Identification of an *N*-acetylglucosamine-6-O-sulfotransferase activity specific to lymphoid tissue: an enzyme with a possible role in lymphocyte homing

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Background: The leukocyte adhesion molecule L-selectin participates in the initial attachment of blood-borne lymphocytes to high endothelial venules (HEVs) during lymphocyte homing to secondary lymphoid organs, and contributes to leukocyte adhesion and extravasation in HEV-like vessels at sites of chronic inflammation. The L-selectin ligands on lymph node HEVs are mucin-like glycoproteins adorned with the unusual sulfated carbohydrate epitope, 6-sulfo sialyl Lewis x. Sulfation of this epitope on the N-acetylglucosamine (GlcNAc) residue confers high-avidity L-selectin binding, and is thought to be restricted in the vasculature to sites of sustained lymphocyte recruitment. The GlcNAc-6-O-sulfotransferase that installs the sulfate ester may be a key modulator of lymphocyte recruitment to secondary lymphoid organs and sites of chronic inflammation and is therefore a potential target for anti-inflammatory therapy.

Results: A GlcNAc-6-O-sulfotransferase activity was identified within porcine lymph nodes and characterized using a rapid, sensitive, and quantitative assay. We synthesized two unnatural oligosaccharide substrates, GlcNAcβ1→6Galα-R and Galβ1→4GlcNAcβ1→6Galα-R, that incorporate structural motifs from the native L-selectin ligands into an unnatural C-glycosyl hydrocarbon scaffold. The sulfotransferase incorporated greater than tenfold more sulfate into the disaccharide than the trisaccharide, indicating a requirement for a terminal GlcNAc. Activity across tissues was highly restricted to the HEVs within peripheral lymph node.

Conclusions: The restricted expression of the GlcNAc-6-O-sulfotransferase activity to lymph node HEVs strongly suggests a role in the biosynthesis of L-selectin ligands. In addition, similar sulfated epitopes are known to be expressed on HEV-like vessels of chronically inflamed tissues, indicating that this sulfotransferase may also contribute to inflammatory lymphocyte recruitment. We identified a concise disaccharide motif, GlcNAcβ1→6-Galα-R, that preserved both recognition and specificity determinants for the GlcNAc-6-O-sulfotransferase. The absence of activity on the trisaccharide Galβ1→4-GlcNAcβ1→6-Galα-R indicates a requirement for a substrate with a terminal GlcNAc residue, suggesting that sulfation precedes further biosynthetic assembly of L-selectin ligands.

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Introduction

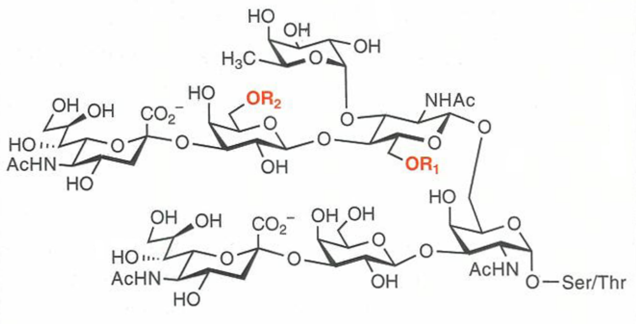
Sulfated carbohydrates mediate a diverse range of highly specific recognition events in both pathogenic and normal processes [1]. For example, sulfation of the carbohydrates on pituitary hormones regulates serum half-life by creating a unique determinant that is selectively recognized by a sulfate-dependent hepatic receptor [2–5]. Moreover, sulfation patterns of heparan sulfate are highly heterogeneous, varying temporally and spatially during normal and pathogenic processes, and unique sulfated motifs are required for cytokine and growth factor binding and signaling [6–9]. Accordingly, the sulfotransferases that install sulfate esters on oligosaccharide molecules are important targets for biological and chemical investigations, and have become the focus of intense research during the past few years.

Recently, a fundamental immunological process was discovered to be dependent upon carbohydrate sulfation [10]. As part of their normal function, lymphocytes exit the bloodstream and migrate into secondary lymphoid tissue by first adhering to the endothelial cells lining the walls of specialized blood vessels [11,12]. This constitutive 'lymphocyte homing' process is initiated in peripheral lymph nodes by the interaction between the leukocyte adhesion molecule L-selectin and sulfated glycoprotein ligands on endothelial cells. In addition, L-selectin mediates a similar leukocyte–endothelial interaction in the vasculature at certain sites of inflammation [13–16]. This initial adhesion event is a requisite first step in the cascade of events that leads to migration of the leukocytes into the tissue, a process termed extravasation. L-selectin is constitutively expressed on circulating lymphocytes, and in both homing and inflammatory recruitment, the attachment of lymphocytes to the blood vessel wall is directed by the presentation of L-selectin ligands on cognate endothelial cells.

In lymph nodes, lymphocyte extravasation is confined within specialized structures termed high endothelial venules (HEVs) [17]. Indeed, a defining feature of HEVs is their ability to initiate and sustain continuous lymphocyte recruitment. HEVs are characteristic of all secondary lymphoid organs except spleen, and HEV-like vessels emerge in chronically inflamed tissues from a variety of human diseases. Interestingly, HEVs in normal lymph nodes and HEV-like vessels at sites of chronic inflammation are known to incorporate a large amount of radiolabeled inorganic sulfate ($^{35}SO_4^{2-}$) relative to typical vascular endothelium in a variety of species, including humans [17].

The broad participation of L-selectin in numerous acute and chronic inflammatory disease states has directed significant attention to the molecular characterization of HEV-associated L-selectin ligands with the hope of identifying possible avenues for therapeutic intervention [18]. Four discrete HEV-associated glycoproteins have been identified as L-selectin ligands in peripheral and mesenteric lymph nodes. Now referred to as GlyCAM-1 [19,20], CD34 [21,22], MAdCAM-1 [23,24], and podocalyxin [25], these glycoproteins all possess mucin-like domains characterized by dense clusters of O-linked glycans that determine L-selectin-binding activity. Furthermore, the oligosaccharides on all three glycoproteins are heavily sulfated, and sulfation is essential for high-avidity L-selectin binding [26–28]. Similar sulfated ligands have been shown to be induced on HEV-like vessels within chronically inflamed tissues, presumably in response to pro-inflammatory signals [29]. Sulfation of these mucins appears to correlate exclusively with their expression on HEVs at sites of leukocyte recruitment as nonsulfated GlyCAM-1 and CD34 glycoforms have been found in other, nonlymphoid tissues, and these glycoforms do not bind L-selectin [30,31].

Figure 1



1: $R_1 = SO_3^-$, $R_2 = H$

2: $R_1 = H$, $R_2 = SO_3^-$

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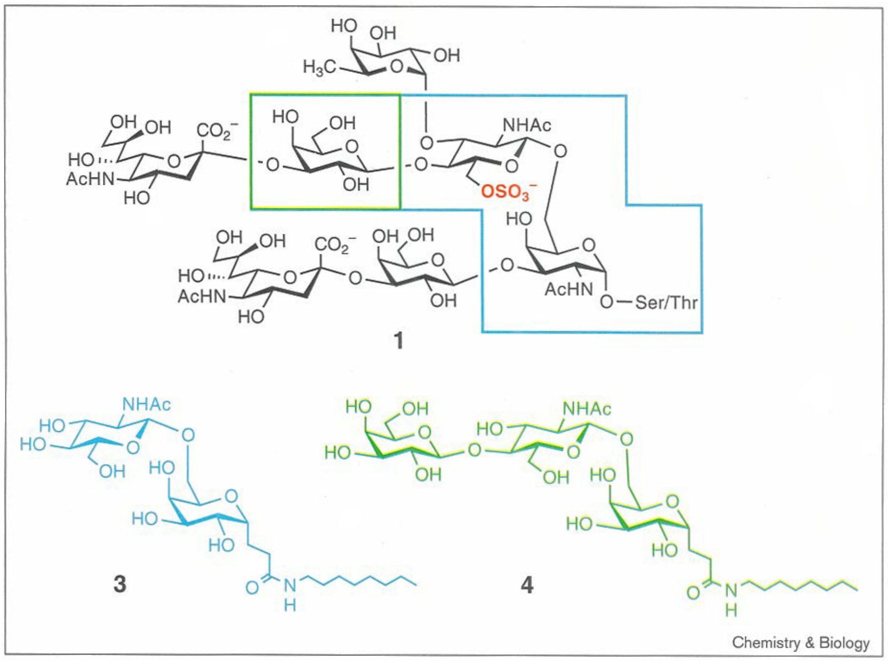
Oligosaccharides bearing the 6-sulfo sialyl Lewis x motif (**1**) and the 6′-sulfo sialyl Lewis x motif (**2**) are found on L-selectin ligands from murine lymph nodes. Evidence for the presence of structure **1** on human lymphoid tissue has also been reported.

An additional link between sulfation and lymphocyte recruitment comes from studies with MECA-79, an antibody that stains lymph node HEVs and binds the L-selectin ligands in a carbohydrate- and sulfate-dependent manner [20,22,24,26]. MECA-79 staining is also observed on HEV-like vessels in a large number of chronically inflamed tissue sites, including various cutaneous inflammatory lesions [32] and inflammatory bowel disease in humans [33], and pancreatic islets of Langerhans in nonobese diabetic (NOD) mice [34]. As L-selectin ligands induced in chronically inflamed tissues cross-react with MECA-79, they are likely to possess sulfated oligosaccharides similar to those on the L-selectin ligands expressed constitutively on lymph node HEVs. Furthermore, MECA-79 cross-reacts with the L-selectin ligands from murine, bovine, porcine and human tissues, indicating that the sulfated oligosaccharide structures, and hence the corresponding sulfotransferases, are also similar among several species [27,35].

Hemmerich *et al.* [36–38] analyzed the oligosaccharides on murine lymph node GlyCAM-1 in detail, leading to the discovery of the novel structures **1** and **2** depicted in Figure 1. Structure **1** contains the sulfated tetrasaccharide epitope 6-sulfo sialyl Lewis x (Siaα2→3Galβ1→4[Fucα1→3]-[SO_3→6]GlcNAc) assembled on the core structure GlcNAcβ1→6[Siaα2→3Galβ1→3]GalNAcαSer/Thr. Structure **2** contains a 6′-sulfo sialyl Lewis x tetrasaccharide (Siaα2→3[SO_3→6]Galβ1→4[Fucα1→3]GlcNAc) assembled on the same core epitope. In combination with the sulfate dependence of the L-selectin–GlyCAM-1 interaction, the identification of these sialyl Lewis x sulfoforms implicated GlcNAc-6-sulfate and/or Gal-6-sulfate as critical determinants for physiological L-selectin binding. The functional significance of HEV sulfate metabolism could be accounted for by these unusual sulfated structures and their role in L-selectin binding. Accordingly, these observations suggest the presence of HEV-specific sulfotransferases that are constitutively expressed in lymph node HEVs and are induced in chronically inflamed tissues.

Figure 2



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Structure **1** and the two synthetic oligosaccharide substrates **3** and **4**. The blue and green boxes designate the core residues from which the disaccharide **3** and the trisaccharide **4** were derived, respectively. Both substrates preserve the GlcNAcβ1→6 linkage found in the native structure while incorporating a 'reducing' end galactose residue in place of the GalNAc and a carbon-linked hydrocarbon tail.

The individual contribution of each sulfate ester to L-selectin-binding affinity has been examined using synthetic sulfated sialyl Lewis x analogs. In a series of studies, oligosaccharides related to sialyl Lewis x bearing GlcNAc-6-sulfate were found to bind to L-selectin with higher affinity than the unsulfated, parent oligosaccharide [39–41]. The contribution of Gal-6-sulfate to L-selectin binding is less clear: no measurable effect on L-selectin binding was observed in some studies [40,41], but in others Gal-6-sulfate was found to increase L-selectin-binding potency [42,43].

In choosing an initial sulfotransferase for biological investigation, we focused our attention on a recent report by Kannagi and coworkers [44]. Using antibodies that selectively recognize a panel of sulfated sialyl Lewis x oligosaccharides, these researchers found evidence for the presence of GlcNAc-6-sulfated sialyl Lewis x on human peripheral lymph node HEVs and demonstrated that this modification was essential for L-selectin binding. By contrast, no evidence for the Gal-6-sulfated epitope was obtained. Thus, it appears that sulfation of GlcNAc within sialyl Lewis x-like oligosaccharides is a feature shared by both human and murine L-selectin ligands. Collectively, these observations have directed our attention to the GlcNAc-6-O-sulfotransferase.

The restricted vascular expression of the unusual sulfated motif 6-sulfo sialyl Lewis x to sites of lymphocyte recruitment, coupled with the requirement for sulfate in L-selectin binding, present compelling evidence for the role of a GlcNAc-6-O-sulfotransferase as a key modulator of lymphocyte–endothelial cell adhesion and a possible target for anti-inflammatory therapy. We report here the characterization of a GlcNAc-6-O-sulfotransferase from porcine peripheral lymph node whose tissue distribution and substrate specificity suggest that it may be involved in the biosynthesis of L-selectin ligands.

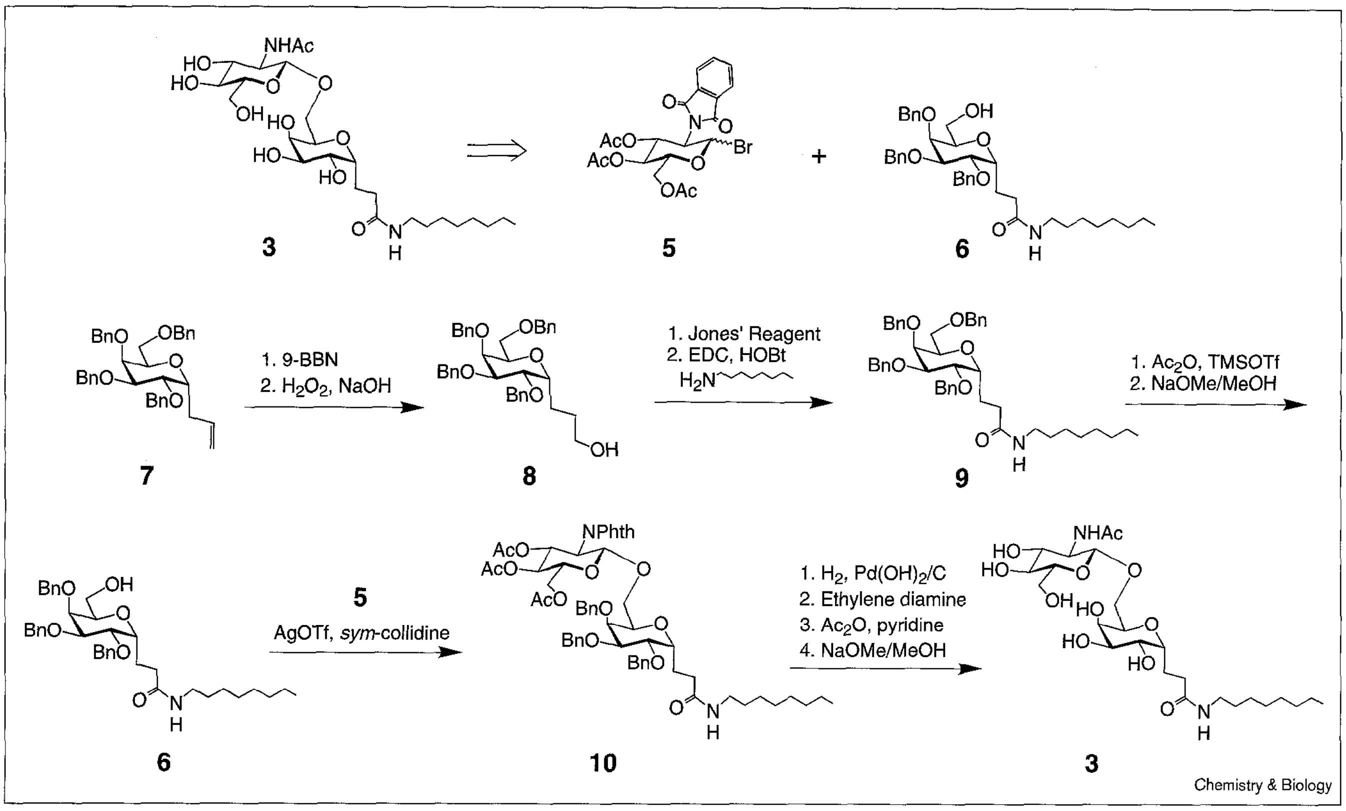
Results

Development of an *in vitro* GlcNAc-6-O-sulfotransferase assay

Two critical components of our biochemical study were the design and synthesis of a highly specific oligosaccharide substrate and the development of a sensitive and quantitative *in vitro* assay. The assay was based on the transfer of radiolabeled ^{35}S-sulfate from 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the activated sulfate donor for all known mammalian sulfotransferases [1,45–48], to the synthetic oligosaccharide substrate. An essential feature of the substrate was the presence of a hydrocarbon tail, which permitted selective adsorption onto reverse-phase silica gel after the enzymatic reaction and separation of the product from the other reaction components such as salts and unreacted ^{35}S-PAPS. Assays of this type are readily adaptable to several high-throughput architectures, including reverse-phase thin layer chromatography (TLC) plates and 96-well extraction plates, and have been useful in the characterization of other carbohydrate sulfotransferases [3,49] and several glycosyltransferases [50].

With the goal of identifying a minimal oligosaccharide substrate for the enzyme, we synthesized two compounds,

Figure 3



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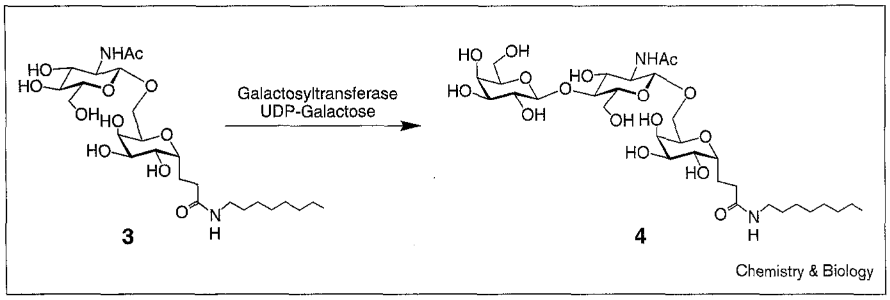
Synthesis of disaccharide substrate **3**.

disaccharide **3** (GlcNAcβ1→6Galα-R) and trisaccharide **4** (Galβ1→4GlcNAcβ1→6Galα-R), that comprise structural motifs found in the native L-selectin ligands (structures **1** and **2**, Figure 2). Disaccharide **3** possesses the target GlcNAc residue linked to a galactose moiety that mimics the peptide-proximal GalNAc found in the native oligosaccharide. Trisaccharide **4** has an additional terminal galactose residue appended to the target GlcNAc. The synthetic substrates share the distinguishing feature of a reducing terminal carbon-linked glycoside (C-glycoside), which was designed to fulfill two purposes. The first is synthetic facility, as α-linked C-glycosides of galactose are readily prepared on a large scale with high stereoselectivity [51] whereas α-linked O-glycosides of GalNAc, the native linkage, are more arduous to prepare. Second, the C-glycosyl linkage is stable to chemical hydrolysis, which simplified the analysis of the sulfated substrate as described in detail below.

The synthesis of compound **3** is outlined in Figure 3. In the retrosynthetic sense, we envisioned a key glycosyl coupling reaction between protected glucosamine analog **5** [52] and C-linked galactose derivative **6**. Intermediate **6** was prepared from the previously reported C-allyl galactoside **7** [53]. Briefly, compound **7** was subjected to a hydroboration reaction to afford alcohol **8**, which was oxidized to the corresponding carboxylic acid and coupled with *n*-octylamine to afford compound **9** bearing the requisite hydrocarbon tail. Regioselective acetoxylation of the primary 6-position using the procedure of Angibeaud and Utille [54], followed by saponification of the acetate ester, provided compound **6** in which the 6-OH was exposed for glycosyl coupling. Reaction with compound **5**, promoted by AgOTf, afforded disaccharide **10**, which was converted to the target substrate **3** by a series of protecting group manipulations. Trisaccharide **4** was prepared in one step from disaccharide **3** by enzymatic galactosylation using UDP-galactose and a commercially available bovine milk galactosyltransferase (GalT) [55] (Figure 4).

The sulfate donor used in the assay, ^{35}S-labeled PAPS, is available from commercial suppliers. However, commercial PAPS has low specific activity and contains significant amounts of 3′,5′-phosphoadenosine, an inhibitor of sulfotransferase activity. We therefore generated carrier-free ^{35}S-PAPS by *in vitro* enzymatic synthesis from carrier-free $^{35}SO_4^{2-}$ and adenosine triphosphate (ATP) according to the procedure of Long and coworkers [56].

Figure 4



Enzymatic synthesis of trisaccharide substrate **4**.

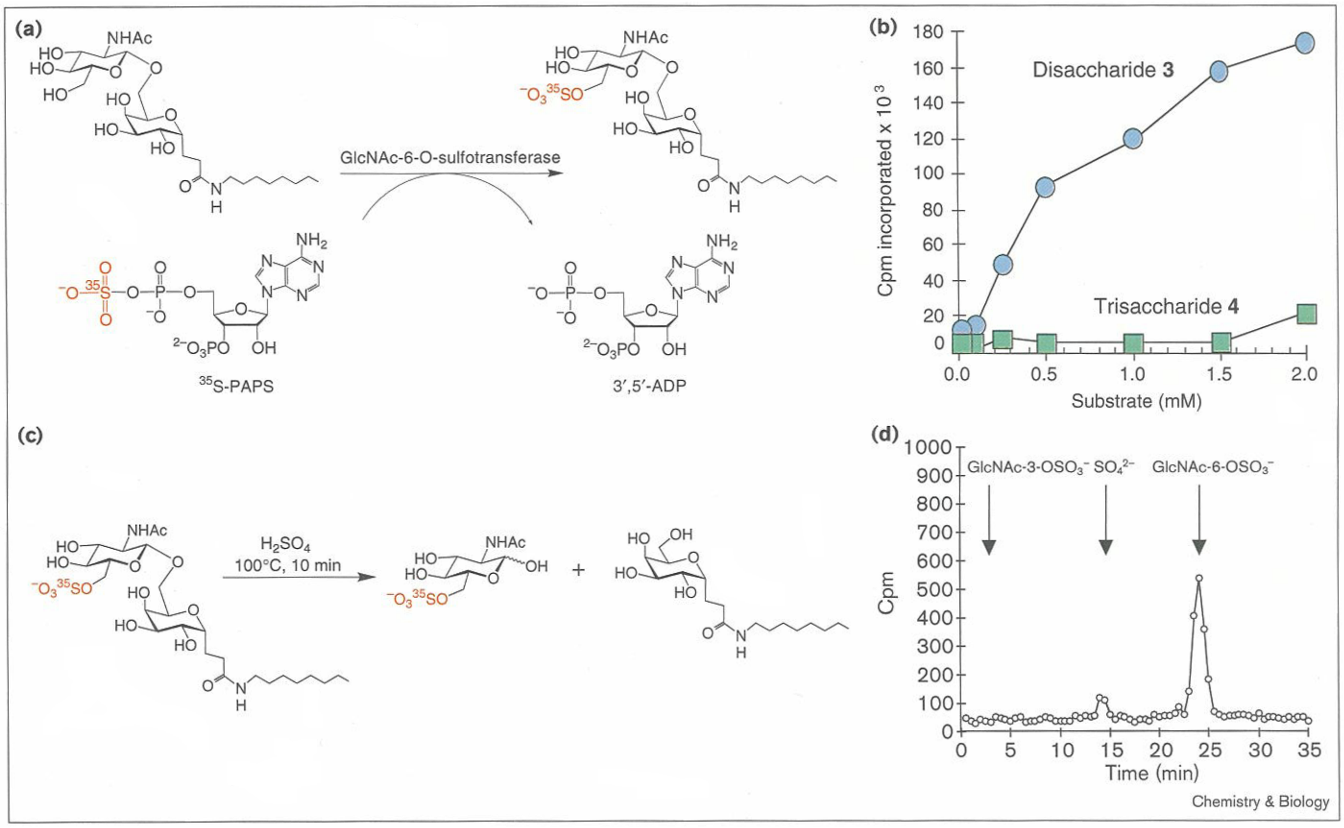
We chose porcine peripheral lymph nodes (PLNs) as a tissue source for the GlcNAc-6-O-sulfotransferase. Porcine tissues are readily available and porcine L-selectin ligands are structurally similar to those from murine, bovine and human lymph nodes, suggesting the expression of similar sulfotransferases across species [27,35]. We assumed the enzyme would be membrane associated and localized within Golgi compartments, similar to other carbohydrate sulfotransferases that have been previously cloned and characterized [5,49,57–63]. We therefore prepared a microsomal extract by centrifugation of a postnuclear supernatant from homogenized PLNs. The resulting supernatant (cytosol) and pellet (microsomes) were tested in the sulfotransferase assay using substrate **3** as follows. Briefly, an aliquot of the crude enzyme preparation was incubated with ^{35}S-PAPS (5 μCi) and oligosaccharide substrate **3**, in the presence of a cocktail of protease and phosphatase inhibitors using conditions similar to those reported by Baenziger and coworkers [5]. The enzymatic reaction mixtures were incubated in a conical microtiter plate, and then loaded onto a 96-well reverse-phase Oasis extraction plate. The unreacted ^{35}S-PAPS was washed from the cartridges with 5% aqueous MeOH, and the ^{35}S-labeled product was then eluted with 70% MeOH. The ^{35}S-labeled product could be quantified by one of two methods. In the first method, a small fraction of the eluate (5 μl) was transferred onto a PVDF membrane and phosphorimaging analysis was performed. In the second method, radioactivity in the eluate could be quantified using a Hewlett-Packard TopCount microtiter plate scintillation counter. In accord with a membrane-bound protein integral to secretory compartments, the majority of the activity was found to be localized to the microsomes (data not shown).

Substrate specificity of the GlcNAc-6-O-sulfotransferase

We compared the relative activities of disaccharide **3** and trisaccharide **4** as substrates for the PLN-derived sulfotransferase (Figures 5a,b). Radiolabeled sulfate was incorporated into compound **3** as a function of acceptor concentration, whereas only a modest amount of radioactivity was incorporated into trisaccharide **4** under the same conditions, corresponding to less than 10% of the activity observed with the disaccharide (Figure 5b). Both substrates have several hydroxyl groups that could serve as sites for enzymatic sulfation by irrelevant sulfotransferases. Thus, it was important to rigorously establish the position(s) of the sulfate ester on the radiolabeled products. Towards this end, we hydrolyzed ^{35}S-sulfated disaccharide **3** by treatment with 100 mM H_2SO_4 at 100°C for 10 min, conditions that cleave glycosidic linkages but leave sulfate esters largely intact (Figure 5c) [36]. The C-glycosyl linkage was resistant to hydrolysis and, consequently, the hydrocarbon tail remained associated with the galactose residue while the GlcNAc residue was liberated. The hydrolysate was analysed by high-pH anion exchange chromatography (HPEAC) using a Dionex high performance liquid chromatography (HPLC) system [64]. The C-glycosyl linkage on the galactose residue greatly simplified the analysis because the lack of a free reducing terminal hydroxyl group resulted in a retention time on a Dionex PA-1 column distinct from any free sugars. Indeed, none of the radioactivity was found to be associated with the C-linked galactose residue, indicating exclusive sulfation on the GlcNAc residue.

The regiochemistry of GlcNAc sulfation was determined by comparison of its retention time to those of authentic samples of GlcNAc-3-sulfate and GlcNAc-6-sulfate. The majority of the radioactivity from the hydrolysate co-eluted with GlcNAc-6-sulfate (Figure 5d), and a small amount of radioactivity co-eluted with an authentic sample of $^{35}SO_4^{2-}$, an expected byproduct from the hydrolysis reaction. No evidence for sulfation elsewhere on GlcNAc was observed (for example, no radioactivity co-eluted with an authentic sample of GlcNAc-3-sulfate). Thus, sulfation of disaccharide **3** by the porcine PLN-derived microsomes occurred exclusively at the 6-position of GlcNAc. Substrate **3** therefore presents a structural motif that is specifically recognized by a GlcNAc-6-O-sulfotransferase. No evidence for GlcNAc-6-sulfate was found on the product of sulfation of trisaccharide **4**. The minute level of radioactivity associated with trisaccharide **4** was identified, after hydrolysis as

Figure 5



The sulfotransferase reaction with synthetic substrate **3** and analysis of the regiochemistry of the sulfated products. **(a)** Enzymatic transfer of sulfate from radiolabeled ^{35}S-PAPS to the disaccharide substrate **3**. In a typical reaction, 5 μCi of radiolabeled PAPS and 500 μM substrate **3** were incubated with Golgi microsomes from porcine peripheral lymph nodes. The sulfated products were separated from the reaction mixture by solid phase extraction, facilitated by the hydrocarbon tail. **(b)** Substrate activity of disaccharide **3** and trisaccharide **4**. Enzymatic sulfation of compound **3** proceeded in a dose-dependent manner. In contrast, trisaccharide **4** was not sulfated at a significant level, suggesting the enzyme activity requires a terminal GlcNAc residue. Data points represent the average of five replicate experiments. The standard error of the mean for each point was between 3 and 10%. **(c)** Hydrolysis of the products of enzymatic sulfation. In order to identify the site of the sulfate ester, the glycosidic bond was selectively cleaved in aqueous H_2SO_4. **(d)** High pH anion-exchange chromatography (HPAEC) was used to analyze radiolabeled products of the hydrolysis. For the disaccharide **3**, only GlcNAc-6-sulfate and free sulfate were detected (retention times of authentic standards are indicated with arrows). The same analysis of trisaccharide **4** revealed only minute levels of free sulfate (data not shown) and no evidence for any other sulfated products.

above, as free ^{35}S-sulfate. Any possible sulfation on the monosaccharide residues was below the limits of detection and therefore deemed insignificant.

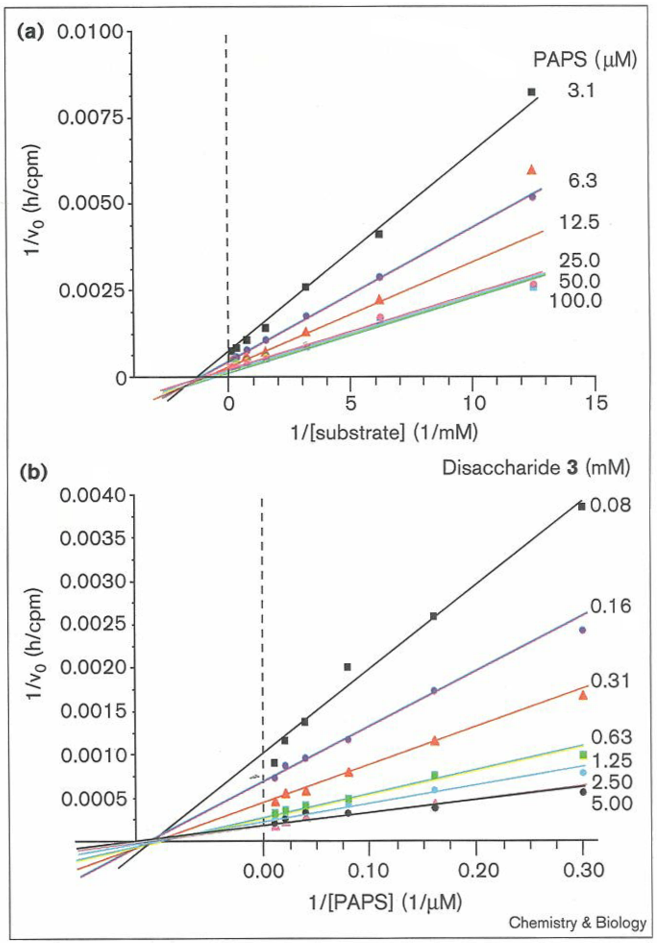
Quantitative parameters of the GlcNAc-6-O-sulfotransferase activity

We next evaluated the dependence of enzyme activity on the concentrations of both compound **3** and PAPS to obtain more detailed quantitative parameters for the enzyme and preliminary mechanistic information. Analysis of double reciprocal plots (Figures 6a,b) revealed K_M values of approximately 1 mM for disaccharide **3** and 7 μM for PAPS, the latter value being comparable to the K_M values for other carbohydrate sulfotransferases [63,65]. The observation of a common intersection point for the lines on both plots supports either a random or ordered mechanism and rules out a ping-pong mechanism involving a sulfoenzyme intermediate. Thus, it is likely that the sulfotransferase catalyzes the direct transfer of sulfate from PAPS to the oligosaccharide acceptor. This information will help guide the future design of sulfotransferase inhibitors.

Tissue and cellular distribution of the GlcNAc-6-O-sulfotransferase activity

Finally, we prepared microsomal extracts from a panel of porcine tissues obtained from the same animal and screened for GlcNAc-6-O-sulfotransferase activity using disaccharide **3** as a substrate. Background incorporation of radiolabeled sulfate into endogenous substrates from the tissue preparation was determined in the absence of substrate **3** and subtracted from the radioactivity incorporated

Figure 6



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Double reciprocal plots for the sulfotransferase reaction. **(a)** Plot of $1/v_0$ versus 1/[disaccharide **3**] indicates a K_M value of 1 mM for disaccharide **3**. **(b)** Plot of $1/v_0$ versus 1/[PAPS] indicates a K_M value for PAPS of 7 μM.

in the presence of substrate **3**. For comparative purposes, the extracts were normalized for total protein content. As shown in Figure 7a, the GlcNAc-6-O-sulfotransferase activity was highly localized to lymph nodes and spleen. By contrast, brain, heart, kidney, liver, lung, ovary, peripheral blood leukocytes (PBLs) and skeletal muscle tissue showed low levels of activity.

Lymph nodes comprise several cell types including lymphocytes, stromal cells and the activated endothelial cells that line the HEVs, referred to as high endothelial venule cells or HECs. L-Selectin ligands have been shown by histological methods to be localized to the HECs, and, accordingly, the GlcNAc-6-O-sulfotransferase responsible for their biosynthesis should be restricted to these cells. To investigate the cellular distribution of the lymph node enzyme, we fractionated porcine lymph nodes into lymphocytes and stromal elements that include HECs, and found the activity to reside predominantly in the crude stroma with only a minor amount in the lymphocyte population (Figure 7b). The stroma were then further fractionated into purified HECs and HEC-depleted stroma by immunomagnetic separation using MECA-79 as an HEC-specific marker. As shown in Figure 7b, the GlcNAc-6-O-sulfotransferase activity was highly concentrated in the HECs, paralleling the expression of MECA-79-reactive L-selectin ligands.

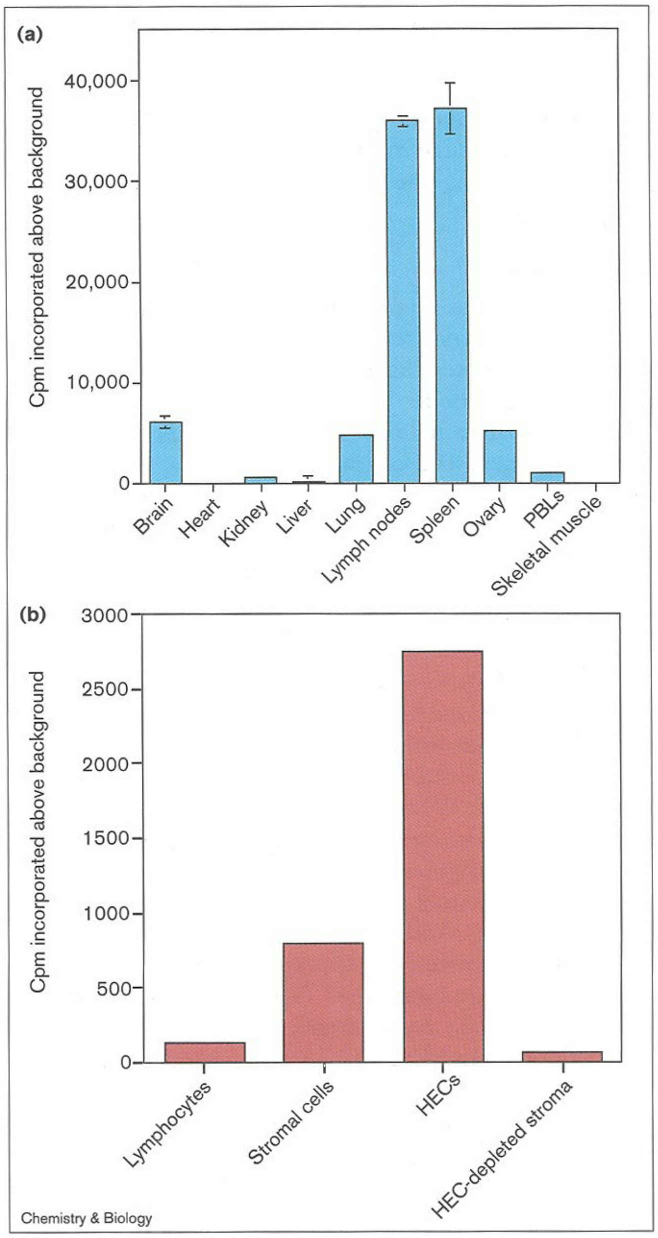
Discussion

Previous work has established that high-avidity L-selectin binding has a strict requirement for the sulfation of its cognate glycoprotein ligands, and that, in both murine and human lymph node endothelium, these ligands bear GlcNAc-6-sulfate within sialyl Lewis x oligosaccharides. We report here the characterization of a GlcNAc-6-O-sulfotransferase activity that is restricted to high endothelial cells within secondary lymphoid tissue, suggesting a possible role in the biosynthesis of L-selectin ligands.

A substrate comparison revealed a greater than tenfold preference for disaccharide **3** over trisaccharide **4**, indicating that the GlcNAc-6-O-sulfotransferase requires a terminal GlcNAc residue and is impeded by the presence of a β1→4-linked Gal residue. This observation has implications for the order of biosynthesis of L-selectin ligands as discussed below. We were concerned that irrelevant sulfotransferases concurrently expressed in the Golgi compartment could recognize the synthetic substrates, thereby leading to competing activities. For example, the heparin sulfate GlcNAc-*N*-deacetylase/*N*-sulfotransferase [57], a Gal-3-O-sulfotransferase [65] and a GalNAc-4-O-sulfotransferase [5] are established Golgi enzymes. To address this, we performed a regiochemical analysis of the sulfated products of disaccharide **3** and trisaccharide **4**. For substrate **3**, sulfation was confined exclusively to the 6-position of GlcNAc, indicating that the synthetic disaccharide presents a motif that preserves both recognition and specificity determinants for GlcNAc-6-O-sulfotransferase activity, and is not recognized by other sulfotransferase activities present in the microsomal preparation. For trisaccharide **4**, only a small amount of free sulfate was observed. The identity of any possible sulfate ester was obscured by the extremely low activity.

Many cell-surface glycoproteins bear 6-sulfated GlcNAc residues, such as the glycosaminoglycan chains of keratan sulfate [66] and N-linked oligosaccharides on thyroglobulin [67], HIV envelope components [68], and porcine zona pellucida [69]. GlcNAc-6-sulfate is presented within a different oligosaccharide context for each of these, and several GlcNAc-6-O-sulfotransferases appear to account for the biosynthesis within such a diverse array of contexts. For example, a GlcNAc-6-O-sulfotransferase activity that sulfates the motif GlcNAcβ1→6Man,

Figure 7



Tissue and cellular distribution of the GlcNAc-6-O-sulfotransferase activity using disaccharide **3** as a substrate. **(a)** The enzyme activity was highly restricted to peripheral lymph nodes and spleen. Solubilized Golgi microsomes were prepared from each tissue and an equal amount of total protein was analyzed. The data represent the average from duplicate experiments and error bars represent the standard error of the mean. **(b)** The GlcNAc-6-O-sulfotransferase activity is restricted to lymph node HECs, with essentially no activity residing in the other cell populations. Whole-cell lysates with identical total protein content were analyzed and similar results were obtained in duplicate experiments.

found within *N*-linked oligosaccharides, has been recently characterized in rat liver [70]. Like the lymph node enzyme described here, the liver-derived enzyme requires a terminal GlcNAc residue and is blocked by a β1→4-linked Gal residue, raising the possibility that the same enzyme generates both products. We detected no significant GlcNAc-6-O-sulfotransferase activity in porcine liver using substrate **3**, however. Thus, if a homologous enzyme exists in porcine liver, it does not recognize the GlcNAcβ1→6Gal determinant and therefore is distinct from the lymph node enzyme. Furthermore, the tissue distribution of the rat activity on GlcNAcβ1→6Man was markedly different from the activity with GlcNAcβ1→6Gal that we observe in porcine tissues, and was found in a broad range of tissues [71]. By contrast, the activity described here is highly localized to lymph nodes and spleen. These observations indicate that the two GlcNAc-6-O-sulfotransferase activities are distinct and that the substrate specificity of each enzyme is influenced by underlying carbohydrate structures proximal to the terminal GlcNAc moiety.

Keratan sulfate is widely distributed in connective tissue [72] and we were concerned that our assay would be permissive for one or more keratan sulfate GlcNAc-6-O-sulfotransferases. Trisaccharide **4**, which possesses the Galβ1→4GlcNAc motif found in keratan sulfate, was inactive as a substrate for the lymph node sulfotransferase, however. Combined with the known activity of the keratan sulfate enzyme on Galβ1→4GlcNAc oligomers [59], this result gives us confidence that our assay is specific for the enzyme of interest.

The occurrence of GlcNAc-6-sulfate within the context of core 2 oligosaccharides (that is, those possessing the structural motif [SO$_3$→6]GlcNAcβ1→6[Galβ1→3]GalNAc) has been observed in only a few examples beyond the L-selectin ligands. Notably, oligosaccharides similar to structure **1** have been identified on respiratory mucins from patients suffering from cystic fibrosis [73]. A corresponding GlcNAc-6-O-sulfotransferase activity from human lung mucosa has been characterized recently and shown to modify only terminal GlcNAc residues, with the β-linked anomer being significantly more active than the α-linked anomer [63]. Although we found low activity in porcine lung on disaccharide **3**, we await the cloning of both sulfotransferases to determine if these are the same or two different enzymes. In addition, the [SO$_3$→6]GlcNAcβ1→6[Galβ1→3]GalNAc motif has also been observed in human colon carcinoma mucins from highly metastatic cells [74], indicating the presence of a related GlcNAc-6-O-sulfotransferase. The relationship between the lung, colon carcinoma and lymph node enzymes warrants further investigation and can be addressed using the assay developed in this study.

The biosynthetic pathway for L-selectin ligands has several potential points of interception, and, consequently, the sequence of biosynthetic assembly of structures **1** and **2** has been a matter of significant interest [75–78]. It has been established that the sialyl Lewis x epitope on the L-selectin ligands is produced by the successive action of an α2→3sialyltransferase and an α1→3fucosyltransferase, most likely FucTVII, on a precursor bearing the Galβ1→4GlcNAc disaccharide [75]. In addition, Crommie and Rosen [75] have shown previously that sulfation of GlyCAM-1 in murine lymph nodes is blocked in the presence of brefeldin A, which collapses the Golgi compartments and endoplasmic reticulum and separates them from the trans-Golgi network. This observation suggested that sulfation occurs in the *trans*-Golgi network and is a late step along the biosynthetic pathway.

In contrast, the substrate specificity that we observed indicates that sulfation of GlcNAc must precede further elaboration of the β1→4Gal residue and, consequently, the other components of the sialyl Lewis x tetrasaccharide. Consistent with this proposal is the observation that several human fucosyltransferases recognize GlcNAc-6-sulfated 3′-sialyl-*N*-acetyllactosamine as a substrate [39,76,78]. Moreover, β1→4galactosyltransferases capable of modifying terminal GlcNAc-6-sulfate have been identified [63,70]. It is possible that a complement of glycosyltransferases successively installs the galactose, sialic acid and fucose residues within the trans-Golgi network, subsequent to GlcNAc sulfation. Alternatively, the GlcNAc-6-O-sulfotransferase might reside in an earlier secretory compartment and operate prior to delivery of the L-selectin ligands to the trans-Golgi network. In this scenario, the results obtained using brefeldin A might reflect a premature exposure of terminal GlcNAc residues to competing galactosyltransferases that prevent sulfation by blocking the GlcNAc epitope with a β1→4Gal residue. Indeed, the expression level of structure **1** under normal conditions might be regulated by the reciprocal activities of the GlcNAc-6-O-sulfotransferase and one or more galactosyltransferases.

We observed a distribution of activity across tissue and cell type that is in accord with a possible role in L-selectin ligand biosynthesis. In a screen of ten porcine tissues, we found the GlcNAc-6-O-sulfotransferase activity to be highly localized to PLNs and spleen. In contrast, activity in porcine brain, heart, kidney, liver, lung, ovary, peripheral blood leukocytes, and skeletal muscle was significantly lower. Furthermore, among the different cellular components of PLNs, HECs were the predominant source of the enzyme activity, whereas lymphocytes and HEC-depleted stromal cells had essentially no activity. This distribution mirrors that of L-selectin ligand expression and MECA-79 staining, which are confined to HECs within PLNs, and is consistent with a role for the GlcNAc-6-O-sulfotransferase in modulating L-selectin-mediated lymphocyte recruitment. Presently, the significance of the GlcNAc-6-O-sulfotransferase activity in spleen is unclear. Although spleen participates in lymphocyte recirculation, the adhesion interactions that mediate this, and the role of L-selectin, are not well understood [79]. Perhaps this GlcNAc-6-O-sulfotransferase activity contributes to the synthesis of uncharacterized sulfomucins involved in homing to spleen.

Finally, the similarity between sulfated L-selectin ligands in lymph node HEVs and those induced in HEV-like vessels in chronically inflamed tissues invites speculation regarding the possible role for the GlcNAc-6-O-sulfotransferase in modulating inflammatory lymphocyte recruitment. Sulfated L-selectin ligands, reactive with MECA-79, are restricted to lymph node HEVs and HEV-like vessels in chronically inflamed organs. Inactive glycoforms of the L-selectin ligands have been observed outside HEV-like vessels and these glycoforms are invariably unsulfated [30,31]. Thus, a GlcNAc-6-O-sulfotransferase may be required to convert inactive glycoforms to functional L-selectin ligands. An intriguing hypothesis is that the GlcNAc-6-O-sulfotransferase is an induced modulator of sustained lymphocyte recruitment in chronically inflamed tissues, a possibility that we are currently exploring.

Significance

L-selectin participates in the initial attachment of blood-borne lymphocytes to high endothelial venules (HEVs) in peripheral lymph nodes and at sites of chronic inflammation. Previous work has established that L-selectin ligands in lymph node HEVs are sulfated glycoproteins, and that sulfation is critical for high-avidity L-selectin binding. *N*-Acetylglucosamine-6-sulfate (GlcNAc-6-sulfate) within the framework of the sialyl Lewis x tetrasaccharide is a component of L-selectin ligands in both murine and human peripheral lymph nodes and contributes to L-selectin binding affinity *in vitro*. The restricted presentation of sulfated L-selectin ligands at sites of sustained lymphocyte recruitment suggests the presence of an HEV-associated GlcNAc-6-O-sulfotransferase. This enzyme might perform a critical function in normal lymphocyte homing and inflammatory lymphocyte recruitment, and therefore represents a potential target for anti-inflammatory therapy. Here, we describe the first investigation of a GlcNAc-6-O-sulfotransferase activity that is restricted to endothelial cells within lymph node HEVs, sharing a similar tissue distribution with the sulfated L-selectin ligands. The design and synthesis of unnatural oligosaccharide substrates and the development of a sensitive and quantitative radiolabel transfer assay performed in a microtiter plate format were critical to this study. We identified a concise disaccharide motif, GlcNAcβ1→6GalαR, derived from native L-selectin ligands, that preserved both recognition and specificity determinants for the GlcNAc-6-O-sulfotransferase. The absence of activity on the related trisaccharide Galβ1→4GlcNAcβ1→6GalαR implicates a requirement for a substrate with a terminal GlcNAc residue, and

further suggests a biosynthetic pathway for L-selectin ligands in which sulfation precedes further elaboration of the glycan. These discoveries set the stage for the design of inhibitors, for the detailed biological investigation of this and related sulfotransferases, and for the study of L-selectin ligand biosynthesis.

Materials and methods

Materials

All chemical reagents were obtained from commercial suppliers and used without further purification. The ^{1}H and ^{13}C NMR spectra were obtained using Bruker AMX-300, -400, or -500 spectrometers. Chemical shifts are reported in δ values relative to tetramethylsilane, and coupling constants (*J*) are reported in Hertz. Fast atom bombardment (FAB+) spectra and elemental analyses were obtained at the U.C. Berkeley Mass Spectral and Microanalytical Laboratory.

Water used for biochemical procedures was doubly distilled and deionized using a Milli-Q system (Millipore). Protease inhibitors and Pefabloc SC were from Boehringer Mannheim or Sigma, ATP sulfurylase and inorganic pyrophosphatase were from Sigma, APS kinase was a generous gift from Professor Sharon Long (Stanford University), porcine lymph nodes were supplied fresh from Pel-Freeze or harvested directly upon slaughter, $^{35}Na_2SO_4$ was obtained from American Radiolabeled Chemicals, PEI cellulose plates were from VWR, and Oasis HLB Extraction plates and Sep-Pak cartridges were obtained from Waters.

*3-(2,3,4,6-Tetra-O-benzyl-α-D-galactopyranosyl) propanol (**8**)*

Under an atmosphere of N2, 1.74 g (14.3 mmol) of 9-BBN dimer and 1 g of 3 Å molecular sieves were cooled to 0°C, and a solution of 6.50 g (11.5 mmol) of compound **7** in 12 ml of tetrahydrofuran (THF) was added via syringe. The solution was heated at reflux for 3 h. The solution was cooled to 0°C and an additional 1.74 g of 9-BBN were transferred to the flask followed by heating at reflux for an additional 3 h. After cooling to 0°C, the excess 9-BBN was quenched with 20 ml of EtOH, then 7 ml of 4 M NaOH and 7 ml of H_2O_2 were added. The reaction was warmed to room temperature (rt) and stirred for 8 h. The solution was diluted with 50 ml of ether, neutralized with of NH_4Cl, then successively washed with H_2O and brine. The combined aqueous washes were extracted with ether and the combined organic layers dried over $MgSO_4$. Following concentration, the product was purified by silica gel chromatography eluting with 6:1 hexanes/ethyl acetate to give 6.93 g (100%) of a clear, colorless syrup; IR (thin film) 3444, 3087, 3062, 3029, 2915, 2869, 1496, 1454, 1369, 1208, 1028 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.63-1.95 (m, 4H), 3.18 (br s, 1 H), 3.55-3.67 (m, 3 H), 3.71 (dd, 1 H, *J*=2.8, 7.3), 3.75-3.84 (m, 2 H), 3.94-4.01 (m, 3 H), 4.46 (d, 1 H, *J*=12.0), 4.52 (d, 1 H, *J*=10.3), 4.54 (d, 1 H, *J*=11.9), 4.56 (d, 1 H, *J*=11.8), 4.64 (app d, 2 H, *J*=11.3), 4.67 (d, 1 H, *J*=12.2), 4.74 (d, 1 H, *J*=13.1), 7.27-7.35 (m, 20 H); ^{13}C NMR: δ 22.1, 22.1, 23.7, 24.1, 26.3, 28.3, 28.3, 29.7, 30.2, 31.0, 31.1, 31.4, 32.0, 34.1, 34.2, 36.1, 43.0, 43.3, 44.8, 62.4, 71.8, 73.1, 73.2, 73.3, 74.5, 127.5, 127.6, 127.7, 127.8, 127.9, 128.0, 128.3, 128.4, 138.2, 138.3, 138.5, 138.6; MSÊ(FAB+) m/z 583 (M+H). Anal. calc'd. for $C_{37}H_{42}O_6$: C, 76.26; H, 7.26. Found: C, 76.10; H, 7.40.

*3-(2,3,4,6-Tetra-O-benzyl-α-D-galactopyranosyl) propanoic acid (**8a**)*

A solution of 6.90 g (11.8 mmol) of **8** in 50 ml of acetone was cooled to 0°C. Over a period of 12 min, 12 ml (24 mmol) of Jones' reagent was added dropwise, after which the reaction flask was warmed to rt for 3 h. Excess Jones' reagent was quenched with 10 ml of isopropanol, and the solution was diluted with 100 ml of ether, washed successively with $NaHCO_3$, H_2O, and brine, then dried over Na_2SO_4. Purification of the concentrated product was achieved by silica gel chromatography eluting with 5:1 hexanes/ethyl acetate to afford 6.13 g (89%) of a clear, colorless syrup; IR (thin film): 3088, 3064, 3031, 2967, 2248, 1710, 1145 cm^{-1}; ^{1}H NMR (500 MHz, CDC^{l3}): δ 1.95 (m, 1 H), 2.03 (m, 1 H), 2.41 (m, 1 H), 2.53 (m, 1 H), 3.68 (dd, 1 H, *J*=4.8, 10.5), 3.78 (m, 2 H), 3.91 (br app s, 1 H), 4.03 (m, 3 H), 4.50 (d, 1 H, *J*=11.9), 4.56 (d, 1 H, *J*=11.9), 4.59 (d, 1 H, *J*=11.2), 4.62 (d, 1 H, *J*=11.4), 4.69 (d, 1 H, *J*=11.8), 4.70 (d, 1 H, *J*=11.6), 4.75 (d, 1 H, *J*=11.9), 4.80 (d, 1 H, *J*=11.6), 7.34-7.37 (m, 20 H); ^{13}C NMR: δ 21.98, 30.39, 67.70, 71.84, 72.87, 73.06, 73.18, 73.37, 74.25, 76.49, 127.38, 127.45, 127.48, 127.51, 127.63, 127.71, 127.81, 128.11, 128.16, 128.25, 128.31, 130.01, 138.17, 138.12, 138.38, 138.45, 179.21; HRMS (FAB+) m/z: 597.2852 (MH+ $C_{37}H_{41}O_7$: requires 597.2852).

*N-Octyl-3-(2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl) propionamide (**9**)*

A solution of 1.49 g (2.50 mmol) of **8a**, 826 ml (3.75 mmol) of *n*-octylamine, 507 mg (3.75 mmol) of hydroxybenzotriazole, and 719 mg (3.75 mmol) of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) in 25 ml of 3:1 CH_3CN/CH_2Cl_2 was stirred under an atmosphere of N_2 at rt. After 12 h, the solution was diluted with 50 ml of $CHCl_3$ and 50 ml of NH_4Cl. The organic layer was collected and washed with H_2O and brine, then dried over $MgSO_4$. Purification was achieved by silica gel chromatography eluting with 5:1 hexanes/ethyl acetate to yield 1.62 g (92%) of a clear, pale yellow syrup; IR (thin film): 3290, 3064, 1653, 1540 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.87 (t, 3 H, *J*=6.7), 1.22-1.34 (m, 12 H), 1.84 (m, 1 H), 1.94 (m, 1 H), 2.20-2.23 (m, 2 H), 3.05 (m, 1 H), 3.12 (m, 1 H), 3.53 (dd, 1 H, *J*=3.3, 10.7), 3.68-3.74 (m, 2 H), 3.95-4.04 (m, 4 H), 4.48-4.67 (m, 8 H), 5.94 (br s, 1 H), 7.25-7.35 (m, 20 H); ^{13}C NMR: δ 14.2, 22.7, 27.0, 29.3, 29.3, 31.9, 33.0, 39.6, 67.6, 69.4, 73.0, 73.1, 74.3, 76.1, 76.9, 127.6, 127.7, 127.8, 127.8, 128.0, 128.1, 128.4, 128.4, 128.5, 128.5, 138.2, 138.3, 138.4, 138.5, 172.9. HRMS (FAB+) m/z 707.4186 (MH+ $C_{45}H_{58}O_6N$ requires 707.4186).

*N-Octyl-3-(2,3,4-tri-O-benzyl-α-D-galactopyranosyl) propionamide (**6**)*

A solution of 800 mg (1.10 mmol) of **9** in 4 ml of CH_2Cl_2 and 4 ml of acetic anhydride was cooled to −42°C. Over the course of an hour, a solution of 220 μl of trimethylsilyl trifluromethanesulfonate (TMSOTf) in 375 μl CH_2Cl_2 was added dropwise via syringe and the solution was stirred for an additional 3 h. The solution was diluted with 10 ml of $CHCl_3$, and then quenched with $NaHCO_3$. The organic phase was washed successively with H_2O and brine, and then dried over $MgSO_4$. Concentration *in vacuo* afforded a clear, colorless syrup which was carried on to the next reaction without further purification. To obtain full characterization, an analytical sample was purified by silica gel chromatography eluting with 10:1 hexanes/ethyl acetate. IR (thin film): 3386, 1737, 1648, 1541, 1454, 1370, 1239, 1098 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88 (t, 3 H, *J*=1.4), 1.27 (m, 10 H), 1.47 (m, 1 H), 1.98 (m, 1H), 2.06 (s, 3 H), 2.15 (m, 1 H), 2.21 (m, 1 H), 3.20 (m, 2 H), 3.54 (app s, 1 H), 3.74 (app s, 1 H), 3.97 (m, 2 H), 4.09 (m, 1 H), 4.22 (app d, 1 H, *J*=12.2), 4.60 (m, 6 H), 4.79 (m, 1 H), 6.03 (s, 1 H), 7.26-7.44 (m, 15 H); ^{13}C NMR : δ 13.58, 20.87, 22.41, 25.49, 26.74, 29.00, 29.03, 29.40, 31.57, 32.87, 39.33, 61.10, 71.98, 72.64, 72.91, 72.97, 73.31, 76.57, 127.36, 127.41, 127.48, 127.58, 127,70, 127.85, 128.05, 128.19, 128.20, 128.22, 137.70, 137.86, 138.05. MS (FAB+): m/z 660 (MH+). Anal Calcd. for $C_{40}H_{53}O_7N$: C, 72.81; H, 8.10; N, 2.12. Found: C, 73.03; H, 8.01; N, 1.97. The product of the previous reaction was dissolved in 10 ml of a 10 mM solution of $NaOCH_3$ in methanol and stirred overnight. The reaction was neutralized by H+ ion exchange resin, and the product was concentrated and purified by silica gel chromatography eluting with 2:1 hexanes/ethyl acetate to give 564 mg (92% from 9) of a clear, colorless syrup; IR (thin film): 3306, 3088, 3064, 3030, 2953, 1641, 1549, 1454, 1092 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.92 (t, 3 H, *J*=6.8), 1.28 (m, 10 H), 1.48 (m, 2 H), 1.81 (m, 1 H), 2.04 (m, 1 H), 2.25 (d app t, 1 H, *J*=6.1, 13.9), 2.37 (m, 1 H), 3.18 (br s, 1 H), 3.19 (m, 2 H), 3.62 (m, 1 H), 3.68 (m, 1 H), 3.76 (m, 1 H), 3.97 (m, 2 H), 4.04 (d app t, 1 H, *J*=2.8, 10.9), 4.14 (m, 1 H), 4.48 (d, 1 H, *J*=12.1), 4.65 (d, 1 H, *J*=11.9), 4.68 (d, 1 H, *J*=12.0), 5.89 (br m, 1 H), 7.19-7.35 (m, 15 H); ^{13}C NMR: δ 14.01, 22.55, 26.88, 29.13, 29.18, 29.49, 31.71, 33.16, 59.93, 68.73, 72.46, 72.99, 73.16, 74.21, 74.30, 75.28, 127.57, 127.60, 127.72, 127.80, 128.00, 128.34, 137.99, 138.13, 138.30, 173.08. HRMS (FAB+) m/z: 618.3795 (MH+ for $C_{38}H_{52}O_6N$ requires

618.3795). Anal. calc'd for $C_{38}H_{51}O_6N$: C, 73.87; H, 8.32; N, 2.27. Found: C, 73.65; H, 8.39; N, 2.04.

*Protected disaccharide **10***

Under an atmosphere of N_2 protected from light, 336 mg (1.30 mmol) of silver trifluoromethanesulfonate (AgOTf), 1 ml of CH_3NO_2, and 200 mg of 4 Å molecular sieve dust were transferred into a flame-dried flask and stirred for 1 h. After the flask was cooled to −20°C, a solution of 537 mg (0.870 mmol) of compound **6**, 651 mg (1.31 mmol) of compound 5 [52] and 105 mg (0.870 mmol) of sym-collidine in 1 ml of CH_3NO_2 was precooled to −20°C and added via syringe. The reaction was warmed to room temperature and stirred for 48 h, then neutralized with 1 mL saturated $NaHCO_3$, diluted with $CHCl_3$, and filtered through Celite. The filtrate was successively washed with ice cold 1 M HCl and brine, dried over Na_2SO_4, concentrated and purified by silica gel chromatography eluting with 3:2 hexanes/ethyl acetate to afford 612 mg (68%) of a clear, colorless syrup. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.27 (m, 3 H), 1.60 (m, 10 H), 1.88 (s, 3 H). 2.05 (s, 3 H), 2.13 (s, 3 H), 3.96 (m, 2 H), 4.12-4.33 (br m, 8 H), 4.38 (dd, *J*=4.1, 12.4, 1 H), 4.67 (dd, *J*=3.4, 11.3, 1 H), 5.19 (app t, 1 H), 5.87 (dd, *J*=9.2, 10.7, 1 H), 6.16 (dd, *J*=9.1, 11.3, 1 H), 7.32 (m, 15 H), 7.74-7.91 (br m, 4 H).

*Disaccharide **3***

Compound **10** was dissolved in a slurry of 100 mg of $Pd(OH)_2$ on carbon, activated by removal of water *in vacuo*, and 3 ml methanol. The reaction mixture was stirred under an atmosphere of H_2 at rt for 3 h, then filtered through Celite and concentrated to give 451 mg (100%) of a clear, colorless syrup. This intermediate was dissolved in 5 ml of butanol and 3.6 g (59 mmol) ethylenediamine were added. The solution was heated to 85°C for 30 h under an atmosphere of N_2. The reaction was diluted with toluene and concentrated *in vacuo* to give a brown syrup that was carried on to subsequent reactions without further purification or characterization. This product was dissolved in a solution of 5 ml 1:2 acetic anhydride/pyridine and 25 mg dimethylaminopyridine (DMAP) and stirred overnight at rt. The reaction was diluted with methylene chloride and washed successively with ice cold 1 N HCl, 1 M $CuSO_4$, saturated $NaHCO_3$, and brine, then dried over Na_2SO_4. Purification was achieved by silica gel chromatography eluting with a gradient of 1:2 hexanes/ethyl acetate to 100% ethyl acetate to afford 237 mg (50% over two steps) of a clear, colorless syrup; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.86 (app t, *J*=8.8, 3 H), 1.27 (m, 14 H), 1.51 (m, 2 H), 1.4 (s, 3 H), 1.96 (s, 3 H), 2.02 (s, 3 H), 2.04 (s, 3 H), 2.05 (s, 3H), 2.08 (s, 3 H), 2.11 (s, 3 H), 3.21 (m, 2 H), 3.39 (app t, *J*=9.8, 1 H), 3.63 (m, 1 H), 3.86 (app d, *J*=10.1, 1 H), 3.92 (d, *J*=8.1, 1 H), 4.11 (m, 3 H), 4.28 (dd, *J*=4.5, 12.4, 1 H), 4.35 (d, *J*=8.3, 1 H), 5.04 (app t, *J*=9.3, 1 H), 5.10 (t, *J*=9.4, 1 H), 5.14 (dd, *J*=3.4, 10.6, 1 H), 5.32 (m, 2 H), 5.60 (d, *J*=12.0, 1 H), 7.47 (app t, 1 H); 13C NMR: δ 14.09, 20.58, 20.62, 20.65, 20.68, 20.70, 20.86, 22.63, 23.36, 27.10, 29.25, 29.30, 29.58, 30.72, 31.83, 39.57, 41.16, 53.90, 61.75, 67.79, 68.11, 68.14, 68.57, 69.14, 71.90, 72.29, 72.97, 80.44, 101.45, 169.20, 169.89, 170.13, 170.18, 170.63, 170.67, 171.29, 171.88.

The preceding compound (75 mg, 0.14 mmol) was dissolved in 5 ml of 10 mM NaOMe in methanol, and stirred overnight at rt. The reaction was neutralized by H+ cation exchange resin and then concentration to afford 52 mg (100%) of disaccharide 3 as an amorphous solid; ^{1}H NMR (500 MHz, D_2O): δ 0.86 (app t, 3H), 1.27 (m, 12 H), 1.50 (br s, 2 H), 1.93 (m, 3 H), 2.02 (s, 3 H), 2.08 (m, 1 H), 2.18 (m, 1 H), 2.3 (m, 1 H), 3.16 (m, 2 H), 3.44 (m, 2 H), 3.54 (app t, 1 H), 3.66 (app t, 1 H), 3.72 (m, 4 H), 3.77 (app t, 1 H), 3.91 (m, 2 H), 3.97 (m, 4 H), 4.51 (d, 1 H, *J*=8.4); ^{13}C NMR (CD_3OD): δ 14.37, 23.08, 23.66, 28.04, 30.33, 30.38, 30.39, 32.95, 33.36, 40.44, 49.56, 49.59, 57.11, 62.75, 69.88, 69.96, 70.40, 71.73, 72.10, 76.11, 77.90, 79.37, 103.05, 175.82, 173.71. HRMS (FAB+) m/z: 551.3180 (MH+ for $C_{25}H_{47}O_{11}N_2$ requires 551.3180).

*Trisaccharide **3***

The galactosyltransferase reaction was performed essentially as described by Oehrlein *et al.* [55]. Disaccharide **3** (18.5 mg, 33.6 µmol), UDP-galactose (25 mg, 41.0 µmol), galactosyltransferase (2.72 U), and calf intestinal alkaline phosphatase (16.3 U) were incubated in 50 mM cacodylate (pH 7.4), 1% BSA, and 30 mM $MnCl_2$ in a final volume of 840 µl at 37°C for 2 h. The product was isolated by loading the reaction onto a C18 cartridge, washing with 10 ml of H_2O, and finally eluting with 5 ml of 70% aqueous methanol. Concentration followed by lyophilization gave 23.9 mg (100%) of a white, amorphous solid; ^{1}H NMR (500 MHz, CD_3OD): δ 0.84 (app t, *J*=6.5, 3H), 1.24 (m, 10 H), 1.44 (m, 2 H), 1.86 (m, 2 H), 1.91 (s, 3 H), 2.10 (m, 1 H), 2.27 (m, 1 H), 3.10 (m, 2 H), 3.36 (m, 1 H), 3.41 (dd, *J*=3.50, 10.0, 1 H), 3.74 (dd, *J*=7.5, 9.5, 1 H), 3.50-3.63 (m, 6 H), 3.69 (dd, *J*=7.5, 10.0, 1 H), 3.74-3.83 (m, 7 H), 3.60 (dd, *J*=2.5, 9.0, 1 H), 3.90 (dd, *J*=4.0, 10.5, 1 H), 4.32 (dd, *J*=8.8, 18.0, 1 H). ^{13}C NMR (CD3OD): δ 13.84, 15.37, 23.12, 24.06, 24.66, 27.51, 29.04, 29.80, 29.86, 31.33, 31.37, 31.39, 31.70, 33.95, 41.44, 50.54, 63.45, 70.98, 71.25, 73.53, 75.24, 75.77, 77.49, 78.10, 81.84, 104.02, 106.04, 158.22, 176.82. HRMS (FAB+) m/z: 713.3708 (MH+ for $C_{31}H_{57}O_{16}N_2$ requires 713.3708).

Isolation of microsomes from fresh porcine tissues

The following buffer and protease inhibitor cocktails (PIC) were used in the isolation procedures. STKM buffer: 250 mM sucrose, 50 mM Tris-Cl pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 1 µl/ml of protease inhibitor cocktails PIC-I and PIC-II. PIC-I: To 2.5 ml of aprotinin solution (5–10 trypsin inhibitory units/ml) were added 3.0 mg of Leupeptin, 12.5 mg of Pefabloc SC, 5 mg of Antipain, 5 mg of Turkey trypsin inhibitor, and 2.5 mg of benzamidine. PIC-II: Chymostatin (10 mg) and Pepstatin (5 mg) were dissolved in 5 ml of DMSO. All buffers were supplemented with 1 µl/ml of each PIC, and all manipulations were carried out at 4°C.

Tissues for the comparative tissue scan were harvested from the same eight month old female Yucatan micropig euthanized by intravenous administration of ketamine HCl. Following dissection, tissues were snap frozen in liquid nitrogen. Additional porcine lymph nodes for enzyme characterization were purchased frozen from Pel-Freeze (Rogers, AZ). Frozen tissues were placed in a motor-driven tissue grinder and crushed to powder in liquid nitrogen. The powdered tissues were suspended in a tenfold volume of SKTM buffer. Tissue suspensions were then homogenized in a 10 ml glass homogenizer with a drill-driven conico-cylindrical teflon pestle (five passes at 800 rpm). The homogenates were centrifuged at 680 × g for 10 min, the supernatant was collected, and the nuclear/mitochondrial pellet was discarded. The supernatant was then centrifuged at 10,000 × g for 10 min, the new supernatant collected and the lysosomal pellet was discarded. The supernatant was then centrifuged at 100,000 × g for 1 h, and the microsomal pellet was collected and used as as a source of enzyme after processing as described below. The supernatant from this final centrifugation step (cytosol) was collected and analyzed as well.

The microsomal pellet was washed by resuspension in five volumes of 150 mM Tris-Cl pH 8.0 supplemented with 1 × PIC-I and PIC-II followed by centrifugation at 100,000 × g for 1 h. The pellet was solubilized in lysis buffer (50 mM HEPES pH 7.5, 2.5% Triton X-100 supplemented with 1 × PIC-I and PIC-2) using a hand-held microfuge tube homogenizer. Lysates were cleared by recentrifugation for 1 h at 100,000 × g, and then 1/4 volume of 50% glycerol was added to each cleared lysate. The total protein concentrations in the lysates and cytosolic supernatants were determined using the Pierce BCA protein assay. Thereafter, each lysate was diluted with lysis buffer to a concentration of 2 mg/ml total protein, and typically 10 µl of the lysate was used in a sulfotransferase assay.

Cellular fractionation of porcine lymph nodes

Porcine lymph nodes were fractionated into lymphocytes, high endothelial cells (HEC) and HEC-depleted stromal cells essentially as described by Girard and Springer [80]. Briefly, 4.2 g of fresh procine peripheral lymph nodes were diced and teased on a stainless steel screen (86 µM pore size, Tylinter) and lymphocytes were washed through the screen by flushing with RPMI. These cells comprised the lymphocytes analyzed for sulfotransferase activity. The remaining stromal elements were digested with collagenase I and DNAse I in RPMI for 15 min to degrade extracellular matrix components, and the digested stroma were re-filtered on the screen to remove additional lymphocytes. After an 1 h digestion with collagenase I and DNAse I, the liberated stromal cells were flushed through the screen with buffer (PBS, 1% BSA, 5 mM EDTA). These cells comprised the stromal cells analyzed for sulfotransferase activity. HEC cells were purified from the stromal cells by immunomagnetic separation using the MAb MECA-79 as an HEC-specific marker. The stromal cells were incubated with MECA-79 for 15 min at 4°C, washed by centrifugation and resuspended in buffer. The cells were then incubated with a biotinylated secondary antibody (anti-Rat IgM) for 15 min at 4°C, washed and resuspended. The labeled cells were incubated with streptavidin-magnetic beads (Miltenyi Biotech, MACS system) for 15 min, and applied to a WHM separation column in a magnetic field. HEC-depleted stromal cells were washed through the column with buffer, and magnetic HEC were subsequently obtained by removing the column from the field and washing with buffer. The separated cell populations were solubilized with lysis buffer for enzyme assays.

Expression and purification of APS kinase

A plasmid containing the APS kinase gene from *R. meliloti* fused to an N-terminal (His)$_6$ sequence was a generous gift from Professor Sharon Long (Stanford University). APS kinase-(His)$_6$ was overexpressed in *E. coli* M15 cells containing the plasmid pLys using standard protocols. The cells were lysed by three quick freeze-thaw cycles, and the enzyme was purified from the lysate by nickel affinity chromatography using Ni-NTA Agarose (Qiagen) eluting with a gradient of imidazole in 5 mM β-mercaptoethanol according to the manufacturer's instructions.

Enzymatic synthesis of ^{35}S-PAPS

Carrier-free 35S-PAPS was synthesized *in vitro* using a procedure similar to that described by Long and coworkers [56]. Briefly, 1 mCi of carrier-free ^{35}S-Na$_2$SO$_4$ and 30 mM ATP were incubated with approximately 45 μg of APS kinase, 0.12 U ATP sulfurylase (Sigma), and 8.3 U inorganic pyrophosphatase (Sigma) in 60 μl Buffer A (20 mM Tris-HCl (pH 8.0), 30 mM KCl, 40 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, 10% glycerol) for 12 h at 30°C. Quantification of conversion was accomplished through separation of reaction components by TLC on polyethyleneimine (PEI)-cellulose eluting with 0.9 M LiCl, followed by phosphorimaging analysis (Model 445 SI, Molecular Dynamics). Incorporation of ^{35}S-SO$_4{}^{2-}$ into ^{35}S-PAPS typically exceeded 98%.

Assay for sulfotransferase activity

Standard assay reactions of 25 μl were carried out in 96-well plates at pH 6.5 in Buffer ST (15 mM MOPSO, 1% Triton-X 100, 40 mM β-mercaptoethanol, 6 mM Mg(OAc)2, 10 mM NaF, 1 mM ATP, 13.3% glycerol). We determined the optimal pH as 6.5 from an activity profile over a pH range of 5.0–8.0. The reactions were performed with 500 μM disaccharide 3, 5 μCi ^{35}S-PAPS, 3 μM PAPS, and microsomal extract containing 15–20 μg total protein, in the presence of the aforementioned protease inhibitors. Reactions were incubated for 2 h at 37iC, then terminated by dilution to 1 ml followed by loading onto Oasis 96-well reverse-phase extraction plates (Waters) at a flow rate of 1 ml/min. Each column in the plate was washed with 8 ml of 5% methanol. Elution and quantification of the sulfated product was achieved by one of two methods: In Method A, the sulfated product was eluted with 150 μl of 100% methanol followed by quantification with a Hewlett Packard TopCount scintillation counter. In Method B, the the sulfated product was eluted with 1 ml of 70% methanol, and 5 μl of eluant from each reaction was spotted onto a PVDF membrane and quantified of using a phosphorimager.

Regiochemical analysis of the products of enzymatic sulfation

The regiochemistry of substrate sulfation was determined using a procedure adapted from that described by Hemmerich *et al.* [36]. Briefly, the eluant of the sulfotransferase reaction was lyophilized then subjected to acid hydrolysis by treatment with 100 mM H$_2$SO$_4$, overlaid with mineral oil, for 10 min at 100°C. The hydrolysate was neutralized with 1 equivalent of 1 M NH$_4$OH, and then analyzed by high-pH anion exchange chromatography (HPAEC, Dionex) on a CarboPac PA-1 column with pulsed amperometric detection. Elutions were performed at 1 ml/min using the following gradient: 50 mM NaOAc in 150 mM NaOH for 5 min, followed by a linear gradient of 50–850 mM NaOAc in 150 mM NaOH over 30 min. Standards for GlcNAc-3-sulfate, GlcNAc-6-sulfate, Gal-4-sulfate, and Gal-6-sulfate (Sigma) were used at 1 mM; Gal-3-sulfate was obtained by acid hydrolysis of sulfatides (Sigma) under the conditions above, and purified by removing lipid byproducts on a C$_{18}$ Sep-Pak cartridge followed by lyophilization. Radiolabeled products were detected by collecting 0.5 ml fractions followed by scintillation counting.

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