Substrate Specificity of the Heparan Sulfate Hexuronic Acid 2-*O*-Sulfotransferase†

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ABSTRACT: The interaction of heparan sulfate with different ligand proteins depends on the precise location of *O*-sulfate groups in the polysaccharide chain. We have previously shown that overexpression in human kidney 293 cells of a mouse mastocytoma 2-*O*-sulfotransferase (2-OST), previously thought to catalyze the transfer of sulfate from 3′-phosphoadenosine 5′-phosphosulfate to C2 of L-iduronyl residues, preferentially increases the level of 2-O-sulfation of D-glucuronyl units [Rong, J., Habuchi, H., Kimata, K., Lindahl, U., and Kusche-Gullberg, M. (2000) *Biochem. J. 346*, 463−468]. In the study presented here, we further investigated the substrate specificity of the mouse mastocytoma 2-OST. Different polysaccharide acceptor substrates were incubated with cell extracts from 2-OST-transfected 293 cells together with the sulfate donor 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate. Incubations with O-desulfated heparin, predominantly composed of [(4)αIdoA(1)−(4)αGlcNSO$_3$(1)-]$_n$, resulted in 2-O-sulfation of iduronic acid. When, on the other hand, an N-sulfated capsular polysaccharide from *Escherichia coli* K5, with the structure [(4)βGlcA(1)−(4)αGlcNSO$_3$(1)-]$_n$, was used as an acceptor, sulfate was transferred almost exclusively to C2 of glucuronic acid. Substrates containing both iduronic and glucuronic acid residues in about equal proportions strongly favored sulfation of iduronic acid. In agreement with these results, the 2-OST was found to have a ∼5-fold higher affinity for iduronic acid-containing substrate disaccharide units ($K_m \sim 3.7$ μM) than for glucuronic acid-containing substrate disaccharide units ($K_m \sim 19.3$ μM).

Heparin and heparan sulfate (HS)[1] form a complex group of glycosaminoglycans, known for their capacity to interact with proteins. Heparin is exclusively synthesized by connective tissue mast cells and is stored in cytoplasmic granules (*1*). HS is synthesized by most, if not all, mammalian cells and thus is ubiquitous, in the extracellular matrix, in basement membranes, and on cell surfaces (*2*, *3*). Due to their interactions with proteins, HSs influence a variety of biological processes, including embryonic development, inflammation, blood coagulation, angiogenesis, and microbial infections (*2*, *4*). While the biological function of heparin has remained elusive, it was recently demonstrated that heparin is needed for the biogenesis and stability of mast cell granulae (*5*, *6*). The biological roles of heparin and HS depend, at least in part, on the occurrence of specific saccharide sequences designed for protein binding (*2*, *3*). The mechanisms that regulate the generation of such sequences are not well understood, but clearly involve modulated action of the various biosynthetic enzymes that catalyze the assembly of heparin and HS.

Heparin and HS are synthesized through basically similar reactions. The first step in polysaccharide assembly is the formation of a nonsulfated precursor polymer by alternating transfer of D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine units (GlcNAc), from the corresponding UDP-sugars, to the nonreducing end of the growing chain. This polymerization step is catalyzed by two bifunctional proteins that both express both GlcA and GlcNAc transferase activities (*7*, *8*). Along with elongation, the polymer is modified through a series of reactions that include N-deacetylation and N-sulfation of GlcN residues, C5-epimerization of GlcA to L-iduronic acid (IdoA), and finally O-sulfation at various positions (see ref *3*). The modification reactions are generally incomplete, and the resultant polysaccharides therefore vary in structure. The *N*-deacetylase/*N*-sulfotransferase reaction is a key regulatory step, since the enzymes associated with the subsequent reactions all require *N*-sulfate groups for substrate recognition. Interspersed between the N-sulfated domains are nonsulfated N-acetylated regions that remain largely unmodified. Several enzymes involved in the process have been purified and cloned from various sources (*7*, *9*−*23*). The *N*-deacetylase/*N*-sulfotransferase, the GlcN 6-*O*-sulfotransferase, and the GlcN 3-*O*-sulfotransferase all exist in several different genetic isoforms which are believed to

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[1] Abbreviations: HS, heparan sulfate; 2-OST, 2-*O*-sulfotransferase; CHO, Chinese hamster ovary; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; HexA, unspecified hexuronic acid; GlcNAc, 2-deoxy-2-acetamido-D-glucose (*N*-acetyl-D-glucosamine); PAPS, adenosine 3′-phosphate 5′-phosphosulfate; aMan$_R$, 2,5-anhydro-D-mannitol (formed by reduction of terminal 2,5-anhydromannose residues with $NaBH_4$); -NSO$_3$, *N*-sulfate group; -OSO$_3$, *O*-sulfate, ester sulfate group (the locations of *O*-sulfate groups are indicated in parentheses); kb, kilobase pair(s); nt, nucleotide(s).

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modify the HS chain in an isoform specific fashion (*24*−*26*). The GlcA C5-epimerase and the IdoA 2-*O*-sulfotransferase (2-OST) have so far only been found as single forms.

The extensive modification of heparin results in the formation of extended regions of -IdoA(2-OSO_3)-GlcNSO_3-(6-OSO_3)- repeating disaccharide units. HS is generally less extensively modified and has a more diverse structure. However, IdoA(2-OSO_3) residues, although less abundant than in heparin, are a major component also in the N-sulfated regions of HS chains. Due to their conformational flexibility, such residues are believed to promote the interactions of polysaccharides with proteins (*27*). Thus, IdoA(2-OSO_3) residues in different structural contexts have been implicated as essential components of binding regions in HS chains for a variety of proteins, including antithrombin (*28*), basic fibroblast growth factor (FGF-2) (*29*, *30*), lipoprotein lipase (*31*), hepatocyte growth factor (*32*), and platelet-derived growth factor (*33*). Most HS species also contain 2-O-sulfated GlcA units, although such residues are relatively rare. The biological roles of sulfated GlcA residues have not been established. Notably, HS enriched in 2-O-sulfated GlcA residues was found in adult human cerebral cortex (*34*) and in a nuclear fraction from hepatocytes (*35*).

We previously cloned and expressed a 2-OST from CHO cells (*18*) and from a mouse mastocytoma cDNA library (*36*). Overexpression of the mouse mastocytoma 2-OST in human kidney 293 cells had a dramatic effect on HS biosynthesis (*36*), resulting in greatly increased levels of GlcA 2-O-sulfation. In the study presented here, we demonstrate that recombinant mouse mastocytoma 2-OST catalyzes the 2-O-sulfation of hexuronyl residues in both -GlcNS-IdoA-GlcNS- and -GlcNS-GlcA-GlcNS- sequences, although with a marked preference for the Ido isomer.

EXPERIMENTAL PROCEDURES

Materials. Bovine lung heparin (Upjohn) and pig intestinal HS (gift from G. van Dedem, Diosynth) were desulfated by treatment with a 9/1 (v/v) dimethyl sulfoxide/methanol mixture for 8 h at 93 °C (*37*). The desulfated products were re-N-sulfated by reaction with trimethylamine sulfur trioxide (*38*). Capsular polysaccharide from *Escherichia coli* K5, with the structure [(4)βGlcA(1)−(4)αGlcNAc(1)-]$_n$, and derivatives of the polysaccharide, in which ∼50 or ∼100% of the *N*-acetyl groups had been chemically replaced with *N*-sulfate groups, were generously given by B. Casu (Ronzoni Institute, Milan, Italy). N-Sulfated K5 polysaccharide in which ∼50% of the GlcA residues had been converted to IdoA by enzymatic C5-epimerization was a gift from P. Oreste (Ricerche Sperimentali Montale, Montale, Italy). 3′-Phosphoadenosine 5′-phospho[^{35}S]sulfate ([^{35}S]PAPS) was prepared according to the procedure described previously (*39*). Extract of baker's yeast (Sigma) containing sulfate-activating enzymes was incubated with carrier-free [^{35}S]sulfate (DuPont NEN) and ATP (Boehringer Mannheim GmbH, Mannheim, Germany). The resultant [^{35}S]PAPS was purified by anion-exchange chromatography on DEAE-Sephacel (Amersham Pharmacia). Unlabeled PAPS was purchased from Sigma. HexA-[^{3}H]aMan$_R$ disaccharides, with and without *O*-sulfate groups in different positions, used as reference compounds were as described in ref *40*; a synthetic GlcA(2-OSO_3)-[^{3}H]aMan$_R$ standard was generated as reported in ref *41*.

RNA Purification and Northern Blot Analysis. Total RNA was isolated from mouse mastocytoma cells (*42*) using the RNeasy purification kit from Qiagen. Total RNA isolated from mouse mastocytoma and a mouse multiple-tissue Northern blot (Clontech) containing poly(A)-selected RNA from several adult mouse tissues were hybridized with probes labeled with [α-^{32}P]dCTP (DuPont NEN) using Ready-To-Go DNA labeling beads (Pharmacia Biotech). The probes that were used were a 217 bp *Sac*I cDNA fragment recognizing the coding region of the mouse mastocytoma 2-OST and a 1.1 kb cDNA clone recognizing human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA (Clontech). The filters were hybridized at 65 °C in ExpressHyb solution (Clontech) first with the 217 bp *Sac*I fragment and subsequently with human G3PDH.

Expression of Mouse 2-OST. The 2-OST expression construct has been previously described (*36*). The expression plasmids were transfected into human embryonic kidney 293 cells using LipofectAMINE (Life Technologies, Inc.), and stably transfected cell clones expressing the mouse mastocytoma 2-OST (2-OST) and control cells transfected with vector alone (Ctrl.) were selected as described previously (*36*). Cells were cultured under an atmosphere of 5% CO_2 in air and 100% relative humidity in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.) supplemented with 10% (v/v) fetal calf serum (Gibco/BRL), 100 μg/mL streptomycin sulfate, 100 units/mL penicillin G, and 400 μg/mL Geneticin (G418 sulfate, Gibco/BRL).

Enzymatic ^{35}S-Sulfation of Polysaccharides. Crude enzyme preparations were extracted from stably transfected cell clones after washing the cell layer twice with cold phosphate-buffered saline followed by detachment of the cells with trypsine and EDTA. After addition of trypsine inhibitor and centrifugation at 1000 rpm, the cell pellet was lysed by incubation at 4 °C for 30 min with 500 μL of 50 mM Tris-HCl buffer (pH 7.4) containing 1% Triton X-100 along with protease inhibitors [1 mM PMSF (Sigma), 2 mM *N*-ethylmaleimide (Aldrich), and 2 mM EDTA and 10 μg/mL pepstatin A (Sigma)]. After centrifugation at 2000 rpm for 15 min, the supernatant was collected. The cell lysates (50 μL) from 2-OST-transfected cells or control cells were incubated at 37 °C for 30 min with 100 μCi of [^{35}S]PAPS (2 mCi/μmol; prepared by mixing radiolabeled and unlabeled PAPS) and 100 μg of polysaccharide acceptors (see Materials) in 500 μL of 50 mM HEPES buffer (pH 7.4) containing 10 mM $MnCl_2$, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 3.5 μM NaF, and 1% Triton X-100. The resultant ^{35}S-labeled polysaccharides were separated from unreacted [^{35}S]PAPS or free [^{35}S]-sulfate by gel filtration on a column (1 cm × 150 cm) of Sephadex G-50 (Amersham Pharmacia Biotech) equilibrated with 0.2 M NH_4HCO_3. Labeled polysaccharide, emerging at the void volume, was recovered and desalted by lyophilization. Sulfotransferase activities were determined as the level of ^{35}S incorporation into each substrate and defined in units of picomoles of sulfate per minute per milligram of protein.

Structural Analysis of ^{35}S-Labeled Polysaccharides. For analysis of disaccharide composition, the labeled samples were treated with HNO_2 at pH 1.5 (*43*). The ^{35}S-labeled deamination products were reduced with $NaBH_4$ and fractionated by gel chromatography on a column (1 cm × 180 cm) of Sephadex G-15 eluted with 0.2 M NH_4HCO_3. The

Table 1: Incorporation of *O*-[^{35}S]Sulfate into Different Substrates[a]

polysaccharide substrate	total *O*-sulfate incorporation: 2-OST [pmol of *O*-sulfate min^{-1} (mg of protein)$^{-1}$]	total *O*-sulfate incorporation: Ctrl. [pmol of *O*-sulfate min^{-1} (mg of protein)$^{-1}$]	total *O*-sulfate incorporation: 2-OST/Ctrl. ratio	relative incorporation of *O*-[^{35}S]sulfate (%)[b]: 2-OST	relative incorporation of *O*-[^{35}S]sulfate (%)[b]: Ctrl.
50% N-sulfated K5 PS	83	17	5.0	82	70
100% N-sulfated K5 PS	48	12	4.0	48	51
de-O-sulfated HS	65	16	4.2	65	66
de-O-sulfated heparin	100	24	4.2	100	100

[a] Human kidney 293 cells were transfected with vector alone (Ctrl.) or with plasmid containing the 2-OST cDNA (2-OST). [^{35}S]Sulfate incorporation into different saccharide substrates was assessed, catalyzed by cell extracts from transfected or control cells. Total O-sulfation was calculated on the basis of the amounts of labeled disaccharides recovered after low-pH deamination determined by anion-exchange HPLC (Figure 2). The activities recorded with native N-acetylated K5 polysaccharide acceptor or in the absence of added exogenous sulfate acceptors were below the detection limit. [b] The values represent the percentage of total incorporated *O*-[^{35}S]sulfate groups compared with those of O-desulfated heparin.

Table 2: Composition of Products Formed on Deaminative Cleavage of *O*-[^{35}S]Sulfate Polysaccharides[a]

polysaccharide substrate	GlcA(2-OSO$_3$)-aMan$_R$ (% of total): 2-OST	GlcA(2-OSO$_3$)-aMan$_R$ (% of total): Ctrl.	GlcA-aMan$_R$(6-OSO$_3$) (% of total): 2-OST	GlcA-aMan$_R$(6-OSO$_3$) (% of total): Ctrl.	IdoA-aMan$_R$(6-OSO$_3$) (% of total): 2-OST	IdoA-aMan$_R$(6-OSO$_3$) (% of total): Ctrl.	IdoA(2-OSO$_3$)-aMan$_R$ (% of total): 2-OST	IdoA(2-OSO$_3$)-aMan$_R$ (% of total): Ctrl.	IdoA(2-OSO$_3$)-aMan$_R$(6-OSO$_3$) (% of total): 2-OST	IdoA(2-OSO$_3$)-aMan$_R$(6-OSO$_3$) (% of total): Ctrl.
50% N-sulfated K5 PS	80	22	7	31	ND[b]	ND	13	48	ND	ND
100% N-sulfated K5 PS	88	36	2	22	ND	ND	10	42	ND	ND
de-O-sulfated HS	ND	ND	ND	2	2	20	96	64	2	14
de-O-sulfated heparin	ND	ND	ND	ND	5	38	95	62	ND	ND

[a] [^{35}S]Sulfate-labeled samples were degraded to disaccharides (deamination at pH 1.5 followed by reduction of products with $NaBH_4$) that were analyzed by anion-exchange HPLC (see Experimental Procedures). Columns designated 2-OST and Ctrl. represent disaccharides generated by cleavage of ^{35}S-labeled products obtained from incubations with extracts from 2-OST and control transfected cells, respectively. [b] ND, none detected.

resultant ^{35}S-labeled disaccharides were pooled and lyophilized and were then analyzed by anion-exchange HPLC using a Whatman Partisil-10 SAX column eluted with KH_2PO_4 (*44*) as described in the legend of Figure 1.

For chemical glucuronyl C5-epimerization, ^{35}S-labeled disaccharides (2.5×10^5 cpm) were mixed with a synthetic GlcA(2-OSO$_3$)-[^{3}H]aMan$_R$ standard (2×10^5 cpm) in 250 μL of hydrazine hydrate (Fluka; 30% H_2O) containing 1% (w/v) hydrazine sulfate, and heated at 95 °C for 20 h (*45*). Excess hydrazine was evaporated and further coevaporated with 0.5 mL of ice-cold water twice. To destroy any hydrazide that was formed, the sample was treated with nitrous acid at pH 3.9. Disaccharides were recovered by gel chromatography on a column (1 cm × 180 cm) of Sephadex G-15 in 0.2 M NH_4HCO_3 and analyzed by anion-exchange HPLC as described above.

Determination of K_m for 2-OST. K_m values for O-desulfated heparin and 100% N-sulfated K5-polysaccharide were determined in 2-OST assay mixtures as described above, in a total volume of 100 μL containing 1 μCi of [^{35}S]PAPS (100 μM) and various amounts of polysaccharide acceptors. The reaction was quenched by the addition of 400 μL of ethanol containing 1.3% (w/v) sodium acetate and 100 μg of heparin as a carrier. The resulting mixtures were kept at −70 °C for 3−4 h to precipitate the polysaccharide and were then centrifuged at 13 000 rpm for 30 min. The supernatant was discarded, and the pellet was dissolved in 100 μL of water. The ^{35}S-labeled polysaccharide was recovered after centrifugation through Sephadex G-25 as described previously (*46*). The fluid recovered in the centrifuge tubes contained the ^{35}S-labeled polysaccharide which was quantified by liquid scintillation counting. Apparent K_m and V_{max} values were calculated using the Enzfitter program (Biosoft).

RESULTS

Substrate Specificity of Mouse 2-OST. To investigate the substrate recognition properties of 2-OST, the following polysaccharides were tested as *O*-[^{35}S]sulfate acceptors: completely desulfated re-N-sulfated (i.e., O-desulfated) heparin, completely desulfated re-N-sulfated intestinal mucosa HS, *E. coli* K5 polysaccharide, and chemically modified K5 polysaccharide with 50 or 100% of the acetyl groups replaced with *N*-sulfate groups (see Experimental Procedures). These polymers were selected because of their differential display of potential substrate residues. Heparin thus can be considered an extended N-sulfated sequence rich in IdoA residues. HS on the other hand is composed of N-sulfated domains, regions of mixed acetyl/sulfate N-substitution, and *N*-acetylated sequences. The latter type of sequence contains GlcA as the only hexuronic acid component, whereas the other domain types contain both GlcA and IdoA units (*47*). Notably, both heparin and HS show significant proportions of GlcA units in N-sulfated polysaccharide sequences (approximately 20−25% of the total hexuronic acid in these regions). N-Sulfated K5 polysaccharide is composed of repeating -GlcA-GlcNSO$_3$- disaccharide units. Extracts from 2-OST-transfected cells yielded an overall 4−5-fold increase in the level of *O*-[^{35}S]sulfate incorporation into each partially or fully N-sulfated polysaccharide compared to that of control cells (Table 1). Calculations based on the compositional analysis of disaccharides revealed that the observed overall increase in the level of [^{35}S]sulfate incorporation was entirely due to an increased level of 2-O-sulfation (see Table 2). The highest level of incorporation [~100 pmol min^{-1} (mg of protein)$^{-1}$] of *O*-sulfate was obtained with the de-O-sulfated heparin substrate. The unmodified bacterial polysaccharide was a poor substrate for the sulfotransferase, in accord with the postulated requirement of *N*-sulfate groups for substrate

recognition by the 2-OST (*3*). Notably, the *O*-sulfate acceptor efficiency of the 50% N-sulfated K5 derivative exceeded that of the fully N-sulfated homologue, and, in fact, approached that of O-desulfated heparin (Table 1). No significant ^{35}S-sulfation of endogenous polysaccharide was observed in control incubations performed in the absence of an added exogenous saccharide substrate.

To analyze the distribution of incorporated *O*-[^{35}S]sulfate groups, the labeled polysaccharides were degraded by nitrous acid deamination at pH 1.5. Such treatment will cleave the glucosaminidic linkages of N-sulfated but not of N-acetylated GlcN units. Consecutive N-sulfated disaccharide units thus will yield disaccharide deamination products, whereas tetrasaccharides and higher oligosaccharides are obtained from regions which contain N-acetylated, resistant GlcN residues. Gel chromatography showed that ≥95% of the depolymerization products were disaccharides, with merely trace amounts of the radioactivity emerging as tetrasaccharides (data not shown). Notably, these findings applied equally to all polysaccharide substrates, irrespective of the extent of N-sulfation. It is concluded that O-[^{35}S]sulfation was restricted to contiguous N-sulfated sequences within the acceptor polysaccharides.

Identification of ^{35}S-labeled disaccharides recovered after incubation of acceptor polysaccharides with the lysate of mock-transfected (control) cells revealed appreciable proportions of labeled GlcN 6-*O*-sulfate as well as HexA 2-*O*-sulfate groups (Figure 1 and Table 2), in accord with the prediction that the cells express both types of *O*-sulfotransferases. Notably, the heparin- and HS-derived substrates both incorporated all labeled *O*-sulfate groups exclusively into IdoA-containing disaccharide units, of the type (-GlcNSO$_3$)-IdoA-GlcNSO$_3$-. The products generated from the K5 polysaccharides gave a more complex pattern. Contrary to the heparin- and HS-derived substrates, the K5-based polymers showed appreciable incorporation of both 2-*O*- and 6-*O*-sulfate groups into GlcA-containing disaccharide units, (-GlcNSO$_3$)-GlcA-GlcNSO$_3$-. Moreover, a major proportion of the 2-*O*-[^{35}S]sulfate residues was bound to IdoA units, indicative of concerted GlcA C5-epimerization and IdoA 2-O-sulfation reactions (Figure 1 and Table 2). In addition to O-sulfated disaccharides, substantial amounts of inorganic [^{35}S]sulfate were released on deamination of the labeled ~50% N-sulfated K5 derivative. This product presumably represents *N*-[^{35}S]sulfate groups introduced through combined *N*-deacetylase/*N*-sulfotransferase reactions (*48*).

The enzyme present in lysate from 2-OST-transfected 293 cells generated radically different sulfation patterns, strongly dominated by 2-O-sulfated HexA units (Figure 1 and Table 2). While the sulfation of the heparin- and HS-derived substrates was almost exclusively directed toward IdoA units, the K5 acceptors were predominantly sulfated at GlcA target residues. The overexpressed 2-OST thus catalyzes the transfer of sulfate groups to IdoA as well as to GlcA residues, the type of target unit selected depending on the structure of the acceptor polysaccharide. The ability of 2-OST to catalyze 2-O-sulfation of both IdoA and GlcA residues was confirmed using the purified recombinant enzyme. A cDNA encoding 2-OST isolated from CHO cells was expressed in COS-7 cells as a FLAG fusion protein and isolated on a FLAG affinity column as described previously (*18*). The purified recombinant 2-OST transferred [^{35}S]sulfate into both O-desulfated heparin and the N-sulfated K5 polysaccharide. Identification of labeled disaccharides generated by nitrous acid cleavage revealed ^{35}S-sulfation of IdoA and GlcA units, respectively (data not shown), in agreement with the results obtained using cell lysate as the enzyme source.

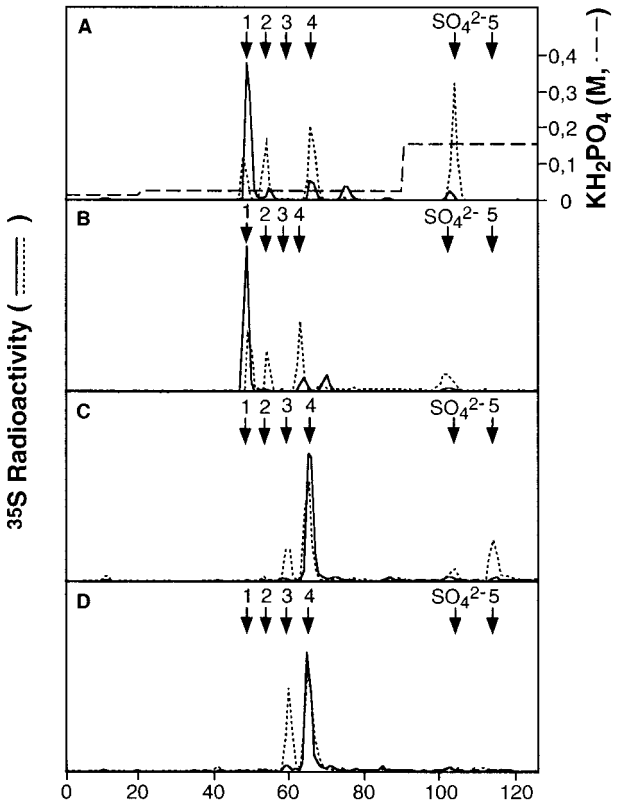

FIGURE 1: Anion-exchange HPLC of ^{35}S-labeled disaccharide fractions. Chemically modified polysaccharide substrates were incubated with [^{35}S]PAPS and cell lysates from 2-OST transfected cells (—) or control cells transfected with vector alone (···) as described in Experimental Procedures. Labeled polysaccharide was isolated and degraded by HNO_2 (pH 1.5), and the resultant disaccharides were reduced and recovered by gel filtration. Samples of isolated disaccharides were analyzed on a Partisil-10 SAX column eluted at a rate of 1 mL/min with KH_2PO_4 solutions with a stepwise increasing concentration (as shown by the dashed line in panel A). Monosulfated disaccharides were eluted with 0.026 M KH_2PO_4 and disulfated disaccharides with 0.15 M KH_2PO_4. The four panels represent the elution profiles of disaccharides from ~50% N-sulfated K5 polysaccharide (A), ~100% N-sulfated K5 polysaccharide (B), de-O-sulfated (re-N-sulfated) pig mucosa HS (C), and de-O-sulfated (re-N-sulfated) bovine lung heparin (D). The elution positions of standard disaccharides are denoted with arrows: 1, GlcA(2-OSO$_3$)-aMan$_R$; 2, GlcA-aMan$_R$(6-OSO$_3$); 3, IdoA-aMan$_R$(6-OSO$_3$); 4, IdoA(2-OSO$_3$)-aMan$_R$; and 5, IdoA(6-OSO$_3$)-aMan$_R$(6-OSO$_3$). SO_4^{2-} represents inorganic sulfate.

Since the initial isolation and cDNA cloning of (CHO cell) 2-OST was based on assays of IdoA 2-O-sulfation, confirming the identity of the alleged GlcA 2-*O*-[^{35}S]sulfate-containing disaccharide was considered essential. This component emerged at the same elution position as a synthetic GlcA(2-OSO$_3$)-[^{3}H]aMan$_R$ disaccharide standard (*41*) on anion-exchange HPLC (Figure 1A,B). The ^{35}S- and ^{3}H-labeled compounds both resisted digestion with β-D-glucuronidase from bovine liver (data not shown), as expected for sulfate-substituted GlcA residues. Chemical C5-epimerization of the GlcA moieties (*49*) and analysis of the products by anion-exchange HPLC indicated similar levels of conversion, ~20%, of both compounds into a product that

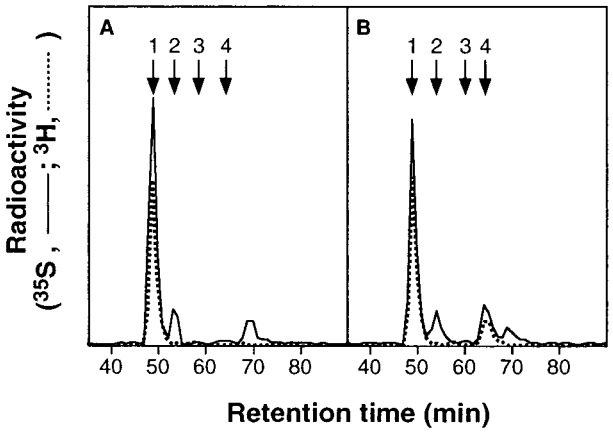

FIGURE 2: Chemical C5-epimerization of GlcA(2-O$^{35}SO_3$)-aMan$_R$. ^{35}S-labeled disaccharides isolated after incubation of cell lysates from 2-OST-transfected cells with 50% N-sulfated K5 polysaccharide and [^{35}S]PAPS were mixed with a synthetic GlcA(2-OSO$_3$)-[^{3}H]aMan$_R$ standard and treated with hydrazine hydrate and finally with nitrous acid at pH 3.9 (as described in Experimental Procedures). Disaccharide mixtures were analyzed on a Partisil-10 SAX column before (A) and after (B) hydrazine treatment. The dashed line represents the ^{3}H-labeled standard disaccharide and the solid line the ^{35}S-labeled sample disaccharides. The reference compounds denoted by arrows 1−4 are the same as those described in the legend of Figure 1. The apparent lack of IdoA(2-O$^{35}SO_3$)-aMan$_R$ disaccharide in panel A reflects the lack of C5-epimerase activity in the cell lysate used as the enzyme source (effect of storing; J.-p. Li, personal communication).

migrated like an IdoA(2-OSO$_3$)-aMan$_R$ standard (Figure 2). Taken together, these findings strongly indicate that the ^{35}S-labeled disaccharide is indeed GlcA(2-O$^{35}SO_3$)-aMan$_R$ and, hence, that the recombinant 2-OST is capable of catalyzing 2-O-sulfation of both -GlcA-GlcNSO$_3$- and -IdoA-GlcNSO$_3$- target sequences.

Hexuronic Acid Target Preference. The results described above show that the recombinant 2-OST can sulfate both C5-epimeric HexA species, but do not reveal any preference regarding target unit. To further elucidate this matter, we analyzed the effect of the enzyme on a K5-derived substrate polysaccharide, enzymatically engineered to contain about equal proportions of GlcA and IdoA residues. This substrate incorporated ~30 pmol of sulfate min^{-1} (mg of protein)$^{-1}$ under standard assay conditions. Analysis by anion-exchange HPLC of ^{35}S-labeled disaccharide deamination products showed a major peak of IdoA(2-O$^{35}SO_3$)-aMan$_R$, but no GlcA-containing isomer (Figure 3). This finding clearly demonstrates that the enzyme preferentially recognizes -GlcNSO$_3$-IdoA-GlcNSO$_3$- target structures.

In view of this result, assessing the basic kinetic properties of the enzyme, in relation to substrate polysaccharides containing either GlcA or IdoA units, was considered important. Since N-sulfated K5 polysaccharide and O-desulfated heparin fulfilled these requirements and, furthermore, were found to serve as highly selective targets for 2-O-sulfation of GlcA and IdoA units, respectively (Figure 1), substrate saturation experiments were conducted using each of these polymers. The resulting curves were clearly different (Figure 4). The apparent K_m values for the N-sulfated K5 polysaccharide and for the O-desulfated heparin substrates were calculated to be ~19 ± 4.9 and ~3.7 ± 0.7 μM disaccharide units, respectively. In addition, the apparent V_{max} value for the heparin-derived substrate was ~3-fold higher

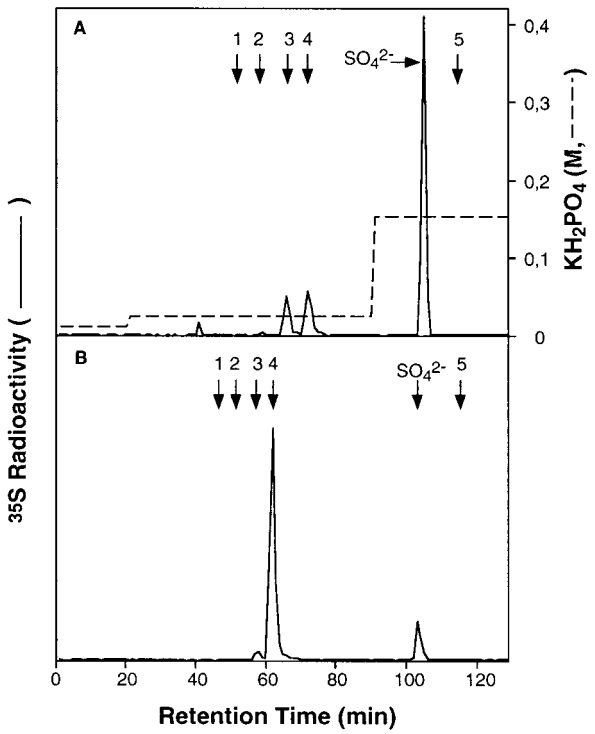

FIGURE 3: Action of 2-OST on partially C5-epimerized N-sulfated K5 polysaccharide. Chemically N-sulfated (~100%) K5 polysaccharide in which ~50% of the HexA residues were IdoA units was incubated with [^{35}S]PAPS and cell lysates from control transfected (A) or 2-OST-transfected (B) cells as described in Experimental Procedures. Labeled polysaccharide was isolated and degraded with HNO_2 (pH 1.5), and the resultant disaccharides were reduced and recovered by gel filtration. Samples of isolated disaccharides were analyzed on a Partisil-10 SAX column. The reference compounds denoted by arrows 1−5 are the same as those described in the legend of Figure 1. SO_4^{2-} represents inorganic sulfate.

than that calculated for the N-sulfated K5 polysaccharide. It is noted that the substrate concentration used under standard assay conditions, 400 μM disaccharide units, would result in enzyme saturation with either substrate.

mRNA Expression of Mouse 2-O-Sulfotransferase. Northern blot analysis of several mouse adult tissues using a cDNA probe specific for mouse 2-OST showed expression of an ~3 kb and, to a lesser extent, an ~5 kb transcript in all tissues that were examined. High levels of expression for 2-OST were found in lung and brain, whereas the message was barely detectable in spleen (Figure 5A). In addition, expression of other size mRNAs was evident. The relative abundance of the different forms varied between tissues, suggesting control of expression. Interestingly, an ~2 kb transcript appeared to be specific for testis. Mouse mastocytoma cells express a predominant ~3 kb mRNA (Figure 5B).

DISCUSSION

The 2-O-sulfation of IdoA residues is a prominent polymer modification reaction in the biosynthesis of heparin and HS (*3*). IdoA(2-OSO$_3$) units thus are abundant throughout the heparin molecule, and in the N-sulfated domains of most HS species (*47*, *50*), and have been directly implicated in binding of a variety of proteins (see the introductory section). Less is known about the distribution and functional role of 2-O-sulfated GlcA residues. They are generally much less

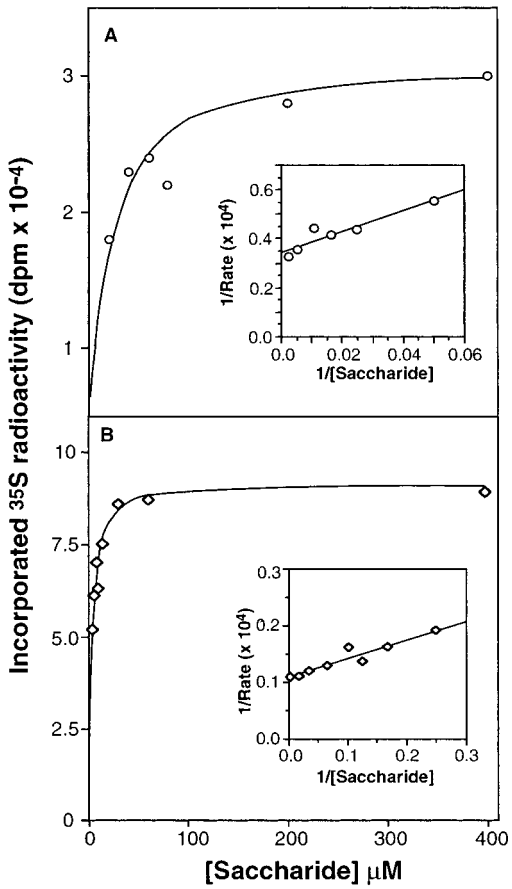

FIGURE 4: Substrate concentration dependence of 2-OST activity. Cell lysate from 2-OST transfected cells was incubated with [^{35}S]PAPS and different concentrations of fully N-sulfated K5 polysaccharide (A) or O-desulfated heparin (B) (see Experimental Procedures). The ^{35}S incorporation values shown represent averages of duplicate incubations. K_m values were calculated from inverted plots as shown in the insets.

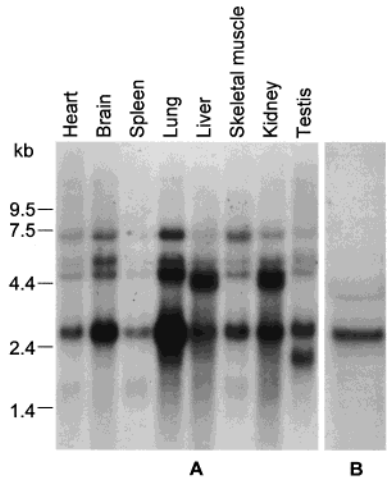

FIGURE 5: Expression of the 2-*O*-sulfotransferase mRNA in mouse tissues. A mouse multiple-tissue Northern blot (Clontech) (A) and mouse mastocytoma total RNA (B) were hybridized with a 217 bp ^{32}P-labeled probe recognizing the coding region of the mouse mastocytoma transcript as described in Experimental Procedures. The filter was washed at 50 °C in 0.1× SSC [SSC is 0.15 M NaCl and 0.015 M sodium citrate buffer (pH 7.0)] and 0.1% SDS. Sizes of RNA markers in kilobases are given.

abundant than the IdoA(2-OSO_3) units and may be virtually absent. There is no correlation between the extent of sulfation of the two types of HexA residues. Still there are indirect observations to suggest that also 2-O-sulfated GlcA residues are endowed with specific biological functions. One such finding is the consistent, abundant occurrence of GlcA(2-OSO_3) units in HS from human cerebral cortex, in particular, the constant proportion of this constituent in HS preparations derived from different subjects (*34*). Moreover, one of the GlcN 3-OST isoforms (3-OST-2) described so far appears to preferentially incorporate sulfate residues at C3 of $GlcNSO_3$ units located adjacent to 2-O-sulfated GlcA residues (*25*). It is of interest to note that this particular 3-OST species seems to be preferentially expressed in the brain (*51*). In view of such observations, it would seem reasonable to predict that 2-O-sulfation of GlcA and IdoA residues is differentially regulated. It was therefore surprising to find that the 2-O-sulfation of GlcA and IdoA residues is catalyzed by a single enzyme. A similar situation apparently applies to the sulfation of HexA residues in dermatan sulfate biosynthesis (*52*). These findings raise the question as to how differential regulation of GlcA and IdoA 2-O-sulfation may be achieved. While we have no definite resolution to this problem, some pertinent experimental observations should be noted.

The 2-O-sulfation of IdoA units may occasionally occur adjacent to *N*-acetylated GlcN residues in heparin (*53*) and HS (*50*), but is largely confined to contiguous N-sulfated domains. The findings of our study indicate that sulfation of GlcA units also occurs preferentially, or exclusively, in the latter regions. What factors determine the selection of target units during biosynthetic polymer modification? Given a choice between the two types of acceptor residues, as in the incubations with partially C5-epimerized, N-sulfated K5 polysaccharide, the 2-OST shows virtually exclusive preference for IdoA targets. The cause of this exclusivity is not obvious, and is not readily explained in terms of the rather modest differences in K_m and V_{max} recorded for polysaccharide substrates containing either of the C5 epimers. Under conditions of substrate saturation, the GlcA residues in (nonepimerized) K5-derived substrates were 2-O-sulfated almost as efficiently as the IdoA units in O-desulfated heparin (Tables 1 and 2 and Figure 1).

These considerations may be relevant to the regulation of HexA 2-O-sulfation in vivo. We recently found, unexpectedly, that overexpression of the 2-OST in 293 cells resulted in a dramatically increased level of 2-O-sulfation of GlcA units, along with decreased levels of N-sulfation and IdoA formation (*36*). In view of our findings presented here, these results may possibly be explained in terms of the mode of interaction of the overexpressed 2-OST with the polysaccharide substrate in the Golgi. If the enzyme encounters the polysaccharide after (or along with) N-deacetylation/N-sulfation but before GlcA C5-epimerization, the situation will be somewhat analogous to the incubations performed in the present study, using partially or completely N-sulfated K5 polysaccharide as a substrate. Also in the native, nonmanipulated cell, the regulation of HexA 2-O-sulfation could indeed depend on the timing of the interaction between the 2-OST and the polysaccharide substrate. An encounter before the C5-epimerization stage would result in sulfation of GlcA units, whereas after C5-epimerization, IdoA would be the preferred target. The partial inhibition of the N-deacetylation/N-sulfation and C5-epimerization reactions observed fol-

lowing 2-OST overexpression (*36*) in fact supports the notion of early contact between the 2-OST and the polysaccharide.

The occurrence of genetically distinct isoforms of the biosynthetic enzymes presumably reflects the need for differential regulation of the corresponding reactions, and thus could be rationalized in terms of structural differences between polysaccharide products, such as heparin and HS. However, no simple relations have emerged from comparative analyses of different cell types. For instance, one of the *N*-deacetylase/*N*-sulfotransferase isoforms, originally thought to be restricted to heparin-producing mast cells, was recently found to be expressed in most other cells and tissues (*54*, *55*). Nevertheless, mice deficient in this particular isoform lack heparin in their mast cells, but produce HS of apparently normal structure (*5*). Contrary to the *N*-deacetylase/*N*-sulfotransferase (*23*, *54*), 6-OST (*26*), and the 3-OST enzyme families (*25*), the GlcA C5-epimerase (*19*) and the 2-OST so far have been found to exist in only one form each. Pending the demonstration of additional forms, we must consider the possibility that the epimerase accounts for the total formation of IdoA units and the 2-OST for the total 2-O-sulfation of GlcA and IdoA residues, in heparin as well as in HS. In accord with this notion, the major ~3 kb 2-OST mRNA species found on Northern blot analysis of mast cell RNA similarly appears to constitute an abundant message also in other mouse tissues (Figure 5).

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