le^x) (Lo-Guidice et al., 1994). A GlcNAc-6-O-sulfotransferase activity was recently identified in microsomal preparations from human bronchial mucosa that could sulfate the simple acceptor GlcNAcβ1→6-O-CH_3 as well as the structure GlcNAcβ1→6[(Siaα2→3)Galβ1→3]GalNAc obtained from bronchial mucosa (Degroote et al., 1997). The activity of this enzyme was completely abolished when the GlcNAc was substituted with β1→4-linked Gal, indicating a requirement for terminal GlcNAc. As was seen for the liver microsomes, the mucosal preparation also contained a β1→4 galactosyltransferase activity capable of modifying terminal GlcNAc-6-sulfate (Degroote et al., 1997), implying that in the synthesis of bronchial mucins, β1→4 galactosylation follows GlcNAc-6-sulfation.

In order to be able to specifically detect a GlcNAc-6-O-sulfotransferase activity in lymph node, Bowman et al. (1998) synthesized two simple acceptor structures (di- and

trisaccharide acceptor, respectively, in Table 1.1, Chapter One) based on the core structure in GlyCAM-1 (Fig. 1.2, Chapter One). These structures preserve the GlcNAcβ1→6 linkage found in native GlyCAM-1, while substituting Gal for GalNAc at the 'reducing' end. Using the disaccharide acceptor, they showed that porcine organs contain a GlcNAc-6-O-sulfotransferase activity that is highly expressed in lymph node and spleen, relative to other tissues. Strikingly the activity is highly enriched in the HEC, with only scant activity detectable in lymph node lymphocytes or HEC-depleted stromal cells.

As was observed for the activities in liver and respiratory mucosa (Degroote et al., 1997; Spiro et al., 1996), the lymph node GlcNAc-6-O-sulfotransferase required a terminal GlcNAc, as it was inhibited >90% by the presence of β1→4 linked Gal. This sulfotransferase activity is unlikely to correspond to the one described by Spiro et al. in liver (Spiro et al., 1996), as there was no detectable activity towards the disaccharide acceptor in porcine liver. Extracts from porcine lungs did catalyze sulfation of the disaccharide acceptor (Bowman et al., 1998), raising the possibility that the GlcNAc-6-O-sulfotransferase detected in this assay is identical to the enzyme detected in bronchial mucosa (Degroote et al., 1997). The relationship between the lung, lymph node and putative colon carcinoma GlcNAc-6-O-sulfotransferases remains to be established.

The biosynthesis of carbohydrate ligands for L-selectin requires the sequential action of a number of glycosyl- and sulfotransferases, and the ordering of their respective activities is of considerable interest, particularly as each step represents a potential point of

interception with possible therapeutic value. Using the metabolic inhibitor BFA, Crommie and Rosen demonstrated that during the biosynthesis of sLe^x in GlyCAM-1, sialylation precedes fucosylation (Crommie and Rosen, 1995). This is in agreement with the fact that no α2→3 sialyltransferase has so far been shown to be capable of sialylating the Le^x tetrasaccharide and that, conversely, several α1→3 FTs can modify Siaα2→3Galβ1→4GlcNAc (reviewed in Natsuka and Lowe, 1994). BFA causes a fusion of the endoplasmic reticulum (ER) with the cis-, medial- and trans-Golgi compartments, but not the trans Golgi network (TGN). BFA can thus help distinguish oligosaccharide processing steps occurring in the TGN from those occurring in the ER and Golgi compartments (Klausner et al., 1992). When BFA was added to lymph node organ cultures, sulfation of GlyCAM-1 was completely inhibited whereas fucosylation was only partially inhibited. These studies therefore implied that sulfation occurs after fucosylation.

As reviewed in the preceding paragraphs, accumulated evidence from *in vitro* enzyme assays indicates that the biosynthesis of sialyl 6-sulfo Le^x in L-selectin ligands (starting form GlcNAc linked β1→6 to GalNAc) occurs by the successive action of a GlcNAc-6-O-sulfotransferase, a β1→4 galactosyltransferase, an α2→3 sialyltransferase and the α1→3 fucosyltransferase, FTVII. It is not clear what the basis is for the discrepancy between these results and those obtained with BFA. However, it is possible that in the BFA treated cells, sulfation of terminal GlcNAc residues was inhibited by the action of β1→4 galactosyltransferases anomalously competing for the same acceptor structure. Indeed, the reciprocal activities of the GlcNAc-6-O-sulfotransferase and β1→4

galactosyltransferases may under normal circumstances constitute a regulatory mechanism for the synthesis of sialyl 6-sulfo Le^x on L-selectin ligands. With respect to sialyl 6'-sulfo Le^x, the inability of FTVII to act on Siaα2$\rightarrow$3[$SO_3\rightarrow$6]Galβ1$\rightarrow$4GlcNAc indicates that fucosylation must come before sulfation of the Gal, and the implied ordering of enzymatic activities is therefore: β1$\rightarrow$4 galactosyltransferase, α2$\rightarrow$3 sialyltransferase, FTVII (or FTIV) and finally Gal-6-O-sulfotransferase.

Summary

In summary, in collaboration with the laboratories of Carolyn Bertozzi of UC Berkeley and Stefan Hemmerich of Roche Bioscience, we have developed two sulfotransferase assays for the specific detection of Gal-6-O-sulfotransferase and GlcNAc-6-O-sulfotransferase activities. Using these assays, we have demonstrated the presence of a Gal-6-O-sulfotransferase activity in murine lymph node and a GlcNAc-6-O-sulfotransferase activity in porcine lymph node. The latter activity was highly enriched in HEC and is a good candidate to be involved in the synthesis of the sialyl 6-sulfo Le^x epitope on L-selectin ligands in vivo.

MATERIALS AND METHODS

Preparation of Microsomal Fractions from Murine Lymph Nodes

Mice were killed by cervical dislocation and mesenteric, cervical and brachial lymph nodes were dissected. All subsequent manipulations were carried out at 4°C. The lymph nodes were minced briefly and then homogenized in STKM buffer (250mM sucrose, 50mM Tris, pH 7.5, 25mM KCl, 5mM$MgCl_2$ and 1µl/ml protease inhibitor cocktails (PIC) I (1.2 mg/ml leupeptin, 2.0 mg/ml antipain, 20 mg/ml turkey trypsin inhibitor, 10 mg/ml benzamidine and 5mg/ml Pefabloc SC from Boehringer Mannheim, dissolved in 4-8 TIU/mg protein of aprotinin) and PIC II (2.0 mg/ml chymostatin and 1 mg/ml pepstatin, dissolved in DMSO)). Homogenization was carried out in a 10 ml glass homogenizer with a motor driven teflon pestle at 800 RPM. The homogenate was centrifuged at 680g for 10 min and the supernatant was recovered. The pellet was rehomogenized in STKM buffer, centrifuged 10 min at 680g and the supernatant was pooled with the first supernatant. The pooled supernatants were centrifuged at 10,000 g for 10 min and the supernatant was recovered by careful pipetting to avoid inclusion of any of the lysosomal pellet. The post-lysosomal supernatant was centrifuged at 100,000 g for 60 min and the supernatant was removed. The microsomal pellet was resuspended in 150mM Tris, pH 7.5, with PICs I and II, and centrifuged at 100,000 g for 60 min to obtain a washed microsomal pellet. The pellet was resuspended in 50mM HEPES, pH 7.3, 2.5% v/v Triton-X 100, 20% glycerol with PICs I and II, and allowed to solubilize

for 60-120 min with rocking. The solubilized microsomal preparation was aliquotted, frozen in liquid nitrogen and stored at -80 °C.

Enzymatic Synthesis of ^{35}S-PAPS

^{35}S-PAPS of high specific activity (1500 Ci/mmol, up to 45 Ci/mmol final) was produced enzymatically from ATP and ^{35}S-SO_4, using ATP sulfurylase (Sigma) and recombinant APS kinase, according to the protocol of Long and coworkers (Ehrhardt et al., 1995). The APS kinase was expressed in *E. coli* from an APS kinase-GST fusion vector prepared by cloning the cDNA for *E. coli* APS kinase, kindly provided to us by Dr. George Markham (Leyh et al., 1988; Smith and Johnson, 1988) into the pGEX vector (Pharmacia). The recombinant APS kinase-GST fusion protein was purified on a glutathione-agarose column (Sigma), according to the manufacturer's protocol. To synthesize ^{35}S-PAPS, 1μCi of carrier-free ^{35}S-Na_2SO_4 and 30mM ATP were incubated with 0.12 U ATP sulfurylase and 8.3 U inorganic pyrophosphatase (Sigma) in 60 μl buffer (20mM Tris, pH8.0, 30mM KCl, 40mM $MgCl_2$, 1mM EDTA, 1mM DTT, 10% glycerol) and APS kinase for 12 h at room temperature. The reaction was stopped by boiling for 5 min. Quantification of conversion was by separation of the reaction components by TLC on polyethylene (PEI)-cellulose, eluting with 0.9M LiCl, followed by autoradiography. Conversion of ^{35}S-Na_2SO_4 into ^{35}S-PAPS was typically quantitative (>95%).

Assay for Sulfotransferase Activity

The acceptor, ^{35}S-PAPS and lymph node extract were incubated at 37°C for one hour in a total volume of 50 µl of 10mM HEPES, pH 7.3, 1mM ATP, 5mM PAPS, 10mM NaF, 6mM $Mg(OAc)_2$, PICs I and II , 1% Triton-X 100 and 10% glycerol. Reactions were terminated by the addition of two volumes of 15mM EDTA, pH 8. The ^{35}S-labeled lipid-conjugated acceptors were separated from other components of the reaction mixture by passage over a reversed phase C-18 column, followed by extensive washing with water (Skelton et al., 1991). Specifically incorporated counts were eluted off the C-18 column with a 40% methanol/H_2O mixture and quantified by liquid scintillation counting.

Determination of the Regiochemistry of Sulfation

Purified ^{35}S-labeled lipid-conjugated acceptors were subjected to acid hydrolysis in 0.1M trifluoroacetic acid at 100°C. The hydrolysate was passed over a C-18 column and the flow through, which contained a mixture of non-lipid-associated mono- and oligosaccharides, was collected and lyophilized twice from water. The lyophilized material was resuspended in 2mM pyridine-HCOOH, pH 3, and applied to an HPLC anion-exchange column which was eluted with a pyridine-HCOOH gradient (2mM to 1M). Peaks eluting in the 100mM to 300mM range (representing monosulfated saccharides) were collected, lyophilized twice from water, and applied to a high pH

anion-exchange column (HPAEC, Dionex), which was eluted with a NaOAc gradient (50-850mM in 150mM NaOH). The specific site of sulfate incorporation in the test compounds was determined by comparing their elution profile with those of known monosulfated monosaccharides (Hemmerich et al., 1994a).

Chapter Three

Cloning of Three Carbohydrate Sulfotransferases that Are Expressed in Lymph Node: One is Highly Expressed in the High Endothelial Cells

Early efforts to identify new genes encoding sulfotransferases relied on purifying the relevant polypeptide to homogeneity, based on following the enzymatic activity with an appropriate assay, and then obtaining peptide sequence to use as a basis for molecular cloning of the corresponding cDNA (Brandan and Hirschberg, 1988; Habuchi et al., 1993; Pettersson et al., 1991; Sakac et al., 1992). This approach was rendered difficult by the requirement for purification on the order of 1,000 - 65,000 fold, without the certainty that sufficient material for peptide sequencing would be recovered. At the outset of our work to develop sulfotransferase assays for a Gal-6-O-sulfotransferase and a GlcNAc-6-O-sulfotransferase, only one vertebrate carbohydrate-modifying sulfotransferase had been identified at the molecular level: the rat liver N-heparan sulfate sulfotransferase (Hashimoto et al., 1992), the cDNA for which also encodes an N-deacetylase (Wei et al., 1993). As of this writing, there are 27 reports of clones encoding 12 different mammalian (and one avian) carbohydrate sulfotransferases, as well reports on two protein tyrosine sulfotransferases (Table 3.1). Table 3.2 shows the amino acid homologies among some of these sulfotransferases. In addition, several more specific sulfotransferase activities have been purified and/or characterized (Bowman et al., 1998; Degroote et al., 1997; Hooper et al., 1995; Lo-Guidice et al., 1995; Spiro et al., 1996;

Wei et al., 1993). Increasingly, new cDNA clones encoding sulfotransferases are being identified based on their homology to related genes. This veritable explosion in available information reflects the growing appreciation for the importance of sulfated carbohydrate motifs as mediators of communication between cells and their environment, including other cells.

Of particular interest to us, in contemplating possible homology-based approaches to identifying novel sulfotransferases involved in the biosynthesis of L-selectin ligands, was a 1995 report by Fukuta et al. (Fukuta et al., 1995) describing the cloning and characterization of a chicken chondroitin/keratan sulfate sulfotransferase (C6/KSST). This enzyme catalyzed sulfation at C-6 in GalNAc of chondroitin sulfate and at C-6 in Gal of keratan sulfate. C6/KSST was furthermore shown to be able to catalyze sulfation at C-6 of Gal in Siaα2→3Galβ1→4GlcNAc (Habuchi et al., 1997), which is a structure found within the capping groups of GlyCAM-1 shown in Fig. 1.2 of Chapter One. Fig. 3.1 illustrates the high degree of similarity between the oligosaccharide acceptors for this enzyme and the core of the GlyCAM-1 capping groups. Thus, the activities of the C6/KSST are inferred to be relevant to the Gal-6sulfate modifications found in GlyCAM-1, CD34 and presumably the other endothelial ligands for L-selectin. We reasoned that human sulfotransferases that modify C-6 of Gal and possibly also C-6 of GlcNAc would likely be homologous to the C6/KSST and that therefore, the cDNA sequence encoding the C6/KSST represented a useful tool in a genetic approach to identifying related human genes.

Table 3.1. Carbohydrate and Tyrosine Sulfotransferases Cloned to Date

Sulfotransferase	Residue	Species	Tissue	Author	Year	Journal	Accession	Notes
HEC/GlcNAc-6-O	GlcNAc	Human		Bistrup	1999		AF131325	
		Murine		Bistrup	1999		AF131326	
GlcNAc-6-O	GlcNAC	Human	Fetal brain	Uchimura	1998	JBiochem 124:670	AB014679	HuGST2
		Human	HUVEC	Li	1999	Genomics 55:345	AF083066	HuGST2
		Mouse	D7Embryo	Uchimura	1998	JBC 273:22577	AB011451	MuGST2
Keratan/Gal-6-O	Gal	Human	Fetal brain	Fukuta	1997	JBC 272:32321	AB003791	
		Human	Chon/HUVEC	Mazany	1998	BiocBiophAc 1407:92	U65637	KS/CSST
		Human	HUVEC	Li	1999	Genomics 55:345	AF090137	
Chondroitin-6-O	GalNAc	Human	Fetal brain	Fukuta	1998	BiocBiophAc1399:57	AB012192	
		Mouse	Teratocarci	Uchimura	1998	Glycobiol 8:489	AB008937	
		Chicken	Chondrocyte	Fukuta	1995	JBC 270:18575	D49915	KS/CSST
"Chromosome X"		Human		Grafman	1998		AL022165	
"Chromosome 22"		Human		Burgess	1998		Z97055	
Heparan-6-O	GlcN	Human	Fetal brain	Habuchi	1998	JBC 273:9208	AB006179	
		Hamster	CHO	Habuchi	1998	JBC 273:9208	AB006180	
Heparan-3-O	GlcN	Human	Brain	Shworak	1997	JBC 272:28008	AF019386	
		Mouse	L cells	Shworak	1997	JBC 272:28008	AF019385	
Heparan-2-O	IduA	Hamster	CHO	Kobayashi	1997	JBC 272:13980	D88811	
Heparan-N-ST	GlcN	Human	Placenta	Dixon	1995	Genomics 26:239	U18918	HepNST1
		Human	HUVEC	Humphries	1998	BiochemJ 332:303	U36601	HepNST2
		Human	Brain	Aikawa	1999	JBC 274:2690	AF074924	HepNST3
		Mouse	Liver	Kusche-Gullb.	1998	JBC 273:11902	AF049894	HepNST1
		Mouse	Mastocytoma	Orellana	1994	JBC 269:2270	U02304	HepNST2
		Mouse	Mastocytoma	Eriksson	1994	JBC 269:10438	X75885	HepNST2
		Rat	Liver	Hashimoto	1992	JBC 267:15744	M92042	HepNST1
HNK-1/GlcA-3-O	GlcA	Human	Fetal brain	Ong	1998	JBC 273:5190	AF033827	
		Rat	Neon. brain	Bakker	1997	JBC 272:29942	AF022729	
GalCer/Gal-3-O	Gal	Human	Renal cancer	Honke	1997	JBC 272:4864	D88667	
NodH/GlcNAc-6-O	GlcNAc	R. meliloti		Debelle	1986	NuclAcRes 14: 7453	X04380	
				Fisher	1987	Genetics 117: 191	M37417	
Tyrosine	Tyr	Human	EST	Ouyang	1998	PNAS 95:2896	AF038009	TPST-1
		Human		Beisswanger	1998	PNAS 95:11134	AF061254	TPST-2
		Mouse	Liver	Ouyang	1998	PNAS 95:2896	AF038008	TPST-1

Table 3.2 Homologies Among Carbohydrate and Tyrosine Modifying Sulfotransferases

Sulfotransferase	HEC	GlcNAc6	Chr X	KSGal6	C6ST	AvC6ST	Chr 22	Hep6O	Hep3O	Hep2O	HepN1	HNK	GalCer	NodH	TyrO
GenBank Acc. #	#aa:386	482	486	411	479	458	284	410	307	356	882 ST dom	356	423	248	370
HuHECGlcNAC6ST		27.81	28.6	33.65	25	28.51	14.47	11.81	16.32	15.19	15.74	14.69	15.28	14.99	15.44
AF131325		16.97	16.26	18.75	21.28	21.38	15.5	20.24	15.54	19.24	17.51	18.81	18.06	17.05	23.54
		9.41	9.47	11.06	7.44	8.42	11.11	9.64	9.84	10.38	8.63	12.37	12.73	8.27	11.39
		54.19	54.33	63.46	53.72	58.31	41.08	41.69	41.7	44.81	41.88	45.87	46.07	40.31	50.37
HuGlcNAc6ST			43.37	28.23	24.75	27.13	15.5	16.02	16.98	13.2	14.7	14.43	16.9	11.32	16.49
AB014679			14.85	18.55	24.17	22.77	11.36	13.35	13.66	16.91	14.49	16.29	15.07	7.41	17.73
AB011451			10.89	7.26	11.98	9.96	7.85	14.37	7.45	9.9	9.11	9.48	12.42	9.67	7.63
			69.11	54.04	60.9	59.86	34.71	43.74	38.09	40.01	38.3	40.2	44.39	28.4	41.85
Hu Chromosome X				29.9	26.63	27.29	16.26	17.85	17.59	14.2	14.31	15.84	18.62	18.27	17.83
AL022165				14.34	19.54	18.53	10.7	12.78	12.27	16.05	13.09	12.76	13.56	11.07	15.98
				9.49	9.58	10.56	5.14	11.76	8.18	7	8.38	11.52	8.91	5.94	8.61
				53.73	55.75	56.38	32.1	42.39	38.04	37.25	35.78	40.12	41.09	35.28	42.42
HuKSGal6ST					35.93	37.42	18.73	14.05	15.5	17.27	18.2	16.9	15.6	16.46	17.1
AB003791					17.68	18.41	15.57	14.75	16.95	17.99	15.78	18.57	18.68	11.14	15.44
U65637					9.45	9.89	8.76	15.22	11.14	10.79	10.68	12.14	11.01	8.72	14.49
					63.06	65.72	43.06	44.02	43.59	46.05	44.66	47.61	45.29	36.32	47.03
HuC6ST						73.9	15.21	15	16.28	16.84	16.49	16.04	15.5	14.94	15.59
AB012192						10.02	12.5	16.88	12.94	13.72	14.2	17.08	18.6	11.83	13.72
						3.76	7.71	10.21	8.98	9.77	8.77	8.75	9.5	4.56	10.81
						87.68	35.42	42.09	38.2	40.33	39.46	41.87	43.6	31.33	40.12
AvC6/KSST							15.47	15.22	16.12	17.86	15.22	17.25	14.94	16.96	16.49
D49915							12.85	17.61	13.94	13.73	17.39	16.59	17.61	11.74	20.13
							7.41	19.78	7.84	9.59	8.04	9.83	19.78	5	8.78
							35.73	52.61	37.9	41.18	40.65	43.67	52.33	33.7	45.4
Hu Chromosome 22								16.34	17.09	18.61	18.26	15.08	16.04	16.67	15.78
Z97055								15.61	21.84	19.17	18.26	15.36	13.44	19.1	15.51
								6.83	10.44	7.5	8.68	10.89	9.91	9.03	9.63
								38.78	49.37	45.28	45.2	41.33	39.39	44.8	40.92
HuHep6O									17.8	20	16.59	14.8	17.35	14.39	15.44
AB006179									14.63	19.04	16.11	18.38	16.44	10.73	17.81
									11.95	9.64	8.65	8.11	10.27	6.83	10.21
									44.38	48.68	41.35	41.29	44.06	31.95	43.46
HuHep3O										14.8	26.27	16.8	16.55	15.48	19.95
AF019386										20.39	22.39	14.29	17.95	18.39	14.29
										11.17	11.04	10.51	9.09	8.03	10.51
										46.36	59.7	41.6	43.59	41.9	44.75
HuHep2O											16.2	17.28	17.4	17.7	14.81
D88811											17.32	20.94	13.92	16.85	22.22
											11.17	11.52	10.21	9.83	11.64
											44.69	49.74	41.53	44.38	48.67
HuHepN1												16.09	15.57	16.46	17.38
ST domain only												15.01	15.33	14.33	16.58
U18918												9.92	7.08	10.06	10.7
												41.02	37.98	40.85	44.66
HNK-1													14.59	16.49	17.54
AF033827													17.88	13.45	15.45
													10.82	8.68	11.26
													43.29	40.62	44.25
HuGalCer														17.92	15.57
D88667														12.26	17.92
														4.48	10.61
														34.66	44.1
NodH															14.82
X04380															13.48
M37417															8.63
															36.93
HuTyr1															
AF038009															

Protein sequences were aligned using the ClustalW algorithm (Thompson et al., 1994).
The Fast Pairwise Alignment Parameters were: Ktupl size=1; window size=5; number of top diagonals=5; gap penalty=3.
Identity at a given residue is indicated in red. High similarity is indicated in green. Low Similarity is indicated in blue.
Overall percent homology is given as the sum of the identity and similarities.

Table 3.2 Homologies Among Carbohydrate and Tyrosine Modifying Sulfotransferases

Sulfotransferase GenBank Acc. #	HEC #aa:386	GlcNAc6 482	Chr X 486	KSGal6 411	C6ST 479	AvC6ST 458	Chr 22 284	Hep6O 410	Hep3O 307	Hep2O 356	HepN1 882 ST dom	HNK 356	GalCer 423	NodH 248	TyrO 370
HuHECGlcNAC6ST		27.81	28.6	33.65	25	28.51	14.47	11.81	16.32	15.19	15.74	14.69	15.28	14.99	15.44
AF131325		16.97	16.26	18.75	21.28	21.38	15.5	20.24	15.54	19.24	17.51	18.81	18.06	17.05	23.54
		9.41	9.47	11.06	7.44	8.42	11.11	9.64	9.84	10.38	8.63	12.37	12.73	8.27	11.39
		54.19	54.33	63.46	53.72	58.31	41.08	41.69	41.7	44.81	41.88	45.87	46.07	40.31	50.37
HuGlcNAc6ST			43.37	28.23	24.75	27.13	15.5	16.02	16.98	13.2	14.7	14.43	16.9	11.32	16.49
AB014679			14.85	18.55	24.17	22.77	11.36	13.35	13.66	16.91	14.49	16.29	15.07	7.41	17.73
AB011451			10.89	7.26	11.98	9.96	7.85	14.37	7.45	9.9	9.11	9.48	12.42	9.67	7.63
			69.11	54.04	60.9	59.86	34.71	43.74	38.09	40.01	38.3	40.2	44.39	28.4	41.85
Hu Chromosome X				29.9	26.63	27.29	16.26	17.85	17.59	14.2	14.31	15.84	18.62	18.27	17.83
AL022165				14.34	19.54	18.53	10.7	12.78	12.27	16.05	13.09	12.76	13.56	11.07	15.98
				9.49	9.58	10.56	5.14	11.76	8.18	7	8.38	11.52	8.91	5.94	8.61
				53.73	55.75	56.38	32.1	42.39	38.04	37.25	35.78	40.12	41.09	35.28	42.42
HuKSGal6ST					35.93	37.42	18.73	14.05	15.5	17.27	18.2	16.9	15.6	16.46	17.1
AB003791					17.68	18.41	15.57	14.75	16.95	17.99	15.78	18.57	18.68	11.14	15.44
U65637					9.45	9.89	8.76	15.22	11.14	10.79	10.68	12.14	11.01	8.72	14.49
					63.06	65.72	43.06	44.02	43.59	46.05	44.66	47.61	45.29	36.32	47.03
HuC6ST						73.9	15.21	15	16.28	16.84	16.49	16.04	15.5	14.94	15.59
AB012192						10.02	12.5	16.88	12.94	13.72	14.2	17.08	18.6	11.83	13.72
						3.76	7.71	10.21	8.98	9.77	8.77	8.75	9.5	4.56	10.81
						87.68	35.42	42.09	38.2	40.33	39.46	41.87	43.6	31.33	40.12
AvC6/KSST							15.47	15.22	16.12	17.86	15.22	17.25	14.94	16.96	16.49
D49915							12.85	17.61	13.94	13.73	17.39	16.59	17.61	11.74	20.13
							7.41	19.78	7.84	9.59	8.04	9.83	19.78	5	8.78
							35.73	52.61	37.9	41.18	40.65	43.67	52.33	33.7	45.4
Hu Chromosome 22								16.34	17.09	18.61	18.26	15.08	16.04	16.67	15.78
Z97055								15.61	21.84	19.17	18.26	15.36	13.44	19.1	15.51
								6.83	10.44	7.5	8.68	10.89	9.91	9.03	9.63
								38.78	49.37	45.28	45.2	41.33	39.39	44.8	40.92
HuHep6O									17.8	20	16.59	14.8	17.35	14.39	15.44
AB006179									14.63	19.04	16.11	18.38	16.44	10.73	17.81
									11.95	9.64	8.65	8.11	10.27	6.83	10.21
									44.38	48.68	41.35	41.29	44.06	31.95	43.46
HuHep3O										14.8	26.27	16.8	16.55	15.48	19.95
AF019386										20.39	22.39	14.29	17.95	18.39	14.29
										11.17	11.04	10.51	9.09	8.03	10.51
										46.36	59.7	41.6	43.59	41.9	44.75
HuHep2O											16.2	17.28	17.4	17.7	14.81
D88811											17.32	20.94	13.92	16.85	22.22
											11.17	11.52	10.21	9.83	11.64
											44.69	49.74	41.53	44.38	48.67
HuHepN1												16.09	15.57	16.46	17.38
ST domain only												15.01	15.33	14.33	16.58
U18918												9.92	7.08	10.06	10.7
												41.02	37.98	40.85	44.66
HNK-1													14.59	18.49	17.54
AF033827													17.88	13.45	15.45
													10.82	8.68	11.26
													43.29	40.62	44.25
HuGalCer														17.92	15.57
D88667														12.26	17.92
														4.48	10.61
														34.66	44.1
NodH															14.82
X04380															13.48
M37417															8.63
															36.93
HuTyr1															
AF038009															

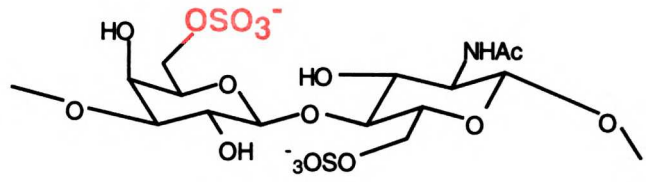
Protein sequences were aligned using the ClustalW algorithm (Thompson et al., 1994).
The Fast Pairwise Alignment Parameters were: Ktupl size=1; window size=5; number of top diagonals=5; gap penalty=3.
Identity at a given residue is indicated in red. High similarity is indicated in green. Low Similarity is indicated in blue.
Overall percent homology is given as the sum of the identity and similarities.

Figure 3.1

Alignment of the core disaccharide motif in GlyCAM-1 with the disaccharide repeat motifs of chondroitin sulfate and keratan sulfate and the trisaccharide 3'-sialyl-N-acetyllactosamine

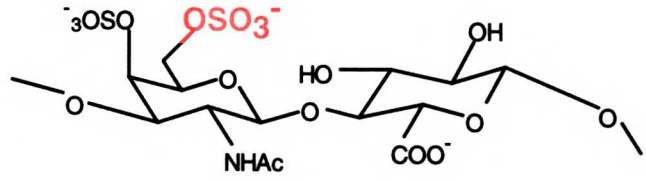
The core disaccharide motif in GlyCAM-1 is aligned with the disaccharide repeat motifs of chondroitin sulfate and keratan sulfate and the trisaccharide 3'-sialyl-N-acetyllactosamine. The sulfate esters at C-6 of galactose (Gal) or N-acetylgalactosamine (GalNAc) within each di- or trisaccharide are indicated in red.

GlyCAM-1



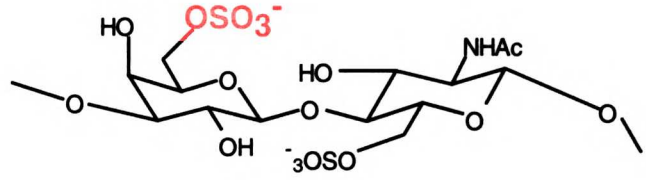
Gal **GlcNAc**

Chondroitin sulfate



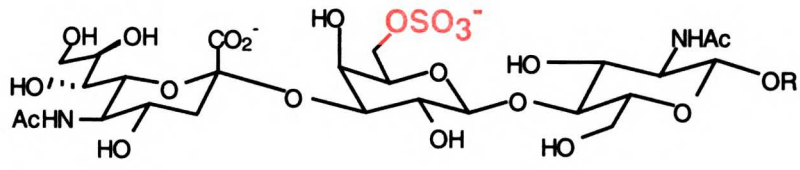
GalNAc **GlcU**

Keratan sulfate



Gal **GlcNAc**

3'-sialyl-N-acetyl-lactosamine



Sia **Gal** **GlcNAc**

We chose to utilize the human expressed sequence tag (EST) databases to search for sequences with homology to the C6/KSST. ESTs are single-pass sequences (usually 150-600 bases) of randomly chosen cDNA clones from libraries prepared from a variety of tissues (Venter et al., 1992). We searched for human ESTs related to the C6/KSST at the protein level by using tblastn, which compares a protein query sequence against a nucleotide sequence database translated in all 6 reading frames. When we used the C6/KSST cDNA sequence to probe the NCBI dbEST and LifeSeq (Incyte, Inc.) human EST databases, a number of ESTs were identified that segregated into three distinct genes, which we initially referred to as "glycosyl sulfotransferases" (GSTs) 1, 2 and 3. A large number of ESTs, representing many different tissues, corresponded to GSTs 1 and 2. In contrast, GST 3 was represented by a single EST, deriving from a breast keratinocyte cell line.

As part of our cloning strategy, the first objective was to identify which tissues most highly expressed transcripts for each of the three genes. We obtained EST clones corresponding to each of the genes, from which we prepared restriction fragments to be used as probes in Northern blots. The fragments ranged in size from 500-730 base pairs (bp). The fragments were labeled with ^{32}P-phosphate and hybridized at high stringency to commercially prepared multiple tissue Northern blots, as described in the Materials and Methods. These blots contain approximately 2μg polyA$^+$ RNA per lane, according to the manufacturer. Please refer to Fig. 3.4 for a representative calibration of the commercially available Northern blots.

Tissue distribution and molecular cloning of KSGal6ST and HuGlcNAc6ST

Figures 3.2, 3.3 and 3.7 show the patterns of expression for GSTs 1, 2 and 3, respectively. A transcript of approximately 2.8 kilobases (kb) corresponding to GST 1 was expressed at high levels in the brain and heart and at low levels in the other tissues on the "standard tissues" blot (Fig. 3.2, left panel). On the "lymphoid tissues" blot (Fig. 3.2, middle panel), high expression was seen in lymph node and spleen, with low levels of expression in thymus, bone marrow and fetal liver. Interestingly, the peripheral blood lymphocytes (PBL) appeared not to express this transcript. One implication of this could be that the expression observed in whole lymph node could be ascribed to the stromal elements of the node. Additional transcripts of 1.5 kb, 4.3 kb and >10 kb appeared to be present in some tissues. As will be described below, we also prepared mRNA from HEC and from HUVEC, which was separated by agarose gel electrophoresis and transferred to nylon in parallel with an aliquot of commercially available human brain mRNA. This blot was first hybridized with a probe specific for GST 3, then stripped under mild conditions and rehybridized with a probe for GST 1. Fig. 3.2 (right panel) shows that a 2.8 kb transcript for GST 1 is expressed in brain and HUVEC, but not in HEC. We have evidence from semiquantitative RT-PCR analysis that transcripts for GST 1 are expressed in HEC (not shown); however, we have not been able to conclusively establish this by Northern analysis (above) or by *in situ* hybridization, as will be discussed below. Additional larger transcripts may be present in HUVEC, however, it is difficult to establish this absolutely, as the mRNA appears to be degraded in this lane.

Figure 3.2

Northern blot analysis of KSGal6ST expression in various tissues

Northern blots containing poly(A)$^+$ RNA from various human tissues (left and center) and from HEC and HUVEC (right) were probed with a 730 bp fragment from the IMAGE Consortium clone 40604 (GenBank accession no. R55609). The blot containing the HEC and HUVEC was stripped and probed with a 300 bp probe for β-actin (lower right panel), as in Fig. 3.7.B.

pancreas
kidney
skeletal
muscle
liver
lung
placenta
brain
heart
fetal liver
bone marrow
PBL
thymus
lymph node
spleen
kb
9.5
7.5
4.4
2.4
1.3
HEC
HUVEC
brain
kb
2.8
actin

GST 2 also had a rather broad tissue distribution, as shown in Fig. 3.3. A transcript of approximately 3.0 kb was expressed at high levels in brain and placenta, and at low levels in the other tissues on the "standard tissue" blot (Fig. 3.3, left panel). GST 2 was expressed uniformly at very high levels in the lymphoid tissues (Fig. 3.3, right panel). A faint transcript of approximately 5.4 kb was apparent in placenta, and possibly also lymph node and thymus.

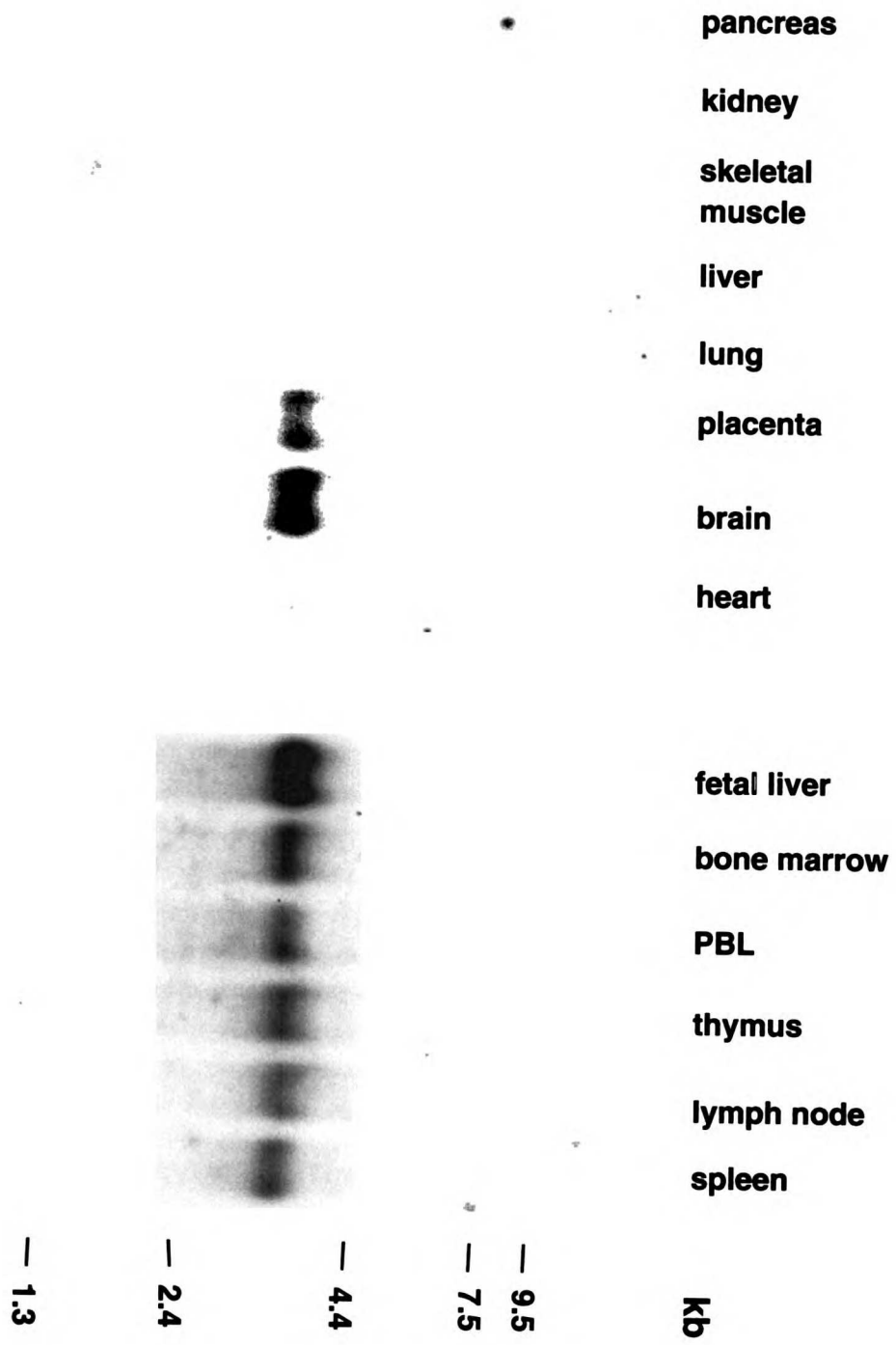
Because of the high expression of GSTs 1 and 2 in brain, we chose a human fetal brain library as source from which to clone cDNAs corresponding to these two genes. This library was kindly made available to us by Dr. Marc Tessier-Lavigne at UCSF. As described in the Materials and Methods, we screened 10^6 colonies at high stringency with the same probes for GSTs 1 and 2 as we had used in the Northern analysis. For both GSTs 1 and 2, 18 independent hybridizing plaques were identified after the second round of screening, all of which were subjected to sequencing.

The sequence for the longest clone corresponding to GST 1 is shown in Fig. 3.4. This sequence contains a single open reading frame (ORF) of 1233 nucleotides (nt) and is apparently full length, as indicated by the presence of an upstream stop codon and a Kozak sequence surrounding the first ATG (Kozak, 1996). The open reading frame predicts a type II transmembrane protein of 411 amino acids (aa) with a short cytoplasmic tail of 6 aa and five potential N-linked glycosylation sites. The predicted protein is 37% identical to the chicken C6/KSST (Table 3.2). While this work was in progress, the same cDNA, slightly longer and with two base changes in the 5'-untranslated region, was

Figure 3.3

Northern blot analysis of HuGlcNAc6ST expression in various tissues

Northern blots containing poly(A)$^+$ RNA from various human tissues were probed with a 673 bp fragment from the Incyte clone 494235.


pancreas
kidney
skeletal muscle
liver
lung
placenta
brain
heart
fetal liver
bone marrow
PBL
thymus
lymph node
spleen
— 1.3
— 2.4
— 4.4
— 7.5
— 9.5
kb

published by Fukuta et al. (1997). They termed the protein keratan sulfate Gal-6-O-sulfotransferase, KSGal6ST, based on their characterization of its enzymatic activities, as will be discussed in Chapter Four. The same cDNA sequence was also recently reported by Mazany et al. and shown in their hands to encode a chondroitin sulfate GalNAc-6-O-sulfotransferase as well as a keratan sulfate Gal-6-O-sulfotransferase (Mazany et al., 1998). We will refer to this protein as KSGal6ST.

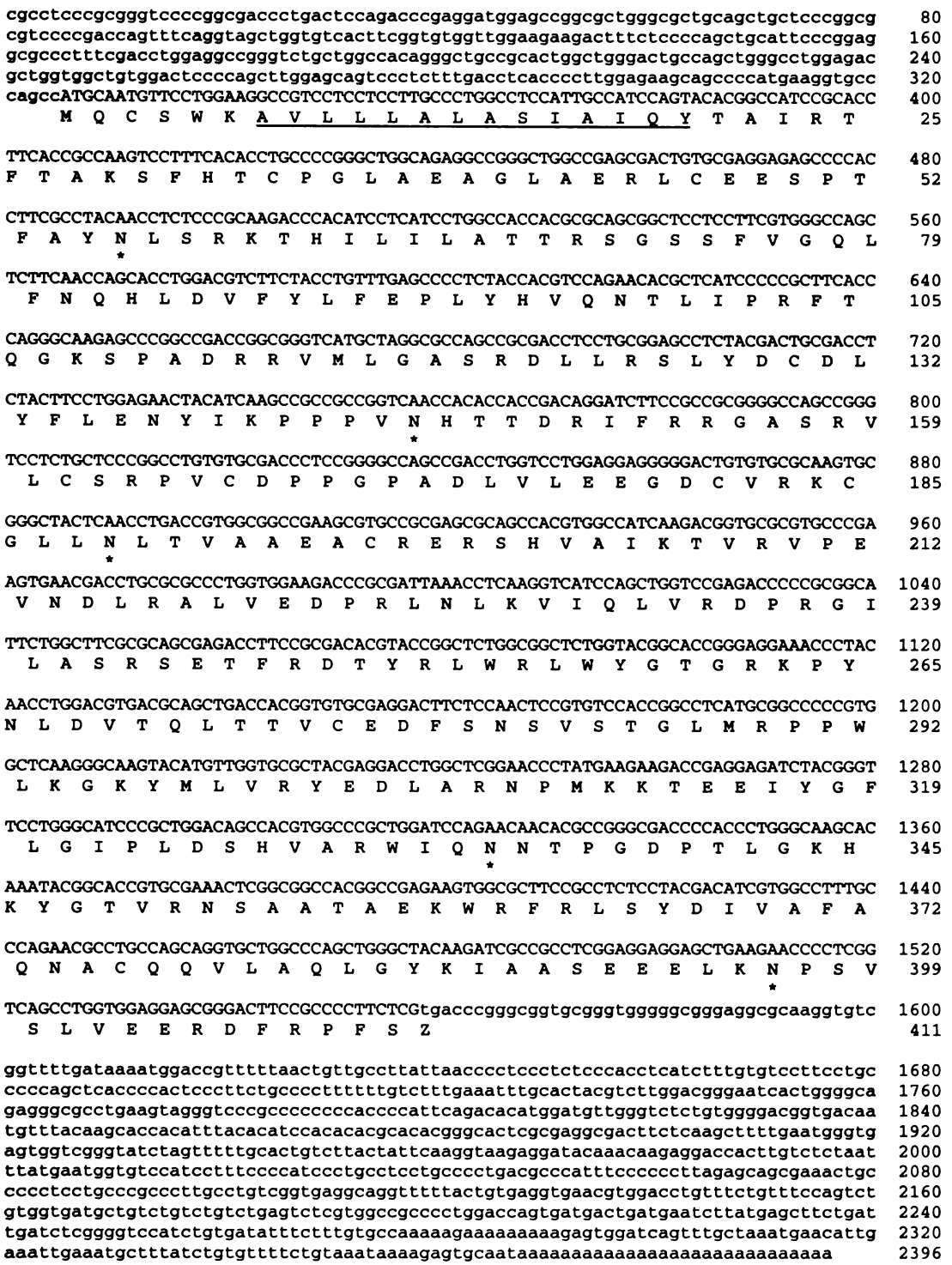
Although we sequenced all 18 of the clones corresponding to GST 2, we did not obtain a full length cDNA sequence. The sequence for the longest clone (clone 17) is shown in Fig. 3.5. This sequence contains a long ORF of 1431 nt, starting at nt 1. However, this ORF does not start with an ATG and as there is no upstream untranslated sequence, it is unlikely to represent an ORF encoding full length GST 2. Indeed, the incompleteness of this sequence was confirmed when two groups reported the sequences corresponding to the sequences for GST 2 recently (Li and Tedder, 1999; Uchimura et al., 1998a; Uchimura et al., 1998b). Fig. 3.6 shows an alignment of our sequence with those of the published human (HuCHST2 and HuGlcNAc6ST, respectively, in Fig. 3.6) (Li and Tedder, 1999; Uchimura et al., 1998b) and mouse (MuGlcNAc6ST in Fig. 3.6) (Uchimura et al., 1998a) GST 2 sequences. In this figure, the sequence for our clone 17 is represented by the sequence for CHST2, as they are identical over the stretch of aa in which sequence is available for clone 17; the sequence for clone 17 starts at position 55 (residue A, indicated in bold) of the CHST2 sequence. We believe we have uncovered two sets of errors in the sequence published for HuGlcNAc6ST (Uchimura et al., 1998b) based on the following observations: The sequences for CHST2/clone 17 and

HuGlcNAc6ST are nearly identical, except for the regions corresponding to aa 102 - 158 and aa 165-167 (numbers refer to Fig. 3.6, "CHST2") of the CHST2, where the reported divergence is considerable. Over the same regions, the sequence for our clone 17 is identical to the CHST2 sequence derived by Li and Tedder (Li and Tedder, 1999). The HuGlcNAc6ST sequence contains an extra aa over this stretch of sequence. With respect to the first set of errors, we noticed that within the 174 nt stretch of sequence corresponding to aa 102-158, the published HuGlcNAc6ST sequence had an extra nt at three different positions (positions 552, 659 and 724 in Uchimura et al., 1998b). The consequence of the presence of these three extra bases and their non-sequential localization is two-fold. First, upon translation, they result in the appearance of an extra aa within this stretch of sequence. Second, they cause three separate frame shifts over the region. This explains the divergence in aa sequence starting at aa 102 in Fig. 3.6 and the convergence starting at aa 159. Fig. 3.6 shows that when the three nt in question are removed from the published HuGlcNAc6ST cDNA sequence, the aa sequence for HuGlcNAc6ST (HuGlcNAc6* in Fig. 3.6) becomes identical to that for CHST2/clone 17 (with the exception of the A at position 136, which in HuGlcNAc6ST is an A). The second error we believe to have uncovered in the published HuGlcNAc6ST sequence concerns aa 165-167 in Fig. 3.6. Here, the aa sequence for our clone 17 is identical to the published HuCHST2 sequence. The HuGlcNAc6ST sequence differs over the three aa, and this would appear to be due to possible transpositions and/or reading errors in the nt sequence for the published HuGlcNAc6ST sequence (Uchimura et al., 1998b).

Figure 3.4

Molecular features of human KSGal6ST

cDNA sequence and predicted protein sequence for human KSGal6ST. The open reading frame is denoted by capital letters and the predicted amino acid sequence is indicated below the nucleotide sequence. The putative transmembrane domain is underlined and five potential N-linked glycosylation sites are indicated by asterisks.


cgcctcccgcgggtccccggcgaccctgactccagacccgaggatggagccggcgctgggcgctgcagctgctcccggcg 80
cgtccccgaccagtttcaggtagctggtgtcacttcggtgtggttggaagaagactttctccccagctgcattcccggag 160
gcgccctttcgacctggaggccgggtctgctggccacagggctgccgcactggctgggactgccagctgggcctggagac 240
gctggtggctgtggactccccagcttggagcagtccctctttgacctcacccctttggagaagcagccccatgaaggtgcc 320
cagccATGCAATGTTCCTGGAAGGCCGTCCTCCTCCTTGCCCTGGCCTCCATTGCCATCCAGTACACGGCCATCCGCACC 400
M Q C S W K A V L L L A L A S I A I Q Y T A I R T 25
TTCACCGCCAAGTCCTTTCACACCTGCCCCGGGCTGGCAGAGGCCGGGCTGGCCGAGCGACTGTGCGAGGAGAGCCCCAC 480
F T A K S F H T C P G L A E A G L A E R L C E E S P T 52
CTTCGCCTACAACCTCTCCCGCAAGACCCACATCCTCATCCTGGCCACCACGCGCAGCGGCTCCTCCTTCGTGGGCCAGC 560
F A Y N L S R K T H I L I L A T T R S G S S F V G Q L 79
TCTTCAACCAGCACCTGGACGTCTTCTACCTGTTTGAGCCCCTCTACCACGTCCAGAACACGCTCATCCCCCGCTTCACC 640
F N Q H L D V F Y L F E P L Y H V Q N T L I P R F T 105
CAGGGCAAGAGCCCGGCCGACCGGCGGGTCATGCTAGGCGCCAGCCGCGACCTCCTGCGGAGCCTCTACGACTGCGACCT 720
Q G K S P A D R R V M L G A S R D L L R S L Y D C D L 132
CTACTTCCTGGAGAACTACATCAAGCCGCCGCCGGTCAACCACACCACCGACAGGATCTTCCGCCGCGGGGCCAGCCGGG 800
Y F L E N Y I K P P P V N H T T D R I F R R G A S R V 159
TCCTCTGCTCCCGGCCTGTGTGCGACCCTCCGGGGCCAGCCGACCTGGTCCTGGAGGAGGGGGACTGTGTGCGCAAGTGC 880
L C S R P V C D P P G P A D L V L E E G D C V R K C 185
GGGCTACTCAACCTGACCGTGGCGGCCGAAGCGTGCCGCGAGCGCAGCCACGTGGCCATCAAGACGGTGCGCGTGCCCGA 960
G L L N L T V A A E A C R E R S H V A I K T V R V P E 212
AGTGAACGACCTGCGCGCCCTGGTGGAAGACCCGCGATTAAACCTCAAGGTCATCCAGCTGGTCCGAGACCCCCGCGGCA 1040
V N D L R A L V E D P R L N L K V I Q L V R D P R G I 239
TTCTGGCTTCGCGCAGCGAGACCTTCCGCGACACGTACCGGCTCTGGCGGCTCTGGTACGGCACCGGGAGGAAACCCTAC 1120
L A S R S E T F R D T Y R L W R L W Y G T G R K P Y 265
AACCTGGACGTGACGCAGCTGACCACGGTGTGCGAGGACTTCTCCAACTCCGTGTCCACCGGCCTCATGCGGCCCCCGTG 1200
N L D V T Q L T T V C E D F S N S V S T G L M R P P W 292
GCTCAAGGGCAAGTACATGTTGGTGCGCTACGAGGACCTGGCTCGGAACCCTATGAAGAAGACCGAGGAGATCTACGGGT 1280
L K G K Y M L V R Y E D L A R N P M K K T E E I Y G F 319
TCCTGGGCATCCCGCTGGACAGCCACGTGGCCCGCTGGATCCAGAACAACACGCCGGGCGACCCCACCCTGGGCAAGCAC 1360
L G I P L D S H V A R W I Q N N T P G D P T L G K H 345
AAATACGGCACCGTGCGAAACTCGGCGGCCACGGCCGAGAAGTGGCGCTTCCGCCTCTCCTACGACATCGTGGCCTTTGC 1440
K Y G T V R N S A A T A E K W R F R L S Y D I V A F A 372
CCAGAACGCCTGCCAGCAGGTGCTGGCCCAGCTGGGCTACAAGATCGCCGCCTCGGAGGAGGAGCTGAAGAACCCCTCGG 1520
Q N A C Q Q V L A Q L G Y K I A A S E E E L K N P S V 399
TCAGCCTGGTGGAGGAGCGGGACTTCCGCCCCTTCTCGtgacccgggcggtgcgggtgggggcgggaggcgcaaggtgtc 1600
S L V E E R D F R P F S Z 411
ggttttgataaaatggaccgtttttaactgttgccttattaacccctccctctcccacctcatctttgtgtccttcctgc 1680
ccccagctcaccccactcccttctgccccttttttgtctttgaaatttgcactacgtcttggacgggaatcactggggca 1760
gagggcgcctgaagtagggtcccgccccccccacccccattcagacacatggatgttgggtctctgtggggacggtgacaa 1840
tgtttacaagcaccacatttacacatccacacacgcacacgggcactcgcgaggcgacttctcaagcttttgaatgggtg 1920
agtggtcgggtatctagtttttgcactgtcttactattcaaggtaagaggatacaaacaagaggaccacttgtctctaat 2000
ttatgaatggtgtccatcctttccccatccctgcctcctgcccctgacgcccatttccccccttagagcagcgaaactgc 2080
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tgatctcggggtccatctgtgatatttctttgtgccaaaaagaaaaaaaaagagtggatcagtttgctaaatgaacattg 2320
aaattgaaatgctttatctgtgttttctgtaaataaaagagtgcaataaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 2396

Figure 3.5

Molecular features of human clone 17 (HuGlcNAc6ST)

Partial cDNA sequence and predicted protein sequence for human clone 17 (HuGlcNAc6ST). The open reading frame is denoted by capital letters and the predicted amino acid sequence is indicated below the nucleotide sequence. The putative transmembrane domain is underlined and four potential N-linked glycosylation sites are indicated by asterisks.

```
CAGGCGCTGGTGTTGTGCGCGGGCTATGCACTGCTGCTGGTGCTCACTATGCTCAACCTCCTGGACTACAAGTGGCACAA   80
Q A L V L C A G Y A L L L V L T M L N L L D Y K W H K   27

GGAGCCGCTGCAGCAGTGCAACCCCGATGGGCCGCTGGGTGCCGCAGCGGGGGCAGCCGGAGGCAGCTGGGGGCGCCCAG  160
 E P L Q Q C N P D G P L G A A A G A A G G S W G R P G   54

GGCCGCCTCCGGCCGGGCCGCCCCGTGCTCATGCCCGTTTGGACCTCCGCACTCCTTACCGCCCTCCCGCTGCCGCCGTC  240
  P P P A G P P R A H A R L D L R T P Y R P P A A A V    80

GGGGCGGCTCCTGCAGCCGCGGCAGGGATGGCGGGGGTTGCGGCCCCTCCAGGCAATGGCACTCGGGGCACCGGGGGCGT  320
G A A P A A A A G M A G V A A P P G N G T R G T G G V   107
                                    *

CGGGGACAAGCGGCAGCTGGTGTACGTGTTCACCACGTGGCGCTCTGGCTCGTCGTTCTTCGGCGAGCTATTCAACCAGA  400
 G D K R Q L V Y V F T T W R S G S S F F G E L F N Q N  134

ATCCCGAGGTGTTCTTTCTCTACGAGCCAGTGTGGCATGTATGGCAAAAACTGTATCCGGGGGACGCCGTTTCCCTGCAG  480
  P E V F F L Y E P V W H V W Q K L Y P G D A V S L Q   160

GGGGCAGCGCGGGACATGCTGAGCGCTCTTTACCGCTGCGACCTCTCTGTCTTCCAGTTGTATAGCCCCGCGGGCAGCGG  560
G A A R D M L S A L Y R C D L S V F Q L Y S P A G S G  187

GGGGCGCAACCTCACCACGCTGGGCATCTTCGGCGCAGCCACCAACAAGGTGGTGTGCTCGTCACCACTCTGCCCCGCCT  640
 G R N L T T L G I F G A A T N K V V C S S P L C P A Y  214
       *

ACCGCAAGGAGGTCGTGGGGTTGGTGGACGACCGCGTGTGCAAGAAGTGCCCGCCACAGCGCCTGGCGCGTTTCGAGGAG  720
  R K E V V G L V D D R V C K K C P P Q R L A R F E E   240

GAGTGCCGCAAGTACCGCACACTAGTCATAAAGGGTGTGCGCGTCTTCGACGTGGCGGTCTTGGCGCCACTGCTGCGAGA  800
E C R K Y R T L V I K G V R V F D V A V L A P L L R D  267

CCCGGCCCTGGACCTCAAGGTCATCCACTTGGTGCGTGATCCCCGCGCGGTGGCGAGTTCACGGATCCGCTCGCGCCACG  880
 P A L D L K V I H L V R D P R A V A S S R I R S R H G  294

GCCTCATCCGTGAGAGCCTACAGGTGGTGCGCAGCCGAGACCCGCGAGCTCACCGCATGCCCTTCTTGGAGGCCGCGGGC  960
  L I R E S L Q V V R S R D P R A H R M P F L E A A G   320

CACAAGCTTGGCGCCAAGAAGGAGGGCGTGGGCGGCCCCGCAGACTACCACGCTCTGGGCGCTATGGAGGTCATCTGCAA 1040
H K L G A K K E G V G G P A D Y H A L G A M E V I C N  347

TAGTATGGCTAAGACGCTGCAGACAGCCCTGCAGCCCCCTGACTGGCTGCAGGGCCACTACCTGGTGGTGCGGTACGAGG 1120
 S M A K T L Q T A L Q P P D W L Q G H Y L V V R Y E D  374

ACCTGGTGGGAGACCCCGTCAAGACACTACGGAGAGTGTACGATTTTGTGGGACTGTTGGTGAGCCCCGAAATGGAGCAG 1200
  L V G D P V K T L R R V Y D F V G L L V S P E M E Q   400

TTTGCCCTGAACATGACCAGTGGCTCGGGCTCCTCCTCCAAGCCTTTCGTGGGATCTGCACGCAATGCCACGCAGGCCGC 1280
F A L N M T S G S G S S S K P F V G S A R N A T Q A A  427
      *                                 *

CAATGCCTGGCGGACCGCCTTCACCTTCCAGCAGATCAACCAGGTGGAGAAGTTTTGCTACCAGCCCATGGCCGTCCTGG 1360
 N A W R T A F T F Q Q I N Q V E K F C Y Q P M A V L G  454

GCTATGAGCGGGTCAACAGCCCTGAGGAGGTCAAAGACCTCAGCAAGACCCTGCTTCGGAAGCCCCGTCTCtaaaagggg 1440
  Y E R V N S P E E V K D L S K T L L R K P R L Z       477

ttcccaggagacctgattccctgtggtgatacctataaagaggatcgtagtgtgtttaaataaacacagtccagactcaa 1520
acggaggaagcccacatattctattatagatatataaataatcacacacacacttgctgtcaatgttttgagtcagtgca 1600
tttcaaggaacagccacaaaatacacacccctaagaaaaggcaagacttgaacgttctgaccaggtgcccctcttcttct 1680
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ggtgggggtggagaaaggggaagtggtccagaaacaaaaagccccattgggcatgataagccgaggaggcattcttctaa 1920
agtagacttttgtgtaaaaagcaaaggttacatgtgagtattaataaagaagataataaataatattctttttaaaaaaa 2000
aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 2080
aaaaaaaaaaaaaaaaaaaaaaaaaaag                                                    2109
```

Figure 3.6

Alignment of the published predicted amino acid sequences for human and mouse GlcNAc6ST

The two published predicted amino acid sequences for human GlcNAc6ST (HuCHST2 and HuGlcNAc6) (Uchimura et al., 1998b; Li and Tedder, 1999) and one for MuGlcNAc6ST (MuGlcNAc6) (Uchimura et al., 1998a) are aligned. Protein sequences were aligned using the ClustalW algorithm (Thompson et al., 1994). The predicted amino acid sequence based on the available cDNA sequence for clone 17 is identical to that for the "CHST2" sequence. The start of the sequence derived from clone 17 (A, position 55) is indicated in bold. Differences between the two published human GlcNAc6ST sequences are shaded in black. Differences between human and mouse GlcNAc6ST sequences are shaded in gray. The sequence corresponding to HuGlcNAc6ST after correction of putative errors in the cDNA sequence is indicated by an asterisk (HuGlcNAc6ST*). See text for details.

```
MuGlcNAc6  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~MKV
HuCHST2    MSRSPQRALP PGALPRLLQA APAAQPRALL PQWPRRPGRR WPASPLGMKV   50
HuGlcNAc6  MSRSPQRALP PGALPRLLQA APAAQPRALL PQWPRRPGRR WPASPLGMKV

MuGlcNAc6  FRRKALVLCA GYALLLVLTM LNLLDYKWHK EPLQQCNPDG PLGAA[ ]GAAG
HuCHST2    FRRKALVLCA GYALLLVLTM LNLLDYKWHK EPLQQCNPDG PLGAAAGAAG  100
HuGlcNAc6  FRRKALVLCA GYALLLVLTM LNLLDYKWHK EPLQQCNPDG PLGAAAGAAG

MuGlcNAc6  [ ]GWGRPGSPP AAPPRAHSRM DPRTPYRPP. AAGVGA[ ]PAA AAG[ ]AG[ ]AA[ ]
HuCHST2    GSWGRPGPPP AGPPRAHARL DLRTPYRPP. AAAVGAAPAA AAGMAGVAAP  149
HuGlcNAc6* SWGRPGPPP AGPPRAHARL DLRTPYRPP. AAAVGA[ ]PAA AAGMAGVAAP
HuGlcNAc6  G*KLGAPRAAS GRAAPCSCPF GPPHSLPPSR CRRRGDTLQP RQGWRGLRPL

MuGlcNAc6  [ ]GNATRGT[ ]G [ ]GDKRQLVYV FTTWRSGSSF FGELFNQNPE VFFLYEPVWH
HuCHST2    PGNGTRGTGG VGDKRQLVYV FTTWRSGSSF FGELFNQNPE VFFLYEPVWH  199
HuGlcNAc6* PGNGTRGTG
HuGlcNAc6  *QAMALGAPE*G VGDKR[ ]YV FTTWRSGSSF FGELFNQNPE VFFLYEPVWH

MuGlcNAc6  VWQKLYPGDA VSLQGAARDM LSALYRCDLS VFQLYSPAGS GGRNLTTLGI
HuCHST2    VWQKLYPGDA VSLQGAARDM LSALYRCDLS VFQLYSPAGS GGRNLTTLGI  249
HuGlcNAc6  VWQKLYPGDA VSLQGAARDM LSALYRCDLS VFQLYSPAGS GGRNLTTLGI
                                                          *

MuGlcNAc6  FGAATNKVVC SSPLCPAYRK EVVGLVDDRV CKKCPPQRLA RFEEECRKYR
HuCHST2    FGAATNKVVC SSPLCPAYRK EVVGLVDDRV CKKCPPQRLA RFEEECRKYR  299
HuGlcNAc6  FGAATNKVVC SSPLCPAYRK EVVGLVDDRV CKKCPPQRLA RFEEECRKYR

MuGlcNAc6  T[ ]VIKGVRVF DVAVLAPLL[ ] DPALDLKVIH LVRDPRAVAS SRIRSRHGLI
HuCHST2    TLVIKGVRVF DVAVLAPLLR DPALDLKVIH LVRDPRAVAS SRIRSRHGLI  349
HuGlcNAc6  TLVIKGVRVF DVAVLAPLLR DPALDLKVIH LVRDPRAVAS SRIRSRHGLI

MuGlcNAc6  RESLQVVRSR DPRAHRMPFL EAAGHKLGAK KEG[ ]GGPADY HALGAMEVIC
HuCHST2    RESLQVVRSR DPRAHRMPFL EAAGHKLGAK KEGVGGPADY HALGAMEVIC  399
HuGlcNAc6  RESLQVVRSR DPRAHRMPFL EAAGHKLGAK KEGVGGPADY HALGAMEVIC

MuGlcNAc6  NSMAKTLQTA LQPPDWLQGH YLVVRYEDLV GDPVKTLRRV YDFVGLLVSP
HuCHST2    NSMAKTLQTA LQPPDWLQGH YLVVRYEDLV GDPVKTLRRV YDFVGLLVSP  449
HuGlcNAc6  NSMAKTLQTA LQPPDWLQGH YLVVRYEDLV GDPVKTLRRV YDFVGLLVSP

MuGlcNAc6  EMEQFALNMT SGSGSSSKPF VVSARNATQA ANAWRTALTF QQIKQVEEFC
HuCHST2    EMEQFALNMT SGSGSSSKPF VVSARNATQA ANAWRTALTF QQIKQVEEFC  499
HuGlcNAc6  EMEQFALNMT SGSGSSSKPF VVSARNATQA ANAWRTALTF QQIKQVEEFC
                  *                       *

MuGlcNAc6  YQPMAVLGYE RVNSPEEVKD LSKTLLRKPR L
HuCHST2    YQPMAVLGYE RVNSPEEVKD LSKTLLRKPR L                      530
HuGlcNAc6  YQPMAVLGYE RVNSPEEVKD LSKTLLRKPR L
```

Taken together, the available sequences for HuGlcNAc6ST/HuCHST2 reveal an ORF predicting two possible polypeptides. The first starts at nt 249 in the published sequence for HuGlcNAc6ST (Uchimura et al., 1998b) and consists of 530 aa, whereas the second starts at nt 390 and consists of 483 aa. In either case, the predicted protein has a type II transmembrane topology and three potential N-linked glycosylation sites. The presence of two possible start sites, each with adequate Kozak sequences surrounding the ATG, predicts two forms of the protein which differ in the length of their cytoplasmic tails. Short cytoplasmic tails appear to be typical of the novel family of sulfotransferases described in this document, as will be discussed below. There is, however, precedent among glycosyltransferases for the possibility of encoding polypeptides with cytoplasmic tails of different lengths from the same gene (Russo et al., 1990; Youakim et al., 1994). In the case of the β1→4 galactosyltransferase described by Russo et al., the length of the cytoplasmic tail appears to correlate with localization of the enzyme to either the cell surface (long tail) or the Golgi network (short tail). The predicted aa sequence for HuGlcNAc6ST/HuCHST2 is 27% identical to the chicken C6/KSST and 28% identical to GST 1 (Table 3.2).

Tissue distribution and molecular cloning of HEC-GlcNAc6ST

In sharp contrast to GSTs 1 and 2, transcripts corresponding to GST 3 could not be detected on the standard Northern blots. As shown in Fig. 3.7.B (left and middle panels), low levels of a 2.4 kb transcript were apparent in lymph node, liver (fetal and adult) and

pancreas. An additional transcript of approximately 6 kb was expressed in liver and pancreas, and at trace levels in lymph node and fetal liver. No other tissue represented on these blots appeared to express this gene.

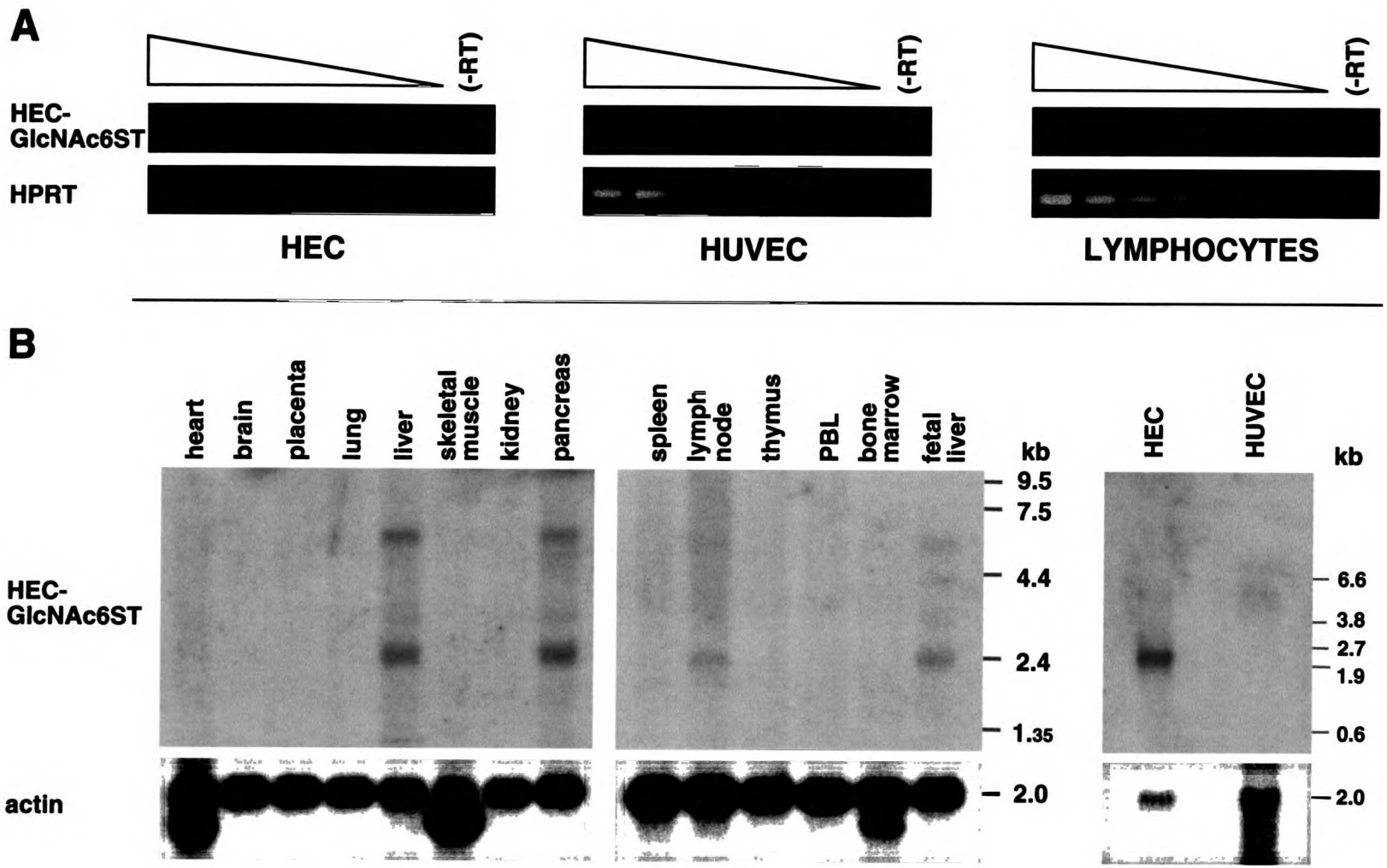
Because of the apparently restricted tissue distribution of the GST 3 transcripts, we wanted to know whether this gene might be expressed in HEC. We chose initially to probe for its expression in HEC by reverse transcription-polymerase chain reaction (RT-PCR), which is a highly sensitive method for detecting the presence of transcripts. Towards this end, we needed to obtain a pure population of HEC. As discussed in Chapter One, the HEC of HEV are uniquely recognized by the mAb MECA-79 (Mackay et al., 1992; Michie et al., 1993; Shailubhai et al., 1997; Streeter et al., 1988b), which recognizes a posttranslational modification on L-selectin ligands (Berg et al., 1991; Hemmerich et al., 1994b). We adapted a published procedure (Girard and Springer, 1995a) for the purification of HEC from human tonsil, based on the immunoselection of these cells with MECA-79. Briefly, fresh surgical specimens of human tonsil were minced and freely associated lymphocytes were flushed out by passage over a fine meshed screen. The resulting preparation was subjected to brief collagenase digestion followed by a secondary screening step. The enriched stromal population was then subjected to collagenase digestion to generate a suspension of stromal cells in singlets and small clusters. Incubation with biotinylated MECA-79 followed by selection with streptavidin-conjugated magnetic beads and passage over a magnetic column resulted in a population that is >99% HEC (Sassetti et al., 1998). The HEC represent less than 0.025% of the starting tonsil cells. Total RNA was prepared from these cells, and in

Figure 3.7

Expression of HEC-GlcNAc6ST transcripts in various tissues including high endothelial cells

(A) Semiquantitative RT-PCR analysis. Fragments of the HEC-GlcNAc6ST and HPRT sequences were amplified by PCR from serial dilutions of cDNA prepared from purified HEC, HUVEC and tonsillar lymphocytes. The reaction products (456 bp and 300 bp, respectively) were analyzed by agarose electrophoresis and ethidium bromide staining. (-RT), PCR reactions in which the template was generated by omission of reverse transcriptase.

(B) Northern blot analysis. Northern blots containing poly(A)$^+$ RNA from various human tissues (left and center) and from HEC and HUVEC (right) were probed with a 500 bp fragment from the HEC-GlcNAc6ST cDNA (upper panels). The blots were stripped and reprobed with a 300 bp probe for β-actin (lower panels).


A
(-RT)
(-RT)
(-RT)
HEC-
GlcNAc6ST
HPRT
HEC
HUVEC
LYMPHOCYTES
B
heart
brain
placenta
lung
liver
skeletal muscle
kidney
pancreas
spleen
lymph node
thymus
PBL
bone marrow
fetal liver
kb
9.5
7.5
4.4
2.4
1.35
HEC
HUVEC
kb
6.6
3.8
2.7
1.9
0.6
HEC-
GlcNAc6ST
actin
2.0
2.0

parallel from tonsillar lymphocytes and HUVEC, which was then reverse transcribed to provide a cDNA template for the PCR reactions.

As shown in Fig. 3.7.A , primers specific for GST 3 (derived from the cDNA sequence of the EST clone, LifeSeq no. 2620445) amplified a fragment of 456 bp from HEC cDNA but not from the lymphocyte cDNA, indicating that the signal deriving from the HEC was unlikely to be due to lymphocyte contamination of the HEC preparation. Furthermore, no GST 3 fragment was amplified from the HUVEC cDNA, implying that GST 3 is not constitutively expressed in endothelial cells. As a further confirmation of this result, we prepared mRNA from HEC and HUVEC which was then separated by agarose gel electrophoresis and transferred to nylon for Northern blot analysis. As shown in Fig. 3.7.B (right panel), a prominent band of 2.4 kb was detected at relatively high levels in mRNA from HEC, but was undetectable in HUVEC mRNA. Trace levels of the 6 kb band appeared to be present in both HEC and HUVEC.

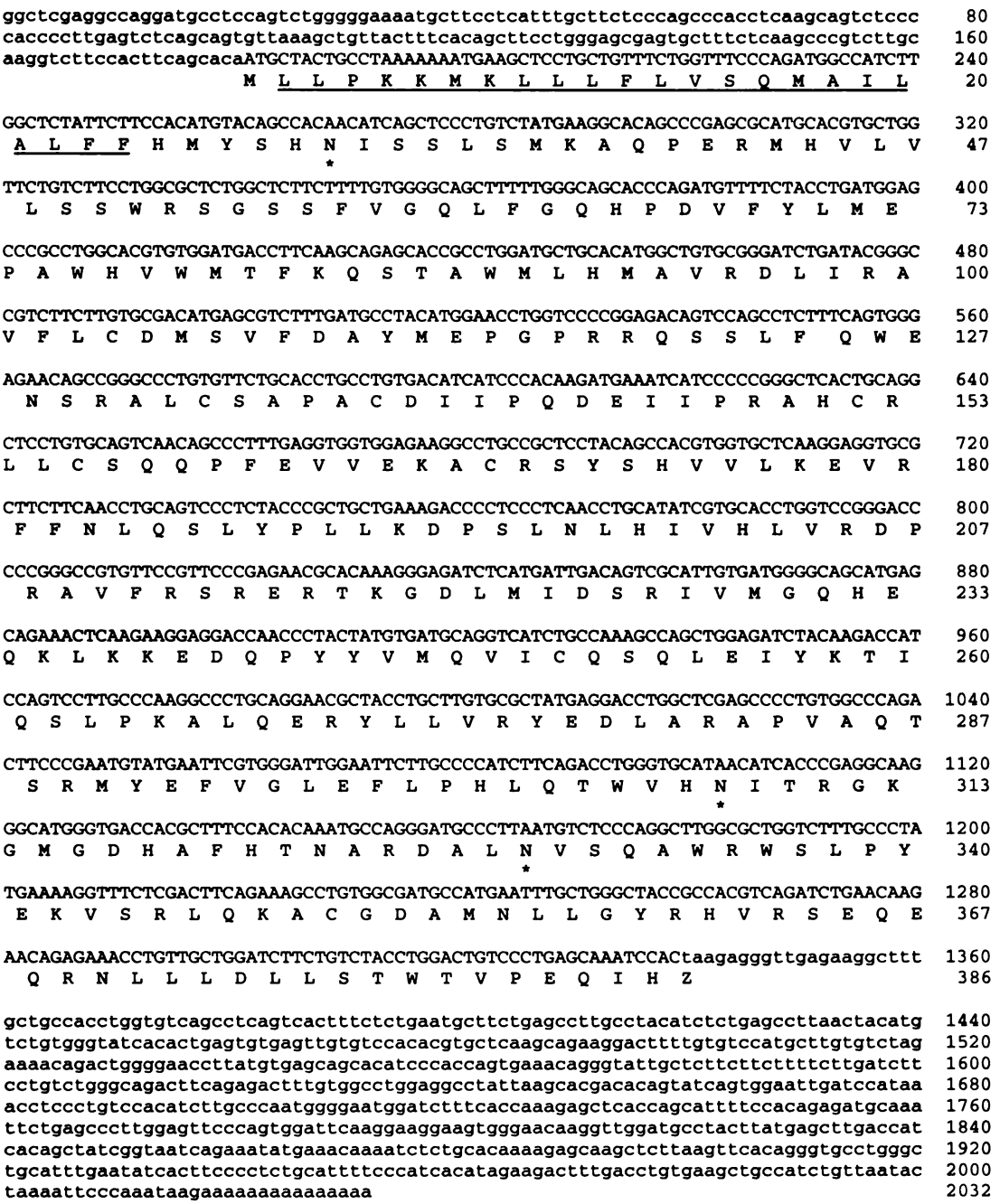
Based on the RT-PCR and Northern results, we decided to clone the GST 3 cDNA from HEC. Since we had a relatively small number of HEC available, we used a PCR-based technique (SMART technology, CLONTECH, Inc.) to produce cDNA from total RNA, from which we prepared a plasmid expression library, as described in detail in Materials and Methods. 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 2000 colony forming units per pool were screened, resulting in the identification of 9 positive pools. After two

additional rounds of screening, a single positive clone was obtained. The cDNA corresponding to this clone contains a single open reading frame of 1158 nt. The cDNA is apparently full-length as indicated by the presence of an upstream stop codon and a Kozak sequence surrounding the first ATG. This open reading frame predicts a type II transmembrane protein of 386 aa with three potential sites for N-linked glycosylation. 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 (LifeSeq 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. The sequence corresponding to LifeSeq clone no. 2617407 is presented here (Fig. 3.8), since the library from which it was cloned was created without a PCR amplification step. The predicted aa sequence of this novel gene is 28.5% identical to C6/KSST (Fukuta et al., 1995), and 34% and 28% identical to KSGal6ST (GST 1) (Fukuta et al., 1997; Mazany et al., 1998) and HuGlcNAc6ST/CHST2 (GST 2) (Li and Tedder, 1999; Uchimura et al., 1998b), respectively (Table 3.2). We have termed this novel gene HEC-specific GlcNAc-6-O-sulfotransferase (HEC-GlcNAc6ST) on the basis of its restricted expression and the characterization described in Chapter Four.

Figure 3.8

Molecular features of human and mouse HEC-GlcNAc6ST

cDNA sequence for human HEC-GlcNAc6ST and predicted protein sequences of human (h) and mouse (m) HEC-GlcNAC6STs (GenBank accession numbers 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 domains are underlined and three potential N-linked glycosylation sites (for each sequence) are indicated by asterisks.


ggctcgaggccaggatgcctccagtctgggggaaaatgcttcctcatttgcttctcccagcccacctcaagcagtctccc 80
cacccctgagtctcagcagtgttaaagctgttactttcacagcttcctgggagcgagtgctttctcaagcccgtcttgc 160
aaggtcttccacttcagcacaATGCTACTGCCTAAAAAAATGAAGCTCCTGCTGTTTCTGGTTTCCCAGATGGCCATCTT 240
M L L P K K M K L L L F L V S Q M A I L 20
GGCTCTATTCTTCCACATGTACAGCCACAACATCAGCTCCCTGTCTATGAAGGCACAGCCCGAGCGCATGCACGTGCTGG 320
A L F F H M Y S H N I S S L S M K A Q P E R M H V L V 47
TTCTGTCTTCCTGGCGCTCTGGCTCTTCTTTTGTGGGGCAGCTTTTTGGGCAGCACCCAGATGTTTTCTACCTGATGGAG 400
L S S W R S G S S F V G Q L F G Q H P D V F Y L M E 73
CCCGCCTGGCACGTGTGGATGACCTTCAAGCAGAGCACCGCCTGGATGCTGCACATGGCTGTGCGGGATCTGATACGGGC 480
P A W H V W M T F K Q S T A W M L H M A V R D L I R A 100
CGTCTTCTTGTGCGACATGAGCGTCTTTGATGCCTACATGGAACCTGGTCCCCGGAGACAGTCCAGCCTCTTTCAGTGGG 560
V F L C D M S V F D A Y M E P G P R R Q S S L F Q W E 127
AGAACAGCCGGGCCCTGTGTTCTGCACCTGCCTGTGACATCATCCCACAAGATGAAATCATCCCCCGGGCTCACTGCAGG 640
N S R A L C S A P A C D I I P Q D E I I P R A H C R 153
CTCCTGTGCAGTCAACAGCCCTTTGAGGTGGTGGAGAAGGCCTGCCGCTCCTACAGCCACGTGGTGCTCAAGGAGGTGCG 720
L L C S Q Q P F E V V E K A C R S Y S H V V L K E V R 180
CTTCTTCAACCTGCAGTCCCTCTACCCGCTGCTGAAAGACCCCTCCCTCAACCTGCATATCGTGCACCTGGTCCGGGACC 800
F F N L Q S L Y P L L K D P S L N L H I V H L V R D P 207
CCCGGGCCGTGTTCCGTTCCCGAGAACGCACAAAGGGAGATCTCATGATTGACAGTCGCATTGTGATGGGGCAGCATGAG 880
R A V F R S R E R T K G D L M I D S R I V M G Q H E 233
CAGAAACTCAAGAAGGAGGACCAACCCTACTATGTGATGCAGGTCATCTGCCAAAGCCAGCTGGAGATCTACAAGACCAT 960
Q K L K K E D Q P Y Y V M Q V I C Q S Q L E I Y K T I 260
CCAGTCCTTGCCCAAGGCCCTGCAGGAACGCTACCTGCTTGTGCGCTATGAGGACCTGGCTCGAGCCCCTGTGGCCCAGA 1040
Q S L P K A L Q E R Y L L V R Y E D L A R A P V A Q T 287
CTTCCCGAATGTATGAATTCGTGGGATTGGAATTCTTGCCCCATCTTCAGACCTGGGTGCATAACATCACCCGAGGCAAG 1120
S R M Y E F V G L E F L P H L Q T W V H N I T R G K 313
GGCATGGGTGACCACGCTTTCCACACAAATGCCAGGGATGCCCTTAATGTCTCCCAGGCTTGGCGCTGGTCTTTGCCCTA 1200
G M G D H A F H T N A R D A L N V S Q A W R W S L P Y 340
TGAAAAGGTTTCTCGACTTCAGAAAGCCTGTGGCGATGCCATGAATTTGCTGGGCTACCGCCACGTCAGATCTGAACAAG 1280
E K V S R L Q K A C G D A M N L L G Y R H V R S E Q E 367
AACAGAGAAACCTGTTGCTGGATCTTCTGTCTACCTGGACTGTCCCTGAGCAAATCCACtaagagggttgagaaggcttt 1360
Q R N L L L D L L S T W T V P E Q I H Z 386
gctgccacctggtgtcagcctcagtcactttctctgaatgcttctgagccttgcctacatctctgagccttaactacatg 1440
tctgtgggtatcacactgagtgtgagttgtgtccacacgtgctcaagcagaaggacttttgtgtccatgcttgtgtctag 1520
aaaacagactggggaaccttatgtgagcagcacatcccaccagtgaaacagggtattgctcttcttcttttcttgatctt 1600
cctgtctgggcagacttcagagactttgtggcctggaggcctattaagcacgacacagtatcagtggaattgatccataa 1680
acctccctgtccacatcttgcccaatggggaatggatctttcaccaaagagctcaccagcattttccacagagatgcaaa 1760
ttctgagcccttggagttcccagtggattcaaggaaggaagtgggaacaaggttggatgcctacttatgagcttgaccat 1840
cacagctatcggtaatcagaaatatgaaacaaaatctctgcacaaaagagcaagctcttaagttcacagggtgcctgggc 1920
tgcatttgaatatcacttcccctctgcattttcccatcacatagaagactttgacctgtgaagctgccatctgttaatac 2000
taaaattcccaaataagaaaaaaaaaaaaaaa 2032

Upon cloning of full length cDNAs corresponding to the three sulfotransferases (HEC-GlcNAc6ST, HuGlcNAc6ST and KSGal6ST), we were in a position to further explore their expression pattern by performing *in situ* hybridizations. We were particularly interested in their possible expression in HEV of lymph node. We initially attempted to hybridize probes based on the human clones to sections of human tonsil, however, this approach was unsuccessful as the background level of hybridization was too high due to the poor quality of the tissue. We therefore turned to the murine system, which provides for the examination of multiple tissues without the background problems associated with using human surgical specimens. Having obtained EST clones corresponding to mouse homologs of all three of the novel human sulfotransferases, we prepared ^{35}S -labeled riboprobes based on these clones and hybridized these to panels of murine tissues, as described in the Materials and Methods.

Despite prolonged exposure of the sections to film (12 weeks), probe corresponding to murine KSGal6ST did not hybridize to any tissue in the panel (not shown), including brain. Since transcripts for human KSGal6ST were abundantly expressed in several tissues by Northern blot, we favor the interpretation that the *in situ* analysis failed due to a problem with the probe. We therefore consider this result to be inconclusive.

Expression of murine GlcNAc6ST was similarly not apparent in any of the tissues represented in the panel (not shown), including brain and pancreas. This is in contrast to the report by Uchimura et al. (Uchimura et al., 1998a), which showed specific hybridization of the probe for murine GlcNAc6ST to the HEV of murine mesenteric

lymph node. These workers apparently did not examine any other tissues by *in situ* hybridization despite high levels of expression in brain, pancreas and ovary by Northern blot. Our failure to detect transcripts corresponding to murine GlcNAc6ST in any of the tissues represented in the panel is therefore likely also due to a poorly hybridizing probe.

In contrast to the results for murine KSGal6ST and GlcNAc6ST, transcripts corresponding to murine HEC-GlcNAc6ST were easily detectable by *in situ* hybridization. Strikingly, HEC-GlcNAc6ST transcripts were detected only in the HEV of lymph node (Fig. 3.9). 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 (not shown). A weak signal was detected in gut intestinal epithelium (not shown). The failure to detect expression in the liver or pancreas, which tissues in the human expressed detectable levels of HEC-GlcNAc6ST (Fig. 3.7), may be due to species differences. The sense control did not yield signal in any tissue. Taken together with the Northern blot and RT-PCR analysis for human HEC-GlcNAc6ST, the *in situ* hybridization analysis firmly establishes a very restricted expression pattern for HEC-GlcNAc6ST and its unequivocal expression in HEC. This characteristic fulfills a very important requirement for sulfotransferases involved in the construction of L-selectin ligands, as they would minimally have to be expressed in HEC, and furthermore could reasonably be expected to exhibit limited expression elsewhere.

Figure 3.9

In situ hybridization to detect HEC-GlcNAc6ST transcripts in mouse lymph node

Sections of C56BL7 mouse lymph node were hybridized with ^{35}S-labeled sense or antisense riboprobes based upon the cDNA 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 HEV, 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 HEV are evident.

A
B
C

Identification of a family of highly related carbohydrate sulfotransferases

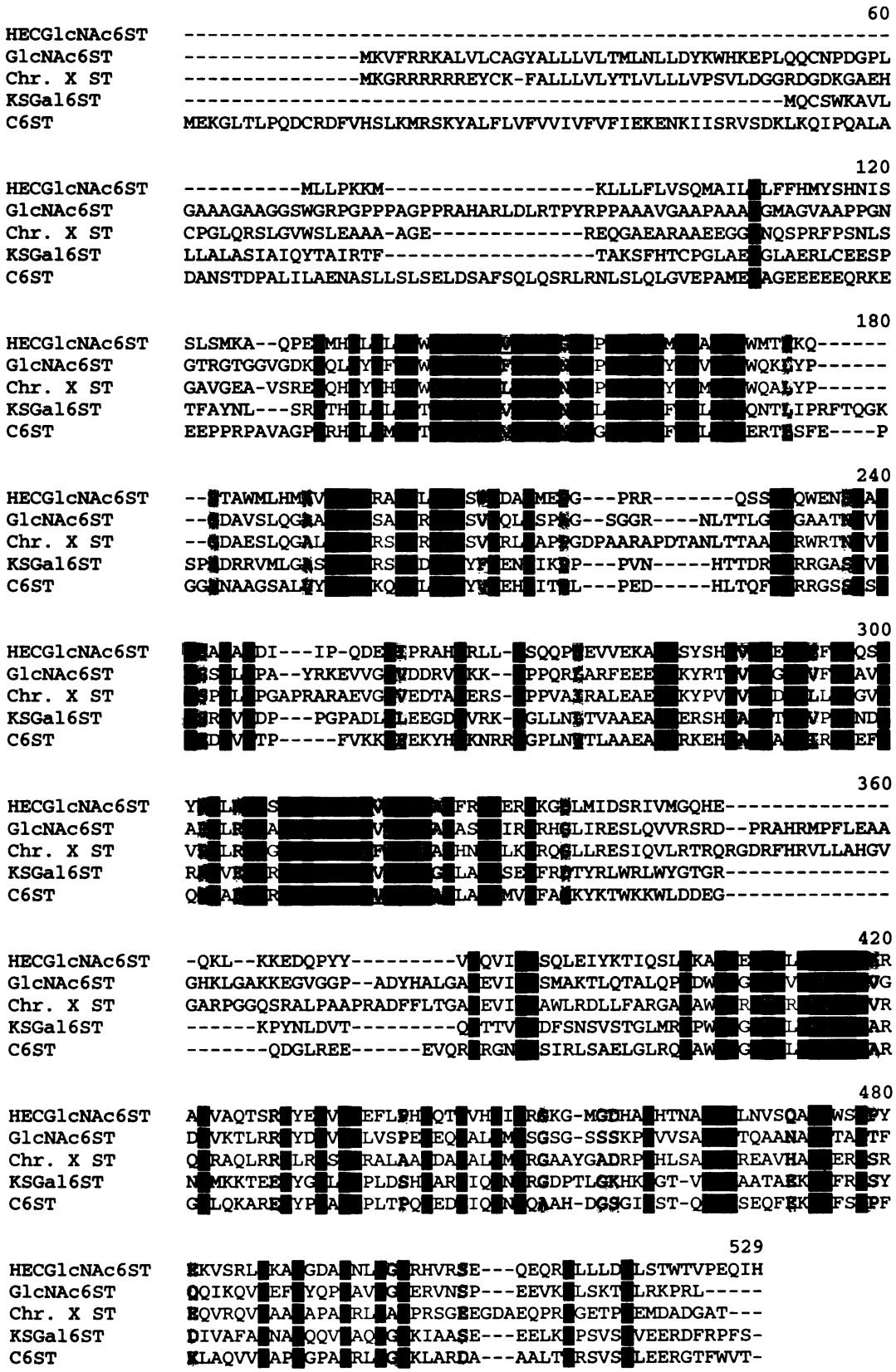
The high levels of homology among the three proteins initially identified (KSGal6ST, HEC-GlcNAc6ST and HuGlcNAc6ST) indicated that they belong to a novel family of carbohydrate sulfotransferases. We and others have since identified additional members of this family. Table 3.2 shows the homologies among all the human carbohydrate modifying sulfotransferases reported to date (see also Table 3.1). Included in this table is one of the two recently reported tyrosylprotein sulfotransferases, as well as the bacterial NodH, which is a GlcNAc-6-O-sulfotransferase. Strikingly, while the level of aa identity among members of the novel family and all other sulfotransferases in Table 3.2 is relatively constant in the range of 11-20%, the percent identity within this novel family ranges from 25-43%, and percent similarity is as high as 70%. Thus, the five human (and one avian) carbohydrate sulfotransferases indicated by the boxed area in Table 3.2 clearly constitute a family of highly related proteins. This family will be referred to as the 6-hydroxyl (6-OH) family, reflecting the fact that they all modify (or, in the case of the Chromosome 11-sulfotransferase, likely will prove to modify) the 6-hydroxyl group of similar monosaccharide constituents (Gal, GalNAc or GlcNAc) of their substrates.

The five members of the family range in size from 386 to 486 aa (the long form of HuGlcNAc6ST is 530 aa). Fig. 3.10 shows an alignment of the five sulfotransferases. They are all type II transmembrane proteins with short cytoplasmic tails ranging from 1 to 23 aa. The alternative long form (530 aa) of HuGlcNAc6ST has a cytoplasmic tail of 54 aa. All of the novel sulfotransferases appear to be glycoproteins, as indicated by the

Figure 3.10

Alignment of the five members of the novel family of carbohydrate sulfotransferases that modify 6-hydroxyl residues

The five predicted amino acid sequences for carbohydrate sulfotransferases that are known (all others) or suspected (Chromosome X sulfotransferase) to modify the 6-hydroxyl groups of their respective substrates (Gal-6, GlcNAc-6 and GalNAc-6) are aligned. Protein sequences were aligned using the ClustalW algorithm (Thompson et al., 1994). Black shading indicates identity among all five sequences at a given residue. High similarity at a residue is indicated by dark gray shading, low similarity is indicated by light gray shading.


60
HECGlcNAc6ST
GlcNAc6ST
Chr. X ST
KSGal6ST
C6ST
120
180
240
300
360
420
529

presence of multiple consensus sites for N-linked glycosylation in each of their coding sequences.

Within the 6-OH family of sulfotransferases, there are three regions of aa sequence in which identity ranges from 35-44% and similarity from 70-78% (Fig. 3.11). Regions one and two (Fig. 3.11.A and 3.11.B) contain elements that conform to the recently described consensus binding motifs for the high energy sulfate donor PAPS (see below). These elements, indicated by the green shading in Fig. 3.11, are found in all sulfotransferases characterized to date (Kakuta et al., 1998a). While there so far is no crystal structure reported for a carbohydrate sulfotransferase, information about the probable configuration of the active site has been obtained from two recent reports on the structure of estrogen sulfotransferase (EstST) co-crystallized with the inactive co-factor adenosine 3',5'-diphosphate and either estrogen (Kakuta et al., 1997) or vanadate (Kakuta et al., 1998b).

The 3'-phosphate binding site is comprised of residues from two conserved regions of EstST, one of which contains the sequence GxxGx**WK** that was demonstrated to play a role in PAPS binding (Komatsu et al., 1994; Lee et al., 1994), and which is located at the C-terminus of the protein. The GxxGx**WK** sequence does not appear to be present in the 6-OH family; however the sequence **WR**, occurring at the C-terminus within a possible weak consensus sequence, is absolutely conserved within this family (Fig. 3.10, not included in Fig. 3.11). The other conserved region involved in binding the 3'-phosphate of PAPS occurs towards the middle of the aa sequences in the 6-OH family, highlighted

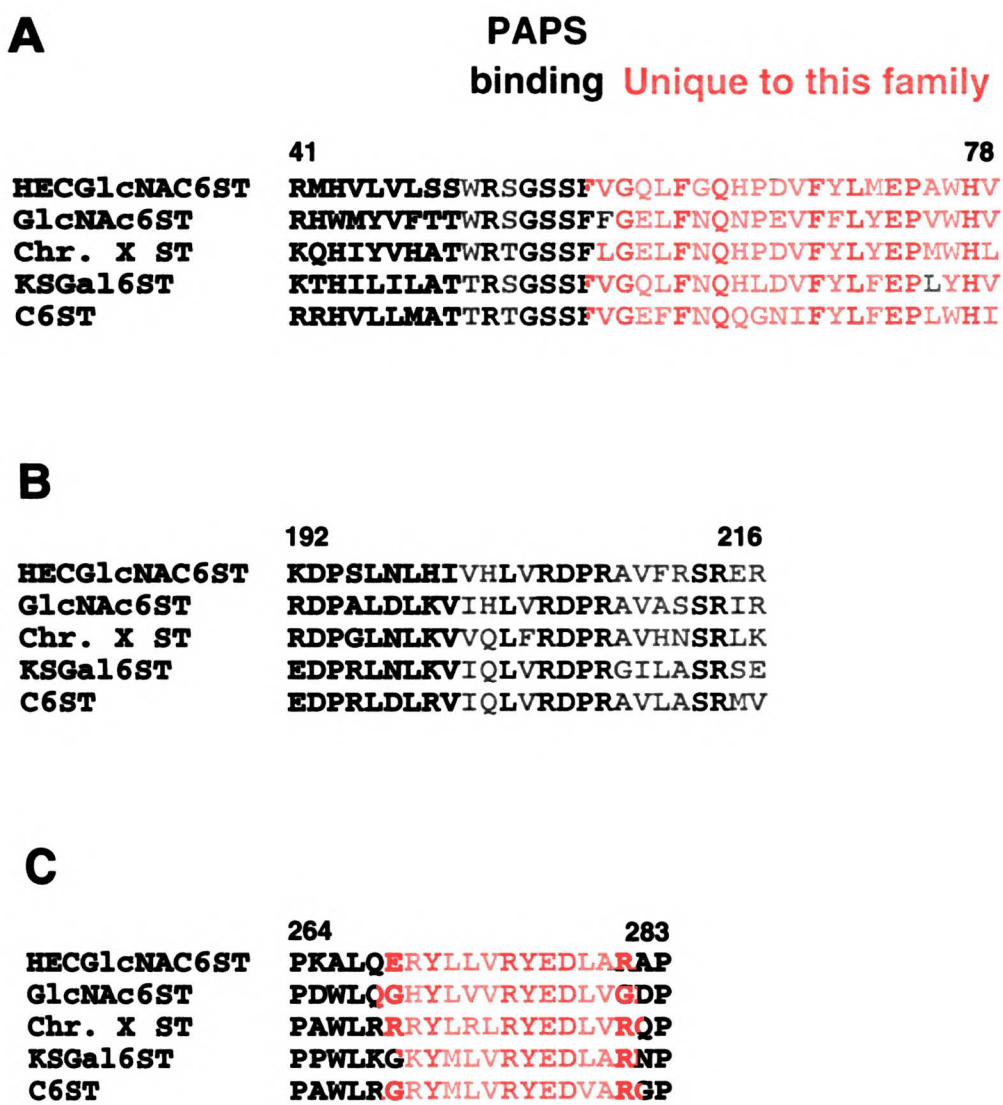
een in Fig. 3.11.B. Here too, all sulfotransferases so far characterized contain ence that conforms to a consensus motif (ITVLRNPADRLVSYYY in Kakuta et al., 3a), however, within the 6-OH family, this motif is extremely highly conserved, with ntral L(V/F)RDPR(A/G)(V/I) sequence that is not found in any other sulfotransferase g. 3.11.B).

sidues interacting with the 5'-phosphate in PAPS are in a characteristic P-loop motif nilar to the one found in ATP and GTP binding proteins (Chiba et al., 1995; Komatsu al., 1994). This loop, termed the PSB loop, is represented by the almost completely nserved sequence (W/T)R(S/T)GSSF(V/L/F) in the 6-OH family (highlighted in green Fig. 3.11.A). Within the PSB loop of EstST is a conserved lysine residue (Lys 48 in stST) which has been shown by mutational analysis to be critically involved in catalysis Kakuta et al., 1998b). The equivalent lysine (Lys 614) in human heparan sulfate N-leacetylase/N-sulfotransferase was also shown to be critical for the sulfotransferase ictivity of this enzyme (Sueyoshi et al., 1998). Taken together, the structural and mutational analyses of EstST suggest an in-line sulfuryl transfer mechanism similar to the one observed for phosphoryl transfer (Kakuta et al., 1997; Kakuta et al., 1998b) by many kinases (Matte et al., 1998). In the 6-OH family, the residue corresponding to Lys 48 in the EstST appears to be the arginine located in position 2 of the PSB loop (Fig. 3.11.A). The K48R mutant in the EstST retained a low level of activity (24% of wild type k_{cat}) (Kakuta et al., 1998b), indicating that the essential character of the residue at that position may be the positive charge of its side chain. This character is therefore conserved within the novel 6-OH family of sulfotransferases.

gure 3.11

lignment of regions of high homology among the five members of the novel family of rbohydrate sulfotransferases that modify 6-hydroxyl residues

Alignment of regions of high conservation among human carbohydrate 6-ulfotransferases, as defined in the legend for Fig. 3.10. Protein sequences were aligned sing the ClustalW algorithm (Thompson et al., 1994). Sequences that conform to the onsensus sequences for binding to PAPS are indicated in green (identity at a given esidue in dark green). Sequences that are unique to this family are indicated in red (identity at a given residue in dark red). See text for details.


A
PAPS
binding
Unique to this family
41
78
HECGlcNAC6ST RMHVLVLSSWRSGSSFVGQLFGQHPDVFYLMEPAWHV
GlcNAc6ST RHWMYVFTTWRSGSSFFGELFNQNPEVFFLYEPVWHV
Chr. X ST KQHIYVHATWRTGSSFLGELFNQHPDVFYLYEPMWHL
KSGal6ST KTHILILATTRSGSSFVGQLFNQHLDVFYLFEPLYHV
C6ST RRHVLLMATTRTGSSFVGEFFNQQGNIFYLFEPLWHI
B
192
216
HECGlcNAC6ST KDPSLNLHIVHLVRDPRAVFRSRER
GlcNAc6ST RDPALDLKVIHLVRDPRAVASSRIR
Chr. X ST RDPGLNLKVVQLFRDPRAVHNSRLK
KSGal6ST EDPRLNLKVIQLVRDPRGILASRSE
C6ST EDPRLDLRVIQLVRDPRAVLASRMV
C
264
283
HECGlcNAC6ST PKALQERYLLVRYEDLARAP
GlcNAc6ST PDWLQQGHYLVVRYEDLVGDP
Chr. X ST PAWLRRRYLRLRYEDLVRQP
KSGal6ST PPWLKGKYMLVRYEDLARNP
C6ST PAWLRGRYMLVRYEDVARGP

ddition to the PAPS binding motifs found in regions one and two of Fig. 3.11 (parts A
B, respectively), regions one and three contain two stretches of sequence of 20 and
aa, respectively, (corresponding to aa 59-78 and 270-280, respectively, in the HEC-
cNAc6ST sequence) that are highly conserved. These stretches are highlighted in red
Fig. 3.11.A and 3.11.C. The sequence (L/V)RYED(L/V)(A/V) in the third region
g.3.11.C) is very highly conserved within the 6-OH family. A similar motif appears to
present in the majority of other sulfotransferases, however, there is a high degree of
vergence. The motif at the C-terminal end of the first region (Fig. 3.11.A), containing
e highly conserved sequence F(Y/F)L(F/Y/M)EP(L/V/A)(W/Y)H(V/I) is unique within
e 6-OH family. It is possible that these two elements contribute to a binding pocket
at interacts with the 6-hydroxyl group of the appropriate oligosaccharide acceptor (Gal,
alNAc, or GlcNAc) to bring it into apposition with the donor phosphosulfate group.

ummary

n summary, we have cloned three carbohydrate sulfotransferases which belong to a
ıovel family of highly related proteins. The proteins in this family share as much as 43%
dentity and 70% similarity at the aa level. This high level of homology may derive from
he fact that the acceptor structures for sulfation by these sulfotransferases are similar:
The sulfotransferases all modify the 6-hydroxyl group of their related monosaccharide
substrates (Gal, GalNAc, GlcNAc). Of the three sulfotransferases described here, one is
a Gal-6-O-sulfotransferase, which has been independently reported by Fukuta et al.

1997) and named KSGal6ST, and by Mazany et al. (1998) as a chondroitin sulfate GalNAc-6-O-sulfotransferase. This sulfotransferase is widely expressed, including in ymph node. We have preliminary evidence from RT-PCR analysis that this enzyme is xpressed in HEC, however this was not confirmed by our Northern or *in situ* ıybridization analyses. The two others are GlcNAc-6-O-sulfotransferases, one of which ıas been reported by others (Li and Tedder, 1999; Uchimura et al., 1998b) and named HuGlcNAc6ST/CHST2. Like KSGal6ST, HuGlcNAc6ST is rather ubiquitously expressed, including high levels of expression in both lymph node and PBL. Although we did not obtain positive evidence for the presence of the murine equivalent of HuGlcNAc6ST transcripts in HEC, Uchimura et al. (Uchimura et al., 1998a) reported its specific localization to these cells. The third sulfotransferase, which we have termed HEC-GlcNAc6ST, contrasts sharply with KSGal6ST and HuGlcNAcST in that it is expressed in a highly restricted fashion. Very high levels of expression was observed in the HEC of human tonsil and mouse lymph node, raising the possibility that this enzyme is specifically involved in the biosynthesis of L-selectin ligands. The next chapter provides a detailed description of the experiments we carried out to characterize the activities of the three newly cloned sulfotransferases.

ATERIALS AND METHODS

orthern Blot Analysis

ıe probe for KSGal6ST consisted of a 730 bp Hind III/BamH I restriction fragment
ɔm IMAGE Consortium clone 40604 (GenBank accession no. R55609) (American
ɣpe Culture Collection, Rockville, MD). The probe for HuGlcNAc6ST consisted of a
3 bp Hind III/EcoR I restriction fragment from LifeSeq clone 424325 (Incyte). The
ɔbe for HEC-GlcNAc6ST consisted of a 496 bp fragment from LifeSeq clone no.
20445 (derived from a human breast epithelial cell line), corresponding to nt 1021-
16 of the cloned cDNA (Fig. 3.8). The probes were labeled with [α-^{32}P]dATP
ıersham) by the random decamer priming method (Strip-EZ DNA Kit, Ambion, Inc.,
ɜtin TX). Multiple Tissue Northern blots (CLONTECH) containing poly(A)$^+$ RNA
ın various human tissues, were hybridized at 60 °C overnight in Rapid-Hyb
ıersham) and then washed twice at room temperature for 15 min in 2X SSC/0.1%
S followed by two 15 min washes at 60 °C in 0.1X SSC/0.1% SDS and
ɔradiography. For the Northern blot to establish expression in HEC, poly(A)$^+$ RNA
prepared from 1.5 x 10^7 HEC and 2.0 x 10^7 HUVEC, respectively. Cell lysis and
ıtion of the poly(A)$^+$ RNA with oligo(dT) latex beads was performed according to
ıanufacturer's protocol (Oligotex Direct Kit, Qiagen, Inc., Valencia, CA).
ɔximately 2 μg poly(A)$^+$ RNA was loaded per lane. The RNA was separated by
rophoresis in a 1% denaturing agarose-formaldehyde gel and transferred to

positively charged nylon filters (Hybond N+). The filters were hybridized and washed as for the Multiple Tissue blots. Blots were stripped using the Strip-EZ DNA Kit (Ambion), according to the manufacturer's protocol.

Semiquantitative RT-PCR Analysis

High endothelial cells (HEC) were purified from human tonsils as previously described (Sassetti et al., 1998). Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA). Total RNA was isolated from HEC, HUVEC and human tonsillar lymphocytes by lysis and extraction with RNAZol (Tel-Test, Inc., Friendswood, TX). Approximately 45 μg RNA was obtained from 7 x 10^6 cells. First strand cDNA was made from 2 μg total RNA primed with random hexamers using AMV reverse transcriptase (RT) (Life Technologies, Inc., Gaithersburg, MD). PCR reactions were carried out in a total volume of 10 μl of 1X KlenTaq buffer (CLONTECH Laboratories, Inc., Palo Alto, CA) containing 400nM primers, 200μM dNTPs, 0.2 μl KlenTaq Advantage DNA polymerase mix (KlenTaq polymerase, CLONTECH) and 1.0 μl of 2-fold serially diluted cDNA as template. Cycling conditions were: 1 min at 94 °C; 30 cycles of 30 sec at 92 °C followed by 1 min 15 sec at 68 °C; one cycle of 10 min at 68 °C. The following primers were used: for HEC-GlcNAc6ST, 5'-AAACTCAAGAAGGAGGACCAACCCTACTATGTGATGC-3' and 5'-GTGGATTTGCTCAGGGACAGTCCAGCTAGACAGAAGAT-3', which amplify a 456 bp fragment corresponding to nt 884-1339 in Fig 3.8.; for hypoxanthine

hosphoribosyltransferase (HPRT), 5'-CCTGCTGGATTACATCAAAGCACTG-3' and
'-TCCAACACTTCGTGGGGTCCT-3'. The resulting amplified DNA was
ectrophoresed and visualized by ethidium bromide.

eparation of an HEC cDNA Expression Library

cDNA expression library was prepared from human HEC using the SMART cDNA
hnology (CLONTECH), which incorporates a long distance PCR amplification step of
t strand cDNA. 1 μg of total HEC RNA prepared as above was mixed with a
dified oligo (dT) primer (1μM) containing a Not I site and a universal site for 3'
ning of the PCR reaction and the SMART oligonucleotide (1μM), which provides a
versal site for 5' priming of the PCR reaction. This mixture was heated at 72 °C for 2
to disrupt RNA secondary structure and first strand cDNA was synthesized by
LV RT in a total volume of 10 μl. 2 μl of this reaction mixture was subjected to 18
es of long-distance PCR primed by the universal primers using KlenTaq polymerase
ONTECH). The PCR reaction mixture was incubated in the presence of proteinase K
hr at 45 °C to destroy the KlenTaq polymerase activity, followed by heat
ivation at 90 °C for 10 min. The ds cDNA was polished by treatment with T4 DNA
nerase at 16 °C for 30 min, followed by ligation to EcoR I/BstX I adaptors
rogen, Carlsbad, CA) overnight at 16 °C in the presence of T4 DNA ligase.
tor-ligated cDNAs were digested with Not I and then phosphorylated. The ds
A was purified by size fractionation, ligated into EcoR I/Not I digested pCDNA1.1

(Invitrogen), and introduced into *E.coli* MC1061/p3 (Invitrogen) by electroporation. The library contained 500,000 independent clones with an average insert size of 1.1 kb.

Molecular Cloning of KSGal6ST and HuGlcNAc6ST

A human fetal brain library (λ ZAP, Stratagene, La Jolla, CA) was the kind gift of Dr. Marc Tessier-Lavigne at the University of California, San Francisco. Approximately 10^6 plaques were transferred in duplicate onto positively charged nylon filters (Hybond N+, Amersham, Arlington Heights, IL) and fixed by UV irradiation (Stratalinker, Stratagene). The probes for KSGal6ST and HuGlcNAc6ST were the same as were used for the Northern blot analysis. The probes were labeled with [α-^{32}P]dCTP (Amersham) by the random hexamer priming method (Mega Prime DNA labeling system, Amersham). Filters were prehybridized in Rapid-Hyb (Amersham) for 1 hr. Radioactive probe was added and filters were hybridized for 12-15 hr at 65 °C and then washed twice for 15 min each at room temperature in 2X standard saline citrate (SSC, 1X = 150mM sodium chloride/15mM sodium citrate, pH 7)/0.1% SDS, followed by two 15 min washes at 65 °C in 0.2X SSC/0.1% SDS. For both KSGal6ST and HuGlcNAc6ST, 18 independent hybridizing plaques were identified after the second round of hybridization. Cloned fragments contained within the Bluescript phagemid were in vivo excised using ExAssist helper phage (Stratagene) and sequenced (Sanger, 1977).

Molecular Cloning of HEC-GlcNAc6ST

HEC-GlcNAc6ST (human) was cloned from the HEC cDNA library by modification of a pool selection procedure (Kolodkin et al., 1997). Briefly, 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 approximately 18 hr at 37 °C. Each pool of 2000 colonies was harvested and grown for an additional 2 hr at 37 °C, and glycerol stocks were made. PCR analysis was performed, using the HEC-GlcNAc6ST specific primers described above, to identify positive pools. One 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. Analysis of individual colonies by PCR resulted in a single positive clone, which was sequenced (Sanger, 1977). To clone the murine HEC-GlcNAc6ST, a 241 bp probe (nt 26 through 267) was amplified from the EST clone (AA522184, Research Genetics Inc., Huntsville, AL) and used as probe for screening a BAC library from the C57BL/6 mouse (Genome Systems Inc., St. Louis, MO). From the single positive clone, DNA was purified and sequenced directly, employing primers derived from EST AA522184 (forward: 5'TGGGTCAGCATGCCTTCCATACTAAC 3'; reverse: 5'TTCTAAGATTCCGGTTGCTTCTCCGTGGAC 3') and then obtaining sequence upstream (1559 nt) and downstream (582 nt). The resulting 1926 nt sequence was confirmed by resequencing in both directions.

In situ Hybridization

Paraffin sections (5μM) from C57BL6 mice were deparaffinized, fixed in 4% paraformaldehyde and treated with proteinase K. After washing in 0.5X SSC, the sections were covered with hybridization solution (50% formamide, 300mM NaCl, 20mM Tris, pH 8.0, 5mM EDTA, 1x Denhardt's, 10% Dextran sulfate, 10mM DTT), prehybridized for 1-3 hr at 55 °C, and hybridized overnight with sense or antisense ^{35}S-labeled riboprobe transcribed from the IMAGE consortium clone 851801 (GenBank accession no. AA522184) (Research Genetics, Inc., Huntsville AL) which had been modified by digestion with Sac I followed by religation. After hybridization, sections were washed at high stringency, dehydrated, dipped in photographic emulsion NTB2 (Eastman Kodak Company, Rochester, NY), stored at 4 °C for 2-8 weeks, developed and counterstained with hematoxylin and eosin.

Chapter Four

Characterization of the Three Cloned Carbohydrate Sulfotransferases: Demonstration of Their Acceptor Specificities, Ability to Sulfate L-selectin Ligands and Contribution to the Generation of L-selectin Ligand Activity

The three cDNAs described in Chapter Three belong to a family of related sulfotransferases (Table 3.2), all the human members of which have been identified within the last two years (Table 3.1). This family comprises members that modify the C-6 position in either Gal, GalNAc or GlcNAc (Table 3.1). As mentioned in Chapter Three, while our work to clone and characterize the three cDNAs was in progress, cDNAs for two of the sulfotransferases were identified independently by others, and their activities characterized. Fukuta et al. reported the cloning of KSGal6ST and characterized it as a keratan sulfate Gal-6-O-sulfotransferase (Fukuta et al., 1997). In their hands, KSGal6ST had two-fold better activity towards keratan sulfate than desulfated keratan sulfate, indicating that the enzyme prefers to sulfate the Gal residue when the adjacent GlcNAc is sulfated at C-6. This enzyme has also been characterized as a chondroitin sulfate GalNAc-6-O-sulfotransferase (Mazany et al., 1998). The mouse and human GlcNAc6STs reported by Uchimura et al. (1998a; 1998b) were shown to catalyze the transfer of sulfate to C-6 of the non-reducing GlcNAc in GlcNAcβ1→3Galβ1→4GlcNAc. The enzymes did not transfer sulfate to the GlcNAc

residue in the presence of β1→4-linked Gal, indicating that they require terminal GlcNAc. As will be discussed below, their characterization also addressed the ability of these enzymes to confer a sulfated sLe^x epitope, sialyl 6-sulfo Le^x, to cells transfected with cDNAs encoding human or murine GlcNAc6ST.

Demonstration of the acceptor specificities of the three sulfotransferases encoded by the cloned cDNAs

We had three objectives in characterizing the three cloned cDNAs. First, we wanted to demonstrate that they encode sulfotransferases, which in the case of KSGal6ST and HuGlcNAc6ST would confirm already published characterizations (Fukuta et al., 1997; Uchimura et al., 1998b). We utilized an *in vitro* sulfotransferase assay developed by Bowman et al. (1998) to determine acceptor specificities of the enzymes. Second, we wanted to know whether they could sulfate L-selectin ligands. Towards this end, we transfected COS cells with cDNAs encoding GlyCAM-1 or CD34 and the three sulfotransferases and measured sulfate incorporation into the recombinant acceptors. Acceptor specificity could also be established in this assay, by procedures previously established in the Rosen laboratory (Hemmerich et al., 1994a). And finally, we were very interested in determining whether the sulfotransferase cDNAs could confer L-selectin ligand activity onto the recombinant ligands. We employed two assays to answer this question. First, we used flow cytometry to determine whether CHO cells transfected with CD34 and either of the three sulfotransferases could elaborate L-selectin ligands as measured by the binding of an L-selectin/IgM chimera. Second, we asked whether

recombinant GlyCAM, produced in COS cells by cotransfection with either sulfotransferase could serve as a rolling ligand for lymphocytes in a flow chamber assay.

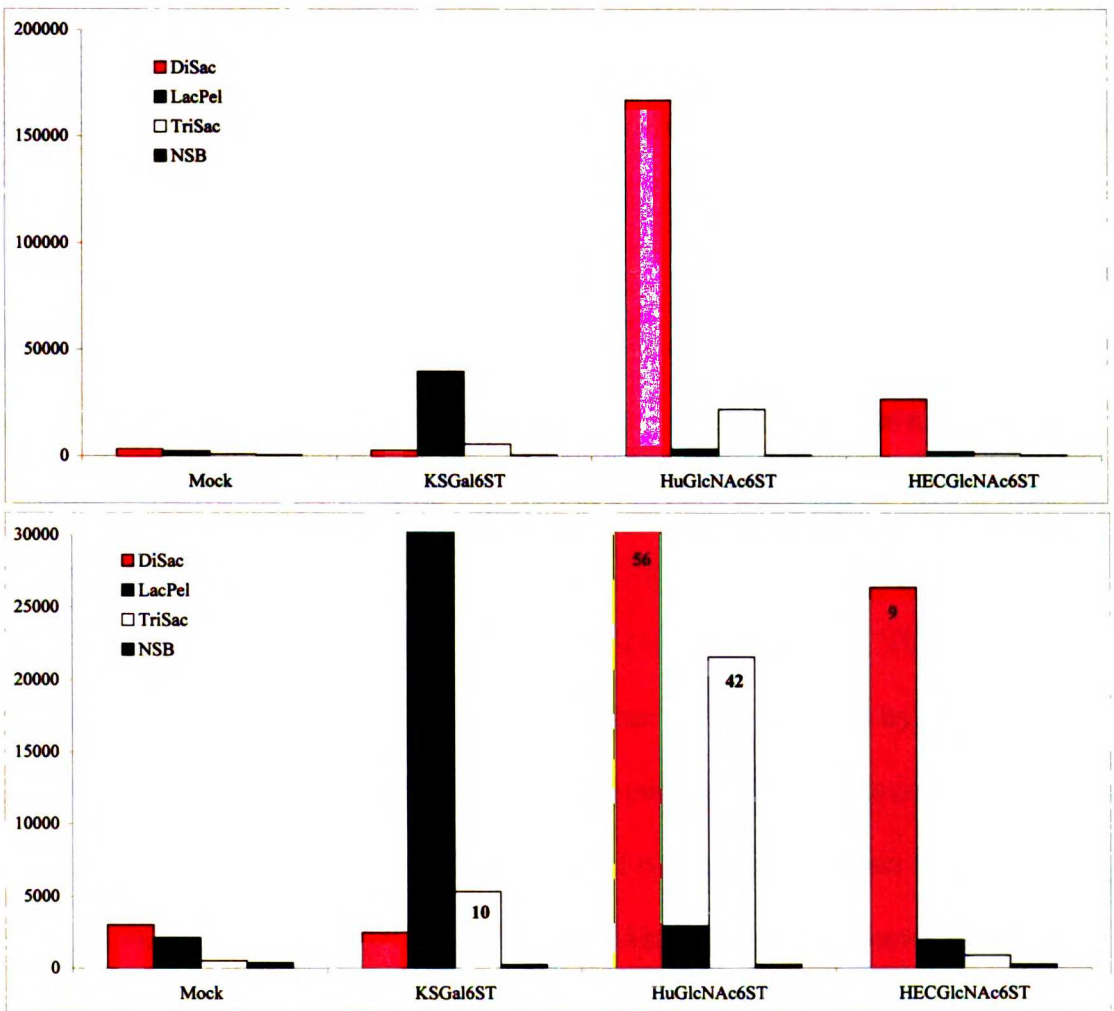
Our initial goal was to demonstrate that the newly cloned cDNAs encoded sulfotransferases and to determine their acceptor specificity. We expressed them in COS cells and tested cellular extracts for their ability to transfer ^{35}S-sulfate from ^{35}S-PAPS to synthetic oligosaccharide acceptors, using an assay recently reported by Bowman et al. (1998). The lipid-conjugated disaccharide and trisaccharide acceptors (Table 1.1) were based on core structures of GlyCAM-1 chains (Chapter One, Fig. 1.2) with the substitution of Gal for GalNAc at the reducing termini. The cDNAs encoding KSGal6ST and HuGlcNAc6ST were first subcloned into expression vectors. COS cells were transfected with cDNAs for each of the three sulfotransferases and detergent extracts of microsomal fractions were prepared. These extracts were incubated with either one of the three acceptors and ^{35}S-PAPS, and the sulfated acceptor was then isolated from the reaction mixture by capture onto a C-18 reversed-phase column followed by elution in methanol. As can be seen in Fig.4.1, KSGal6ST transferred sulfate to both the acceptor lactose (LacPel, Galβ1→4Glc; 19 fold over the mock transfectant microsomes) and the trisaccharide acceptor Galβ1→4GlcNAcβ1→6Galα-R (trisaccharide, TriSac; 10 fold over mock), but had no activity towards the disaccharide acceptor GlcNAcβ1→6Galα-R (disaccharide, DiSac).

In contrast, extracts from COS cells transfected with HEC-GlcNAc6ST transferred substantial radioactivity to the disaccharide acceptor (Fig 4.1; 9 fold over mock) whereas

Figure 4.1

Sulfation of Synthetic Acceptors by KSGal6ST, HuGlcNAc6ST and HEC-GlcNAc6ST

Microsomal extracts from COS cells transfected with cDNAs encoding KSGal6ST, HuGlcNAc6ST or HEC-GlcNAc6ST, or vector cDNA (Mock) were reacted with [^{35}S]-PAPS and the lipid-conjugated disaccharide acceptors lactose (LacPel) or GlcNAcβ1→6GalαR (DiSac), or the trisaccharide acceptor Galβ1→4GlcNacβ1→6GalαR (see Table 1.1, Chapter One). After a 2 hour incubation at 37°C, radiolabeled acceptor was isolated by reversed-phase chromatography and incorporation was quantified by liquid scintillation counting. Each datapoint represents the average of duplicate determinations. In the bottom panel, the scale has been changed to show the fold differences in the low range (to 30,000 CPM). The bold numbers written in the bars indicate the fold activity of the given extract relative to that of the mock transfectant extract.


200000
150000
100000
50000
0
DiSac
LacPel
TriSac
NSB
Mock
KSGal6ST
HuGlcNAc6ST
HECGlcNAc6ST
30000
25000
20000
15000
10000
5000
0
DiSac
LacPel
TriSac
NSB
10
56
42
9
Mock
KSGal6ST
HuGlcNAc6ST
HECGlcNAc6ST

the activity towards the trisaccharide acceptor was barely above the control generated by the extract from mock-transfected cells. This requirement for terminal GlcNAc mirrors that of the HEC-specific GlcNAc-6-O-sulfotransferase activity recently described by Bowman et al. (1998), as well as those of recently described liver (Spiro et al., 1996) and lung mucosa (Degroote et al., 1997) GlcNAc-6-O-sulfotransferase activities. HuGlcNAc6ST utilized the disaccharide acceptor very efficiently (Fig. 4.1; 56 fold over mock) and also exhibited a low level of activity towards the trisaccharide (Fig. 4.1; 42 fold over mock), indicating that at least with our acceptors, this enzyme might not have an absolute requirement for terminal GlcNAc, as had been indicated by the experiments of Uchimura et al. (1998b).

The regiochemistry of sulfation by the three enzymes was established by HPAEC using the protocols of Bowman et al. (1998) (data not shown). This analysis confirmed the designation by Fukuta et al. of KSGal6ST as a Gal-6-O-sulfotransferase (Fukuta et al., 1997). Similarly, HEC-GlcNAc6ST and HuGlcNAc6ST were both shown to be GlcNAc-6-O-sulfotransferases, confirming the report by Uchimura et al. (1998b) with respect to HuGlcNAc6ST and establishing the specificity of the novel enzyme HEC-GlcNAc6ST.

Sulfation of GlyCAM-1 and CD34 by HEC-GlcNAc6ST, HuGlcNAc6ST and KSGal6ST

The experiments described in the preceding section demonstrated that the three cloned cDNAs encode sulfotransferases that can modify simple oligosaccharide substrates in an *in vitro* sulfotransferase assay. Based on their activities towards these simple acceptors, they are referred to as GlcNAc-6-O-sulfotransferases (HEC-GlcNAc6ST and HuGlcNAc6ST) and one as a Gal-6-O-sulfotransferase sulfotransferase (KSGal6ST).

Our next objective was to determine whether the three sulfotransferases were capable of sulfating L-selectin ligands. This capability is one of the important criteria for establishing the involvement of a candidate sulfotransferase in the biosynthesis of these ligands. However, as has been demonstrated for the fucosyltransferases, this ability does not in and of itself constitute conclusive evidence of the biological role of a given enzyme. For example, transfection of cDNA for FTIII into COS cells can direct the expression of sLe^x on the surface of these cells (Lowe et al., 1990) and can furthermore cause the cells to bind E-selectin (Larsen et al., 1992). FTIII is therefore capable of appropriately modifying ligands for at least one selectin. Sako et al. exploited this property in the cloning of the cDNA for PSGL-1, which was based on the expression by COS cell transfectants of functional P-selectin ligand, as measured by the binding of a P-selectin/IgG chimera (Sako et al., 1993). They cotransfected the cDNA for FTIII into COS cells together with a cDNA library from HL-60 cells, which were known to be able to synthesize a functional ligand for P-selectin (Moore et al., 1992). Thus, FTIII clearly

is capable of modifying a bona fide P-selectin ligand in a cell line. However, based on the expression pattern of FTIII and on gene inactivation studies in mice for the FTVII gene, it is now clear that the essential FTs for synthesis of P- and E-selectin ligands in leukocytes are FTVII and FTIV, but not FTIII (Lowe et al., 1990; Maly et al., 1996).

To test whether the novel sulfotransferases could sulfate L-selectin ligands, we took advantage of two chimeric cDNA constructs encoding GlyCAM-1/IgG and CD34/IgG. These constructs were made by subcloning GlyCAM-1 (full length) and human CD34 (extracellular domain), respectively, into the pIG1 vector (Simmons, 1993). When transfected into COS cells, these cDNAs direct the expression and secretion of the chimeric proteins into the conditioned medium (CM) of the cells. Purification of the protein is facilitated by the presence of the IgG Fc portion, which mediates high affinity interactions with protein A. By incubating the cells with ^{35}S -sulfate after transfection, we could thus obtain radiolabeled GlyCAM-1/IgG or CD34/IgG for assessment of the relative abilities of the three sulfotransferases to sulfate these recombinant ligands. Furthermore, since GlyCAM-1/IgG is abundantly secreted into the CM, we could generate enough material to establish the regiochemistry of sulfation by hydrolysis and HPAEC analysis.

Sulfation of GlyCAM-1/IgG by HEC-GlcNAc6ST, HuGlcNAc6ST and KSGal6ST

COS cells were transfected with cDNAs encoding 1) GlyCAM-1/IgG and 2) one of the three sulfotransferases. The transfected cells were cultured in the presence of ^{35}S -sulfate, and radiolabeled GlyCAM-1/IgG was purified from the CM by passage over protein A-sepharose, followed by SDS-PAGE. As shown in Fig 4.2.A., there was substantial incorporation of radioactivity into GlyCAM-1/IgG when either KSGal6ST or HEC-GlcNAc6ST cDNA (but not empty vector) was included in the cotransfection mixture. HuGlcNAc6ST cDNA also conferred substantial sulfation of GlyCAM-1/IgG in COS cells (data not shown).

Table 4.1. Level of sulfation of GlyCAM-1/IgG conferred by KSGal6ST, HuGlcNAc6ST and HEC-GlcNAc6ST.

Transfection	Specific Activity [cpm/μg]
FT	2367 ± 142
FT+KSGal6ST	19262 ± 1824
FT+HuGlcNAc6ST	9624 ± 1804
FT+HEC-GlcNAc6ST	10068 ± 1347

COS cells were transfected with plasmids encoding GlyCAM-1/IgG, C2GnT, FTVII and either KSGal6ST, HuGlcNAc6ST, HEC-GlcNAc6ST (FT+ST) or empty plasmid (FT). Transfected cells were cultured in the presence of [^{35}S]-sulfate. Recombinant GlyCAM-1/IgG chimeras were purified and the specific radioactivity was determined. The data points represent the mean ± range in two independent experiments.

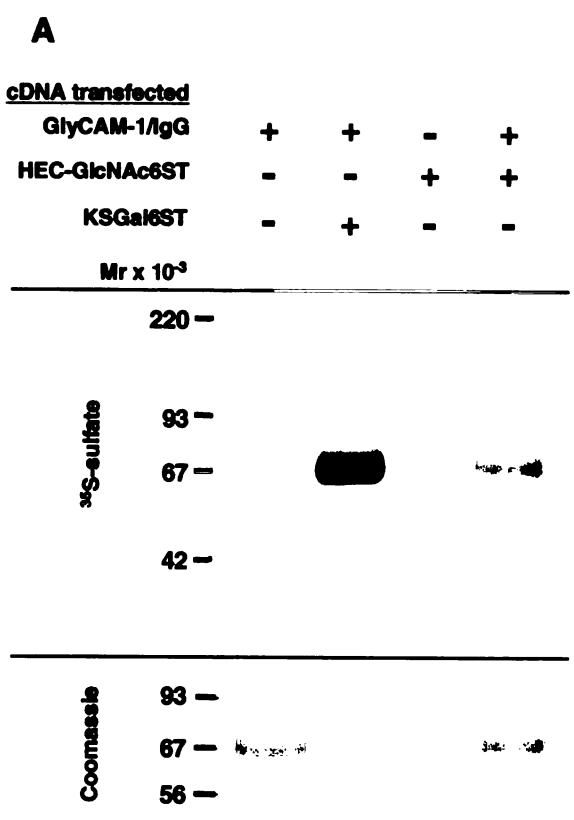
Figure 4.2

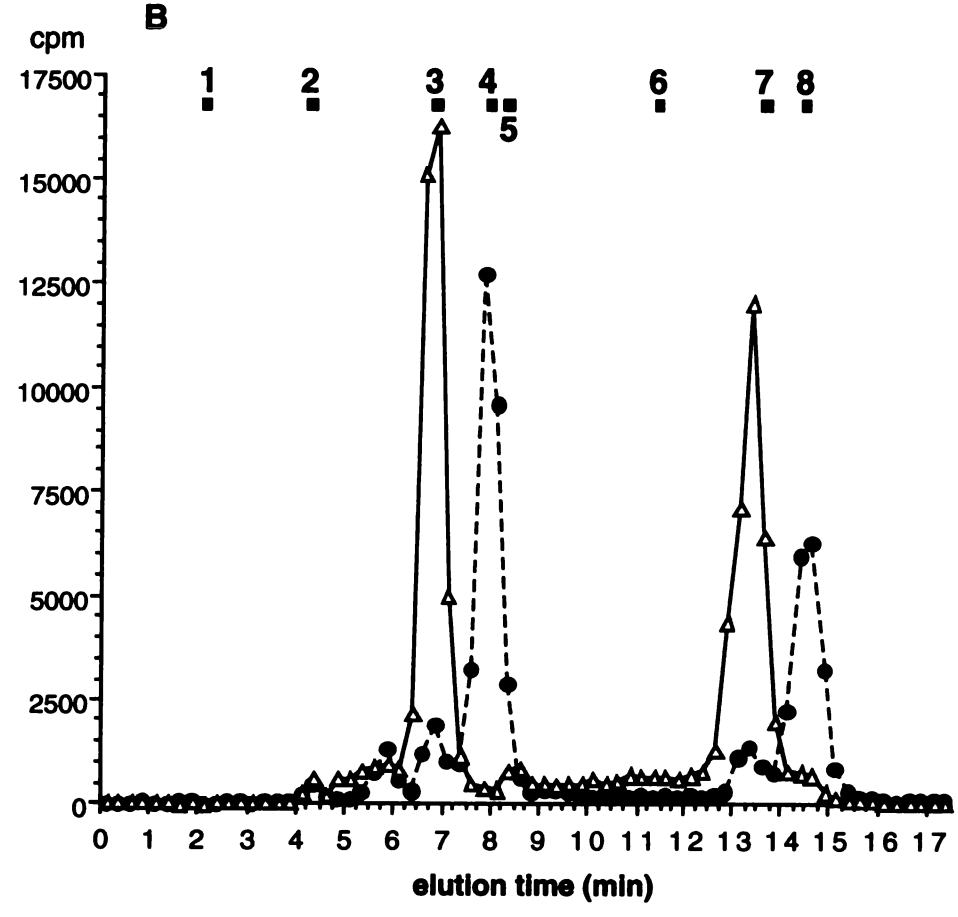
Sulfation of GlyCAM-1/IgG by KSGal6ST and HEC-GlcNAc6ST

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 [^{35}S]-sulfate and recombinant GlyCAM-1/IgG was isolated from the conditioned medium. 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 (—Δ—) or KSGal6ST (--•--), were analyzed by HPAEC after acid hydrolysis. The following standards are indicated: **1**, GlcNAc-3SO_3^-; **2**, [^{35}S]-SO_4^{2-}; **3**, Galβ1→4[SO_3^-→6]GlcNAc; **4**, [SO_3^-→6]Galβ1→4GlcNAc; **5**, Gal-4SO_3^-; **6**, Gal-3SO_3^-; **7**, GlcNAc-6SO_3^-; **8**, Gal-6SO_3^-.


A
cDNA transfected
GlyCAM-1/IgG + + - +
HEC-GlcNAc6ST - - + +
KSGal6ST - + - -
Mr x 10-3
220
93
67
42
35S-sulfate
93
67
56
Coomassie


B
cpm
17500
15000
12500
10000
7500
5000
2500
0
1 2 3 4 5 6 7 8
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
elution time (min)

The activities of each sulfotransferase towards GlyCAM-1/IgG were compared directly by transfecting and radiolabeling COS cells as above, in parallel, with equal amounts of each sulfotransferase cDNA. Radiolabeled GlyCAM-1/IgG was isolated and quantified by ELISA to normalize for amount of acceptor, and then subjected to scintillation counting. As can be seen in Table 4.1, the specific activity of the two GlcNAc-6-O-sulfotransferases towards GlyCAM-1/IgG appears to be approximately the same, as measured by amount of sulfate incorporation per μg of sulfotransferase cDNA. In contrast, KSGal6ST sulfated GlyCAM-1/IgG with approximately two-fold better efficiency. We do not know what the basis is for this apparent difference in ability to sulfate GlyCAM-1/IgG, however, based on the *in vitro* results discussed above, one possibility is that a limiting factor is the availability of acceptor structures for the two GlcNAc-6-O-sulfotransferases in the appropriate cellular compartment. Both of the GlcNAc-6-O-sulfotransferases require (or in the case of HuGlcNAc6ST, greatly prefer) a terminal GlcNAc, and sulfation of GlcNAc by these enzymes in vivo therefore by inference must precede galactosylation. Indeed, several β1→4galactosyltransferases have been identified that are capable of modifying terminal GlcNAc-6-sulfate (Degroote et al., 1997; Seko et al., 1998; Spiro et al., 1996), and one of these in fact has almost no activity in the absence of the sulfate ester on C-6 of GlcNAc (Seko et al., 1998). Under this view, β1→4galactosylation of GlcNAc can be considered to be competitive with sulfation, whereas the converse is not true: 6-sulfated GlcNAc is a substrate for at least one β1→4galactosyltransferase. With respect to KSGal6ST, it is capable of sulfating C-6 of Gal in the presence or absence of an adjacent 6-sulfate ester on GlcNAc (Fukuta et al., 1997). Thus, while the pool of available substrates for either HEC-GlcNAc6ST or

HuGlcNAc6ST is subject to reduction by competitive β1→4galactosyltransferases possibly present within the same compartment, sulfation of the galactose residue by KSGal6ST is not restricted dynamically in this manner. We can not exclude the possibility that the observed greater level of sulfation of GlyCAM-1/IgG by KSGal6ST reflects artificial effects on the activities of the three sulfotransferases consequent to their being expressed in COS cells and/or utilizing a substrate that may traverse the compartments of the secretory pathway with different kinetics than native GlyCAM-1.

Analysis of sulfated GlyCAM-1/IgG carbohydrates to determine the regiospecificity of sulfation by HEC-GlcNAc6ST, HuGlcNAc6ST and KSGal6ST

To confirm that the substrate specificities for each of the three sulfotransferases towards GlyCAM-1/IgG oligosaccharides reflected those observed with the *in vitro* acceptors, we subjected the sulfated GlyCAM-1/IgG to hydrolysis and HPAEC analysis as was previously done for native GlyCAM-1 (Hemmerich et al., 1994a). In brief, by slight modifications to our established procedures (Hemmerich et al., 1994a), the isolated GlyCAM-1/IgG was hydrolyzed in acid and the hydrolysate was subjected to gel filtration to obtain low molecular weight fragments. These fragments were separated on DEAE-sepharose to obtain singly charged oligosaccharides which were then separated by gel filtration to obtain singly charged monosaccharides. These monosaccharides were subjected to HPAEC to determine the position of the sulfate esters. As shown in Fig. 4.2.B, transfection with KSGal6ST resulted in sulfated mono- and disaccharides that

comigrated with [SO_3→6]Gal and [SO_3→6]Galβ1→4GlcNAc, establishing that this enzyme has Gal-6-O-sulfotransferase activity on an L-selectin ligand. In contrast, transfection with HEC-GlcNAc6ST resulted in products that corresponded to [SO_3→6]GlcNAc and Galβ1→4[SO_3→6]GlcNAc (Fig 4.2.B). The HPAEC profile for sulfated monosaccharides produced in cells transfected with HuGlcNAc6ST was identical to the one obtained for HEC-GlcNAc6ST (data not shown). Thus, these enzymes were confirmed to be GlcNAc-6-O-sulfotransferases (our *in vitro* data, above, and Uchimura et al., 1998b) and shown to be capable of sulfating an L-selectin ligand.

Sulfation of CD34/IgG by HEC-GlcNAc6ST and KSGal6ST

Since GlyCAM-1 is a murine protein we considered the remote possibility that the sulfation of GlyCAM-1/IgG in COS cells by the three sulfotransferases could reflect irrelevant activities that would not act on a human L-selectin ligand. We therefore undertook a similar analysis with human CD34. COS cells were transfected as described above with cDNAs encoding CD34/IgG and either KSGal6ST or HEC-GlcNAc6ST. The cells were incubated in the presence of ^{35}S-sulfate, and radiolabeled CD34/IgG was purified from the CM as above. However, when we subjected 20% of the isolated material to scintillation counting, the radioactive signal was barely detectable. As we had previously observed that the CD34/IgG cDNA does not direct high levels of CD34/IgG secretion in COS cells, we were concerned that the absence of radioactive signal might reflect an absence of CD34/IgG in the CMs. To control for this, we decided to verify the

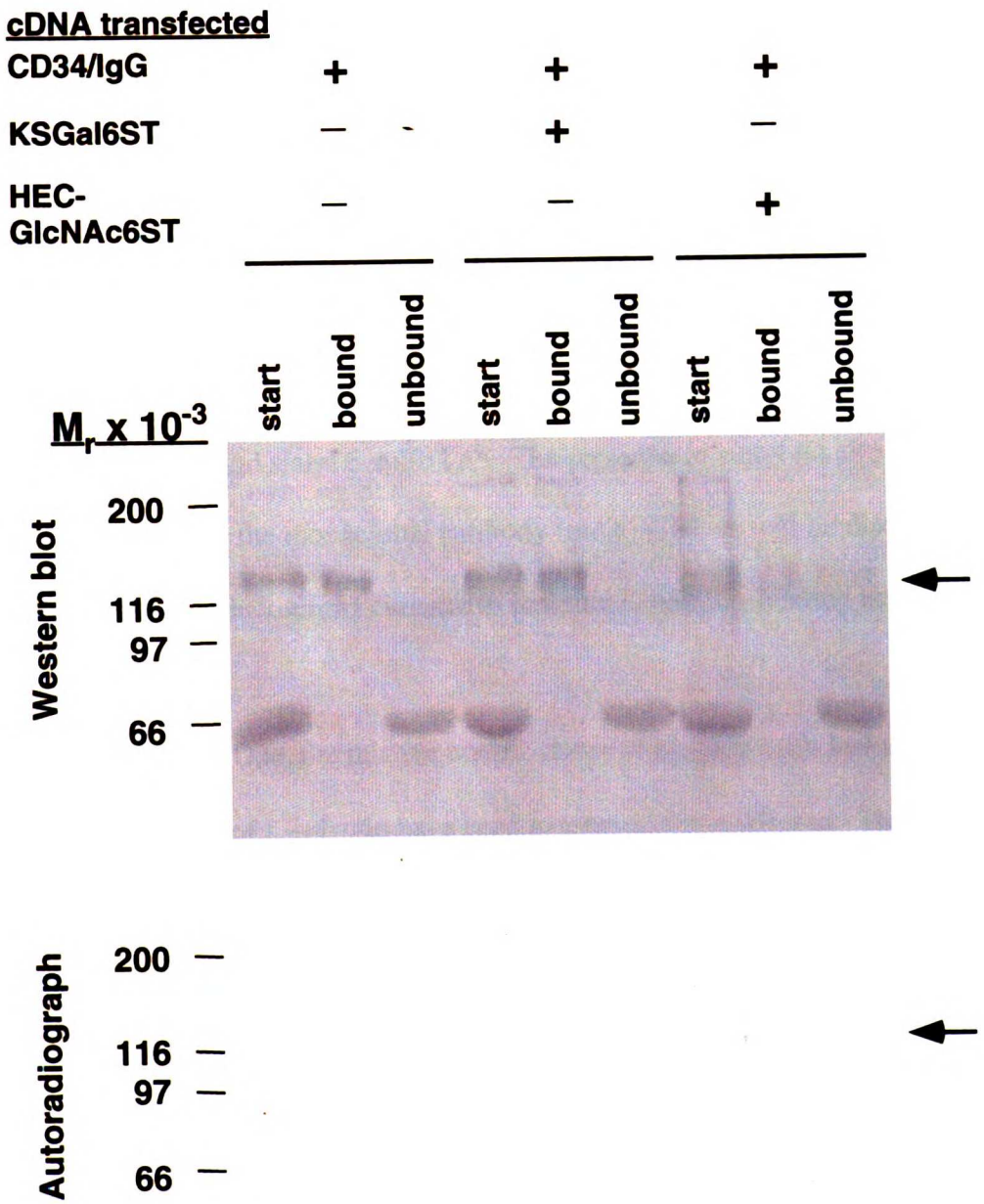
presence of CD34/IgG protein by Western blot. As can be seen in Fig 4.3, top panel, CD34/IgG protein was produced by all the transfectants, albeit at low levels, and was efficiently captured by the protein A-sepharose. Fig. 4.3, bottom panel, shows an autoradiograph of the same Western blot membrane after 4 weeks of exposure. Although the bands are faint, they clearly show specific sulfation of CD34/IgG in the presence of either KSGal6ST or HEC-GlcNAc6ST. The presence of a sulfate labeled band in the "unbound" lane for the HEC-GlcNAc6ST transfection mirrors the apparent incomplete capture of the CD34/IgG, as indicated by the Western blot.

In summary of the *in vitro* assays and COS cell sulfation experiments, we have shown that all three of the newly cloned cDNAs encode sulfotransferases. One of them, KSGal6ST, is a Gal-6-O-sulfotransferase, as had been previously reported (Fukuta et al., 1997), that appears to have activity towards both keratan sulfate (Fukuta et al., 1997) and chondroitin sulfate (Mazany et al., 1998). The two others are GlcNAc-6-O-sulfotransferases that appear to require terminal GlcNAc for their activity. This profile mirrors that of recently described GlcNAc-6-O-sulfotransferases activities in HEC, liver and lung mucosa (Bowman and Bertozzi, 1999; Degroote et al., 1997; Spiro et al., 1996). All three of the enzymes can sulfate the L-selectin ligands GlyCAM-1 and CD34 (KSGal6ST and HEC-GlcNAc6ST) in COS cells and, as shown with GlyCAM-1, with the appropriate regiospecificity.

Figure 4.3

Sulfation of CD34/IgG by KSGal6ST and HEC-GlcNAc6ST

COS cells were transfected with combinations of plasmids encoding CD34/IgG, KSGal6ST and HEC-GlcNAc6ST, as indicated. Transfected cells were cultured in the presence of [^{35}S]-sulfate and recombinant CD34/IgG was isolated from the conditioned medium and analyzed by SDS-PAGE and Western blot. Top panel shows Western blot detecting CD34 protein (arrow). Bottom panel shows autoradiograph of the same Western blot.


cDNA transfected
CD34/IgG
KSGal6ST
HEC-GlcNAc6ST
+ + +
– + –
– – +
start bound unbound start bound unbound start bound unbound
Mr x 10-3
Western blot
200
116
97
66
Autoradiograph
200
116
97
66

Characterization by flow cytometry: Demonstration of the ability of the cloned cDNAs to confer the sialyl 6-sulfo Le^x epitope and L-selectin binding activity onto CHO cells

The demonstration that all three of the newly cloned sulfotransferases could sulfate GlyCAM-1/IgG and CD34/IgG in COS cells raised the issue of what the functional implications are of the presence of the sulfate esters on these recombinant ligands. Based on our analysis of the GlyCAM-1 capping structures, we were particularly interested in whether the sulfotransferases could catalyze sulfation of these ligands to give sialyl 6'-sulfo Le^x and sialyl 6-sulfo Le^x. The presence of sialyl 6-sulfo Le^x could be directly assessed with the monoclonal antibody (mAb) G72, as will be discussed below. We used an L-selectin/IgM chimera to measure L-selectin binding activity.

As discussed in Chapter One, the relative contributions of sialyl 6-sulfo Le^x and sialyl 6'-sulfo Le^x to the binding of L-selectin have been extensively investigated. The evidence to date favors neither structure as significantly more potent than the other, as the effects demonstrated have not been large and the data are not consistent. Several groups synthesized sulfated variants of sLe^x and tested their ability to interact directly with L-selectin by immobilizing them on ELISA plates and then detecting binding with an L-selectin/IgG chimera (Galustian et al., 1997; Yoshino et al., 1997). In these assays, evidence was found for a contribution from sialyl 6-sulfo Le^x (Galustian et al., 1997) and from sialyl 6',6-disulfo Le^x (Yoshino et al., 1997). Direct binding to L-selectin was also tested by Tsuboi et al., who generated sialyl 6-sulfo Le^x and sialyl 6'-sulfo Le^x at the cell surface of CHO cells and tested the ability of the cells to bind to immobilized L-

selectin/IgG chimera (Tsuboi et al., 1996). In this assay, sialyl 6'-sulfo Le^x supported higher levels of cell binding than sLe^x (20% versus 10% cells bound) and furthermore, sialyl 6-sulfo Le^x appeared to have a counter-adhesive effect. Competition studies utilizing sulfated sLe^x variants have also been carried out, in which the binding of an L-selectin/IgG chimera to GlyCAM-1 (Koenig et al., 1997) or to PNAd (Scudder et al., 1994) was inhibited. Evidence for a contribution from the sulfate esters of both sialyl 6'-sulfo Le^x (Koenig et al., 1997) and sialyl 6-sulfo Le^x (Scudder et al., 1994) was found. Two additional studies tested the ability of sulfated analogs of sLe^x to inhibit the binding of L-selectin to GlyCAM-1 (Bertozzi et al., 1995; Saunders et al., 1996). Saunders et al. utilized 3'-sulfo Le^x (Glc) as a substitute for sLe^x. This structure differs from sLe^x in two aspects: GlcNAc is replaced with Glc and the sialic acid is replaced with sulfate at C-3 of Gal. In agreement with the results of Scudder et al., who found that sulfation at C-6 of GlcNAc enhanced L-selectin binding by 4-fold (Scudder et al., 1994), 6-sulfation of 3'-sulfo Le^x (Glc) enhanced the inhibitory potency against L-selectin by 3-fold. However, surprisingly, 6'-sulfated 3'-sulfo Le^x was no better an inhibitor than 3'-sulfo Le^x, contrasting with the enhanced efficacy of inhibition from the analogous modification on sLe^x (Koenig et al., 1997). One possible explanation for this discrepancy is that 3'-sulfation (i.e., at Gal) of Le^x does not truly mimic 3'-sialylation. In a set of experiments employing the same inhibition assay, Bertozzi et al. (1995) found that several sulfated variants of lactose (Table 1.1, Chapter One) were equal to or more potent than sLe^x itself. For example, 6'6-disulfo lactose competed 2-fold better than sLe^x, demonstrating that the relevant sulfate modifications by themselves, in the absence of any contribution from sialic acid or fucose, can confer a significant degree of binding to L-selectin. In

summary, neither the direct binding nor the inhibition studies have revealed significant differences between sialyl 6'-sulfo Le^x and sialyl 6-sulfo Le^x in terms of their ability to bind L-selectin.

In order to study the expression of sulfated sLe^x epitopes on HEV, Mitsuoka et al. generated a panel of mAb directed against simple (synthetic) and complex sLe^x determinants (Mitsuoka et al., 1997). Two of these, G152 and G72, recognize synthetic sialyl 6-sulfo Le^x in a sialic acid-, fucose- and sulfate-dependent manner. G152 and G72 both stained HEV in human lymphoid organs very strongly and inhibited the binding of an L-selectin/IgG chimera to human lymph node sections (Mitsuoka et al., 1997). These studies presented no positive evidence for the presence of sialyl 6'-sulfo Le^x or sialyl 6',6-disulfo Le^x on HEV on human lymph node. However, the data were not definitive with respect to these questions.

We chose to probe for the presence of the G72 epitope and L-selectin ligand activity by flow cytometry. Flow cytometry is a rapid, sensitive and quantitative method which allows for the simultaneous measurement of the expression of several epitopes on individual cells. The experimental strategy was to transfect a suitable cell line with cDNAs encoding an L-selectin ligand and either of the three sulfotransferases, and then stain the cells with monoclonal antibodies that detect specific epitopes or with L-selectin itself, as a measure of the generation of functional ligand activity. We chose to use CHO cells because they proved to be reliably transfectable and easy to manipulate for flow

cytometry. Indeed, we achieved sufficiently high transfection efficiencies that we were able to perform these experiments utilizing transiently transfected cells.

The recipient CHO cells, kindly donated to us by Dr. Geoffrey Kansas, were stably expressing the core 2 β1→6-N-acetylglucosaminyltransferase (the "core 2 branching enzyme", C2GnT) as well as FTVII. The core 2 structure (Galβ1→3[GlcNAcβ1→6]GalNAcα →Ser/Thr, Fig. 1.2, Chapter One) is the most prevalent among core structures in mammalian O-glycans (Schachter and Brockhausen, 1989), and the C2GnT is a key enzyme regulating branching diversity (Bierhuizen and Fukuda, 1992; Williams and Schachter, 1980). The core 2 structure was found to be present in all GlyCAM-1 O-glycans (Hemmerich et al., 1995, Fig. 1.2) and is therefore inferred to be required for the elaboration of the sulfated sLe^x capping structures. Similarly, the FTVII activity has recently been shown to be required for the generation of L-selectin ligands in HEV (Maly et al., 1996). As native CHO cells possess neither the C2GnT nor the FTVII activities endogenously (Bierhuizen and Fukuda, 1992), the double stably transfected line (CHO/C2GnT/FTVII) provided us with a useful starting population for our transient transfection approach. The CHO/C2GnT/FTVII cells will be referred to as "CHO" cells, unless otherwise indicated.

The CHO cells were transiently transfected with cDNAs encoding CD34 and each sulfotransferase, alone or in various combinations. We were initially concerned that C2GnT and FTVII might not be expressed at sufficiently high levels in the stable transfectants, and we therefore included cDNAs encoding these two enzymes in the

transfection mix. Their presence did not seem to diminish the overall expression efficiency (as measured by levels of CD34 expression, see Fig. 4.4) and we therefore decided to keep these two cDNAs included in the transfection mixtures for all subsequent experiments. As will be discussed below, expression of sLe^x did not vary significantly among different transfectants, indicating that C2GnT and FTVII activities were constant. The CHO cells were grown for two days after transfection at which time they were harvested in 0.6mM EDTA, quantified and stained with antibodies and/or the L-selectin/IgM chimera, as described in the Materials and Methods.

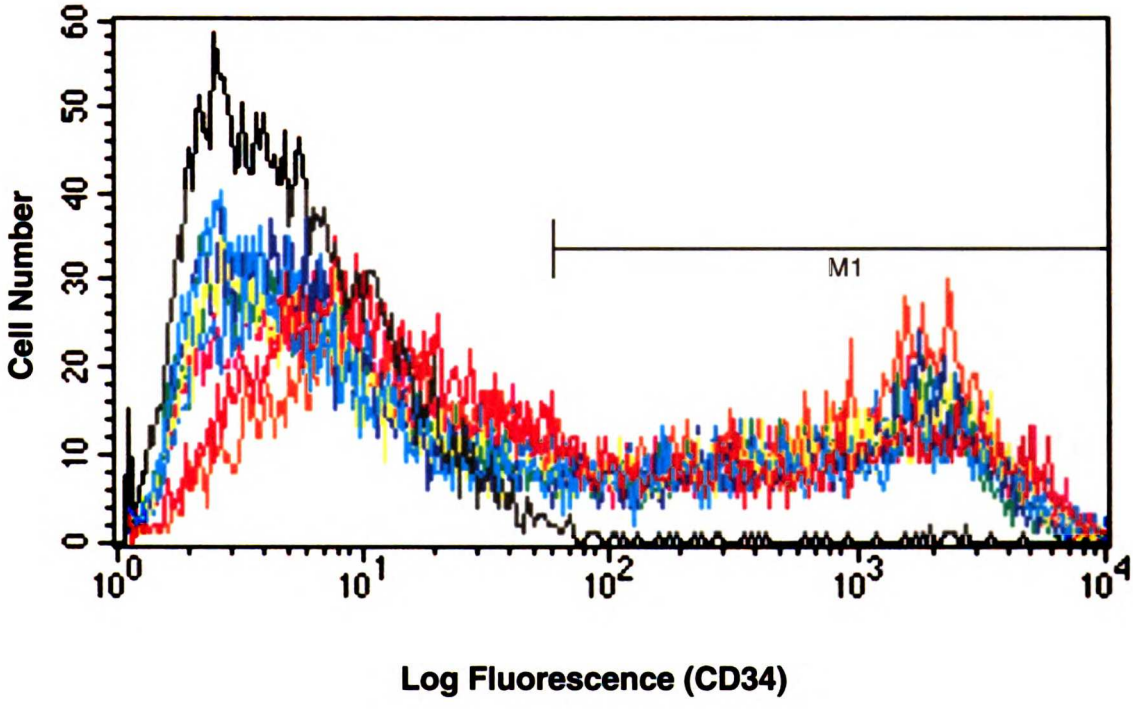
Expression of CD34 as a measure of transfection efficiency

When the CHO cells were transfected by the Lipofectamine protocol (Felgner et al., 1987), we consistently achieved transfection efficiencies in the range of 25-40%, as measured by the expression of CD34 on the cell surface. The level of CD34 expression did not vary substantially within a given experiment, irrespective of the number or amounts of other cDNAs cotransfected with CD34 (Table 4.2). A representative experiment is shown in Fig 4.4. In this experiment, the average transfection efficiency is 38±5.4% ($CD34^+$ cells), and the cells expressing the highest levels of CD34 were also transfected with the highest levels of sulfotransferase cDNAs. Thus, CD34 appeared to be a reasonable and reliable measure of overall transfection efficiency.

Figure 4.4

Expression of CD34 in cells transfected with combinations of sulfotransferases

CHO/FTVII/C2GnT cells were transfected with cDNAs encoding C2GnT, FTVII, CD34 and combinations of KSGal6ST, HEC-GlcNAc6ST or HuGlcNAc6ST, as indicated in Table 4.2. Cells were stained with anti-CD34 mAb. The region M1 indicates cells staining positive for CD34, as defined by the isotype control antibody (<0.5% of cells stained with the control antibody are included in M1). Please refer to Table 4.2 for the percentage of positive cells and mean fluorescence intensity (MFI) of the cells in each population of transfectants.


Cell Number
0
10
20
30
40
50
60
M1
10^0
10^1
10^2
10^3
10^4
Log Fluorescence (CD34)

Expression of sialyl 6-sulfo Le^x

We were in a position to determine whether HuGlcNAc6ST and HEC-GlcNAc6ST could confer the sialyl 6-sulfo Le^x epitope onto CHO cells, as Dr. Kannagi of the Aichi Cancer Center in Nagoya, Japan had provided us with the G72 mAb. The CHO cells were transfected with cDNAs encoding CD34, C2GnT, FTVII and either of the three sulfotransferases and then stained with the G72 mAb, as described in the Materials and Methods. As shown in figure 4.5.A, CHO cells transfected with either HuGlcNAc6ST or HEC-GlcNAc6ST expressed sialyl 6-sulfo Le^x. In the case of HuGlcNAc6ST, this result confirms those of Uchimura et al. (1998a; 1998b). The expression of sialyl 6-sulfo Le^x was not dependent on the presence of CD34, as omission of this cDNA from the transfection mix resulted in a modest decrement in, but not elimination of, G72 staining (Fig 4.5.B and 4.5.C). This finding is in contrast to the requirements for binding of L-selectin to sulfated sLe^x structures on CHO cells, as will be discussed below.

Compared to the HuGlcNAc6ST-transfected population, more cells (approximately 2-fold more on average, in four experiments) in the HEC-GlcNAc6ST-transfected population expressed the sialyl 6-sulfo Le^x epitope (Fig 4.5.A). It is not clear whether this is due to overall greater efficiency of sulfation by HEC-GlcNAc6ST. The two sulfotransferases sulfated the L-selectin ligand GlyCAM-1 with approximately equal efficiency (Table 4.1), however, the majority of sulfate in the present experiments was not associated with another L-selectin ligand CD34 (Fig 4.5.B and 4.5.C). It is possible

Figure 4.5

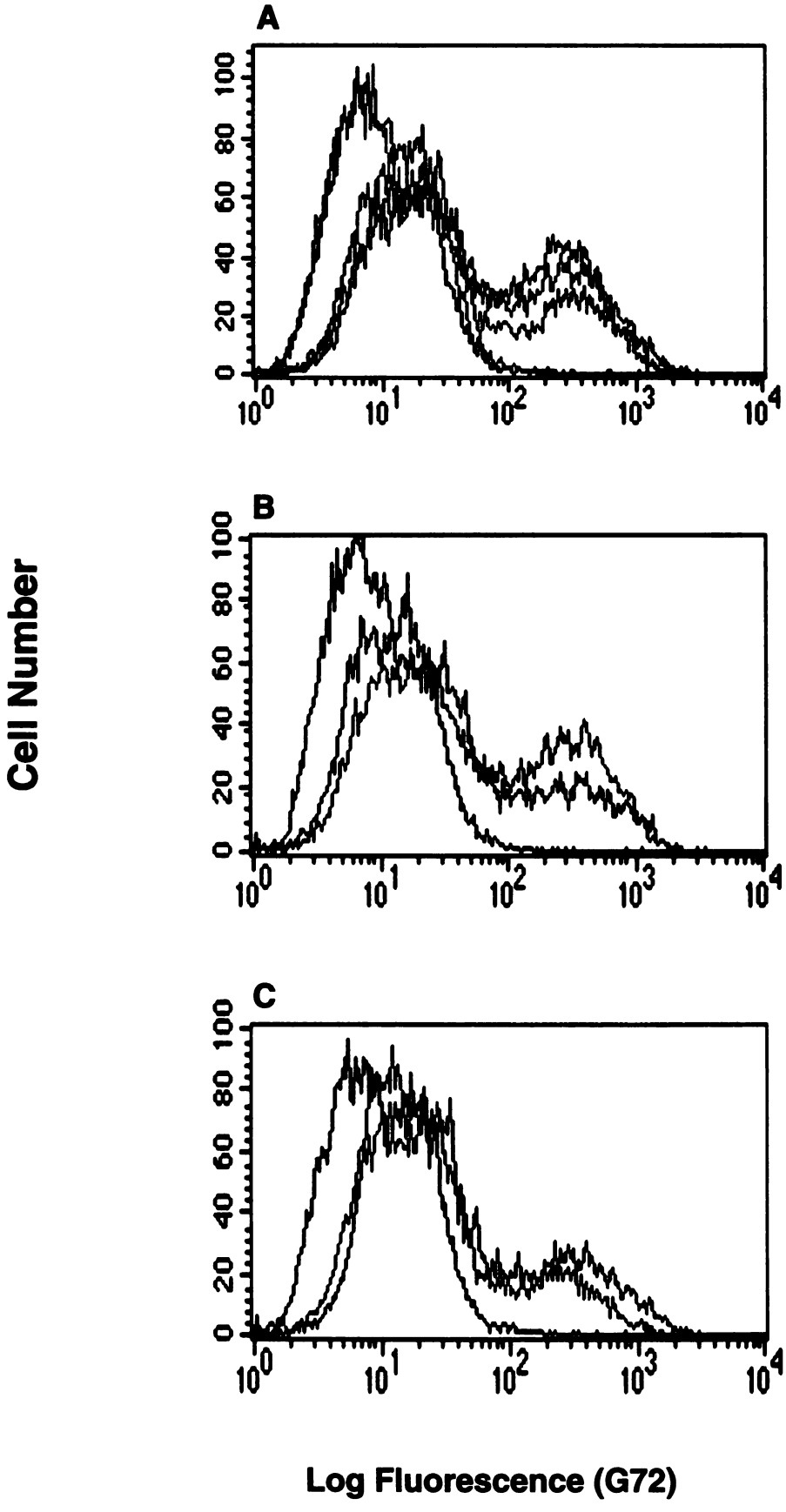
Generation of sialyl 6-sulfo Le^x by transfection with HEC-GlcNAc6ST or HuGlcNAC6ST cDNA

CHO/FTVII/C2GnT cells were transfected with a cDNA encoding HEC-GlcNAc6ST or HuGlcNAc6ST, with or without CD34 cDNA. Cells were stained with the G72 mAb to detect the presence of sialyl 6-sulfo Le^x.

(A) Histogram shows G72 staining for the transfections with HEC-GlcNAc6ST (—) or HuGlcNAc6ST (—) or both (—), or staining of the isotype control antibody (—).

(B) Histogram shows G72 staining for the transfections with HEC-GlcNAc6ST with (—) or without (—) CD34 cDNA, or staining of the isotype control antibody for the transfection with CD34 cDNA (—).

(C) Histogram shows G72 staining for the transfections with HuGlcNAc6ST with (—) or without (—) CD34 cDNA, or staining of the isotype control antibody for the transfection with CD34 cDNA (—).


A
B
C
Cell Number
0
20
40
60
80
100
10^0
10^1
10^2
10^3
10^4
Log Fluorescence (G72)

that HEC-GlcNAc6ST sulfates the endogenous acceptors in CHO cells more efficiently than HuGlcNAc6ST.

Cotransfection of HEC-GlcNAc6ST and HuGlcNAc6ST did not result in higher levels of expression of sialyl 6-sulfo Le^x on the CHO cells (Fig 4.5.A). In four separate experiments, the cotransfected population never exceeded the HEC-GlcNAc6ST-transfected population in terms of either percent positive cells or mean fluorescence intensity (MFI). This result could be due to the presence of non-saturating amounts of the G72 mAb, as we were using cell culture supernatant as a source. Alternatively, a possible mechanistic explanation for this phenomenon is inhibition of the activity of one sulfotransferase by the other, or mutually inhibitory activities of both sulfotransferases. This is implausible in view of the fact that they are unlikely to utilize a completely overlapping set of carbohydrate substrates (Baenziger, 1994; Hooper et al., 1996). One approach to answering this question would be to generate purified GlyCAM-1/IgG sulfated in COS cells by one or the other sulfotransferase and then use this material as a substrate for sulfation by the other sulfotransferase in an *in vitro* assay. If GlyCAM-1/IgG sulfated by either of the two sulfotransferases is a poorer substrate than unsulfated GlyCAM-1/IgG for *in vitro* sulfation by the other sulfotransferase, then this could be an indication of inhibition at the level of substrate availability.

Cells transfected with KSGal6ST did not stain with the G72 mAb (not shown), confirming that this enzyme has no GlcNAc-6-O-sulfotransferase activity.

Summary

In summary, both HuGlcNAc6ST and HEC-GlcNAc6ST were capable of conferring sialyl 6-sulfo Le^x expression to CHO cells by transfection. This epitope did not require CD34 and was presumably presented by glycoconjugates endogenous to CHO cells. HEC-GlcNAc6ST appeared to be slightly more efficient than HuGlcNAc6ST at generating sialyl 6-sulfo Le^x. The basis for this is not clear, as the two enzymes sulfated GlyCAM-1 with equal efficiency.

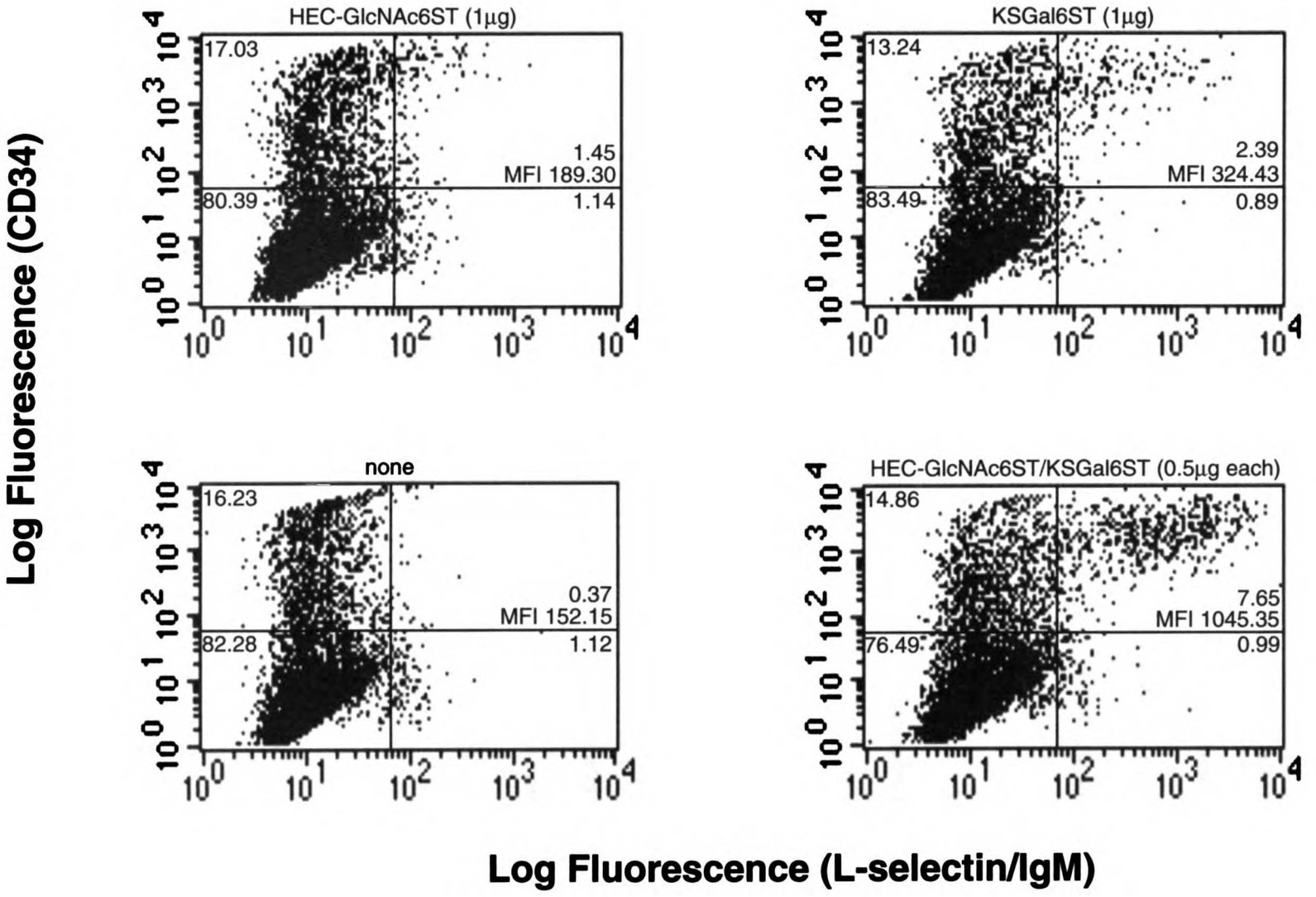
Expression of L-selectin binding activity

The ability of sulfated recombinant ligands to bind L-selectin was also evaluated by flow cytometry. The CHO cells were transfected as before with cDNAs encoding CD34, C2GnT, FTVII and each sulfotransferase, alone or in combination with the others. The cells were harvested and quantified and then stained with an L-selectin/IgM chimera. The cDNA encoding this chimera, a mouse L-selectin/human-Fc fusion construct, was donated to us by Dr. John Lowe at the University of Michigan. The pentameric nature of the IgM fusion partner enhances the avidity of the chimeric molecule and therefore allows for the detection of binding that is of intrinsically low affinity, as is the case with L-selectin/ligand interactions. Figure 4.6 shows that transfection with either HEC-GlcNAc6ST or KSGal6ST conferred binding to the L-selectin/IgM chimera. The profile of staining for HuGlcNAc6ST transfectants was nearly identical to that of HEC-

Figure 4.6

L-selectin reactivity conferred to CD34 by transfection with cDNAs for HEC-GlcNAc6ST and KSGal6ST

CHO/FTVII/C2GnT cells were transfected with different combinations of cDNAs encoding human CD34, HEC-GlcNAc6ST and KSGal6ST. Cells were stained with the L-selectin/IgM chimera to detect ligand activity. Two color analysis showing CD34 expression (y-axis, staining with anti-CD34-PE mAb) and L-selectin ligand activity (x-axis, FITC) in cells transfected with CD34 cDNA and cDNAs encoding HEC-GlcNAc6ST and KSGal6ST, alone or in combination as indicated. The horizontal bar is set such that all cells staining with the isotype matched control for the CD34 mAb are included in the lower quadrants. The vertical bar is set to indicate the L-selectin/IgM staining of cells in which no sulfotransferase cDNA was included in the transfection mixture (lower left panel). The fraction of positive cells (as a percentage of the total) in each quadrant is indicated. The mean fluorescence intensities (MFI) for the cells in the upper right quadrants are indicated. The CD34 mAb did not interfere with staining by the L-selectin/IgM chimera.


HEC-GlcNAc6ST (1μg)
17.03
1.45
MFI 189.30
80.39
1.14
KSGal6ST (1μg)
13.24
2.39
MFI 324.43
83.49
0.89
none
16.23
0.37
MFI 152.15
82.28
1.12
HEC-GlcNAc6ST/KSGal6ST (0.5μg each)
14.86
7.65
MFI 1045.35
76.49
0.99
Log Fluorescence (CD34)
Log Fluorescence (L-selectin/IgM)

GlcNAc6ST transfectants (data not shown), indicating that both of these sulfotransferases are able to modify CD34 at the GlcNAc residues relevant for L-selecting binding. KSGal6ST appeared to exert the greater effect, both in terms of the proportion of positive cells and their MFI. Although not formally proven, this effect might be due to the apparent greater efficiency of sulfation by KSGal6ST (Table 4.1).

While each of the three sulfotransferases was clearly able to confer L-selectin binding onto CD34, the combination of a Gal-6-Osulfotransferase and a GlcNAc-6-O-sulfotransferase cDNA markedly enhanced the binding. This is illustrated in Fig 4.6 for KSGal6ST and HEC-GlcNAc6ST co-transfectants. At an equal amount of total sulfotransferase cDNA, a greater proportion of the cotransfected population bound the L-selectin chimera. Moreover, the signal resulting from the combination clearly exceeded the sum of the signals from the single transfectants. This effect was evident over a range of total sulfotransferase cDNA concentrations, as shown in Tables 4.2 and 4.3. It remains to be established whether this apparent synergy in ligand activity was due to cooperative effects in the activities of the two sulfotransferases towards their respective monosaccharide acceptors, leading to overall higher sulfation levels on the ligand. We favor the interpretation that the apparent synergy reflects a requirement for both the Gal-6-sulfate and GlcNAc-6-sulfate moieties in the generation of optimal L-selectin binding activity. It is possible that this synergy arises through dual recognition of separate monosulfated chains, for example one chain capped by sialyl 6-sulfo Le^x and the other by sialyl 6'-sulfo Le^x. This mechanism would fit a model of selectin binding proposed by

Table 4.2. L-selectin/IgM and CD34 staining of CHO cells transfected with combinations of sulfotransferase cDNAs.

μg cDNA transfected		*L-sel-IgM Staining*	*CD34 Staining*	
KSGal6ST	HEC-GlcNAc6ST	MFI	MFI	% positive
0	0	0	660	36.63
1.0	0	364	742	35.04
1.5	0	391	731	38.95
0	1.0	114	748	49.71
0	1.5	163	700	33.51
0.5	0.5	935	743	37.68
1.0	0.5	917	684	32.41
0.5	1.0	830	703	35.50

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 mean fluorescence intensity (MFI) for staining with a CD34 mAb (Qbend-10) for the population that was positive for CD34 (percentage positive cells in each transfected population is indicated), as defined by staining with the class-matched control antibody.

Varki (Varki, 1994), in which a specific cluster of adjacent O-linked chains comprises the full recognition determinant. Alternatively, individual chains containing both modifications may underlie the synergistic effect. In this regard, it should be noted that most of the sulfated chains within GlyCAM-1 contain two or more sulfates, although the structure of these chains has not been solved (Hemmerich et al., 1995). It is not yet clear whether sialyl 6',6-disulfo Le^x (Figure 1.2, Table 1.1) exists as a capping group on these

multi-sulfated chains or whether there are extended chains containing sulfates on internal Gal or GlcNAc residues.

The combination of all three sulfotransferase cDNAs yielded slightly enhanced L-selectin binding (in terms of percent positive cells and MFI), relative to that obtained with the KSGal6ST/HEC-GlcNAc6ST double transfectants (Fig 4.7). One interpretation of this finding is that, at least to some degree, the GlcNAc residues utilized as substrates by the two GlcNAc-6-O-sulfotransferases are found in different contexts, each of which can contribute to L-selectin binding when sulfated at C-6 of the GlcNAc residue. For example, one of the two GlcNAc-6-O-sulfotransferases might have a greater propensity than the other for sulfating internal GlcNAc residues (subterminal to sLe^x or sulfo sLe^x), thereby leading to overall greater sulfation of the chains and presumably to greater global presentation of binding motifs for L-selectin.

All of the above described binding to the L-selectin/IgM chimera was specific to the L-selectin domain, as indicated by its calcium dependence (Fig 4.8.A and 4.8.B, blue traces) and complete inhibition by MEL-14, a function-blocking anti-L-selectin mAb (Fig 4.8.A and 4.8.B, green traces).

Figure 4.7

Enhanced L-selectin reactivity conferred to CD34 by cotransfection with HEC-GlcNAc6ST, HuGlcNAc6ST and KSGal6ST cDNAs

CHO/FTVII/C2GnT cells were transfected with different combinations of cDNAs encoding human CD34, HEC-GlcNAc6ST, HuGlcNAc6ST and KSGal6ST. Cells were stained with the L-selectin/IgM chimera to detect ligand activity. Two color analysis showing CD34 expression (y-axis, staining with CD34-PE mAb) and L-selectin ligand activity (x-axis, FITC) in cells transfected with CD34 cDNA and cDNAs encoding HEC-GlcNAc6ST, HuGlcNAc6ST and KSGal6ST, alone or in combination as indicated. The horizontal bar is set such that all cells staining with the isotype matched control for the CD34 mAb are included in the lower quadrants. The vertical bar is set to indicate the L-selectin/IgM staining of cells in which no sulfotransferase cDNA was included in the transfection mixture (lower left panel). The fraction of positive cells (as a percentage of the total) in each quadrant is indicated. The mean fluorescence intensities (MFI) for the cells in the upper right quadrants are indicated. The CD34 mAb did not interfere with staining by the L-selectin/IgM chimera.

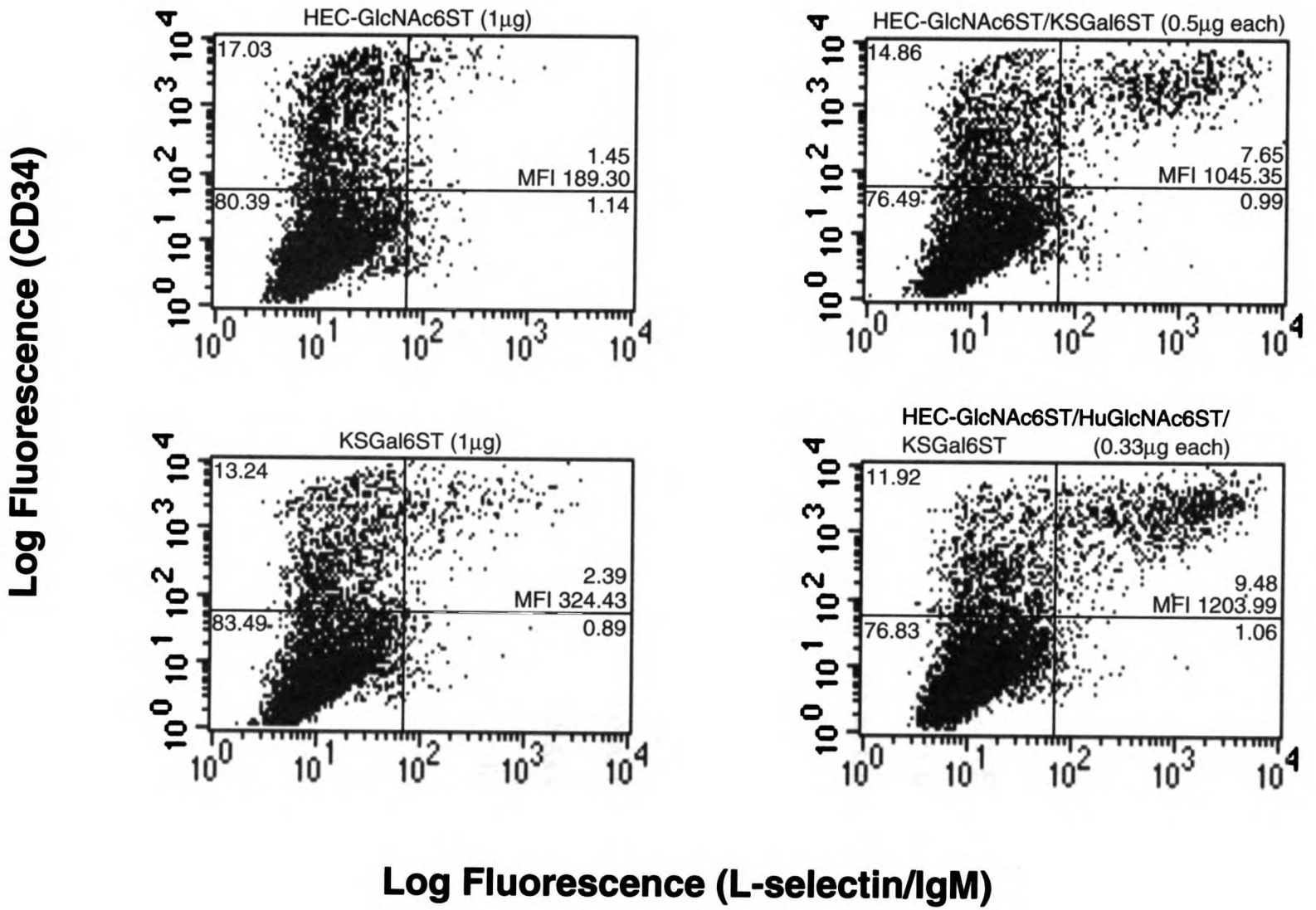

HEC-GlcNAc6ST (1µg)
17.03
1.45
MFI 189.30
80.39
1.14
HEC-GlcNAc6ST/KSGal6ST (0.5µg each)
14.86
7.65
MFI 1045.35
76.49
0.99
KSGal6ST (1µg)
13.24
2.39
MFI 324.43
83.49
0.89
HEC-GlcNAc6ST/HuGlcNAc6ST/
KSGal6ST (0.33µg each)
11.92
9.48
MFI 1203.99
76.83
1.06
Log Fluorescence (CD34)
Log Fluorescence (L-selectin/IgM)

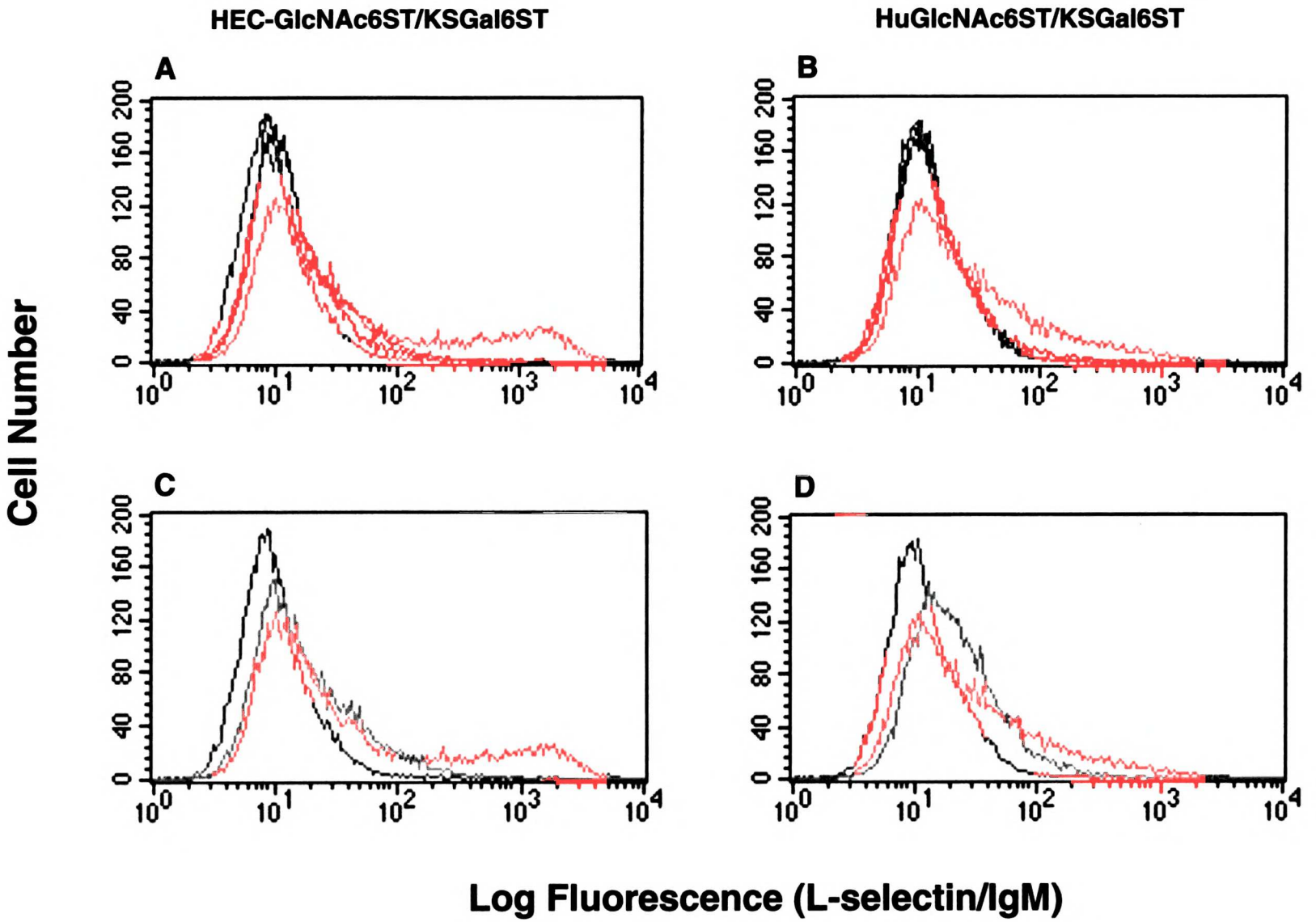
Figure 4.8

Specificity of L-selectin reactivity and requirement for CD34

CHO/FTVII/C2GnT cells were transfected with cDNAs encoding C2GnT, FTVII, KSGal6ST and HEC-GlcNAc6ST (A and C) or HuGlcNAc6ST (B and D), in the presence or absence of human CD34 cDNA. Cells were stained with the L-selectin/IgM chimera to detect ligand activity. Histograms showing L-selectin/IgM staining for the following transfections:

(A and B) CD34/HEC-GlcNAc6ST or HuGlcNAc6ST /KSGal6ST cDNAs (—); CD34/HEC-GlcNAc6ST or HuGlcNAc6ST /KSGal6ST cDNAs with staining done in the presence of anti-L-selectin mAb (—) or EDTA (—); no sulfotransferase cDNAs (—).

(C and D) CD34/HEC-GlcNAc6ST or HuGlcNAc6ST /KSGal6ST cDNAs in the absence (—) or presence (—) of anti-L-selectin mAb; HEC-GlcNAc6ST or HuGlcNAc6ST /KSGal6ST without CD34 cDNA (—).


HEC-GlcNAc6ST/KSGal6ST
HuGlcNAc6ST/KSGal6ST
A
B
C
D
Cell Number
Log Fluorescence (L-selectin/IgM)
0
40
80
120
160
200
10^0
10^1
10^2
10^3
10^4

Expression of the HECA-452 epitope as a control for global glycosylation levels

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 sLe^x related structures and is widely used as a reporter for glycosylation modifications (sialylation and fucosylation) pertinent to selectin ligands (Tu et al., 1999; Wagers et al., 1997). Because this antibody reacts equally well with sulfated (at Gal-6, GlcNAc-6 or both) and non-sulfated sLe^x structures (Mitsuoka et al., 1998b), it was of particular utility for detecting the overall presence of sLe^x on the cell surface of the transfectants. As shown in Table 4.3, the expression of the HECA-452 epitope was not significantly altered (<25% variation) by transfection with the sulfotransferase cDNAs in any combination, whereas the synergetic action of KSGal6ST and HEC-GlcNAc6ST was maintained.

Requirement for a mucin scaffold for L-selectin binding

As mentioned in the Chapter One, a shared property of the HEV-associated ligands identified to date is the presence of a mucin region (Bargatze et al., 1995; Puri et al., 1995; Rosen and Bertozzi, 1994; Sassetti et al., 1998). This domain provides the potential for multivalent presentation of carbohydrate determinants, a feature that is thought to be important for enhancing the avidity of L-selectin interactions (Nicholson et al., 1998). Tsuboi et al. stated (Tsuboi et al., 1996, data not shown) that the binding of an

L-selectin/IgG chimera to sialyl 6'-sulfo Le^x at the cell surface of CHO cells was dependent on the presence of CD34. Our experiments with the G72 mAb demonstrated that the sialyl 6-sulfo Le^x (and by inference the sialyl 6'-sulfo Le^x) epitope could be presented by macromolecules present endogenously in CHO cells (Fig. 4.5). We were therefore interested in whether the binding of the L-selectin/IgM chimera to the CHO cell sulfotransferase transfectants required CD34. As shown in Fig. 4.8.C for KSGal6ST/HEC-GlcNAc6ST cotransfectants, omission of CD34 cDNA from the transfection mixture resulted in the nearly complete loss of staining with the chimera. The effect appeared to be less pronounced when HuGlcNAc6ST was the co-transfection partner of KSGal6ST (Fig. 4.8.D); however, this result may not be significant as the baseline L-selectin binding is lower for this pair of sulfotransferases. We consider it likely that the obligate presence of CD34 for L-selectin binding activity depends on its mucin character. Clearly, future work should be directed at determining the extent to which other mucins can provide this postulated scaffolding function. Candidate mucins to investigate include known ligands for L-selectin, such as GlyCAM-1 (Lasky et al., 1992), podocalyxin (Sassetti et al., 1998), MadCAM-1 (Berg et al., 1993) and PSGL-1 (Guyer et al., 1996; Sako et al., 1993; Spertini et al., 1996; Walcheck et al., 1996). Other mucins of interest include CD44 (Gunthert et al., 1991; Nottenburg et al., 1989; Screaton et al., 1992; Stamenkovic et al., 1989), CD 45 (Barclay et al., 1987; Saga et al., 1986; Streuli et al., 1987) and CD43 (Cyster et al., 1990; Killeen et al., 1987; Pallant et al., 1989), all of which are implicated in leukocyte/endothelial and leukocyte/leukocyte interactions. They have been shown to be sulfated in cell lines (Giordanengo et al.,

1995b; Maiti et al., 1998; Wilson and Rider, 1992), although it is not clear to what extent this sulfation is carbohydrate associated.

Table 4.3. L-selectin/IgM and HECA452 staining of CHO cells transfected with combinations of sulfotransferase cDNAs.

μg cDNA transfected		MFI	
KSGal6ST	HEC-GlcNAc6ST	L-sel-IgM Staining	HECA-452 Staining
0	0	0	665
1.0	0	197	697
0	1.0	60	496
0.5	0.5	836	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 mean fluorescence intensity (MFI) of L-selectin/IgM staining in the L-selectin/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.

Summary

In summary, all three of the sulfotransferases were capable of conferring specific L-selectin binding onto CD34. KSGal6ST appeared to exert the greater effect, however, this may be due to its apparent greater efficiency of sulfation. HEC-GlcNAc6ST and

HuGlcNAc6ST had nearly identical staining profiles, indicating that they are both capable of sulfating GlcNAc residues that participate in L-selectin binding. The binding of the L-selectin/IgM chimera was strongly enhanced upon cotransfection of a Gal-6-O-sulfotransferase and a GlcNAc-6-O-sulfotransferase, indicating that the Gal-6 and GlcNAc-6 sulfate esters synergize in generating ligand activity. Whether this apparent synergy results from sulfation of separate chains or disulfation of the same chain remains to be shown. A slight additive effect was apparent when the two GlcNAc-6-O-sulfotransferases were cotransfected with KSGal6ST, implying at least a partially divergent set of carbohydrate substrates for HEC-GlcNAc6ST and HuGlcNAc6ST. Finally, the binding of the L-selectin/IgM chimera was dependent on the presence of the CD34 polypeptide, a requirement likely to reflect the contribution of the mucin domain of CD34 to the proper presentation of the sulfated carbohydrate binding motifs.

Characterization in the parallel-plate flow chamber: Effects of sulfation on the kinetics of L-selectin-ligand interactions

The flow cytometry assays described above demonstrated that the three sulfotransferases could contribute to the generation of L-selectin ligand activity in an equilibrium binding assay. However, L-selectin normally functions in tethering and rolling of lymphocytes on HEV under shear flow conditions in the vasculature (Lawrence et al., 1995; Warnock et al., 1998). We were interested in determining what impact sulfation at Gal-6 or GlcNAc-6 of an L-selectin ligand would have on the kinetic properties of L-selectin-ligand bonds. Towards this end we employed a parallel-plate flow chamber assay similar

to the one first used by Lawrence and Springer (Lawrence and Springer, 1991), the geometry of which allows for the generation of a flow profile that mimics that found in a blood vessel. Briefly, a substrate or ligand of interest is coated down on the lower plate of the chamber, which is then sealed and mounted onto an inverted microscope. A syringe pump is attached at the inlet manifold, with which the velocity of the buffer flowing through the chamber can be controlled, thereby exposing the cells in the buffer to a range of shear stresses as they interact with the substrate.

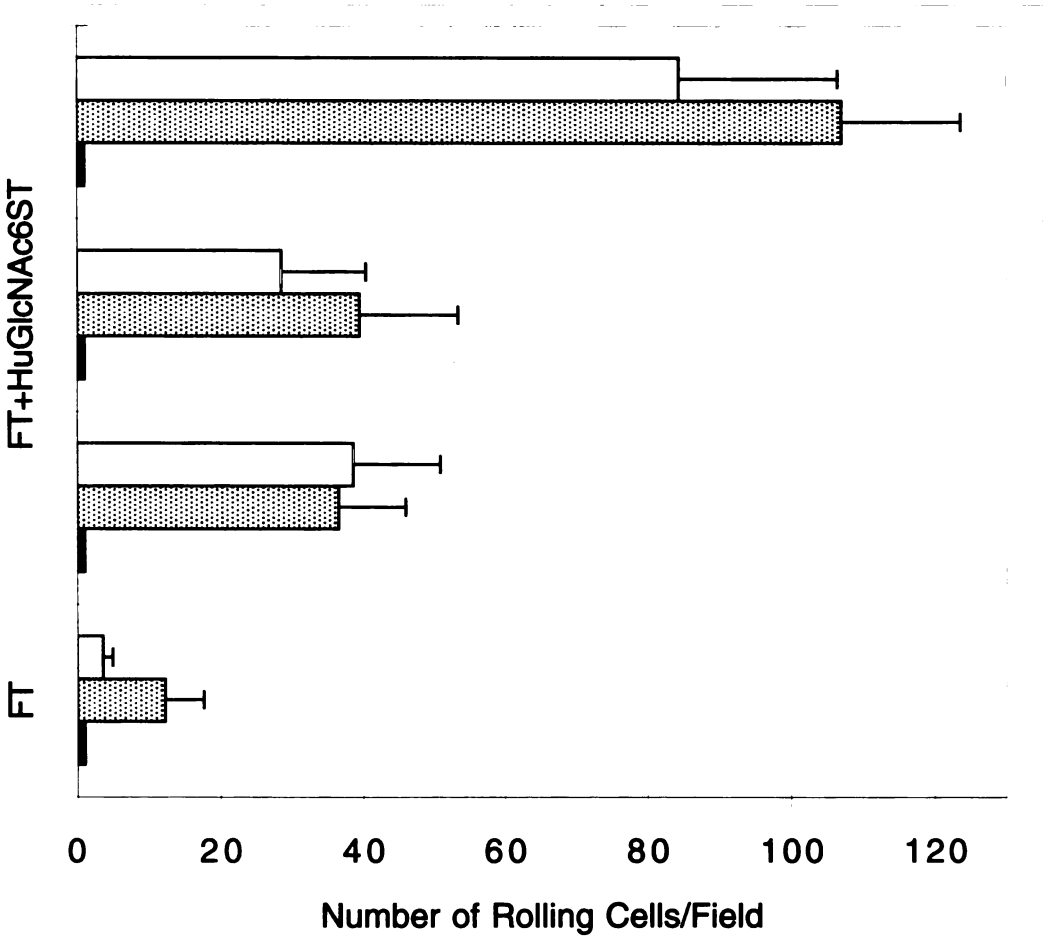
In order to determine the contribution of sulfation to ligand activity under flow conditions, we examined the rolling of PBL or Jurkat cells on sulfated and non-sulfated GlyCAM-1/IgG which was immobilized on the bottom plate of the flow chamber. The different forms of GlyCAM-1/IgG were produced by transfecting COS cells as before with cDNAs for GlyCAM-1/IgG, FTVII and C2GnT, and a cDNA encoding a sulfotransferase. The recombinant proteins were purified from the CM on protein A-sepharose and quantified by ELISA in order to be able to obtain equal site densities in the flow chamber.

As can be seen in Fig. 4.9, GlyCAM-1/IgG produced in the absence of any sulfotransferase (FT) supported a very low level of rolling of PBL or Jurkat cells. The number of rolling cells (PBL or Jurkat) was markedly increased on GlyCAM-1/IgG modified with either Gal-6-sulfate (FT + KSGal6ST) or GlcNAc-6-sulfate (FT+HEC-GlcNac6ST or FT+HuGlcNAc6ST). The rolling was dependent on fucose, as no cells interacted with any form of sulfated GlyCAM-1/IgG produced in the absence of

Figure 4.9

Rolling of PBL and Jurkat cells on various GlyCAM-1/IgG chimeras under flow conditions

Purified recombinant GlyCAM-1/IgG chimeras were coated at equal site densities. PBL (white bars) and Jurkat cells (grey bars) at $2x10^6$ cells/ml were perfused through the flow chamber at a wall shear stress of 1.25 or 1 dyn/ cm^2, respectively. At 2 min of flow, the number of rolling cells was determined. For inhibition studies (black bars), cells were preincubated with anti-human L-selectin mAb (DREG56), fucoidin, EDTA, or the immobilized GlyCAM-1/IgG was treated with sialidase. The values represent the mean ± SD of the number of rolling cells in at least two independent experiments, each performed in duplicate using two different fields of view. Statistical analysis using an unpaired two-tailed Student's t-test showed that the enhanced binding of PBL and Jurkat cells to sulfated GlyCAM-1/IgG was statistically significant in all cases ($p < 0.0001$).


FT+HuGlcNAc6ST
FT
0
20
40
60
80
100
120
Number of Rolling Cells/Field

fucosylation (data not shown). For both the PBL and Jurkat cells, the Gal-6-sulfated GlyCAM-1/IgG yielded stronger effects than the GlcNAc-6-sulfated GlyCAM-1/IgG. This difference may be due to the fact that KSGal6ST apparently sulfates GlyCAM-1/IgG with twice the efficiency of either of the GlcNAc-6-O-sulfotransferases, as discussed above. The rolling interactions on GlyCAM-1/IgG were specific to L-selectin as indicated by a number of controls, indicated in the black bars of Fig. 4.9: Preincubation of the cells with anti-L-selectin antibody, fucoidin or EDTA (Jurkat cells), or treatment of the immobilized GlyCAM-1/IgG with sialidase (Jurkat cells).

Sulfation of fucosylated GlyCAM-1/IgG stabilizes L-selectin mediated rolling adhesion in shear flow

In order for a cell to develop adhesive contacts with the substrate (for example, the endothelium) that allow rolling, the rate of bond formation and breakage must be rapid and balanced (Lawrence and Springer, 1991). When the rate of receptor-ligand bond dissociation is not balanced by the rate of new bond formation, the cells either become firmly adherent or they detach (Alon et al., 1997). Tethering, or capture of a cell from the flow, requires a rapid association rate (k_{on}), whereas the maintenance of rolling interactions requires rapid association as well as dissociation (k_{off}) rates.

We measured three parameters of cell-substrate interactions in the flow chamber, that reflect kinetic as well as equilibrium properties of L-selectin-ligand interactions. 1) Tethering rate: In the flow chamber, the term stable tethers refers to those adhesive

interactions between the cell and the substrate which result in continuous rolling (for ≥ 1 s). Stable tethering requires a high k_{on} to allow for capture of the cell from the flow but is also dependent on k_{off}, since the cell needs to develop rolling interactions in order to be considered stably tethered (Alon et al., 1997). 2) Rolling velocity: Cell rolling is characterized by motion that appears to represent receptor-ligand dissociation events (Alon et al., 1997; Kaplanski et al., 1993; Lawrence and Springer, 1991; Zhao et al., 1995). It has been proposed that when the bond at the rear end of the contact zone between the cell and the substrate dissociates, the hydrodynamic drag force transports the cell forward until the bond that has now moved to the rear of the contact zone restrains the cell. This restraint causes the cell to pause before it is propelled further by the shear force (Alon et al., 1997). The duration of the pause is determined by the bond dissociation kinetics (k_{off}) of the rear-most bond. Indeed, the k_{off} values derived for L-, P-, and E-selectin-ligand interactions correlate well with their relative rolling velocities, implying that rolling velocity is limited by the rate of receptor-ligand dissociation (Alon et al., 1997). 3) Resistance to shear-induced detachment: While tethering and velocity reflect kinetic properties of L-selectin-ligand interactions, resistance to detachment depends on the ratio between k_{on} and k_{off}, i.e., K_{eq} ($K_{eq} = k_{on}/k_{off}$) (Alon et al., 1997). As such, it can be considered a measurement of the overall strength of the rolling adhesions.

As can be seen from Fig. 4.10, the tethering of Jurkat cells on GlyCAM-1/IgG bearing sulfate at C-6 of either GlcNAc (FT+HuGlcNAc6ST; FT+HEC-GlcNAc6ST) or Gal (FT+KSGal6ST) was enhanced as much as 3-fold and 6-fold (at 8 dyn/cm^2), respectively, relative to that on non-sulfated GlyCAM-1/IgG (FT). As discussed, this effect may be

due to a reduced k_{off} as well as an increase in k_{on}. In confirmation of previous reports (Alon et al., 1997; Lawrence et al., 1997; Puri et al., 1998), we observed that tethering of the Jurkat cells occurred only at shear stresses at or above 0.4-0.6 dyn/cm^2, indicating a threshold requirement for shear stress. Interestingly, the Gal-6-sulfate modification resulted in a shift of the threshold towards lower shear stress. Fig. 4.11 shows that sulfation at C-6 of either Gal (FT+KSGal6ST) or GlcNAc (FT+HuGlcNAc6ST; FT+HEC-GlcNAc6ST) significantly reduced the rolling velocity of the interacting cells, relative to that of non-sulfated GlyCAM-1/IgG (FT), over range of shear stresses. When considered in the context of the model correlating velocity with the rate of receptor-ligand dissociation, the slower rolling velocities on the sulfated GlyCAM-1/IgG can be explained by a reduction in the k_{off}. Taken together, the tethering and velocity data suggest that sulfation of GlyCAM-1/IgG promotes rolling interactions under physiological flow conditions by decreasing k_{off} and possibly also increasing k_{on}. The shift to a lower shear threshold requirement for the Gal-6-sulfated GlyCAM-1/IgG (Fig. 4.11) may be explained by a decreased k_{off} (Alon et al., 1997), an increase in the density of functional sites (Lawrence et al., 1997) or changes in the mechanical properties of the L-selectin-ligand bond (Puri et al., 1998).

Since it appeared that sulfation of GlyCAM-1/IgG caused a decrease in k_{off}, and possibly also an increase in k_{on}, we anticipated that this would be reflected in the K_{eq}, as manifested by a stabilization of rolling adhesions. Indeed, we found that sulfation of GlyCAM-1/IgG led to an increase in the resistance of the Jurkat cells to shear-induced detachment, as shown in Fig. 4.12. This finding therefore is consistent with the flow

Figure 4.10

Jurkat cell tethering to various GlyCAM-1/IgG chimeras in shear flow

Jurkat cells ($1x10^6$ cells/ml) were perfused into the chamber and the fraction of cells that came into close proximity with the substrate and tethered stably onto different GlyCAM-1/IgG chimeras was determined. The data points represent the mean ± SD of the frequency of stable tethers in three independent experiments, each performed in duplicate using two different fields of view. Statistical analysis using an unpaired two-tailed Student's t-test showed that the enhanced tethering frequency of Jurkat cells to sulfated GlyCAM-1/IgG as compared to non-sulfated GlyCAM-1 was statistically significant (for KSGal6ST: $p < 0.004$ in the range of 0.4-1.5 dyn/ cm^2; for HuGlcNAc6ST: $p < 0.0002$ in the range of 0.6-1 dyn/ cm^2; for HEC-GlcNAc6ST: $p < 0.002$ in the range of 0.6-1.25 dyn/ cm^2).

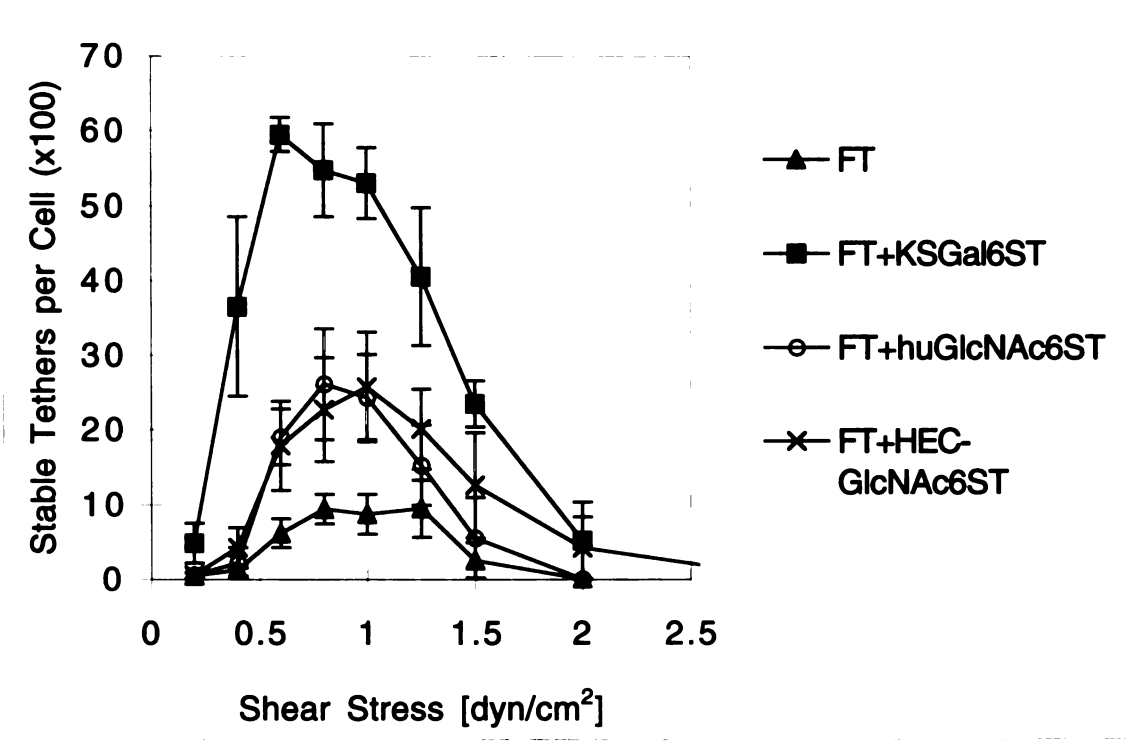

70
60
50
40
30
20
10
0
Stable Tethers per Cell (x100)
0
0.5
1
1.5
2
2.5
Shear Stress [dyn/cm2]
FT
FT+KSGal6ST
FT+huGlcNAc6ST
FT+HEC-GlcNAc6ST

Figure 4.11

Comparison of Jurkat cell rolling velocities on various GlyCAM-1/IgG chimeras

Jurkat cells ($2x10^6$cells/ml) were tethered onto different GlyCAM-1/IgG chimeras at a wall shear stress of 1 dyn/ cm^2 for 2 min. Wall shear stress was then increased progressively to a maximum of 35 dyn/ cm^2, and rolling velocities were measured. The data points represent the mean rolling velocity ± the standard error of the mean of three to four independent experiments, each performed in duplicate using two different fields of view. Statistical analysis using an unpaired two-tailed Student's t-test showed that the reduced rolling velocity of Jurkat cells on sulfated GlyCAM-1/IgG as compared to non-sulfated GlyCAM-1 is statistically significant (for KSGal6ST, HuGlcNAc6ST, HEC-GlcNAc6ST: $p < 0.0001$ at ≥ 1 dyn/ cm^2).

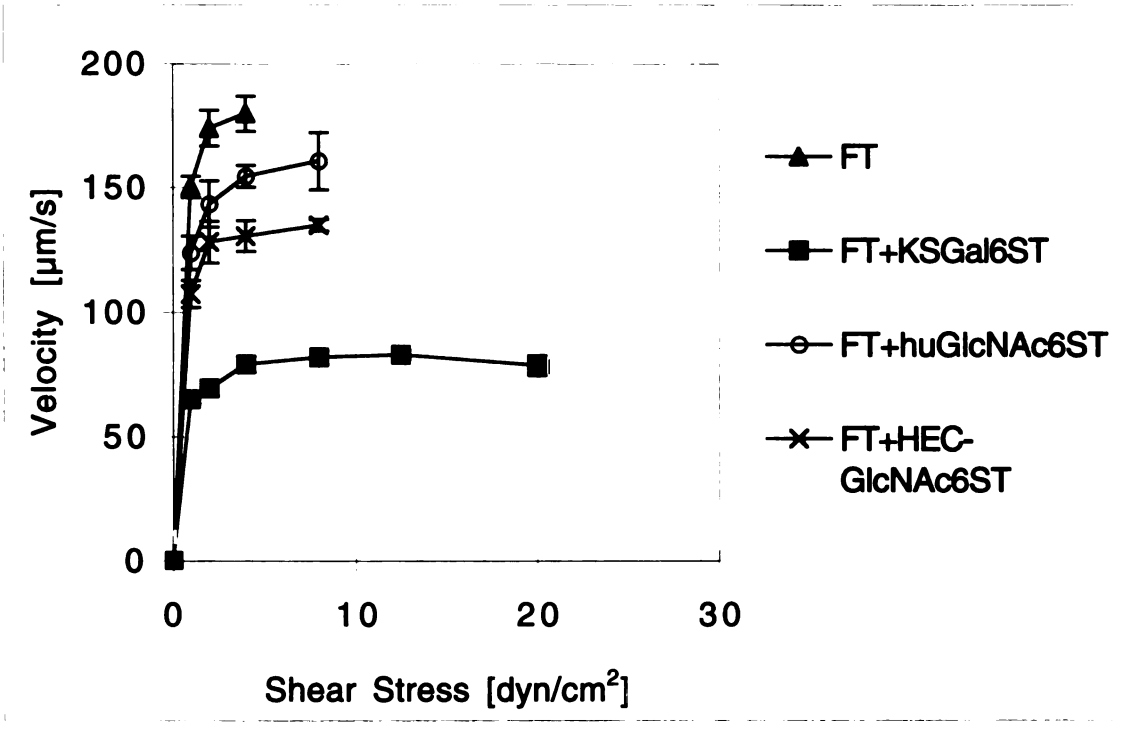
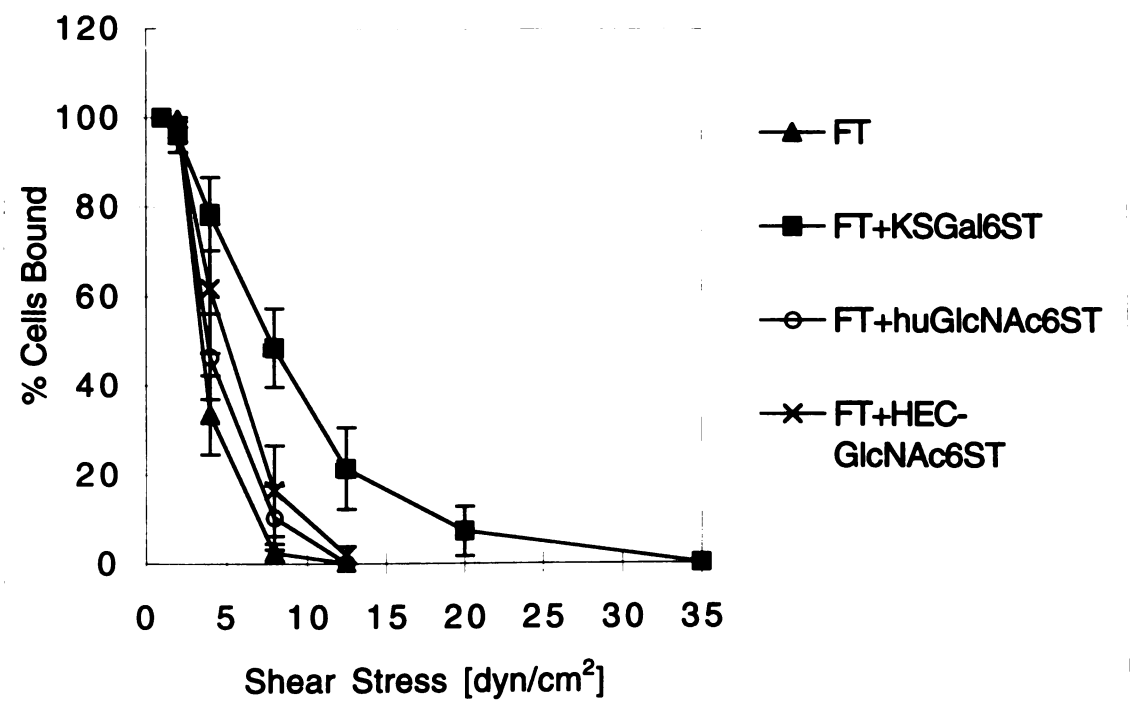

200
150
100
50
0
Velocity [µm/s]
0
10
20
30
Shear Stress [dyn/cm2]
FT
FT+KSGal6ST
FT+huGlcNAc6ST
FT+HEC-GlcNAc6ST

Figure 4.12

Resistance of Jurkat cells to shear-induced detachment when rolling on various GlyCAM-1/IgG chimeras

Jurkat cells ($2x10^6$ cells/ml)were allowed to tether onto different GlyCAM-1/IgG chimeras at a wall shear stress of 1 dyn/ cm^2 for 2 min. Wall shear stress was then increased progressively to a maximum of 35 dyn/ cm^2, and the number of rolling cells remaining bound at each shear stress was determined and expressed as the percentage of the maximum number of adherent cells. The data points represent the mean ± SD of three to four independent experiments, each performed in duplicate in two different fields of view. Statistical analysis using an unpaired two-tailed Student's t-test showed that the increased binding to sulfated GlyCAM-1/IgG as compared to non-sulfated GlyCAM-1 was statistically significant as follows: (for KSGal6ST: $p < 0.007$ over the range of 4-20 dyn/ cm^2; for HuGlcNAc6ST: $p < 0.05$ at 8 dyn/ cm^2; for HEC-GlcNAc6ST: $p < 0.01$ over the range of 4-8 dyn/ cm^2).


120
100
80
60
40
20
0
% Cells Bound
0
5
10
15
20
25
30
35
Shear Stress [dyn/cm2]
FT
FT+KSGal6ST
FT+huGlcNAc6ST
FT+HEC-GlcNAc6ST

cytometry data discussed above, which showed that transfection of CHO cells with either a Gal-6- or a GlcNAC-6-O-sulfotransferase conferred increased binding of an L-selectin/IgM chimera to CD34. Whether the synergistic effect of the two sulfate esters seen in the equilibrium binding experiments will be reflected in flow chamber measurements requires further investigation.

Summary

In summary, the data obtained from the flow chamber experiments indicate that sulfation of L-selectin ligands affect several aspects of the L-selectin-ligand interactions. In terms of the kinetic parameters of the interactions, sulfation caused an increase in the tethering rate of cells and a decrease in the rolling velocity of the interacting cells, both of which are probably due to a decrease in k_{off} and possibly an increase in k_{on}. Furthermore, the overall strength of rolling adhesions was enhanced, indicating a shift in the K_{eq} of the interaction consistent with the inferred changes in the kinetic rate constants. The flow chamber data therefore are in agreement with the equilibrium binding data obtained by flow cytometry and provide further evidence that sulfation of L-selectin ligands enhances the interaction with L-selectin. In the context of lymphocyte homing, sulfation of these ligands could therefore promote the entire process by affecting several integral elements: 1) by increasing the extent of tethering; 2) by increasing the strength of rolling adhesions; and 3) by facilitating the transition from rolling to arrest by decreasing the rolling velocity of the cells.

The following individuals are gratefully acknowledged for their collaboration in the characterization of the sulfotransferases: Sunil Bhakta and Dr. Stefan Hemmerich of Roche Biosciences who carried out the *in vitro* sulfotransferase assays and all the hydrolysis and HPAEC analysis to demonstrate acceptor specificity; Kendra Bowman and Dr. Carolyn Bertozzi at UC, Berkeley, for synthesizing the oligosaccharide acceptors and for their assistance with the HPAEC analysis; Carmen Tam and Dr. Michael D. Gunn of UC San Francisco who performed the *in situ* hybridizations; Dr. Kirsten Tangemann, a postdoctoral fellow in the Rosen laboratory, who performed all the flow chamber rolling assays; and Yevgeniy Belov, a UC, Berkeley, undergraduate who assisted with the flow cytometry analysis to demonstrate L-selectin binding to the CHO cells transfectants.

MATERIALS AND METHODS

Sulfotransferase Assay and Hydrolysis of Sulfated Acceptors

The synthesis of the sulfotransferase assay acceptors GlcNAcβ1→6Galα1-R and Galβ1→4GlcNAcβ1→6Galα1-R, as well as the sulfotransferase assay, have been described (Bowman et al., 1998). In the current studies, the source of enzymatic activity were detergent extracts of microsomal fractions (5 μl per assay) prepared from COS-7 cells transfected with cDNAs encoding HEC-GlcNAc6ST (pCDNA1.1), HuGlcNAc6ST (pCDNA3.1), KSGal6ST (pCDNA3.1), or the empty vector (pCDNA1.1) using Lipofectamine (Life Technologies) in Opti-MEM (Life Technologies). Hydrolysis of the sulfated disaccharide lipid conjugates for release of [^{35}S]-sulfate-labeled monosaccharides was performed as described (Bowman et al., 1998).

Sulfation of GlyCAM-1/IgG and CD34/IgG in COS Cells

For generation of recombinant GlyCAM-1/IgG or CD34/IgGfusion protein, COS-7 cells were grown to 80% confluency in a T162 culture flask (Corning-Costar, Corning, NY) and transfected with 8 μg of a plasmid encoding GlyCAM-1/IgG or CD34/IgG and 8 μg of plasmid encoding either HEC-GlcNAc6ST (pCDNA1.1), HuGlcNAc6ST (pCDNA3.1), KSGal6ST (pCDNA3.1), or the empty vector (pCDNA1.1) using Lipofectamine (Life Technologies) in Opti-MEM (Life Technologies). The cDNAs

encoding the GlyCAM-1/IgG and CD34/IgG chimeras were constructed by amplifying the entire coding sequence of murine GlyCAM-1 (Lasky et al., 1992) or extracellular domain of human CD34 (Simmons et al., 1992) by PCR and cloning the resulting fragment into the pIG1 vector (Simmons, 1993). The subcloning of CD34 into pIG1 was done by Christopher Sassetti, a graduate student in Steve Rosen's laboratory. Cells were grown for 12 hours post-transfection in DMEM containing 10% FBS, then cell layers were washed once with PBS and cultured for 72 h in serum free medium (Endothelial SFM, Gibco BRL, 16 ml per flask) supplemented with $Na_2{}^{35}SO_4$ (0.25 mCi/ml, 1400 Ci/mmol, ICN). Recombinant GlyCAM-1/IgG fusion protein was isolated from the conditioned medium (CM) by affinity chromatography on protein A-agarose (Watson et al., 1990). The protein was then transferred into 50 mM ammonium bicarbonate on a Centricon 30 concentrator (Amicon, Inc., Beverly, MA). 1% of each sample was analyzed by SDS-PAGE and autoradiography and the remaining samples were lyophilized for subsequent acid hydrolysis and positional analysis. For the Western blot of the sulfated CD34/IgG, the proteins in the gel were transferred in a Hoefer TE series electroblotter to Problott (Applied Biosystems, Inc., Foster City, CA) membrane in 10mM 3-[cyclohexylamino]-1-propane-sulfonic acid (CAPS), 10% methanol at 25 V for 16 h. The membrane was blocked in 25mM Tris, pH7.5, 0.5M HCl (TN buffer) and 5% non-fat dry milk (blocking solution) for 1 h. The membrane was then incubated in 1 μg/ml Qbend-10 (Coulter Corporation, Miami, FL) in blocking solution for 1 h, followed by 3 x 10 min washing in TN buffer containing 0.1% Tween 20 (TNT buffer). The membrane was then incubated in 1:1,000 diluted horseradish peroxidase-conjugated goat anti-rat IgG (human adsorbed) (CalTag Laboratories, Inc., Burlingame, CA) in blocking

solution for 1 h, followed by 2 h washing in PBS/0.1% Tween 20, with 6 changes of wash buffer. The membrane was developed using an ECL substrate (Amersham Corp.), according to the manufacturer's protocol.

Analysis of Sulfated GlyCAM-1/IgG Carbohydrates

The lyophilized recombinant GlyCAM-1/IgG samples were hydrolyzed in 0.2 M H_2SO_4 and the hydrolysates were prepared for analysis by high pH anion exchange chromatography (HPAEC) essentially as described earlier (Hemmerich et al., 1994a), with the following modifications: 1) after 30 min hydrolysis in 0.2 M H_2SO_4 at 96°C, and prior to the initial gel filtration step, excess sulfate was precipitated by addition of an equivalent amount of $Ba(AcO)_2$; 2) the second ion exchange column (DEAE-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 & 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 chromatographic analysis system and a Carbopac 1 column (Dionex Corporation, Sunnyvale, CA) essentially a described (Hemmerich et al., 1994a & 1995), with the following modification: The column was eluted isocratically with 150 mM NaOH in 400 mM NaOAc at 1 ml/min. The authentic carbohydrate standards used in this analysis (at 10 nmol per standard per run) were obtained or prepared as described (Hemmerich et al., 1994a & 1995). The lag between the pulsed amperometric detection (PAD) and the fraction collector was determined by aligning the PAD signal from commercial Gal-6-sulfate (Sigma Inc., St.

Louis, MO) with the fraction containing the peak-cpm from [^{3}H]-Gal-6-sulfate (generated from [^{3}H-Gal]-GlyCAM-1 as described in Hemmerich et al., 1994a).

Flow Cytometry

CHO/FTVII/C2GnT cells were grown to 80% confluency in T75 flasks (Nalge Nunc) (6 x 10^6 cells per flask) and then transfected with plasmids encoding C2GnT (1μg, pCDNA1.1), FTVII (1μg, pCDNA3.1), human CD34 (2μg, pRK5), sulfotransferases (at the concentrations indicated for each experiment) and vector plasmid (pCDNA3.1) to achieve 8 μg total DNA per flask, using Lipofectamine. 48 hr after transfection, the cells were harvested in 0.6mM EDTA in PBS without calcium and magnesium, washed once in assay buffer (0.1% BSA in PBS), and resuspended at 4 x 10^6/ml in assay buffer. 25 μl of this cell suspension was added to wells of 96 well plates containing 50 μl of L-selectin/IgM 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 tertiary staining reagents and/or directly conjugated primary mAbs. For the sialyl 6-sulfo Le^x or overall sLe^x determinations, cells were incubated with or G72 mAb or HECA 452 (Pharmingen) respectively, diluted in assay buffer, and then reacted with rabbit anti-mouse IgG-FITC (Zymed labs) (for G72) or mouse anti-rat IgM-FITC (Pharmingen) (for HECA 452). The cells were incubated for 30 min, washed twice and fixed in 1.5% paraformaldehyde in

PBS, pH 7, for 20 min before transfer into 300 μl of assay buffer. Cells were analyzed on a FACSort (Becton Dickinson, Franklin Lakes, NJ) flow cytometer using CELLQuest software (Becton Dickinson). To produce the L-selectin/IgM chimera (Maly et al., 1996), COS cells (10 cm plates) were transfected with L-selectin/IgM cDNA in pCDM8 (8μg cDNA, 50 μl Lipofectamine per plate) and incubated in Opti-MEM for 10 days, at which time the CM was harvested and clarified by centrifugation. The CM was concentrated to half its original volume, titered and used neat. Biotinylated goat anti-human IgM(μ) and streptavidin-fluorescein isothiocyanate (FITC) were purchased from CalTag Laboratories. The L-selectin mAb Mel-14 (Gallatin et al., 1983) was purified by ammonium sulfate precipitation from hybridoma cell culture supernatant, followed by immunoaffinity purification with mouse L-selectin/IgG. The control for Mel-14 was a rat IgG2a myeloma (Zymed Laboratories, Inc., South San Francisco, CA). For the two color analysis, we used mouse anti-CD34 phycoerythrin (PE) (QBend10-PE; Coulter Corporation) and the isotype-matched control, mouse IgG1-PE (CalTag).

Laminar Flow Assays

The following individuals generously provided reagents: T. K. Kishimoto (DREG56), John Lowe (FTVII cDNA), Minoru Fukuda (C2GnT cDNA), G.S. Kansas (300.19 B-cells transfected with E-selectin), and Arthur Weiss (Jurkat cells). Cells were maintained in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 5% heat-inactivated FCS (Hyclone, Logan, UT) with 100 μM 2-

mercaptoethanol added to the 300.19 cells. Human PBL were isolated as previously described (Tangemann et al., 1998). To equalize coating densities of the GlyCAM-1/IgG chimeras, ELISAs were performed . Proteins were coated onto 96 well polystyrene plates (Costar, Corning, NY) in Tris-buffered saline, pH 9 at 4°C. After blocking with BSA, the chimeras were detected with biotinylated anti-GlyCAM-1 peptide Ab (Hemmerich et al., 1994b) or biotinylated anti-human IgG (Fc specific) and streptavidin-conjugated alkaline phosphatase. For flow experiments, polystyrene dishes coated similarly with the chimeras as in the ELISA assays were incorporated as the lower wall of a parallel plate flow chamber (Tangemann et al., 1998). Cells were perfused through the flow chamber at 1-2x10^6 cells/ml. For inhibition studies, cells were pretreated with 5 µg/ml DREG56, 10 µg/ml fucoidin (Sigma Chemicals, St. Louis, MO; 10 min, 22 °C) or resuspended in Ca^{2+}, Mg^{2+} free Hanks buffer with 5 mM EDTA. For sialidase experiments, coated substrates were incubated with 5 mU/ml *Vibrio cholera* sialidase (Oxford Glycosystems, Rosedale, NY) for 30 min in 50 mM sodium acetate, 4 mM $CaCl_2$, 0.1% BSA, pH 5.5 or with buffer alone for the control. Experiments were analyzed as previously described (Tangemann et al., 1998). For tethering, cells were perfused through the chamber over a range of shear stresses (3 to 0.2 dyn/cm^2). The fraction of cells that came into close proximity with the substrate and tethered stably (rolling for > 1s after initial attachment) was determined. For velocity and detachment determinations (Tangemann et al., 1998), cells were infused for 2 min at 1 dyn/ cm^2 after which shear stress was increased in 1.5 to twofold increments up to 35 dyn/ cm^2 at intervals of 5 s. Cell displacement was followed for 1 - 3 seconds to determine rolling

velocities. In detachment assays, the number of rolling cells was determined at each shear stress and calculated as the percentage of the peak value.

Summary and Concluding Remarks

Our objective in the work described in this dissertation was the molecular identification of the sulfotransferases that participate in the biosynthesis of endothelial ligands for L-selectin. 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-6-sulfate in the context of sialyl Lewis x (sialyl 6'-sulfo Le^x, sialyl 6-sulfo Le^x, respectively). Gal-6-sulfate and GlcNAc-6-sulfate was also found in approximately equal representations in the functional glycoforms of CD34, another ligand for L-selectin (Hemmerich and Rosen, unpublished observations). Sialyl 6-sulfo Le^x was recently demonstrated to be prominently displayed on the HEV of human peripheral lymph node (Mitsuoka et al., 1998), further implicating a GlcNAc-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 HEC.

Using homology-based techniques, we have cloned three carbohydrate sulfotransferases. Two of these are GlcNAc-6-O-sulfotransferases (HEC-GlcNAc6ST and HuGlcNAc6ST) and one is a Gal-6-O-sulfotransferase (KSGal6ST). These three sulfotransferases, together with several other recently identified enzymes (Tables 3.1 and 3.2, Fig. 3.10, Chapter Three), constitute a novel family of carbohydrate modifying

sulfotransferases that appear to utilize the 6-hydroxyl group of Gal, GalNAc or GlcNAc as acceptors.

All three of the sulfotransferases were able to sulfate GlyCAM-1 and CD34 with the appropriate regiospecificity (GlyCAM-1). The two GlcNAc6STs were capable of generating sialyl 6-sulfo Le^x on the cell surface of CHO cells, establishing that recombinant HEC-GlcNAc6ST or HuGlcNAc6ST can be used to generate a highly specific sulfated structure that is present on lymph node HEV (Mitsuoka et al., 1998).

We used two different assays to show that each enzyme was capable of imparting L-selectin ligand activity onto recombinant GlyCAM-1 and CD34: Flow cytometry, in which we assayed the binding of an L-selectin/IgM chimera to transfected CHO cells, and the flow chamber, in which sulfated recombinant GlyCAM-1, when coated down as a rolling ligand, was shown to enhance the adhesive interactions of lymphocytes, relative to non-sulfated GlyCAM-1.

Optimal ligand activity was shown in the flow cytometry assay to be dependent on the protein scaffold provided by CD34, a characteristic likely to reflect the requirement for a mucin domain which has been demonstrated for native L-selectin ligands (Puri et al., 1995). The ability of other mucins to perform this postulated scaffold function and their possible distinct usage of the different sulfotransferases are questions for future investigation.

We furthermore demonstrated in the flow cytometry assay an apparent synergistic effect in ligand activity of the Gal-6-sulfate ester and the GlcNAc-6-sulfate ester, arguing that both esters are required for optimal binding to L-selectin. It is not yet known whether this synergy arises through dual recognition of separate monosulfated chains, for example one chain capped by sialyl 6-sulfo Le^x and the other by sialyl 6'-sulfo Le^x, or through individual chains containing both modifications. Most of the sulfated chains within GlyCAM-1 contain two or more sulfates. It is not yet clear whether sialyl 6',6-disulfo Le^x (Fig. 1.2, Chapter One) exists as a capping group on these multi-sulfated chains or whether there are extended chains containing sulfates on internal Gal or GlcNAc residues. Additional analysis of natural ligands will be required to resolve these important structural questions. Also, it will be of great interest to determine whether the synergistic effect seen in the equilibrium binding experiments will be reflected in flow chamber measurements.

Lymphocyte homing to lymph nodes and other secondary lymphoid organs is a multistep process in which lymphocytes must tether and roll on HEV before firmly arresting via chemokine-induced integrin activation (Girard and Springer, 1995; Butcher and Picker, 1996; Tangemann et al., 1998). The results obtained with the flow chamber indicate that sulfation of L-selectin ligands could promote the overall process at several levels: 1) by increasing the extent of tethering; 2) by increasing the strength of rolling adhesions; and 3) by facilitating the transition from rolling to arrest by decreasing overall rolling velocity.

While HuGlcNAc6ST and KSGal6ST are broadly expressed, HEC-GlcNAc6ST showed a highly restricted, although not absolute, localization to HEC. On the basis of its activities and highly restricted expression pattern, HEC-GlcNAc6ST is likely to be specifically involved in the biosynthesis of the sialyl 6-sulfo Le^x epitope and related structures within L-selectin ligands. It may be necessary to inactivate the gene in mice by gene knockout to define the biological roles of this enzyme. In analogy with HEC-GlcNAc6ST, one might predict the existence of an HEC-specific, or more highly restricted, Gal-6-sulfotransferase that sulfates L-selectin ligands *in vivo* .

L-selectin dependent leukocyte trafficking into lymphoid organs and inflammatory sites is likely to be subject to complex regulation, which may be effected at the level of L-selectin ligand biosynthesis through the availability of protein scaffolds or by posttranslational modification. Given the dramatic sulfate dependency of the interaction between L-selectin and its ligands, and the high ^{35}S-sulfate incorporation seen in HEV and HEV-like vessels (Girard and Springer, 1995a), the expression or activity of the relevant sulfotransferases could represent critical control points for this process. Endothelial sulfotransferases that are implicated in inflammatory leukocyte trafficking would clearly assume importance as potential targets for anti-inflammatory therapeutics.

REFERENCES

Ager, A. (1987). Isolation and culture of high endothelial cells from rat lymph nodes. J. Cell Sci. 87, 133-144.

Alon, R., Chen, S., Puri, K. D., Finger, E. B., and Springer, T. A. (1997). The kinetics of L-selectin tethers and the mechanics of selectin-mediated rolling. J. Cell Biol. 138, 1169-1180.

Anderson, A. O., and Shaw, S. (1993). T cell adhesion to endothelium: the FRC conduit system and other anatomic and molecular features which facilitate the adhesion cascade in lymph node. seminars in IMMUNOLOGY 5, 271-282.

Anderson, N. D., Anderson, A. O., and Wyllie, R. G. (1976). Specialized structure and metabolic activities of high endothelial venules in rat lymphatic tissues. Immunology 31, 455-473.

Andrews, P., Ford, W., and Stoddart, R. (1980). Metabolic studies of high-walled endothelium of postcapillary venules in rat lymph nodes. CIBA Symp 71, 211-230.

Ariga, T., Kohriyama, T., Freddo, L., Latov, N., Saito, M., Kon, K., Ando, S., Suzuki, M., Hemling, M. E., Rinehart, K. L., Jr., and et al. (1987). Characterization of sulfated

glucuronic acid containing glycolipids reacting with IgM M-proteins in patients with neuropathy. J. Biol. Chem. 262, 848-53.

Baenziger, J. U. (1994). Protein-specific glycosyltransferases: How and why they do it! FASEB J. 8, 1019-1025.

Baeuerle, P. A., and Huttner, W. B. (1986). Chlorate - a potent inhibitor of protein sulfation in intact cells. Biochem. Biophys. Res. Commun. 141, 870-877.

Bakker, H., Friedmann, I., Oka, S., Kawasaki, T., Nifant'ev, N., Schachner, M., and Mantei, N. (1997). Expression cloning of a cDNA encoding a sulfotransferase involved in the biosynthesis of the HNK-1 carbohydrate epitope. J. Biol. Chem. 272, 29942-6.

Barclay, A. N., Jackson, D. I., Willis, A. C., and Williams, A. F. (1987). Lymphocyte specific heterogeneity in the rat leucocyte common antigen (T200) is due to differences in polypeptide sequences near the NH2- terminus. Embo J. 6, 1259-64.

Bargatze, R. F., and Butcher, E. C. (1993). Rapid G protein-regulated activation event involved in lymphocyte binding to high endothelial venules. J. Exp. Med. 178, 367-372.

Bargatze, R. F., Jutila, M. A., and Butcher, E. C. (1995). Distinct roles of L-selectin and integrins α4B7 and LFA-1 in lymphocyte homing to Peyer's patch-HEV in situ: the multistep model confirmed and refined. Immunity 3, 99-108.

Baumhueter, S., Singer, M. S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S. D., and Lasky, L. A. (1993). Binding of L-Selectin to the Vascular Sialomucin, CD34. Science 262, 436-438.

Berg, E. L., McEvoy, L. M., Berlin, C., Bargatze, R. F., and Butcher, E. C. (1993). L-selectin-mediated lymphocyte rolling on MAdCAM-1. Nature 366, 695-698.

Berg, E. L., Robinson, M. K., Warnock, R. A., and Butcher, E. C. (1991). The human peripheral lymph node vascular addressin is a ligand for LECAM-1, the peripheral lymph node homing receptor. J. Cell Biol. 114, 343-349.

Berlin, C., Berg, E. L., Briskin, M. J., Andrew, D. P., Kilshaw, P. J., Holzmann, B., Weissman, I. L., Hamann, A., and Butcher, E. C. (1993). $\alpha 4\beta 7$ integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. Cell 74, 185-195.

Bertozzi, C. R., Fukuda, S., and Rosen, S. D. (1995). Sulfated dissaccharide inhibitors of L-selectin; deriving structural leads from a physiological selectin ligand. Biochemistry 34, 14271-14278.

Bevilacqua, M. P. (1993). Endothelial-leukocyte adhesion molecules. Annu Rev Immunol 11, 767-804.

Bevilacqua, M. P., and Nelson, R. M. (1993). Selectins. J. Clin. Invest. 91, 379-387.

Bierhuizen, M. F., and Fukuda, M. (1992). Expression cloning of a cDNA encoding UDP-GlcNAc:Gal β 1-3-GalNAc-R (GlcNAc to GalNAc) β 1-6GlcNAc transferase by gene transfer into CHO cells expressing polyoma large tumor antigen. Proc. Natl. Acad. Sci. U S A 89, 9326-330.

Binns, R. M., Licence, S. T., Wooding, F. B., and Duffus, W. P. (1992). Active lymphocyte traffic induced in the periphery by cytokines and phytohemagglutinin: three different mechanisms? Eur. J. Immunol. 22, 2195-203.

Bowman, K. G., and Bertozzi, C. R. (1999). Carbohydrate sulfotransferases: mediators of extracellular communication. Chem. Biol. 6, R9-R22.

Bowman, K. G., Hemmerich, S., Bakhta, S., Singer, M. S., Bistrup, A., Rosen, S. D., and Bertozzi, C. R. (1998). Identification of an N-acetylglucamine-6-0-sulfotransferase activity specific to lymphoid tissue: an enzyme with a possible role in lymphocyte homing. Chem. and Biol. 5, 447-460.

Brandan, E., and Hirschberg, C. B. (1988). Purification of rat liver N-heparan-sulfate sulfotransferase. J. Biol. Chem. 263, 2417-2422.

Brockhausen, I., Yang, J. M., Burchell, J., Whitehouse, C., and Taylor-Papadimitriou, J. (1995). Mechanisms underlying aberrant glycosylation of MUC1 mucin in breast cancer cells. Eur. J. Biochem. 233, 607-17.

Brown, G. M., Huckerby, T. N., Morris, H. G., Abram, B. L., and Nieduszynski, I. A. (1994a). Oligosaccharides derived from bovine articular cartilage keratan sulfates after keratanase II digestion: Implications for keratan sulfate structural fingerprinting. Biochemistry 33, 4836-4846.

Brown, G. M., Huckerby, T. N., and Nieduszynski, I. A. (1994b). Oligosaccharides derived by keratanase II digestion of bovine articular cartilage keratan sulphates. Eur. J. Biochem. 224, 281-308.

Brown, W. R., Barclay, A. N., Sunderland, C. A., and Williams, A. F. (1981). Identification of a glycophorin-like molecule at the cell surface of rat thymocytes. Nature 289, 456-60.

Brustein, M., Kraal, G., Mebius, R. E., and Watson, S. R. (1992). Identification of a soluble form of a ligand for the lymphocyte homing receptor. J. Exp. Med. 176, 1415-1419.

Cahill, R. N., Frost, H., and Trnka, Z. (1976). The effects of antigen on the migration of recirculating lymphocytes through single lymph nodes. J. Exp. Med. 143, 870-888.

Camp, R. L., Scheynius, A., Johansson, C., and Pure, E. (1993). CD44 is necessary for optimal contact allergic responses but is not required for normal leukocyte extravasation. J. Exp. Med. 178, 497-507.

Capon, C., Wieruszeski, J. M., Lemoine, J., Byrd, J. C., Leffler, H., and Kim, Y. S. (1997). Sulfated lewis X determinants as a major structural motif in glycans from LS174T-HM7 human colon carcinoma mucin. J. Biol. Chem. 272, 31957-68.

Carlsson, S. R., and Fukuda, M. (1986). Isolation and characterization of leukosialin, a major sialoglycoprotein on human leukocytes. J. Biol. Chem. 261, 12779-86.

Carlsson, S. R., Sasaki, H., and Fukuda, M. (1986). Structural variations of O-linked oligosaccharides present in leukosialin isolated from erythroid, myeloid, and T-lymphoid cell lines. J. Biol. Chem. 261, 12787-95.

Chaiyaroj, S. C., Angkasekwinai, P., Buranakiti, A., Looareesuwan, S., Rogerson, S. J., and Brown, G. V. (1996). Cytoadherence characteristics of Plasmodium falciparum isolates from Thailand: evidence for chondroitin sulfate a as a cytoadherence receptor. Am. J. Trop. Med. Hyg. 55, 76-80.

Chandrasekaran, E. V., Jain, R. K., Larsen, R. D., Wlasichuk, K., DiCioccio, R. A., and Matta, K. L. (1996). Specificity analysis of three clonal and five non-clonal α 1,3-L-

fucosyltransferases with sulfated, sialylated, or fucosylated synthetic carbohydrates as acceptors in relation to the assembly of 3'-sialyl-6'-sulfo Lewis x (the L-selectin ligand) and related complex structures. Biochemistry 35, 8925-33.

Chiba, H., Komatsu, K., Lee, Y. C., Tomizuka, T., and Strott, C. A. (1995). The 3'-terminal exon of the family of steroid and phenol sulfotransferase genes is spliced at the N-terminal glycine of the universally conserved GXXGXXK motif that forms the sulfonate donor binding site. Proc. Natl. Acad. Sci. U S A 92, 8176-9.

Chou, D. K., Ilyas, A. A., Evans, J. E., Costello, C., Quarles, R. H., and Jungalwala, F. B. (1986). Structure of sulfated glucuronyl glycolipids in the nervous system reacting with HNK-1 antibody and some IgM paraproteins in neuropathy. J. Biol. Chem. 261, 11717-25.

Chou, D. K., Prasadarao, N., Koul, O., and Jungalwala, F. B. (1991). Developmental expression of HNK-1-reactive antigens in rat cerebral cortex and molecular heterogeneity of sulfoglucuronylneolactotetraosylceramide in CNS versus PNS. J. Neurochem. 57, 852-9.

Clark, R. A., Alon, R., and Springer, T. A. (1996). CD44 and hyaluronan-dependent rolling interactions of lymphocytes on tonsillar stroma. J. Cell Biol. 134, 1075-87.

Clark, R. A., Fuhlbrigge, R. C., and Springer, T. A. (1998). L-Selectin ligands that are O-glycoprotease resistant and distinct from MECA-79 antigen are sufficient for tethering and rolling of lymphocytes on human high endothelial venules. J. Cell Biol. 140, 721-31.

Corfield, A. P., Warren, B. F., Bartolo, D. C., Wagner, S. A., and Clamp, J. R. (1992). Mucin changes in ileoanal pouches monitored by metabolic labelling and histochemistry. Br. J. Surg. 79, 1209-12.

Crommie, D. (1994). The biosynthesis of O-linked oligosaccharides of GlyCAM-1 and the oligomeric structure of L-selectin. Ph.D Thesis, UCSF

Crommie, D., and Rosen, S. D. (1995). Biosynthesis of GlyCAM-1, a mucin-like ligand for L-selectin. J. Biol. Chem. 270, 22614-22624.

Cyster, J., Somoza, C., Killeen, N., and Williams, A. F. (1990). Protein sequence and gene structure for mouse leukosialin (CD43), a T lymphocyte mucin without introns in the coding sequence. Eur. J. Immunol. 20, 875-81.

de Smet, W., Walter, H., and van Hove, L. (1993). A new CD43 monoclonal antibody induces homotypic aggregation of human leucocytes through a CD11a/CD18-dependent and -independent mechanism. Immunology 79, 46-54.

DeGrendele, H. C., Estess, P., Picker, L. J., and Siegelman, M. H. (1996). CD44 and its ligand hyaluronate mediate rolling under physiologic flow: a novel lymphocyte-endothelial cell primary adhesion pathway. J. Exp. Med. 183, 1119-30.

DeGrendele, H. C., Estess, P., and Siegelman, M. H. (1997). Requirement for CD44 in activated T cell extravasation into an inflammatory site. Science 278, 672-5.

Degroote, S., Lo-Guidice, J.-M., Strecker, G., Ducourouble, M.-P., Roussel, P., and Lamblin, G. (1997). Characterization of an N-acetylglucosamine-6-O-sulfotransferase from human respiratory mucosa active on mucin carbohydrate chains. J. Biol. Chem. 272, 29493-29501.

Denarie, J., and Cullimore, J. (1993). Lipo-oligosaccharide nodulation factors: a minireview new class of signaling molecules mediating recognition and morphogenesis. Cell 74, 951-4.

Diacovo, T. G., Puri, K. D., Warnock, R. A., Springer, T. A., and von Andrian, U. H. (1996). Platelet-mediated lymphocyte delivery to high endothelial venules. Science 273, 252-255.

Dong, J. F., Li, C. Q., and Lopez, J. A. (1994). Tyrosine sulfation of the glycoprotein Ib-IX complex: identification of sulfated residues and effect on ligand binding. Biochemistry 33, 13946-53.

Dou, C. L., and Levine, J. M. (1995). Differential effects of glycosaminoglycans on neurite growth on laminin and L1 substrates. J. Neurosci. 15, 8053-66.

Dowbenko, D., Kikuta, A., Fennie, C., Gillett, N., and Lasky, L. A. (1993). Glycosylation-dependent cell adhesion molecule 1 (GlyCAM 1) mucin is expressed by lactating mammary gland epithelial cells and is present in milk. J. Clin. Invest. 92, 952-960.

Drickamer, K. (1993). Ca^{+2} -dependent carbohydrate-recognition domains in animal proteins. Curr. Opin. Struct. Biol. 3, 393-400.

Drickamer, K. (1995). Multiplicity of lectin-carbohydrate interactions. Nat. Struct. Biol. 2, 437-9.

Dwir, O., Shimron, F., Chen, C., Singer, M. S., Rosen, S. D., and Alon, R. (1998). GlyCAM-1 supports leukocyte rolling in flow: Evidence for greater dynamic stability of L-selectin rolling of lymphocytes than on neutrophils. Cell Adh. Comm. 6, 349-370.

Edward, D. P., Thonar, E. J., Srinivasan, M., Yue, B. J., and Tso, M. O. (1990). Macular dystrophy of the cornea. A systemic disorder of keratan sulfate metabolism. Ophthalmology 97, 1194-200.

Ehrhardt, D. W., Atkinson, E. M., Faull, K. F., Freedberg, D. I., Sutherlin, D. P., Armstrong, R., and Long, S. R. (1995). In vitro sulfotransferase activity of NodH, a nodulation protein of *Rhizobium meliloti* required for host-specific nodulation. J.Bacteriol. 177, 6237-6245.

Etzioni, A., Frydman, M., Pollack, S., Avidor, I., Phillips, M. L., Paulson, J. C., and Gershoni, B. R. (1992). Brief report: Recurrent severe infections caused by a novel leukocyte adhesion deficiency. N. Engl. J. Med. 327, 1789-1792.

Falany, C. N. (1997). Enzymology of human cytosolic sulfotransferases. FASEB J. 11, 206-16.

Farooqui, A. A. (1980). 3'-phosphoadenosine 5'-phosphosulphate metabolism in mammalian tissues. Int. J. Biochem. 12, 529-36.

Faveeuw, C., Gagnerault, M. C., and Lepault, F. (1994). Expression of homing and adhesion molecules in infiltrated islets of Langerhans and salivary glands of nonobese diabetic mice. J. Immunol. 152, 5969-5978.

Feizi, T. (1993). Oligosaccharides that mediate mammalian cell-cell adhesion. Curr. Opin. Struct. Biol. 3, 701-710.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U S A 84, 7413-7.

Freemont, A. (1987). Molecules controlling lymphocyte-endothelial interactions in lymph nodes are produced in vessels of inflamed synovium. Ann. Rheum. Dis. 46, 924-928.

Freemont, A. (1988). Functional and biosynthetic changes in endothelial cells of vessels in chronically inflamed tissues: Evidence for endothelial control of lymphocyte entry into diseased tissues. J. Pathol. 155, 225-230.

Fuhlbrigge, R. C., Alon, R., Puri, K. D., Lowe, J. B., and Springer, T. A. (1996). Sialylated, fucosylated ligands for L-selectin expressed on leukocytes mediate tethering and rolling adhesions in physiologic flow conditions. J.Cell Biol. 135, 837-848.

Fukuda, M., Carlsson, S. R., Klock, J. C., and Dell, A. (1986). Structures of O-linked oligosaccharides isolated from normal granulocytes, chronic myelogenous leukemia cells, and acute myelogenous leukemia cells. J. Biol. Chem. 261, 12796-806.

Fukuta, M., Inazawa, J., Torii, T., Tsuzuki, K., Shimada, E., and Habuchi, O. (1997). Molecular cloning and characterization of human keratan sulfate Gal-6-sulfotransferase. J. Biol. Chem. 272, 32321-8.

Fukuta, M., Uchimura, K., Nakashima, K., Kato, M., Kimata, K., Shinomura, T., and Habuchi, O. (1995). Molecular cloning and expression of chick chondrocyte chondroitin 6-sulfotransferase. J. Biol. Chem. 270, 18575-18580.

Gallatin, W., Weissman, I., and Butcher, E. (1983). A cell-surface molecule involved in organ-specific homing of lymphocytes. Nature 304, 30-34.

Galustian, C., Lawson, A. M., Komba, S., Ishida, H., Kiso, M., and Feizi, T. (1997). Sialyl-lewis x sequence 6-O-sulfated at N-acetylglucosamine rather than at galactose is the preferred ligand for L-selectin and de-N-acetylation of the sialic acid enhances its binding strength. Biochem. Biophys. Res. Com. 240, 748-751.

Giblin, P. A., Hwang, S. T., Katsumoto, T. R., and Rosen, S. D. (1997). Ligation of L-selectin on T lymphocytes activates β-1 integrins and promotes adhesion to fibronectin. J. Immunol. 159, 3498-3507.

Giordanengo, V., Limouse, M., Desroys du Roure, L., Cottalorda, J., Doglio, A., Passeron, A., Fuzibet, J. G., and Lefebvre, J. C. (1995a). Autoantibodies directed against CD43 molecules with an altered glycosylation status on human immunodeficiency virus type 1 (HIV-1)- infected CEM cells are found in all HIV-1+ individuals. Blood 86, 2302-11.

Giordanengo, V., Limouse, M., Peyron, J. F., and Lefebvre, J. C. (1995b). Lymphocytic CD43 and CD45 bear sulfate residues potentially implicated in cell to cell interactions. Eur. J. Immunol. 25, 274-8.

Girard, J.-P., and Springer, T. A. (1995a). Cloning from purified high endothelial venule cells of hevin, a close relative of the antiadhesive extracellular matrix protein SPARC. Immunity 2, 113-123.

Girard, J.-P., and Springer, T. A. (1995b). High endothelial venules: specialized endothelium for lymphocyte migration. Immunol. Today 16, 449-457.

Girard, J. P., Baekkevold, E. S., and Amalric, F. (1998). Sulfation in high endothelial venules: cloning and expression of the human PAPS synthetase. FASEB J. 12, 603-612.

Giuffrè, L., Cordey, A. S., Monai, N., Tardy, Y., Schapira, M., and Spertini, O. (1997). Monocyte adhesion to activated aortic endothelium: Role of L-selectin and heparan sulfate proteoglycans. J. Cell Biol. 136, 945-956.

Gunthert, U., Hofmann, M., Rudy, W., Reber, S., Zoller, M., Haussmann, I., Matzku, S., Wenzel, A., Ponta, H., and Herrlich, P. (1991). A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell 65, 13-24.

Guyer, D. A., Moore, K. L., Lynam, E. B., Schammel, C. M. G., Rogelj, S., McEver, R. P., and Sklar, L. A. (1996). P-selectin glycoprotein ligand-1 is a ligand for L-selectin in neutrophil aggregation. Blood 88, 2415-2421.

Habuchi, O., Matsui, Y., Kotoya, Y., Aoyama, Y., Yasuda, Y., and Noda, M. (1993). Purification of chondroitin 6-sulfotransferase secreted from cultured chick embryo chondrocytes. J. Biol. Chem. 268, 21968-21974.

Habuchi, O., Suzuki, Y., and Fukuta, M. (1997). Sulfation of sialyl lactosamine oligosaccharides by chondroitin 6-sulfotransferase. Glycobiology 7, 405-412.

Hänninen, A., Taylor, C., Streeter, P. R., Stark, L. S., Sarte, J. M., Shizuru, J. A., Simell, O., and Michie, S. A. (1993). Vascular addressins are induced on islet vessels during insulitis in nonobese diabetic mice and are involved in lymphoid binding to islet endothelium. J. Clin. Invest. 92, 2509-2515.

Hashimoto, Y., Orellana, A., Gil, G., and Hirschberg, C. B. (1992). Molecular cloning and expression of rat liver N-heparan sulfate sulfotransferase. J. Biol. Chem. 267, 15744-15750.

Hemmerich, S., Bertozzi, C. R., Leffler, H., and Rosen, S. D. (1994a). Identification of the sulfated monosaccharides of GlyCAM-1, an endothelial derived ligand for L-selectin. Biochemistry 33, 4820-4829.

Hemmerich, S., Butcher, E. C., and Rosen, S. D. (1994b). Sulfation-dependent recognition of HEV-ligands by L-selectin and MECA 79, an adhesion-blocking mAb. J. Exp. Med. 180, 2219-2226.

Hemmerich, S., Leffler, H., and Rosen, S. D. (1995). Structure of the O-glycans in GlyCAM-1, an endothelial-derived ligand for L-selectin. J. Biol. Chem. 270, 12035-12047.

Hemmerich, S., and Rosen, S. D. (1994). 6'-sulfated, sialyl Lewis X is a major capping group of GlyCAM-1. Biochemistry 33, 4830-4835.

Higgins, E. A., Siminovitch, K. A., Zhuang, D. L., Brockhausen, I., and Dennis, J. W. (1991). Aberrant O-linked oligosaccharide biosynthesis in lymphocytes and platelets from patients with the Wiskott-Aldrich syndrome. J. Biol. Chem. 266, 6280-90.

Higuchi, Y., Herrera, P., Muniesa, P., Huarte, J., Belin, D., Ohashi, P., Aichele, P., Orci, L., Vassalli, J. D., and Vassalli, P. (1992). Expression of a tumor necrosis factor alpha transgene in murine pancreatic beta cells results in severe and permanent insulitis without evolution towards diabetes. J. Exp. Med. 176, 1719-1731.

Hoke, D., Mebius, R. E., Dybdal, N., Dowbenko, D., Gribling, P., Kyle, C., Baumhueter, S., and Watson, S. R. (1995). Selective modulation of the expression of L-selectin ligands by an immune response. Curr. Biol. 5, 670-678.

Holley, J. A., and Yu, R. K. (1987). Localization of glycoconjugates recognized by the HNK-1 antibody in mouse and chick embryos during early neural development. Dev. Neurosci. 9, 105-19.

Honke, K., Tsuda, M., Hirahara, Y., Ishii, A., Makita, A., and Wada, Y. (1997). Molecular cloning and expression of cDNA encoding human 3'-phosphoadenylylsulfate:galactosylceramide 3'-sulfotransferase. J. Biol. Chem. 272, 4864-4868.

Honke, K., Tsuda, M., Hirahara, Y., Miyao, N., Tsukamoto, T., Satoh, M., and Wada, Y. (1998). Cancer-associated expression of glycolipid sulfotransferase gene in human renal cell carcinoma cells. Cancer Res. 58, 3800-5.

Hooper, L. V., Hindsgaul, O., and Baenziger, J. U. (1995). Purification and characterization of the GalNAc-4-sulfotransferase responsible for sulfation of GalNAcβ1,4GlcNAc-bearing oligosaccharides. J. Biol. Chem. 270, 16327-16332.

Hooper, L. V., Manzella, S. M., and Baenziger, J. U. (1996). From legumes to leukocytes: Biological roles for sulfated carbohydrates. FASEB J. 10, 1137-1146.

Hortin, G. L. (1990). Sulfation of tyrosine residues in coagulation factor V. Blood 76, 946-52.

Hortin, G. L., Farries, T. C., Graham, J. P., and Atkinson, J. P. (1989). Sulfation of tyrosine residues increases activity of the fourth component of complement. Proc. Natl. Acad. Sci. U S A 86, 1338-42.

Hulme, E., C., and Birdsall, N. J. M. 1992. Strategy and tactics in receptor-binding studies. In Receptor-ligand interactions. A practical approach. Edited by E. C. Hulme. 63-176. Oxford: Oxford University Press.

Hwang, S. T., Singer, M. S., Giblin, P. A., Yednock, T. A., Bacon, K. B., Simon, S. I., and Rosen, S. D. (1996). GlyCAM-1, a physiological ligand for L-selectin, activates β2 integrins on naive peripheral lymphocytes. J. Exp. Med. 184, 1343-1348.

Ilyas, A. A., Chou, D. K., Jungalwala, F. B., Costello, C., and Quarles, R. H. (1990). Variability in the structural requirements for binding of human monoclonal anti-myelin-associated glycoprotein immunoglobulin M antibodies and HNK-1 to sphingoglycolipid antigens. J. Neurochem. 55, 594-601.

Imai, Y., Lasky, L. A., and Rosen, S. D. (1992). Further characterization of the interaction between L-selectin and its endothelial ligands. Glycobiology 2, 373-381.

Imai, Y., Lasky, L. A., and Rosen, S. D. (1993). Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin. Nature 361, 555-557.

Imai, Y., and Rosen, S. D. (1993). Direct demonstration of heterogeneous, sulfated O-linked carbohydrate chains on an endothelial ligand for L-selectin. Glycoconj. J. 10, 34-39.

Imai, Y., Singer, M. S., Fennie, C., Lasky, L. A., and Rosen, S. D. (1991). Identification of a carbohydrate-based endothelial ligand for a lymphocyte homing receptor. J. Cell Biol. 113, 1213-1221.

Ise, I., Yamaguchi, K., Sato, K., Kitamura, F., Tamatani, T., and Miyasaka, M. (1988). Molecular mechanisms underlying lymphocyte recirculation. I. Functional, phenotypical and morphological characterization of high endothelial cells cultured in vitro. Europ. J. Immunol. 18, 1235-1244.

Jain, R. K., Vig, R., Rampal, R., Chandrasekaran, E. V., and Matta, K. L. (1994). Total Synthesis of 3'-O-Sialyl, 6'-O-Sulfo Lewis ^{x}, NeuAcα2→3(6-O-SO_3Na)Galβ1→4(Fucα1→3)-GlcNAcβ–OMe: A Major Capping Group of GlyCAM-1. J. Am. Chem. Soc. 116, 12123-12124.

Jung, U., Ramos, C. L., Bullard, D. C., and Ley, K. (1998). Gene-targeted mice reveal importance of L-selectin-dependent rolling for neutrophil adhesion. Am. J. Physiol. 274, H1785-91.

Kakuta, Y., Pedersen, L. G., Carter, C. W., Negishi, M., and Pedersen, L. C. (1997). Crystal structure of estrogen sulphotransferase [letter]. Nat. Struct. Biol. 4, 904-8.

Kakuta, Y., Pedersen, L. G., Pedersen, L. C., and Negishi, M. (1998a). Conserved structural motifs in the sulfotransferase family. Trends Biochem. Sci. 23, 129-30.

Kakuta, Y., Petrotchenko, E. V., Pedersen, L. C., and Negishi, M. (1998b). The sulfuryl transfer mechanism. Crystal structure of a vanadate complex of estrogen sulfotransferase and mutational analysis. J. Biol. Chem. 273, 27325-30.

Kamerling, J. P., Rijkse, I., Maas, A. A., van Kuik, J. A., and Vliegenthart, J. F. (1988). Sulfated N-linked carbohydrate chains in porcine thyroglobulin. FEBS Lett. 241, 246-50.

Kansas, G. S. (1996). Selectins and their ligands: current concepts and controversies. Blood 88, 3259-3287.

Kansas, G. S., Saunders, K. B., Ley, K., Zakrzewicz, A., Gibson, R. M., Furie, B. C., Furie, B., and Tedder, T. F. (1994). A role for the epidermal growth factor-like domain of P-selectin in ligand recognition and cell adhesion. J. Cell Biol. 124, 609-618.

Kansas, G. S., Spertini, O., Stoolman, L. M., and Tedder, T. F. (1991). Molecular mapping of functional domains of the leukocyte receptor for endothelium, LAM-1. J. Cell Biol. 114, 351-358.

Kaplanski, G., Farnarier, C., Tissot, O., Pierres, A., Benoliel, A. M., Alessi, M. C., Kaplanski, S., and Bongrand, P. (1993). Granulocyte-endothelium initial adhesion. Analysis of transient binding events mediated by E-selectin in a laminar shear flow. Biophys. J. 64, 1922-33.

Karsan, A., Cornejo, C. J., Winn, R. K., Schwartz, B. R., Way, W., Lannir, N., Gershoni-Baruch, R., Etzioni, A., Ochs, H. D., and Harlan, J. M. (1998). Leukocyte Adhesion Deficiency Type II is a generalized defect of de novo GDP-fucose biosynthesis. Endothelial cell fucosylation is not required for neutrophil rolling on human nonlymphoid endothelium. J. Clin. Invest. 101, 2438-45.

Kershaw, D. B., Beck, S. G., Wharram, B. L., Wiggins, J. E., Goyal, M., Thomas, P. E., and Wiggins, R. C. (1997). Molecular cloning and characterization of human podocalyxin-like protein. Orthologous relationship to rabbit PCLP1 and rat podocalyxin. J. Biol. Chem. 272, 15708-14.

Kiessling, L. L., and Pohl, N. L. (1996). Strength in numbers: non-natural polyvalent carbohydrate derivatives. Chem. Biol. 3, 71-7.

Kikuta, A., and Rosen, S. D. (1994). Localization of ligands for L-selectin in mouse peripheral lymph node high endothelial cells by colloidal gold conjugates. Blood 84, 3766-3775.

Killeen, N., Barclay, A. N., Willis, A. C., and Williams, A. F. (1987). The sequence of rat leukosialin (W3/13 antigen) reveals a molecule with O-linked glycosylation of one third of its extracellular amino acids. Embo J. 6, 4029-34.

Kincade, P. W., Zheng, Z., Katoh, S., and Hanson, L. (1997). The importance of cellular environment to function of the CD44 matrix receptor. Curr. Opin. Cell Biol. 9, 635-42.

Klaassen, C. D., and Boles, J. W. (1997). Sulfation and sulfotransferases 5: the importance of 3'- phosphoadenosine 5'-phosphosulfate (PAPS) in the regulation of sulfation. FASEB J. 11, 404-18.

Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992). Brefeldin A: insights into the control of membrane traffic and organelle structure. J Cell Biol 116, 1071-80.

Knibbs, R. N., Craig, R. A., Natsuka, S., Chang, A., Cameron, M., Lowe, J. B., and Stoolman, L. M. (1996). The fucosyltransferase FucT-VII regulates E-selectin ligand synthesis in human T cells. J. Cell Biol. 133, 911-920.

Koenig, A., Jain, R., Vig, R., Norgard-Sumnicht, K. E., Matta, K. L., and Varki, A. (1997). Selectin inhibition: Synthesis and evaluation of novel sialylated, sulfated and fucosylated oligosaccharides, including the major capping group of GlyCAM-1. Glycobiology 7, 79-93.

Kolodkin, A. L., Levengood, D. V., Rowe, E. G., Tai, Y. T., Giger, R. J., and Ginty, D. D. (1997). Neuropilin is a semaphorin III receptor. Cell 90, 753-62.

Komatsu, K., Driscoll, W. J., Koh, Y. C., and Strott, C. A. (1994). A P-loop related motif (GxxGxxK) highly conserved in sulfotransferases is required for binding the activated sulfate donor. Biochem. Biophys. Res. Commun. 204, 1178-85.

Kozak, M. (1996). Interpreting cDNA sequences: some insights from studies on translation. Mamm. Genome 7, 563-74.

Kraal, G. (1992). Cells in the marginal zone of the spleen. Int. Rev. Cytol. 132, 31-74.

Kuhns, W., Jain, R. K., Matta, K. L., Paulsen, H., Baker, M. A., Geyer, R., and Brockhausen, I. (1995). Characterization of a novel mucin sulphotransferase activity synthesizing sulphated O-glycan core 1,3-sulphate-Galβ1-3GalNAcα-R. Glycobiology 5, 689-97.

Lander, A. D. 1998. Proteoglycans. In Guidebook to the Extracellular Matrix and Adhesion Proteins. Edited by T. Kreis and R. Vale.

Larsen, G. R., Sako, D., Ahern, T. J., Shaffer, M., Erban, J., Sajer, S. A., Gibson, R. M., Wagner, D. D., Furie, B. C., and Furie, B. (1992). P-selectin and E-selectin. Distinct but overlapping leukocyte ligand specificities. J. Biol. Chem. 267, 11104-11110.

Lasky, L. A. (1995). Selectin-carbohydrate interactions and the initiation of the inflammatory response. Annu. Rev. Biochem. 64, 113-139.

Lasky, L. A., Singer, M. S., Dowbenko, D., Imai, Y., Henzel, E. J., Fennie, C., Gillett, N., Watson, S. R., and Rosen, S. D. (1992). An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell 69, 927-938.

Lawrence, M. B., Berg, E. L., Butcher, E. C., and Springer, T. A. (1995). Rolling of lymphocytes and neutrophils on peripheral node addressin and subsequent arrest on ICAM-1 in shear flow. Eur. J. mmunol. 25, 1025-1031.

Lawrence, M. B., Kansas, G. F., Kunkel, E. J., and Ley, K. (1997). Threshold levels of fluid shear promote leukocyte adhesion through selectins (CD62L,P,E). J. Cell Biol. 136, 717-727.

Lawrence, M. B., and Springer, T. A. (1991). Leukocytes roll on a selectin at physiologic flow rates: distinction form and prerequisite for adhesion through integrins. Cell 65, 859-873.

Lee, M. S., and Sarvetnick, N. (1994). Induction of vascular addressins and adhesion molecules in the pancreas of IFN-gamma transgenic mice. J. Immunol 152, 4597-4603.

Lee, Y. C., Komatsu, K., Driscoll, W. J., and Strott, C. A. (1994). Structural and functional characterization of estrogen sulfotransferase isoforms: distinct catalytic and high affinity binding activities. Mol. Endocrinol. 8, 1627-35.

Lee, Y. C., and Lee, R. T. (1995). Carbohydrate-protein interactions: Basis of glycobiology. Accounts Chem. Res. 28, 321-327.

Lefebvre, J. C., Giordanengo, V., Limouse, M., Doglio, A., Cucchiarini, M., Monpoux, F., Mariani, R., and Peyron, J. F. (1994). Altered glycosylation of leukosialin, CD43, in HIV-1-infected cells of the CEM line. J. Exp. Med. 180, 1609-17.

Lesley, J., Hyman, R., and Kincade, P. W. (1993). CD44 and its interaction with extracellular matrix. Adv. Immunol. 54, 271-335.

Levinovitz, A., Mühlhoff, J., Isenmann, S., and Vestweber, D. (1993). Identification of a glycoprotein ligand for E-selectin on mouse myeloid cells. J. Cell Biol. 121, 449-459.

Leyh, T. S., Taylor, J. C., and Markham, G. D. (1988). The sulfate activation locus of Escherichia coli K12: cloning, genetic, and enzymatic characterization. J. Biol. Chem. 263, 2409-16.

Leyte, A., van Schijndel, H. B., Niehrs, C., Huttner, W. B., Verbeet, M. P., Mertens, K., and van Mourik, J. A. (1991). Sulfation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. J. Biol. Chem. 266, 740-6.

Li, X., and Tedder, T. F. (1999). CHST1 and CHST2 sulfotransferases expressed by human vascular endothelial cells: cDNA cloning, expression, and chromosomal localization. Genomics 55, 345-7.

Lindahl, U., Kusche-Gullberg, M., and Kjellen, L. (1998). Regulated diversity of heparan sulfate. J. Biol. Chem. 273, 24979-82.

Liu, J., Shworak, N. W., Fritze, L. M. S., Edelberg, J. M., and Rosenberg, R. D. (1996). Purification of heparan sulfate D-glucosaminyl 3-O-sulfotransferase. J. Biol. Chem. 271, 27072-82.

Lo-Guidice, J. M., Périni, J. M., Lafitte, J. J., Ducourouble, M. P., Roussel, P., and Lamblin, G. (1995). Characterization of a sulfotransferase from human airways

responsible for the 3-*O*-sulfation of terminal galactose in *N*-acetyllactosamine-containing mucin carbohydrate chains. J. Biol. Chem. 270, 27544-27550.

Lo-Guidice, J. M., Wieruszeski, J. M., Lemoine, J., Verbert, A., Roussel, P., and Lamblin, G. (1994). Sialylation and sulfation of the carbohydrate chains in respiratory mucins from a patient with cystic fibrosis. J. Biol. Chem. 269, 18794-18813.

Long, S. R., and Staskawicz, B. J. (1993). Prokaryotic plant parasites. Cell 73, 921-35.

Low, K., Orberger, G., Schmitz, B., Martini, R., and Schachner, M. (1994). The L2/HNK-1 carbohydrate is carried by the myelin associated glycoprotein and sulphated glucuronyl glycolipids in muscle but not cutaneous nerves of adult mice. Eur. J. Neurosci. 6, 1773-81.

Lowe, J. B., Stoolman, L. M., Nair, R. P., Larsen, R. D., Berhend, T. L., and Marks, R. M. (1990). ELAM-1-dependent cell adhesion to vascular endothelium determined by a transfected human fucosyltransferase cDNA. Cell 63, 475-484.

Mackay, C. R., Marston, W., and Dudler, E. (1992). Altered patterns of T cell migration through lymph nodes and skin following antigen challenge. Eur. J. Immunol. 22, 2205-2210.

Maiti, A., Maki, G., and Johnson, P. (1998). TNF-alpha induction of CD44-mediated leukocyte adhesion by sulfation. Science 282, 941-3.

Maly, P., Thall, A. D., Petryniak, B., Rogers, C. E., Mith, P. L., Marks, R. M., Kelly, R. J., Gersten, K. M., Cheng, G., Saunders, T. L., Camper, S. A., Camphausen, R. T., Sullivan, F. X., Isogai, Y., Hindsgaul, O., von Andrian, U. H., and Lowe, J. B. (1996). The Fuc-TVII alpha(1,3)fucosyltransferase Fuc-TVII controls lymphocyte homing, and blood leukocyte emigration through an essential role in L-,E- and P-selectin ligand biosynthesis. Cell 86, 643-653.

Mandon, E. C., Milla, M. E., Kempner, E., and Hirschberg, C. B. (1994). Purification of the Golgi adenosine 3'-phosphate 5'-phosphosulfate transporter, a homodimer within the membrane. Proc. Natl. Acad. Sci. U S A 91, 10707-11.

Marchese, P., Murata, M., Mazzucato, M., Pradella, P., De Marco, L., Ware, J., and Ruggeri, Z. M. (1995). Identification of three tyrosine residues of glycoprotein Ib alpha with distinct roles in von Willebrand factor and alpha-thrombin binding. J. Biol. Chem. 270, 9571-8.

Matte, A., Tari, L. W., and Delbaere, L. T. (1998). How do kinases transfer phosphoryl groups? Structure 6, 413-9.

Mazany, K. D., Peng, T., Watson, C. E., Tabas, I., and Williams, K. J. (1998). Human chondroitin 6-sulfotransferase: cloning, gene structure, and chromosomal localization. Biochim. Biophys. Acta. 1407, 92-7.

McEver, R. P. (1994). Selectins. Curr. Opin. Immunol. 6, 75-84.

McEvoy, L. M., Sun, H., Frelinger, J. G., and Butcher, E. C. (1997). Anti-CD43 inhibition of T cell homing. J. Exp. Med. 185, 1493-8.

Michie, S. A., Streeter, P. R., Bolt, P. A., Butcher, E. C., and Picker, L. J. (1993). The human peripheral lymph node vascular addressin. Amer. J. Path. 143, 1688-1698.

Mitsuoka, C., Kawakami-Kimura, N., Kasugai-Sawada, M., Hiraiwa, N., Toda, K., Ishida, H., Kiso, M., Hasegawa, A., and Kannagi, R. (1997). Sulfated sialyl Lewis X, the putative L-selectin ligand, detected on endothelial cells of high endothelial venules by a distinct set of anti-sialyl Lewis X antibodies. Biochem. Biophys. Res. Commun. 230, 546-51.

Mitsuoka, C., Sawada-Kasugai, M., Ando-Furui, K., Izawa, M., Nakanishi, H., Nakamura, S., Ishida, H., Kiso, M., and Kannagi, R. (1998b). Identification of a major carbohydrate capping group of the L-selectin ligand on high endothelial venules in human lymph nodes as 6-sulfo sialyl Lewis X. J. Biol. Chem. 273, 11225-33.

Moore, K. L., Stults, N. L., Diaz, S., Smith, D. F., Cummings, R. D., Varki, A., and Mc Ever, R. P. (1992). Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. J. Cell Biol. 118, 445-456.

Mortell, K. H., Weatherman, R. V., and Kiessling, L. L. (1996). Recognition specificity of neoglycopolymers prepared by ring-opening metathesis polymerization. J. Am. Chem. Soc. 118, 2297-2298.

Nakazawa, K., Suzuki, S., and Wada, K. (1995). Proteoglycan synthesis by corneal explants from developing embryonic chicken. J. Biochem. (Tokyo) 117, 707-18.

Nathan, C., Xie, Q. W., Halbwachs-Mecarelli, L., and Jin, W. W. (1993). Albumin inhibits neutrophil spreading and hydrogen peroxide release by blocking the shedding of CD43 (sialophorin, leukosialin). J. Cell Biol. 122, 243-56.

Natsuka, S., and Lowe, J. B. (1994). Enzymes involved in mammalian oligosaccharide biosynthesis. Curr. Opin. Struct. Biol. 4, 683-691.

Nicholson, M. W., Barclay, A. N., Singer, M. S., Rosen, S. D., and van deer Merwe, P. A. (1998). Affinity and kinetic analysis of L-selectin (CD62L) binding to GlyCAM-1. J. Biol. Chem. 273, 763-770.

Niehrs, C., Beisswanger, R., and Huttner, W. B. (1994). Protein tyrosine sulfation, 1993--an update. Chem. Biol. Interact. 92, 257-71.

Noguchi, S., and Nakano, M. (1992). Structure of the acidic N-linked carbohydrate chains of the 55-kDa glycoprotein family (PZP3) from porcine zona pellucida. Eur. J. Biochem. 209, 883-94.

Nottenburg, C., Rees, G., and St. John, T. (1989). Isolation of mouse CD44 cDNA: structural features are distinct from the primate cDNA. Proc. Natl. Acad. Sci. U S A 86, 8521-5.

Ong, E., Yeh, J. C., Ding, Y., Hindsgaul, O., and Fukuda, M. (1998). Expression cloning of a human sulfotransferase that directs the synthesis of the HNK-1 glycan on the neural cell adhesion molecule and glycolipids. J. Biol. Chem. 273, 5190-5.

Onrust, S. V., Hartl, P. M., Rosen, S. D., and Hanahan, D. (1996). Modulation of L-selectin ligand expression during an immune response accompanying tumorigenesis in transgenic mice. J. Clin. Invest. 97, 54-64.

Ozeran, J. D., Westley, J., and Schwartz, N. B. (1996). Identification and partial purification of PAPS translocase. Biochemistry 35, 3695-703.

Pabst, R. (1988). The spleen in lymphocyte migration. Imm. Today 9, 43-45.

Pallant, A., Eskenazi, A., Mattei, M. G., Fournier, R. E., Carlsson, S. R., Fukuda, M., and Frelinger, J. G. (1989). Characterization of cDNAs encoding human leukosialin and localization of the leukosialin gene to chromosome 16. Proc. Natl. Acad. Sci. U S A 86, 1328-32.

Pettersson, I., Kusche, M., Unger, E., Wlad, H., Nylund, L., Lindahl, U., and Kjellen, L. (1991). Biosynthesis of heparin. Purification of a 110-kDa mouse mastocytoma protein required for both glucosaminyl N-deacetylation and N-sulfation. J. Biol. Chem. 266, 8044-9.

Pfeiffer, G., Stirm, S., Geyer, G., Strube, K.-H., Bergwerff, A. A., Kamerling, J. P., and Vliegenthart, J. F. P. (1992). Structure elucidation of sulphated oligosaccharides from recombinant human tissue plasminogen activator expressed in mouse epithelial cells. Glycobiology 2, 411-418.

Picker, L. J., and Butcher, E. C. (1992). Physiological and molecular mechanisms of lymphocyte homing. Annu. Rev. Immunol. 10, 561-591.

Piller, F., Le Deist, F., Weinberg, K. I., Parkman, R., and Fukuda, M. (1991). Altered O-glycan synthesis in lymphocytes from patients with Wiskott- Aldrich syndrome. J. Exp. Med. 173, 1501-10.

Pittman, D. D., Wang, J. H., and Kaufman, R. J. (1992). Identification and functional importance of tyrosine sulfate residues within recombinant factor VIII. Biochemistry 31, 3315-25.

Pouyani, T., and Seed, B. (1995). PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. Cell 83, 333-343.

Prasadaro, N., Koul, O., Tobet, S. A., Chou, D. K., and Jungalwala, F. B. (1990). Developmental expression of HNK-1-reactive antigens in the rat cerebellum and localization of sulfoglucuronyl glycolipids in molecular layer and deep cerebellar nuclei. J. Neurochem. 55, 2024-30.

Price, T. H., Ochs, H. D., Gershoni-Baruch, R., Harlan, J. M., and Etzioni, A. (1994). In vivo neutrophil and lymphocyte function studies in a patient with leukocyte adhesion deficiency type II. Blood 84, 1635-1639.

Puri, K. D., Chen, S., and Springer, T. A. (1998). Modifying the mechanical property and shear threshold of L-selectin adhesion independently of equilibrium properties. Nature 392, 930-3.

Puri, K. D., Finger, E. B., Gaudernack, G., and Springer, T. A. (1995). Sialomucin CD34 is the major L-selectin ligand in human tonsil high endothelial venules. J. Cell Biol. 131, 261-270.

Puri, K. D., Finger, E. B., and Springer, T. A. (1997). The faster kinetics of L-selectin than of E-selectin and P-selectin rolling at comparable binding strength. J. Immunol. 158, 405-13.

Raouf, A. H., Tsai, H. H., Parker, N., Hoffman, J., Walker, R. J., and Rhodes, J. M. (1992). Sulphation of colonic and rectal mucin in inflammatory bowel disease: reduced sulphation of rectal mucus in ulcerative colitis. Clin. Sci. (Colch) 83, 623-6.

Remold-O'Donnell, E., Kenney, D. M., Parkman, R., Cairns, L., Savage, B., and Rosen, F. S. (1984). Characterization of a human lymphocyte surface sialoglycoprotein that is defective in Wiskott-Aldrich syndrome. J. Exp. Med. 159, 1705-23.

Remold-O'Donnell, E., Zimmerman, C., Kenney, D., and Rosen, F. S. (1987). Expression on blood cells of sialophorin, the surface glycoprotein that is defective in Wiskott-Aldrich syndrome. Blood 70, 104-9.

Roche, P., Debellé, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Dénarié, J., and Promé, J. C. (1991). Molecular basis of symbiotic host specificity in rhizobium meliloti: signals. Cell 67, 1131-1143.

Rosen, S. D., and Bertozzi, C. R. (1994). The selectins and their ligands. Curr. Opin. Cell Biol. 6, 663-673.

Rosen, S. D., Chi, S.-I., True, D. D., Singer, M. S., and Yednock, T. A. (1989). Intravenously injected sialidase inactivates attachment sites for lymphocytes on high endothelial venules. J. Immunol. 142, 1895-1902.

Rosen, S. D., Singer, M. S., Yednock, T. A., and Stoolman, L. M. (1985). Involvement of sialic acid on endothelial cells in organ-specific lymphocyte recirculation. Science 228, 1005-1007.

Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997). Heparan sulfate proteoglycans of the cardiovascular system. Specific structures emerge but how is synthesis regulated? J. Clin. Invest. 99, 2062-70.

Russo, R. N., Shaper, N. L., and Shaper, J. H. (1990). Bovine β 1,4-galactosyltransferase: two sets of mRNA transcripts encode two forms of the protein with different amino-terminal domains. In vitro translation experiments demonstrate that both the short and the long forms of the enzyme are type II membrane-bound glycoproteins. J. Biol. Chem. 265, 3324-31.

Saga, Y., Tung, J. S., Shen, F. W., and Boyse, E. A. (1986). Sequences of Ly-5 cDNA: isoform-related diversity of Ly-5 mRNA [published erratum appears in Proc Natl Acad Sci U S A 1987 Apr;84(7):1991]. Proc. Natl. Acad. Sci. U S A 83, 6940-4.

Saitoh, O., Piller, F., Fox, R. I., and Fukuda, M. (1991). T-lymphocytic leukemia expresses complex, branched O-linked oligosaccharides on a major sialoglycoprotein, leukosialin. Blood 77, 1491-9.

Sakac, D., Zachos, M., and Lingwood, C. A. (1992). Purification of the testicular galactolipid: 3'-phosphoadenosine 5'- phosphosulfate sulfotransferase. J. Biol. Chem. 267, 1655-9.

Sako, D., Chang, X. J., Barone, K. M., Vachino, G., White, H. M., Shaw, G., Veldman, G. M., Bean, K. M., Ahern, T. J., Furie, B., Cumming, D. A., and Larsen, G. R. (1993). Expression cloning of a functional glycoprotein ligand for P-selectin. Cell 75, 1179-1186.

Sako, D., Comess, K. M., Barone, K. M., Camphausen, R. T., Cumming, D. A., and Shaw, G. D. (1995). A sulfated peptide segment at the amino terminus of PSGL-1 is critical for P-selectin binding. Cell 83, 323-331.

Salmi, M., Granfors, K., MacDermott, R., and Jalkanen, S. (1994). Aberrant binding of lamina propria lymphocytes to vascular endothelium in inflammatory bowel diseases. Gastroenterology 106, 596-605.

Salmivirta, M., Lidholt, K., and Lindahl, U. (1996). Heparan sulfate: a piece of information. FASEB J. 10, 1270-9.

Sanchez-Mateos, P., Campanero, M. R., del Pozo, M. A., and Sanchez-Madrid, F. (1995). Regulatory role of CD43 leukosialin on integrin-mediated T-cell adhesion to endothelial and extracellular matrix ligands and its polar redistribution to a cellular uropod. Blood 86, 2228-39.

Sanger, F., Nicklens, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.

Sassetti, C., Tangemann, K., Singer, M. S., Kershaw, D. B., and Rosen, S. D. (1998). Identification of podocalyxin as an HEV ligand for L-selectin: parallels to CD34. J. Exp. Med. 187, 1965-1975.

Saunders, W. J., Katsumoto, T. R., Bertozzi, C. R., Rosen, S. D., and Kiessling, L. L. (1996). Selectin-carbohydrate interactions: an investigation into the relevant modifications of the Lewis x trisaccharides. Biochem. 35, 14862-14867.

Schachner, M., Martini, R., Hall, H., and Orberger, G. (1995). Functions of the L2/HNK-1 carbohydrate in the nervous system. Prog Brain Res 105, 183-8.

Schachter, H., and Brockhausen, I. (1989). The biosynthesis of branched O-glycans. Symp Soc. Exp. Biol. 43, 1-26.

Schumacher, S. v. (1899). Ueber Phagocytose und die Abfuhrwege der Leucocyten in den Lympdrusen. Arch. mikrosk. Anat. 76, 421.

Screaton, G. R., Bell, M. V., Jackson, D. G., Cornelis, F. B., Gerth, U., and Bell, J. I. (1992). Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc. Natl. Acad. Sci. U S A 89, 12160-4.

Scudder, P. R., Shailubhai, K., Duffin, K. L., Streeter, P. R., and Jacob, G. S. (1994). Enzymatic synthesis of a 6-sulfated sialyl-Lewis^x which is an inhibitor of L-selectin binding to peripheral addressin. Glycobiol. 4, 929-933.

Seko, A., Hara-Kuge, S., Yonezawa, S., Nagata, K., and Yamashita, K. (1998). Identification and characterization of N-acetylglucosamine-6-O-sulfate- specific beta1,4-galactosyltransferase in human colorectal mucosa. FEBS Lett. 440, 307-10.

Sen, J., Goltz, J. S., Stevens, L., and Stein, D. (1998). Spatially restricted expression of pipe in the Drosophila egg chamber defines embryonic dorsal-ventral polarity. Cell 95, 471-81.

Shailubhai, K., Streeter, P., Smith, C. E., and Jacob, G. S. (1997). Sulfation and sialylation requirements for a glycoform of CD34, a major endothelial ligand for L-selectin in porcine peripheral lymph nodes. Glycobiology 7, 305-314.

Shelley, C. S., Remold-O'Donnell, E., Rosen, F. S., and Whitehead, A. S. (1990). Structure of the human sialophorin (CD43) gene. Identification of features atypical of genes encoding integral membrane proteins. Biochem. J. 270, 569-76.

Shilatifard, A., Merkle, R. K., Helland, D. E., Welles, J. L., Haseltine, W. A., and Cummings, R. D. (1993). Complex-type N-linked oligosaccharides of gp120 from human immunodeficiency virus type 1 contain sulfated N-acetylglucosamine. J. Virol. 67, 943-952.

Siegelman, M. H., Cheng, I. C., Weissman, I. L., and Wakeland, E. K. (1990). The mouse lymph node homing receptor is identical with the lymphocyte cell surface marker Ly-22: Role of the EGF domain in endothelial binding. Cell 61, 611-622.

Simmons, D. L. 1993. Cloning cell surface molecules by transient expression in mammalian cells. In Cellular Interactions in Development- A Practical Approach. Edited by D. A. Hartley. 93-127. Oxford: IRL Press.

Simmons, D. L., Satterthwaite, A. B., Tenen, D. G., and Seed, B. (1992). Molecular cloning of a cDNA encoding CD34, a sialomucin of human hematopoietic stem cells. J. Immunol. 148, 267-271.

Skelton, T. P., Hooper, L. V., Srivastava, V., Hindsgaul, O., and Baenziger, J. U. (1991). Characterization of a sulfotransferase responsible for the 4-O-sulfation of terminal beta-

N-acetyl-D-galactosamine on asparagine-linked oligosaccharides of glycoprotein hormones. J. Biol. Chem. 266, 17142-50.

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene 67, 31-40.

Sperling, A. I., Green, J. M., Mosley, R. L., Smith, P. L., DiPaolo, R. J., Klein, J. R., Bluestone, J. A., and Thompson, C. B. (1995). CD43 is a murine T cell costimulatory receptor that functions independently of CD28. J. Exp. Med. 182, 139-46.

Spertini, O., Cordey, A., Monai, N., Giuffrè, L., and Schapira, M. (1996). P-selectin glycoprotein ligand 1 is a ligand for L-selectin on neutrophils, monocytes, and $CD34^+$ hematopoietic progenitor cells. J. Cell Biol. 135, 523-531.

Spiro, R. G., and Bhoyroo, V. D. (1998). Characterization of a spleen sulphotransferase responsible for the 6-O- sulphation of the galactose residue in sialyl-N-acetyl-lactosamine sequences. Biochem. J. 331, 265-71.

Spiro, R. G., Yasumoto, Y., and Bhoyroo, V. (1996). Characterization of a rat liver Golgi sulphotransferase responsible for the 6-O-sulphation of N-acetylglucosamine residues in beta-linkage to mannose: role in assembly of sialyl-galactosyl-N-acetylglucosamine 6-sulphate sequence of N-linked oligosaccharides. Biochem. J. 319, 209-16.

Springer, T. A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76, 301-314.

Stamenkovic, I., Amiot, M., Pesando, J. M., and Seed, B. (1989). A lymphocyte molecule implicated in lymph node homing is a member of the cartilage link protein family. Cell 56, 1057-62.

Steegmaler, M., Levinovitz, A., Isenmann, S., Borges, E., Lenter, M., Kocher, H. P., Kleuser, B., and Vestweber, D. (1995). The E-selectin-ligand ESL-1 is a variant of a receptor for fibroblast growth factor. Nature 373, 615-620.

Streeter, P., Berg, E., Rouse, T., Bargatze, R., and Butcher, E. (1988a). A tissue-specific endothelial cell molecule involved in lymphocyte homing. Nature 331, 41-43.

Streeter, P. R., Rouse, B. T. N., and Butcher, E. C. (1988b). Immunohistologic and functional characterization of a vascular addressin involved in lymphocyte homing into peripheral lymph nodes. J. Cell. Biol. 107, 1853-1862.

Streuli, M., Matsuyama, T., Morimoto, C., Schlossman, S. F., and Saito, H. (1987). Identification of the sequence required for expression of the 2H4 epitope on the human leukocyte common antigens. J. Exp. Med. 166, 1567-72.

Sueyoshi, T., Kakuta, Y., Pedersen, L. C., Wall, F. E., Pedersen, L. G., and Negishi, M. (1998). A role of Lys614 in the sulfotransferase activity of human heparan sulfate N-deacetylase/N-sulfotransferase. FEBS Lett 433, 211-4.

Tangemann, K., Giblin, P., Gunn, M. D., and Rosen, S. D. (1998). A high endothelial cell derived chemokine induces rapid, efficient, and subset-selective arrest of rolling T-lymphocytes on a reconstituted endothelial substrate. J. Immunol. 161, 6330-6337.

Tedder, T. F., Steeber, D. A., Chen, A., and Engel, P. (1995). The selectins: vascular adhesion molecules. FASEB J. 9, 866-873.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-80.

True, D. D., Singer, M. S., Lasky, L. A., and Rosen, S. D. (1990). Requirement for sialic acid on the endothelial ligand of a lymphocyte homing receptor. J. Cell Biol. (in press)

Tsuboi, S., Isogai, Y., Hada, N., King, J. K., Hindsgaul, O., and Fukuda, M. (1996). 6'-Sulfo sialyl Lex but not 6-sulfo sialyl Lex expressed on the cell surface supports L-selectin-mediated adhesion. J. Biol. Chem. 271, 27213-6.

Tu, L., Delahunty, M. D., Ding, H., Luscinskas, F. W., and Tedder, T. F. (1999). The cutaneous lymphocyte antigen is an essential component of the L- selectin ligand induced on human vascular endothelial cells. J. Exp. Med. 189, 241-52.

Uchimura, K., Muramatsu, H., Kadomatsu, K., Fan, Q. W., Kurosawa, N., Mitsuoka, C., Kannagi, R., Habuchi, O., and Muramatsu, T. (1998a). Molecular cloning and characterization of an N-acetylglucosamine-6-O- sulfotransferase. J. Biol. Chem. 273, 22577-83.

Uchimura, K., Muramatsu, H., Kaname, T., Ogawa, H., Yamakawa, T., Fan, Q. W., Mitsuoka, C., Kannagi, R., Habuchi, O., Yokoyama, I., Yamamura, K., Ozaki, T., Nakagawara, A., Kadomatsu, K., and Muramatsu, T. (1998b). Human N-acetylglucosamine-6-O-sulfotransferase involved in the biosynthesis of 6-sulfo sialyl Lewis X: molecular cloning, chromosomal mapping, and expression in various organs and tumor cells. J. Biochem (Tokyo) 124, 670-8.

Varki, A. (1994). Selectin ligands. Proc. Natl. Acad. Sci. (USA) 91, 7390-7397.

Vavasseur, F., Dole, K., Yang, J., Matta, K. L., Myerscough, N., Corfield, A., Paraskeva, C., and Brockhausen, I. (1994). O-glycan biosynthesis in human colorectal adenoma cells during progression to cancer. Eur. J. Biochem. 222, 415-24.

Venter, J. C., Adams, M. D., Martin-Gallardo, A., McCombie, W. R., and Fields, C. (1992). Genome sequence analysis: scientific objectives and practical strategies. Trends Biotechnol. 10, 8-11.

Von Andrian, U. H., Berger, E. M., Ramezani, L., Chambers, J. D., Ochs, H. D., Harlan, J. M., Paulson, J. C., Etzioni, A., and Arfors, K. E. (1993). In vivo behavior of neutrophils from two patients with distinct inherited leukocyte adhesion deficiency syndromes. J. Clin. Invest. 91, 2893-2897.

Vos, J. P., Lopes-Cardozo, M., and Gadella, B. M. (1994). Metabolic and functional aspects of sulfogalactolipids. Biochim. Biophys. Acta 1211, 125-49.

Wagers, A. J., Stoolman, L. M., Kannagi, R., Craig, R., and Kansas, G. S. (1997). Expression of leukocyte fucosyltransferases regulates binding to E- selectin: relationship to previously implicated carbohydrate epitopes. J. Immunol. 159, 1917-29.

Walcheck, B., Moore, K. L., McEver, R. P., and Kishimoto, T. K. (1996). Neutrophil-neutrophil interactions under hydrodynamic shear stress involve L-selectin and PSGL-1. J. Clin. Invest. 98, 1081-1087.

Warnock, R. A., Askari, S., Butcher, E. C., and von Andrian, U. H. (1998). Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. J. Exp. Med. 187, 205-216.

Watson, S. R., Imai, Y., Fennie, C., Geoffroy, J. S., Rosen, S. D., and Lasky, L. A. (1990). A homing receptor-IgG chimera as a probe for adhesive ligands of lymph node high endothelial venules. J. Cell Biol. 110, 2221-2229.

Wei, Z., Swiedler, S. J., Ishihara, M., Orellana, A., and Hirschberg, C. B. (1993). A single protein catalyzes both N-deacetylation and N-sulfation during the biosynthesis of heparan sulfate. Proc. Natl. Acad. Sci. U S A 90, 3885-8.

Wernecke, H., Lindner, J., and Schachner, M. (1985). Cell type specificity and developmental expression of the L2/HNK-1 epitopes in mouse cerebellum. J. Neuroimmunol. 9, 115-30.

Wilkins, P. P., Moore, K. L., McEver, R. P., and Cummings, R. D. (1995). Tyrosine sulfation of P-selectin glycoprotein ligand-1 is required for high affinity binding to P-selectin. J. Biol. Chem. 270, 22677-22680.

Williams, D., and Schachter, H. (1980). Mucin synthesis. I. Detection in canine submaxillary glands of an N- acetylglucosaminyltransferase which acts on mucin substrates. J. Biol. Chem. 255, 11247-52.

Wilson, A. P., and Rider, C. C. (1992). Evidence that leukosialin, CD43, is intensely sulfated in the murine T lymphoma line RDM-4. J. Immunol. 148, 1777-1783.

Wogensen, L., Huang, X., and Sarvetnick, N. (1993). Leukocyte extravasation into the pancreatic tissue in transgenic mice expressing interleukin 10 in the islets of Langerhans. J. Exp. Med. 178, 175-185.

Yamauchi, S., Hirahara, Y., Usui, H., Takeda, Y., Hoshino, M., Fukuta, M., Kimura, J. H., and Habuchi, O. (1999). Purification and characterization of chondroitin 4-sulfotransferase from the culture medium of a rat chondrosarcoma cell line. J. Biol. Chem. 274, 2456-63.

Yamori, T., Kimura, H., Stewart, K., Ota, D. M., Cleary, K. R., and Irimura, T. (1987). Differential production of high molecular weight sulfated glycoproteins in normal colonic mucosa, primary colon carcinoma, and metastases. Cancer Res. 47, 2741-7.

Yednock, T. A., and Rosen, S. D. (1989). Lymphocyte homing. Adv. Immunol. 44, 313-378.

Yoshino, K., Ohmoto, H., Kondo, N., Tsujishita, H., Hiramatsu, Y., Inoue, Y., and Kondo, H. (1997). Studies on selectin blockers .4. Structure-function relationships of sulfated sialyl Lewis X hexasaccharide ceramides toward E-, P-, and L-selectin binding. J. Med. Chem. 40, 455-462.

Youakim, A., Dubois, D. H., and Shur, B. D. (1994). Localization of the long form of beta-1,4-galactosyltransferase to the plasma membrane and Golgi complex of 3T3 and F9 cells by immunofluorescence confocal microscopy. Proc. Natl. Acad. Sci. U S A 91, 10913-7.

Zakrzewicz, A., Grafe, M., Terbeek, D., Bongrazio, M., Auch-Schwelk, W., Walzog, B., Graf, K., Fleck, E., Ley, K., and Gaehtgens, P. (1997). L-selectin-dependent leukocyte adhesion to microvascular but not to macrovascular endothelial cells of the human coronary system. Blood 89, 3228-35.

Zhao, Y., Chien, S., and Skalak, R. (1995). A stochastic model of leukocyte rolling. Biophys. J. 69, 1309-20.

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