

Activities and expression pattern of the carbohydrate sulfotransferase GlcNAc6ST-3 (I-GlcNAc6ST): functional implications

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In recent years, a family of five GlcNAc-6-O-sulfotransferases, called the GlcNAc6STs, has been molecularly cloned. One of these, GlcNAc6ST-2 (originally named HEC-GlcNAc6ST or LSST), shows a very restricted expression at the mRNA level in high endothelial cells (HECs) of lymph nodes high endothelial venules (HEVs). This enzyme has been shown to be involved in elaborating the 6-sulfo sLex structure on a set of mucin-like acceptors within HECs, thus providing a critical recognition determinant for L-selectin during the process of lymphocyte homing to lymph nodes. Limited information has been available about the closely related sulfotransferase known as GlcNAc6ST-3 (I-GlcNAc6ST). Here, employing transfection experiments with a series of glycoprotein acceptors, we report that this sulfotransferase has a marked preference for sulfating O-linked sugars of mucin-type acceptors, whereas other sulfotransferases in the family (GlcNAc6ST-1, GlcNAc6ST-2) and a Gal-6-O-sulfotransferase exhibit strong activity on both mucin-type acceptors and glycoproteins with predominantly N-linked chains. PCR analysis of cDNAs derived from a panel of tissues and purified cell populations confirms the strong expression of GlcNAc6ST-3 in gut-associated tissues and extends the expression to include lymphocytes. In contrast to GlcNAc6ST-2, GlcNAc6ST-3 transcripts are present minimally, if at all, in HECs; moreover, this enzyme is not able to generate the 6-sulfo sLex epitope in transfected cells. These latter findings argue that GlcNAc6ST-3 is not involved in generating HEV-expressed ligands for L-selectin.

Key words: GlcNAc-6-O-sulfotransferase/homing/L-selectin/mucin/sulfation

Introduction

Carbohydrate sulfation and protein sulfation are important extracellular modifications that contribute to a variety of

biological recognition events at the cell surface and in the extracellular matrix (reviewed in Hooper *et al.*, 1996; Bowman and Bertozzi, 1999; Fukuda *et al.*, 2001). Just as the kinases that mediate the diverse phosphorylation events within the cytosol have received great attention in recent years, the Golgi-associated sulfotransferases that impart specific sulfation modifications are now stimulating considerable interest. Over the last several years, approximately 30 Golgi-associated sulfotransferases have been identified at the molecular level in humans (reviewed in Habuchi, 2000; Hemmerich and Rosen, 2000; Fukuda *et al.*, 2001). Several subfamilies have been delineated that show enhanced sequence conservation and related catalytic activities. One of these subfamilies, known as the GlcNAc-6-O-sulfotransferases (GlcNAc6STs), catalyze the transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate to the C-6 position of GlcNAc (Fukuda *et al.*, 2001). The nomenclature for the five members of this group is diverse, reflecting the participation of numerous groups in the discovery and characterization of these enzymes (Table I). We employ the GlcNAc6ST nomenclature, which designates the enzymes in order of their cloning.

GlcNAc6ST-1 was the first member to be identified at the molecular level (Uchimura *et al.*, 1998a,b). It is expressed in a wide variety of human tissues, as judged by northern analysis and the frequency of expressed sequence tags (ESTs) in the NCBI human database. By contrast, GlcNAc6ST-2, also known as HEC-GlcNAc6ST (Bistrup *et al.*, 1999) or L-selectin ligand sulfotransferase (LSST) (Hiraoka *et al.*, 1999), shows a very narrow expression pattern in high endothelial cells (HECs) of high endothelial venules (HEVs) of secondary lymphoid organs. Additionally, it is ectopically expressed in certain adenocarcinomas (Seko *et al.*, 2002; Uchimura *et al.*, 2002). GlcNAc6ST-2 is essential to the process of L-selectin-mediated lymphocyte homing by elaborating a critical GlcNAc-6-sulfate modification on the HEV-expressed ligands for L-selectin (Hemmerich *et al.*, 2001a). This modification is found in the context of 6-sulfo sLex (Sia α 2 \rightarrow 3Gal β 1 \rightarrow 4[Fuc1 \rightarrow 3][SO $_3$ \rightarrow 6]GlcNAc) on O-linked chains borne by a series of sialomucins, including CD34, podocalyxin, MAdCAM-1, and GlyCAM-1 (Hemmerich *et al.*, 1995; Mitsuoka *et al.*, 1998; Yeh *et al.*, 2001).

GlcNAc6ST-2 is also essential for the formation of the epitope of MECA-79, a monoclonal antibody that stains HEV and blocks L-selectin-dependent adherence of lymphocytes (Berg *et al.*, 1991; Hemmerich *et al.*, 1994, 2001a; Yeh *et al.*, 2001). The closest relatives of GlcNAc6ST-2 are GlcNAc6ST-3 and GlcNAc6ST-5, also known as I-GlcNAc6ST (Lee *et al.*, 1999) and C-GlcNAc6ST (Akama *et al.*, 2000; Hemmerich *et al.*, 2001b), respectively,

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Table 1. The family of GlcNAc-6-O-sulfotransferases

Systematic name	Other designations	References
GlcNAc6ST-1	GlcNAc6ST, GST-2, CHST2	Uchimura <i>et al.</i> , 1998a,b; Li and Tedder, 1999
GlcNAc6ST-2	HEC-GlcNAc6ST, LSST, GST-3, CHST4	Bistrup <i>et al.</i> , 1999; Hiraoka <i>et al.</i> , 1999
GlcNAc6ST-3	I-GlcNAc6ST, GST-4 α , CHST5	Lee <i>et al.</i> , 1999
GlcNAc6ST-4	C6ST-2, GST-5, CHST7	Bhakta <i>et al.</i> , 2000; Kitagawa <i>et al.</i> , 2000; Uchimura <i>et al.</i> , 2000
GlcNAc6ST-5	C-GlcNAc6ST, GST-4 β , CHST6	Akama <i>et al.</i> , 2000; Hemmerich <i>et al.</i> , 2001b

because of their prominent expression in the intestine and cornea, respectively. These three sulfotransferases map to the same region of chromosome 16 and show the highest degree of sequence homology within the GlcNAc6ST subfamily (Hemmerich *et al.*, 2001b). Mutations in the gene for GlcNAc6ST-5 underlie macular corneal dystrophy in humans, which is attributable to the normal role of this enzyme in transferring sulfate to C-6 of GlcNAc within keratan sulfate (Akama *et al.*, 2000, 2001). The remaining member of the family, GlcNAc6ST-4, like GlcNAc6ST-1, shows a broad expression pattern in human tissues (Bhakta *et al.*, 2000; Kitagawa *et al.*, 2000; Uchimura *et al.*, 2000). This enzyme is also reported to be a chondroitin 6-O-sulfotransferase that transfers sulfate to C-6 of GalNAc (Kitagawa *et al.*, 2000).

The present study focuses on GlcNAc6ST-3 because of the limited functional and biochemical information available about this enzyme. In comparing GlcNAc6ST-3 with GlcNAc6ST-1 and GlcNAc6ST-2, we find striking differences with respect to the acceptor specificities and the sulfated products that can be formed. Moreover, we have expanded the survey of tissue expression for GlcNAc6ST-3 by using a more extensive panel of tissue cDNAs and including isolated cell populations. The pertinence of our findings to L-selectin ligand synthesis is discussed.

Results

Sulfation of different glycoprotein acceptors

As already reviewed, GlcNAc6ST-2, a close relative of GlcNAc6ST-3, has been established to impart L-selectin ligand activity to several mucins (Bistrup *et al.*, 1999; Hiraoka *et al.*, 1999; Hemmerich *et al.*, 2001a; Yeh *et al.*, 2001). Therefore, we wanted to compare these two enzymes in their ability to sulfate O-linked and N-linked chains. We chose a series of glycoproteins with predominantly O-linked chains or N-linked chains as acceptors. The former consisted of mucin scaffolds (CD34, GlyCAM-1, and MAdCAM-1) that serve as L-selectin ligands when expressed in HEVs (reviewed in Rosen, 1999), as well as a novel chemokine (fractalkine) that possesses a large mucin domain (Bazan *et al.*, 1997). The latter group was made up of ICAM-1 and NCAM-1, containing, respectively, eight and six potential sites for N-linked glycosylation. We also included in the comparison another member of the GlcNAc6ST family (GlcNAc6ST-1) and an example of a

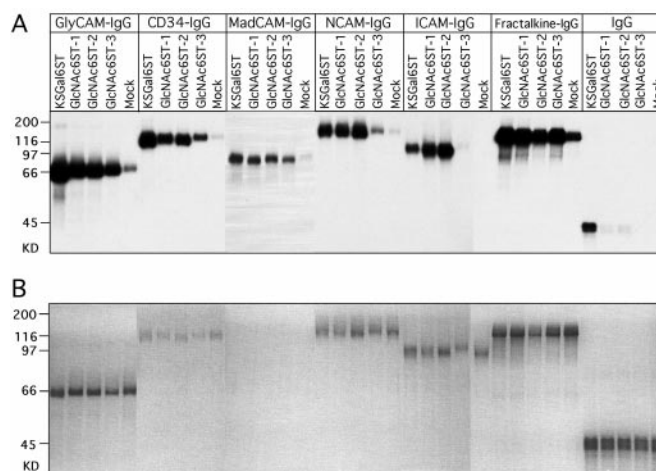


Fig. 1. Sulfation of various glycoproteins by GlcNAc6STs and KSGal6ST. COS cells were transfected with combinations of plasmids encoding GlyCAM-1/IgG, CD34/IgG, MAdCAM/IgG, NCAM/IgG, ICAM/IgG, Fractalkine/IgG or IgG, and each sulfotransferase as indicated. Transfected cells were cultured in the presence of [35 S] sulfate. The recombinant IgG chimeric proteins were purified from the conditioned medium by protein A. (A) The purified proteins were analyzed by SDS-PAGE followed by autoradiography. (B) The proteins were visualized by Coomassie staining.

Gal-6-O-sulfotransferase, that is, KSGal6ST (Fukuta *et al.*, 1997).

COS-7 cells were transfected with a cDNA for each of these sulfotransferases together with a cDNA encoding one of the acceptor glycoproteins in the form of an Fc chimera. The Fc region itself (denoted as IgG) contains one potential site for N-linked glycosylation. During the transfection, the cells were labeled with [35 S]-SO $_4$. The chimeric proteins were purified on protein A-agarose resins and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We verified by Coomassie blue staining (Figure 1B) that expression levels of the different acceptor proteins were similar. Differences in sulfation levels could therefore be taken to be due to differential activity of the enzymes. As documented previously and confirmed in Figure 1A, COS cells possess endogenous sulfotransferase activity that resulted in detectable levels of sulfation on several of the acceptors in the absence of exogenously provided activity (Tangemann *et al.*, 1999). The GlcNAc6STs imparted only trace levels of sulfate to

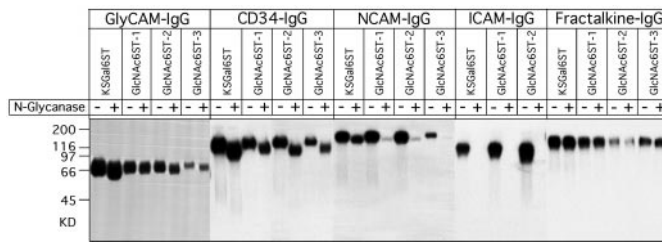


Fig. 2. N-glycanase treatment of glycoproteins sulfated by GlcNAc6STs and KSGal6ST. The purified glycoproteins sulfated by GlcNAc6STs and KSGal6ST (as described in *Materials and methods* and Figure 1) were incubated in the presence (+) or absence (-) of N-glycanase and analyzed by SDS-PAGE and autoradiography.

the IgG component of the chimeras as judged by the weak labeling seen for IgG construct. KSGal6ST, GlcNAc6ST-1, and GlcNAc6ST-2 catalyzed strong sulfation in all acceptors of both classes. In contrast, GlcNAc6ST-3 demonstrated a marked preference for the mucin containing acceptors in that only very weak (relative to the mock transfection) label was incorporated into NCAM-1, ICAM-1, or IgG.

To distinguish sulfation of N-linked versus O-linked chains, we subjected the various Fc chimeras to N-glycanase treatment after their sulfation in transfected COS cells. As shown in Figure 2, label that was incorporated into NCAM-1 and ICAM-1 was almost completely abolished by N-glycanase digestion, whereas label incorporated into the mucins was substantially resistant to treatment by this enzyme. Thus, our choice of acceptors for the two general categories was validated.

Because of the marked selectivity of GlcNAc6ST-3 in sulfating mucin-type glycoproteins, we carried out a further analysis of the acceptor specificity of this enzyme. The core 1 structure (Gal β 1 \rightarrow 3GalNAc α 1 \rightarrow) and the core 2 branch (GlcNAc β 1 \rightarrow 6[Gal β 1 \rightarrow 3]GalNAc α \rightarrow) are found on O-linked glycans that are linked to core proteins through Ser or Thr. Although COS cells possess core 2 branching enzyme activity catalyzing the transfer of GlcNAc in a β 1 \rightarrow 6 position to GalNAc on core 1, Chinese hamster ovary (CHO) cells lack this activity (Bierhuizen and Fukuda, 1992). We therefore used CHO cells to test the dependency of GlcNAc6ST-3 on mucin-like acceptors by transfecting the GlcNAc6ST-3 cDNA into these cells in the absence or presence of a cDNA for the core 2 branching enzyme (Core2GlcNAcT-I) (Bierhuizen and Fukuda, 1992). As expected, sulfation of CD34 and fractalkine was dramatically enhanced in the presence of the core 2 branch (Figure 3). In contrast, equivalent, albeit very weak, sulfation of ICAM-1 was seen irrespective of the presence of the core 2 branch.

Expression of GlcNAc6ST-3 transcripts

Among the GlcNAc-6-O-sulfotransferase family, GlcNAc6ST-3 is most closely related to GlcNAc6ST-2 and GlcNAc6ST-5 (reviewed in Hemmerich and Rosen, 2000; Fukuda *et al.*, 2001). As already discussed, significant functional information is available about these latter two enzymes, but biological data concerning

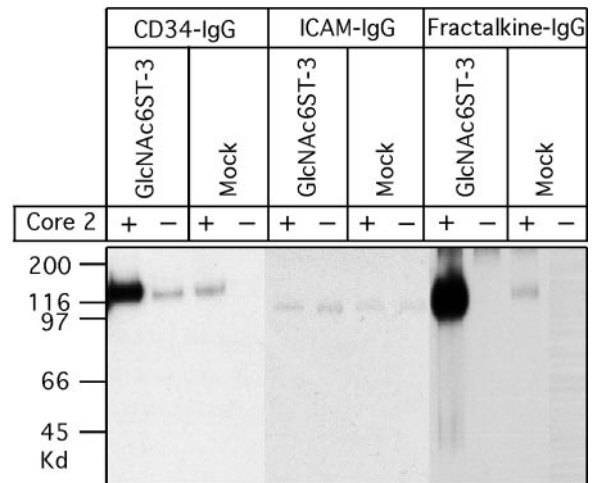


Fig. 3. Dependency of GlcNAc6ST-3 on the core 2 structure. CHO cells were transfected with combinations of plasmid encoding CD34/IgG, ICAM/IgG, or vector only (Mock), and GlcNAc6ST-3. A plasmid encoding core 2 GlcNAcT-I was either present (+) or absent (-) during transfection. Transfected cells were cultured in the presence of [35 S] sulfate. The recombinant IgG chimeric proteins were purified from the conditioned medium by protein A. The purified proteins were analyzed by SDS-PAGE followed by autoradiography.

GlcNAc6ST-3 has been lacking. To gain insights about function, we therefore expanded the screen for tissue expression of GlcNAc6ST-3 mRNA. We used a gene expression cDNA panel, generated from 24 different human tissues, to determine the relative representation of transcripts for GlcNAc6ST-3. After performing semiquantitative polymerase chain reaction (PCR), we found the highest level of transcripts in small intestine and colon, which was consistent with our previous northern analysis (Figure 4A). Extending the previous analysis (Lee *et al.*, 1999), we also detected transcripts at lower levels in brain, stomach, thyroid, adrenal, ovary, uterus, and peripheral blood mononuclear cells. We employed the same cDNA panel to determine the expression pattern of GlcNAc6ST-5 (Figure 4B). Strikingly, GlcNAc6ST-5 was not expressed in any tissues of the gut. As with GlcNAc6ST-3, transcripts for GlcNAc6ST-5 were detected in both brain and peripheral blood mononuclear cells.

To confirm expression of GlcNAc6ST-3 in mononuclear leukocytes and to gain information about subtypes, we performed semi-quantitative PCR on cDNAs isolated from purified subpopulations. The cDNAs were normalized based on expression of G3PDH. As shown in Figure 4C, transcripts corresponding to GlcNAc6ST-3 were detected in T cells (CD4 $^{+}$ and CD8 $^{+}$) as well as B cells (CD19 $^{+}$).

As reviewed, GlcNAc6ST-2 has a demonstrated function in the elaboration of L-selectin ligands in HECs of lymph nodes. We next asked whether GlcNAc6ST-3, like GlcNAc6ST-2, was expressed in isolated HECs. Here we took advantage of the MECA-79 monoclonal antibody, which selectively stains HEVs in secondary lymphoid organs. HECs were isolated from collagenase-dissociated human tonsils by affinity to MECA-79 and a magnetic

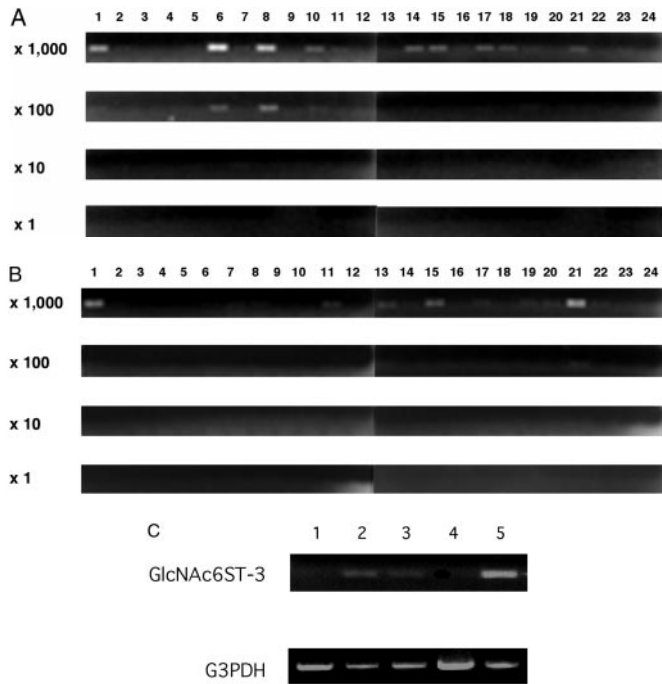


Fig. 4. Expression of GlcNAc6ST-3 and GlcNAc6ST-5 transcripts in human tissues. Fragments of GlcNAc6ST-3 (A, C) or GlcNAc6ST-5 (B) or G3PDH sequences were amplified by PCR from serial dilutions of cDNA from various human tissues. (A and B) 1, brain; 2, heart; 3, kidney; 4, spleen; 5, liver; 6, colon; 7, lung; 8, small intestine; 9, muscle; 10, stomach; 11, testis; 12, placenta; 13, salivary; 14, thyroid; 15, adrenal; 16, pancreas; 17, ovary; 18, uterus; 19, prostate; 20, skin; 21, PBL; 22, bone marrow; 23, fetal brain; 24, fetal liver. (C) 1, mononuclear cells; 2, resting CD8⁺ cells; 3, resting CD4⁺ cells; 4, resting CD14⁺ cells; 5, resting CD19⁺ cells. The reaction products (GlcNAc6ST-3: 353 bp; GlcNAc6ST-5: 467 bp; G3PDH: 983 bp) were analyzed by agarose gel electrophoresis and ethidium bromide staining.

bead immunoselection (see *Materials and methods*). In parallel, T and B cells (CD3⁺ and CD19⁺ cells, respectively) were isolated from tonsillar cell suspensions using fluorescence-activated cell sorting. Semi-quantitative PCR was performed on cDNAs generated from these specimens. As shown in Figure 5B, we observed strong expression of GlcNAc6ST-2 in the isolated HECs, consistent with our previous observations (Bistrup *et al.*, 1999). Interestingly, low levels of GlcNAc6ST-2 transcripts were also detected in the cDNAs derived from the populations of B and T cells, which were estimated to be >99% pure by flow cytometry analysis. A converse expression pattern was observed for GlcNAc6ST-3 (Figure 5A): B and T cells showed significant expression, whereas only a trace level was detectable in HECs. This minimal expression was very likely attributable to contamination of the HECs with adherent lymphocytes. We also found only barely detectable levels of GlcNAc6ST-3 transcripts in HECs isolated from mouse lymph node or Peyer's patches (data not shown).

Generation of the 6-sulfo sLex epitope

Previous work has shown that both GlcNAc6ST-1 and GlcNAc6ST-2 can generate the 6-sulfo sLex epitope in

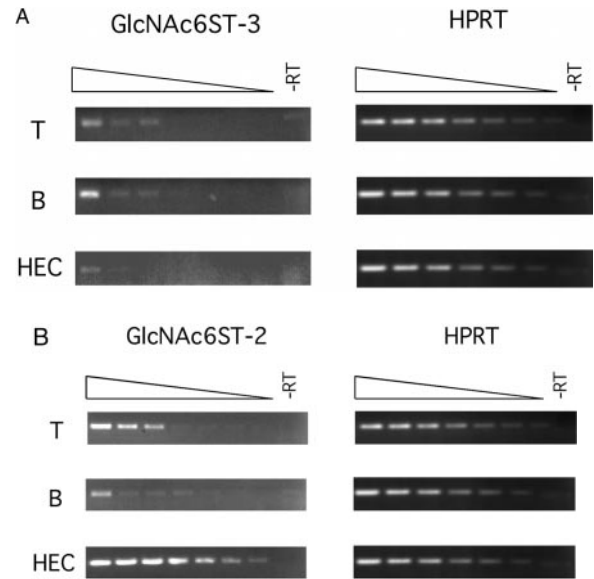


Fig. 5. Expression of GlcNAc6ST-2 and GlcNAc6ST-3 transcripts in T lymphocytes, B lymphocytes, and HECs from human tonsil. Fragments of GlcNAc6ST-3 (A), GlcNAc6ST-2 (B), and HPRT (A, B) sequences were amplified by PCR from serial dilutions of cDNA prepared from purified human tonsillar T-lymphocytes, B-lymphocytes, and HEC. The reaction products (GlcNAc6ST-2: 467 bp, GlcNAc6ST-3: 257 bp, and HPRT: 300 bp) were analyzed by agarose gel electrophoresis and ethidium bromide staining. RT:PCR reactions in which the template was generated by omission of reverse transcriptase.

transfected cells (Bistrup *et al.*, 1999; Kimura *et al.*, 1999; Kanamori *et al.*, 2002; Uchimura *et al.*, 2002). This determinant can be detected by two monoclonal antibodies of nearly identical specificity: G72 (which does not require fucose for activity) and G152 (which exhibits an absolute dependency on fucosylation) (Mitsuoka *et al.*, 1998). We determined the ability of GlcNAc6ST-3 to produce the 6-sulfo sLex on the surface of transfected cells. To provide for the generation of sLex on a core 2 branch, we utilized CHO cells (known as CHO/FTVII/Core2GlcNAcT) that stably expressed Core2GlcNAcT-I and fucosyltransferase VII activities (Bistrup *et al.*, 1999). We transiently transfected these cells with a cDNA encoding CD34 and a cDNA for either GlcNAc6ST-2 or GlcNAc6ST-3. Using flow cytometry with the G152 monoclonal antibody, we probed for the 6-sulfo sLex epitope on the cell surface. As shown in Figure 6A, the CHO/FTVII/C2GnT cells transfected with GlcNAc6ST-2 were very strongly positive for the G152 epitope. In contrast, transfection with GlcNAc6ST-3 did not result in G152 staining above background. To confirm that GlcNAc6ST-3 did, in fact, cause increased sulfation of CD34, we labeled CHO cells with [³⁵S]-SO₄ after transfection with a CD34 cDNA and a cDNA for GlcNAc6ST-2 or GlcNAc6ST-3. Lysates were analyzed by SDS-PAGE and autoradiography. Transfection with either sulfotransferase resulted in enhanced sulfation of CD34 relative to that in control cells (not shown). However, only GlcNAc6ST-2 could generate the 6-sulfo sLex determinant on CD34, as detected by immunoprecipitation with the G72 monoclonal antibody (Figure 6B).

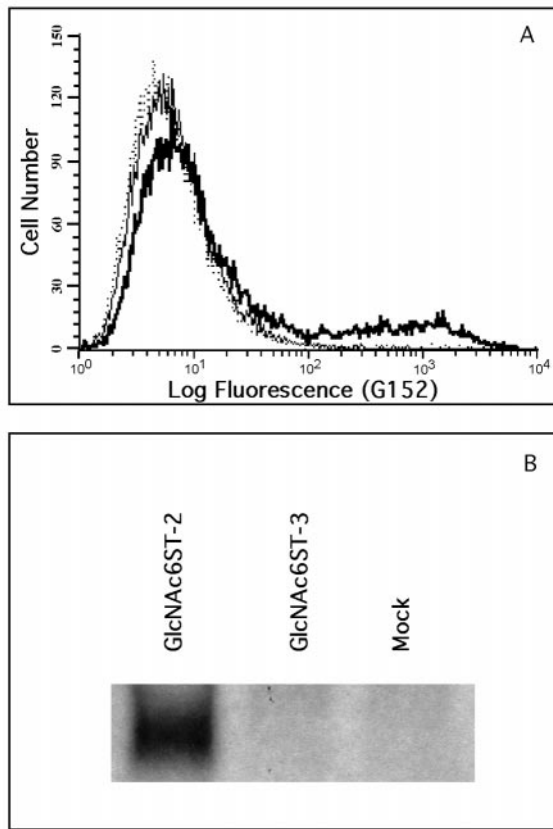


Figure 6. Generation of 6-sulfo sLex by GlcNAc6ST-2 but not GlcNAc6ST-3. (A) CHO/FTVII/Core2GlcNAcT-I cells were transfected with cDNAs encoding CD34 and either GlcNAc6ST-2 or GlcNAc6ST-3. Cells were stained with the G152 monoclonal antibody and analyzed by flow cytometry. Histogram shows staining for the transfections with GlcNAc6ST-2 (bold) or GlcNAc6ST-3 (stippled), or staining with the isotype control antibody for the GlcNAc6ST-2 transfected cells (solid). (B) CHO cells were transfected with cDNAs encoding FTVII, core 2 GlcNAcT-I, full-length CD34, and either GlcNAc6ST-2 or GlcNAc6ST-3 or vector alone (mock). Transfected cells were cultured in the presence of [³⁵S]sulfate, and lysates were prepared. Equal amounts of protein were subjected to immuno-precipitation with the G72 monoclonal antibody. The precipitates were analyzed by SDS-PAGE.

Discussion

The GlcNAc-6-sulfate modification is found on keratan sulfate and heparan sulfate glycosaminoglycans, as well as on smaller glycans of N- and O-linked carbohydrate chains in various glycoproteins. Among the mucins that carry this modification are respiratory mucins of cystic fibrosis patients (Lo-Guidice *et al.*, 1994), HEV-expressed ligands for L-selectin (Hemmerich *et al.*, 1995; Mitsuoka *et al.*, 1998), and human colon carcinoma mucins (Capon *et al.*, 1997).

GlcNAc-6-O-sulfotransferase activities have been demonstrated in extracts from a variety of tissues and tumors (Carter *et al.*, 1988; Goso and Hotta, 1993; Spiro *et al.*, 1996; Bowman *et al.*, 1998; Nakazawa *et al.*, 1998; Hasegawa *et al.*, 2000; Seko *et al.*, 2000; Delmotte *et al.*, 2001). Underlying these activities are members of the GlcNAc6ST subfamily of carbohydrate sulfotransferases, of which five have been cloned to date. This subfamily is

distinct with respect to sequence homology and activities from the heparan sulfate GlcNSO₃-6-O-sulfotransferase (HS6ST) subfamily of three enzymes that are responsible for addition of sulfate to the 6-position of GlcN or GlcNAc in heparan sulfate chains (reviewed in Fukuda *et al.*, 2001). It is strongly suspected that the existence of multiple sulfotransferases for a given modification underlies higher-order specificities, involving the elaboration of sulfate modifications within the context of different oligosaccharides (Bowman and Bertozzi, 1999; Fukuda *et al.*, 2001). Further acceptor specificity may derive from features of the protein scaffold. An emerging view is that different sulfotransferases acting in concert with specific glycosyltransferases provide for a huge diversity of sulfate-based determinants ("sulfotopes") that enable a broad range of biological recognition events (Bowman and Bertozzi, 1999).

Among the members of the GlcNAc6ST subfamily, two members have been extensively characterized with respect to their function, as reviewed in the *Introduction*. GlcNAc6ST-2 is strongly implicated as one of the contributing enzymes in the synthesis of the 6-sulfo sLex determinant on L-selectin ligands, as well as the formation of the MECA-79 epitope carried by these ligands. Akama *et al.* (2000, 2001) have demonstrated the involvement of GlcNAc6ST-5 in the elaboration of the glycosaminoglycan chains of keratan sulfate. Although GlcNAc6ST-3 is highly homologous to GlcNAc6ST-5, and it is likely that their genes arose by gene duplication, GlcNAc6ST-3 is not able to generate highly sulfated keratan sulfate (Akama *et al.*, 2001). Also, when we compared the tissue distributions of GlcNAc6ST-3 and GlcNAc6ST-5, notable differences were found. The former was strongly expressed in tissues of the gut, whereas the latter was not. A commonality was their expression in peripheral blood mononuclear cells and brain.

To address the issue of the acceptor specificity for GlcNAc6ST-3, we analyzed the ability of several members of the GlcNAc6ST subfamily to sulfate N-linked versus O-linked chains in a series of glycoproteins. Compared to its closest relatives in the GlcNAc6ST family, GlcNAc6ST-3 was much more selective for O-linked chains of mucin-type acceptors with minimal activity in sulfating glycoproteins with predominantly N-linked glycosylation. No such preference was found for GlcNAc6ST-2, despite the fact that the functionally relevant HEV-expressed acceptors for this enzyme are mucin-type glycoproteins (Puri *et al.*, 1995; Hemmerich *et al.*, 2001a). As expected, the sulfation of O-linked chains by GlcNAc6ST-3 within CHO cells required the presence of the core 2 branch. Our findings are compatible with the recent biochemical analyses in which cell-free sulfotransferase assays were performed with a series of recombinantly expressed GlcNAc6STs and defined oligosaccharides acceptors (Bowman *et al.*, 2001; Seko *et al.*, 2002; Uchimura *et al.*, 2002). GlcNAc6ST-1 and GlcNAc6ST-2 were able to sulfate GlcNAc on a core 2 acceptor (Bowman *et al.*, 2001; Uchimura *et al.*, 2002), but both were also active on GlcNAc-Man structures found in N-Linked chains. In contrast, GlcNAc6ST-3 was found to act on the core 2 acceptor but exhibited very limited activity on GlcNAc-Man structures, consistent with our observation that this enzyme preferred mucin-type over N-linked acceptors in

cotransfection assays. Our findings with respect to the acceptor preference for GlcNAc6ST-2 appear to be at variance with those of Hiraoka *et al.* (1999) who studied the mouse homolog of this enzyme. They, like us, found that this enzyme could direct the sulfation of IgG chimeras of GlyCAM-1, MAdCAM-1, and CD34, but they observed no detectable activity above background on NCAM-1/IgG. Whether this discrepancy is attributable to the species difference or to technical issues (e.g., level of enzyme or acceptor glycoprotein) remains to be determined.

Our previous analysis of GlcNAc6ST-2 gene-targeted mice has established a clear role for this enzyme in the elaboration of HEV ligands for L-selectin and in the generation of the MECA-79 epitope on these ligands (Hemmerich *et al.*, 2001a). However, the phenotype of the null mice was only partial in that L-selectin ligands and MECA-79 reactivity were still present in HEVs, albeit to a greatly reduced extent and restricted to the abluminal aspects of the HEVs. Also, we have observed a reduced but still substantial level of sulfation in HEV-expressed ligands from GlcNAc6ST-2 null mice (Van Zante and Rosen, unpublished data). One of the candidates for these residual activities has been GlcNAc6ST-3. Two of our present findings render this possibility remote. First, we found only a trace level of GlcNAc6ST-3 transcripts in isolated HECs, which was very likely attributable to contaminating lymphocytes in the HEC preparation. Second, our transfection experiments indicate that GlcNAc6ST-3 is unable to direct the synthesis of the 6-sulfo sLex determinant, a critical structure for L-selectin ligands. A more likely candidate to account for the residual ligand and MECA-79 reactivity in GlcNAc6ST-2 null mice is GlcNAc6ST-1, which is known to be expressed in HEVs (Uchimura *et al.*, 1998a). This enzyme can participate in the generation of functional L-selectin ligands, the 6-sulfo sLex determinant, and the MECA-79 epitope (Kimura *et al.*, 1999; Kanamori *et al.*, 2002; Uchimura *et al.*, 2002). It should be noted that GlcNAc6ST-1 is also implicated in the generation of the 6-sulfo sLex determinant in the mouse embryo (Fan *et al.*, 1999).

We found evidence for GlcNAc6ST-2 expression in B and T cells, although at a markedly lower level than in HECs (Figure 5B). This low level of expression likely explains the failure to detect this transcript by *in situ* hybridization in the lymphocyte-rich regions of lymph nodes (Bistrup *et al.*, 1999; Hiraoka *et al.*, 1999). Ohmori *et al.* (2000) reported the presence of transcripts for GlcNAc6ST-2 in the human lymphoid leukemia line Nawalma. However, our results provide the first direct evidence of its presence in primary lymphocytes. The enzyme may account for the occurrence of the 6-sulfo sLex epitope on subpopulations of lymphocytes (Kannagi and Kanamori, 1999).

A novel finding of the present study is the expression of GlcNAc6ST-3 in mononuclear cells. This was established in sets of cDNAs derived by three independent means (Figures 4A, C, and 5A). The preference of GlcNAc6ST-3 to sulfate mucin acceptors focuses attention on leukocyte cell surface molecules with mucin domains. In fact, several such molecules (CD43, CD44, and CD45) are reported to be sulfated (Giordanengo *et al.*, 1995; Maiti *et al.*, 1998;

Brown *et al.*, 2001), although the nature of the sulfated moieties has not been reported. In the case of CD44, ligand binding activity (i.e., hyaluronic acid binding) depends on its sulfation (Maiti *et al.*, 1998; Brown *et al.*, 2001). PSGL-1, another sialomucin, is broadly distributed on leukocytes (reviewed in McEver and Cummings, 1997) and carries tyrosine sulfation. On NK subpopulations, PSGL-1 is further decorated in a cell type-specific manner with the PEN5 epitope, which is thought to involve a GlcNAc-6-sulfate modification (Andre *et al.*, 2000). This epitope is of functional interest because it is implicated in L-selectin ligand activity on these cells. The potential involvement of GlcNAc6ST-3 in modulating the function of cell surface mucins on leukocytes deserves further attention.

The present study confirms and extends the original finding that GlcNAc6ST-3 is strongly expressed in the gastrointestinal tract. Studies by Seko *et al.* (2000, 2002) indicate that GlcNAc6ST-3 corresponds to the GlcNAc-6-O-transferase activity detected in normal human colon mucosa. In view of the preference of GlcNAc6ST-3 for mucin-type acceptors, demonstrated herein, a role in the modification of gut-associated mucins should be considered. A variety of secreted and membrane-bound mucins are found in the gut (reviewed in Kim and Gum, 1995). Normal functions and pathological roles ascribed to mucins are varied, including mechanical protection, lubrication, facilitating metastasis of carcinoma cells, and providing attachment sites for microbes. Most recently, a gene targeting approach has implicated the Muc2 mucin in the suppression of colorectal cancer (Velcich *et al.*, 2002). Mucins exhibit an enormous diversity of sulfated O-linked chains (Lo-Guidice *et al.*, 1994; Capon *et al.*, 1997). Mucin function undoubtedly will depend on these sulfation modifications whether through influence on overall physicochemical properties or via sulfotopes involved in specific recognition events. Further work on the contribution of GlcNAc6ST-3 to the structure and function of gut-associated mucins under both normal and pathological circumstances is clearly warranted.

Materials and methods

Sulfation of defined glycoproteins by GlcNAc6STs

cDNAs encoding Fc chimeras were constructed by amplifying the coding sequence corresponding to the entire coding region of GlyCAM-1 or the extracellular domains of human CD34 and human fractalkine by PCR and cloning the resulting fragments into the pIG1 vector to yield fusion proteins with the hinge, CH2 and CH3 regions of human IgG1 at the C-terminus (Simmons, 1993). COS cells were transfected with vectors encoding one of these fusion proteins or the Fc alone plus a cDNA for one of the following sulfotransferases: KSGal6ST (pCDNA3.1), GlcNAc6ST-1 (pCDNA3.1), GlcNAc6ST-2 (pCDNA1.1), GlcNAc6ST-3 (pCDNA3.1), or the empty vector (pCDNA3.1). COS cells were transfected at 80% confluency using Lipofectamine (Life Technologies) in Opti-MEM (Life Technologies, Carlsbad, CA). Cells were grown for 6 h after transfection in Opti-MEM, then cultured for 72 h in serum-free medium (Dulbecco's modified Eagle's medium, Gibco BRL,

Carlsbad, CA) supplemented with Na₂[³⁵S]SO₄ (0.25 mCi/ml, 1400 Ci/mmol ICN). The recombinant fusion proteins were isolated from the conditioned medium by affinity binding on protein A-agarose (Watson *et al.*, 1990). The proteins were separated by 10% SDS-PAGE and were visualized by Coomassie staining and autoradiography.

For the N-glycanase experiments, Fc-fusion proteins were produced as above. Each 2–5 µl of protein A-agarose-bound protein was boiled for 2 min in 20 mM sodium phosphate, pH 7.5, 50 mM ethylenediamine tetra-acetic acid (EDTA), 0.02% sodium azide, 0.5% SDS, and 5% β-mercaptoethanol. As a control, 25 µg of α1-acid glycoprotein was subjected to N-glycanase treatment. The denatured samples were treated with or without 10 U/ml of recombinant Peptide-N-Glycosidase F (Glyko, Novato, CA) in the presence of 20 mM sodium phosphate, pH 7.5, 50 mM EDTA, 0.02% sodium azide, 1% Nondiet P-40 (Sigma, St. Louis, MO) and 1% β-mercaptoethanol in a volume of 30 µl for 16 h at 37°C. The resulting samples were analyzed by SDS-PAGE as mentioned.

For the experiments to determine the presence of the 6-sulfo sLex determinant, CHO cells were transfected with cDNAs encoding full-length CD34, Core2GlcNAcT-I (pCDNA1.1), FTVII (pCDNA3.1), and either GlcNAc6ST-2 or -3 or the empty vector (pCDNA3.1). The transfected cells were cultured for 3 days in the presence of Na₂[³⁵S]SO₄. Whole cell lysates (0.1% Triton-X 100 in PBS) were prepared and equalized for protein content. Equal aliquots of each sample were separated by 10% SDS-PAGE and visualized by Coomassie blue staining and autoradiography. In parallel, equal aliquots were incubated with 8 µg G72 monoclonal antibody immobilized on anti-murine IgM Sepharose (Zymed, San Francisco, CA). Bound proteins were separated by 10% SDS-PAGE and visualized by Coomassie staining and autoradiography. To determine the presence of the 6-sulfo sLex determinant by flow cytometry, CHO cells stably expressing Core2GlcNAcT-I and FTVII were transiently transfected by Lipofectamine as previously described (Bistrup *et al.*, 1999) with cDNAs encoding Core2GlcNAcT-I and FTVII and either GlcNAc6ST-2 or -3 or the empty vector (pCDNA3.1). Two days after transfection, cells were analyzed by flow cytometry as previously described (Bistrup *et al.*, 1999) using the G152 monoclonal antibody (Mitsuoka *et al.*, 1998) or a mouse IgM (Pharmingen, San Diego, CA) isotype control.

For the core 2 dependency experiments, CHO cells were transfected with a cDNA for GlcNAc6ST-3 (or the empty vector), together with a plasmid encoding one of a series of the Ig fusion proteins (CD34/IgG, ICAM/IgG NCAM/IgG or fractalkine/IgG) with or without a plasmid encoding Core2GlcNAcT-I (pCDNA1.1). Transfection methods and analysis of the recombinant fusion proteins were as already described for the COS cell transfections.

Reverse transcriptase PCR experiments

Primers for each GlcNAc6ST were designed to avoid cross-reactivity among the GlcNAc6ST cDNAs. The following primers were used:

- GlcNAc6ST-2: 5'-ATAAAGCTTGTGGATTTGTTCA-GGGACATTCCAGGTAGACAGAAGAT-3' and 5'-AA-

AACTCAAGAAGGAGGACCAACCCTACTACTATG-TGATGC-3', which amplify a 467-bp fragment;

- GlcNAc6ST-3: 5'-AACATCACCCACGGGTCGGGG-ATCGGCAA-3' and 5'-GTCAGGCCGATGCCAG-CTGAAGTGGTCTG-3', which amplify a 257-bp fragment;
- GlcNAc6ST-5: 5'-GGTGGATGATGCCCAAGTGAAG-CCGTTCA-3' and 5'-TCCATAACATCACCCACGGG-TCTGGACCT-3', which amplify a 262-bp fragment;
- HPRT: 5'-CCTGCTGGATTACATCAAA-GCACTG-3' and 5'-TCCAACACTTCGTGGGGTCCT-3', which amplify a 300-bp fragment;
- G3PDH: 5'-TGAAGGTCGGAGTCAACGGATTTG-GT-3' and 5'-CATGTGGGCCATGAGGTCCACCAC-3', which amplify a 983-bp fragment.

Each PCR reaction was carried out in a total volume of 10 µl of 1× Klen Taq buffer (Clontech, Palo Alto, CA) containing 500 µM dNTPs, 400 nM primers, 0.2 µl Klen Taq Advantage DNA polymerase mix (Clontech), and twofold serially diluted cDNA as template. Thermocycling conditions were as follows: 1 cycle of 3 min at 94°C, 30 s at 65°C (60°C for HPRT), 1 min at 68°C and 38 cycles (33 cycles for HPRT, 22 cycles for G3PDH) of 30 s at 94°C, 1 min at 6°C (60°C for HPRT), 1 min at 68°C, followed by 5 min at 68°C. The resulting amplified DNA was electrophoresed and visualized with ethidium bromide.

To verify the specificity of the PCR reactions, each of three primer pairs were tested against all three GlcNAc6ST cDNAs (20 ng of each cDNAs per reaction). Only the homologous combinations yielded PCR products of the predicted size. In addition the following PCR reactions were verified by directly sequencing the PCR product isolated from the agarose gel: GlcNAc6ST-3 product from CD19⁺ cells (B cells) cDNA; GlcNAc6ST-2 product from tonsillar B cell cDNA; and GlcNAc6ST-2 product from tonsillar T cell cDNA.

Tissue expression of GlcNAc6ST-3 and GlcNAc6ST-5 were determined using the Rapid-Scan Gene Expression Panel Human-24 (Origene Technologies, Rockville, MD). According to the manufacturer's description, poly A⁺ RNA was used to synthesize first-strand cDNA, employing oligo(dT) primers. The amount of first-strand cDNAs from each tissue was normalized to contain an equivalent concentration of β-actin reverse transcripts. The cDNA pools from the 24 human tissues were diluted in water at four different concentrations in steps of 10-fold dilutions with the lowest concentration at approximately 1 pg/ml. Fragments were amplified by PCR using the primers for GlcNAc6ST-3 already given. PCR conditions were as follows: 1 cycle of 3 min at 94°C, 30 s at 68°C, 1 min at 68°C and 35 cycles of 30 s at 94°C, 30 s at 68°C, 1 min at 68°C, followed by 5 min at 68°C.

cDNAs derived from isolated purified populations of peripheral blood mononuclear cells were obtained from Clontech (K1428-1). Mononuclear cells were purified from peripheral blood on a Percoll gradient. Populations of CD4⁺ T cells, CD8⁺ T cells, and CD19⁺ cells (B cells) were obtained by immunomagnetic separation using

antibody conjugated Dynabeads (Dynal, Lake Success, NY). According to the manufacturer, the purity of the populations was >95%, as evaluated by immunostaining the preparations. PCR was performed as described.

HECs were purified from human tonsils by immunomagnetic selection with MECA-79 by a modification of a previously described procedure (Girard and Springer, 1995; Sasseti *et al.*, 2000). Surgical specimens were digested with a combination of collagenase A (Boehringer-Mannheim, Indianapolis, IN) and dispase I (Roche Diagnostics, Indianapolis, IN), and the resulting cell suspension (2×10^8) was incubated with 10 μ g MECA-79 in 1 ml staining buffer (phosphate buffered saline [PBS] containing 1% bovine serum albumin) at 4°C for 20 min. Cells were collected by centrifugation, washed with staining buffer, and incubated with 10 μ g of biotinylated mouse anti-rat IgM (Caltag, Burlingame, CA) at 4°C for 20 min. Finally, cells were collected, washed, and incubated with 40 μ l of streptavidin-conjugated beads (Dynal) at 4°C for 20 min. MECA-79 positive cells were selected using a Dynal cell separation magnet and extensive washing as directed by the manufacturer. The purity of the resulting HEC preparation was 95% as determined by microscopic examination of cellular morphology with the major contaminant being adherent lymphocytes.

To purify T and B lymphocytes, tonsillar lymphocytes were prepared by mincing surgical specimens of human tonsil and flushing the loose lymphocytes through a 100 μ m cell strainer with cold RPMI 1640 medium. The filtered cells were pelleted for 5 min at 1000 rpm and washed with PBS. T and B lymphocytes were purified by sorting after incubation with anti-CD3-FITC (Pharmingen) or anti-CD19-PE (Caltag). The purity of the sorted populations was >99%. Total RNA was isolated from each purified cell population (3×10^6 T cells and 1×10^7 B cells) by lysis and extraction with RNazol (Tel-Test, Friendswood, TX). To avoid genomic DNA contamination, the purified RNA was digested with RNase-free Dnase I (Gibco BRL) followed by ethanol precipitation. First strand cDNA was synthesized from 5 μ g of total RNA primed with random hexamers using AMV reverse transcriptase (Gibco BRL). PCR reactions were carried out as described.

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Abbreviations

CHO, Chinese hamster ovary; EDTA, ethylenediamine tetra-acetic acid; EST, expressed sequence tag; FTVII, fucosyltransferase VII; HEC, high endothelial cell; HEV, high endothelial venule; LSST, L-selectin ligand sulfotransferase; PBS, phosphate buffered saline; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sLex, sialyl Lewis x.

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