Substrate specificity of 6-O-endosulfatase (Sulf-2) and its implications in synthesizing anticoagulant heparan sulfate

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Heparan sulfate (HS) 6-O-endosulfatase (Sulf) catalyzes the hydrolysis of 6-O-sulfo groups from HS polysaccharides. The resultant HS has reduced sulfation levels and displays altered biological activities. The Sulfs have been associated with several cancers and developmental problems and could function as a tool for editing specific HS structures. Here, we characterize the substrate specificity of human Sulf-2 using site-specifically radiolabeled synthetic polysaccharides. The enzyme was expressed and harvested from the conditioned medium of Chinese hamster ovary cells transfected with Sulf-2 expression plasmids. The uniquely [35S]sulfated polysaccharides were prepared using purified recombinant HS biosynthetic enzymes. We found that Sulf-2 is particularly effective in removing the 6-O-sulfo group residing in the trisulfated disaccharide repeating unit comprising 2-O-sulfated uronic acid and N-sulfated 6-O-sulfo glucosamine, but can also hydrolyze sulfo groups from N- and 6-O-sulfated disaccharides. In addition, we found that Sulf-2 treatment significantly decreases HS’s ability to bind to platelet factor 4 (PF4), a chemokine, while binding to antithrombin is maintained. Because HS–PF4 complexes are the initiating cause of heparin-induced thrombocytopenia, this finding provides a promising strategy for developing heparin therapies with reduced side effects. Further understanding of Sulf-2 activity will help elucidate HS structure–function relationships and provide a valuable tool in tailoring HS-based anticoagulant drugs.

Keywords: heparan sulfate / heparin / substrate specificity / sulfatase

Introduction

Heparan sulfate (HS) is a glycosaminoglycan present on the surface of all animal tissues as well as within the extracellular matrix. As the body’s most negatively charged molecule, it interacts with numerous proteins and has a role in many physiological processes, including cell proliferation, coagulation and the immune response (Rosenberg et al. 1997; Esko and Selleck 2002; Taylor and Gallo 2006). A special highly sulfated form of HS, heparin, is isolated from porcine intestine and is the world’s most widely used anticoagulant drug. Heparin activates antithrombin (AT), which prevents the action of thrombin on fibrinogen and the formation of blood clots (Rosenberg et al. 1997). Heparin is very effective but has several concerning drawbacks, including contamination issues like those causing the wide-spread heparin recall in 2008 (Guerrini et al. 2009) and potentially deadly side effects like heparin-induced thrombocytopenia.

HS exists as a linear polysaccharide attached to a protein core by a tetrasaccharide linkage region. The carbohydrate chain is made up of repeating disaccharide units of either gluconic or iduronic acid (GlcA/IdoA) and glucosamine (GlcN). These disaccharides can be decorated at several positions with sulfo groups, and the GlcN residue can be unsubstituted, acetylated or sulfated. These modifications occur during HS biosynthesis within the cell. The location of the sulfo groups and the position of IdoA are crucial for the biological functions of HS.

HS biosynthesis occurs primarily in the Golgi apparatus by series of specialized enzymes (Tumbull et al. 2001). First, a core linkage region consisting of xylose-galactose-galactose-GlcA is attached to the core protein, and HS polymerase synthesizes a linear polysaccharide chain consisting of alternating GlcA and GlcNAc (N-acetyl glucosamine) units (Lind et al. 1998). Next, a series of biosynthetic enzymes modify the carbohydrate chain. N-deacetylase/N-sulfotransferase (NST) converts GlcNAc to GlcNS, and C5-epimerase (C5-epi) converts some GlcA residues to IdoA. 2OST (2-O-sulfotransferase) adds sulfation to the 2-O position of uronic acid residues, and 6OST and 3OST sulfate GlcNS sugars (Lindahl et al. 1998; Feyerabend et al. 2006). After synthesis, mature HS chains can be modified by other enzymes such as hepananase and sulfatases. HS and heparin differ in their amount of sulfation (0.6 vs 2.6 sulfo groups per disaccharide) and their iduronic acid content (20 vs 80%) (Peterson et al. 2009).

A novel class of sulfatase enzymes known as Sulfs (6-O-endosulfatases) was found to have endosulfatase activity...
and remove 6-O-sulfo groups from HS (Dhoot et al. 2001; Morimoto-Tomita et al. 2002). Two Sulf enzymes (Sulf-1 and Sulf-2) have been identified, cloned and expressed in several cell lines (Dhoot et al. 2001; Morimoto-Tomita et al. 2002; Ohto et al. 2002; Nagamine et al. 2005; Ai et al. 2006). The two Sulfs appear to be functionally redundant and have varied roles in cell signaling, development and cancer. The Sulfs promote some signaling pathways, such as Wnts (Dhoot et al. 2001; Ai et al. 2003; Tang and Rosen 2009), bone morphogenetic protein (Viviano et al. 2004) and glial cell-derived neurotrophic factor (Ai et al. 2007) while inhibiting others, such as fibroblast growth factor-2 (Dai et al. 2005; Lamannaa et al. 2007) and transforming growth factor-β (Yue et al. 2008). Gene knockdown (Dhoot et al. 2001) and knock-out studies (Ai et al. 2007; Holst et al. 2007; Lum et al. 2007; Ratzka et al. 2008) have shown the importance of Sulfs in development; these mice show aberrant growth, muscle innervation, skeletal tissue and lung development. In cancer, Sulfs are believed to possess both pro-oncogenic (Morimoto-Tomita et al. 2002; Nawroth et al. 2007; Lai et al. 2008) and tumor suppressing (Danenberg et al. 2003; Lai et al. 2004; Dai et al. 2005) activities. Overexpression of Sulf-2 in particular was recently found to promote carcinogenesis in non-small-cell lung carcinomas, pancreatic cancer and hepatocellular carcinoma (Lemjabbar-Alaoui et al. 2010; Rosen and Lemjabbar-Alaoui 2010). Sulf-2 also regulates receptor tyrosine kinase pathways and tumor growth in glioblastoma (Phillips et al. 2012), making it an attractive target for cancer therapy.

The Sulfs are understood to cleave 6-O-sulfo groups from trisulfated (N-2-O- and 6-O-) disaccharides (Morimoto-Tomita et al. 2002). However, the extent of their substrate specificity and their ability to recognize other disaccharides is not well understood, largely due to the fact that polysaccharides with defined sulfation types were not available. In previous studies, only substrates containing this trisulfated motif have been used to test Sulf-2 activity (Morimoto-Tomita et al. 2002; Saad and Leary 2003). Recently, we demonstrated the control of the sulfation types in HS polysaccharides using bio-synthetic enzymes (Chen et al. 2007), enabling us to investigate the substrate specificity of Sulfs in greater detail. Here, we report the substrate specificity of human Sulf-2 using enzymatically synthesized [35S]sulfated polysaccharides that allowed us to test Sulf-2’s ability to recognize other disaccharide motifs. In addition, for the first time, we have investigated the effect of Sulf-2 treatment on the ability of HS to bind to AT and PF4. The interactions between HS and these two proteins are important for controlling the anticoagulant activity and side effects of heparin, respectively. Further characterization of Sulf-2 and its effect on HS-protein interactions will serve as a valuable tool for modulating HS structures and generating specific bioactive polysaccharides for medical applications.

Results

Expression of an active Sulf-2 enzyme

The Sulf-2 enzyme was transiently expressed in Chinese hamster ovary (CHO) cells with the goal of obtaining the Sulf-2 protein for the substrate specificity study. The recombinant Sulf-2 enzyme was detected in both the cell lysate as well as in the conditioned medium as measured by western analysis (Figure 1D). The recombinant Sulf-2 enzyme was harvested from the conditioned medium of transfected CHO cells. To test the activity of the crude protein, [35S]HS isolated from CHO cells that had been metabolically labeled with sodium [35S]sulfate was subjected to Sulf-2 treatment. The resultant HS was digested with heparin lyases to disaccharides for disaccharide compositional analyses. The high-performance liquid chromatography (HPLC) chromatograms of the disaccharide analysis of Sulf-2-treated, empty vector-treated and untreated [35S]HS samples (Figure 1) show a clear decrease in the trisulfated ΔUA2S-GlcNS6S peak in the Sulf-2-treated sample with an increase in the corresponding free [35S]sulfate and ΔUA2S-GlcNS peaks. This suggested that the enzyme was in fact active, and it could act to desulfate 6-O-sulfo groups located on UA2S-GlcNS6S disaccharides as described in previously published reports (Morimoto-Tomita et al. 2002; Saad and Leary 2003).

Preparation of substrates with different sulfation groups

Having determined that Sulf-2 was active, we sought to determine with greater detail which polysaccharide substrates could function as substrates for the enzyme. To this end, a series of polysaccharides carrying different sulfation types with or without IdoA were prepared using an enzymatic approach, as demonstrated in Figure 2. A [35S]-label was strategically introduced to a specific site by a sulfotransferase, facilitating the identification of which sulfo groups were removed by Sulf-2. For example, construct 1, IdoA2S-[6-O-[35S]GlcNS6S, carried the 6-O-[35S]sulfo group at the N,6-O-sulfo glucosamine (GlcNS6S) unit. Thus, a release of [35S]sulfo groups from construct 1 after Sulf-2 treatment unambiguously indicated that a 6-O-desulfation reaction occurred. Construct 1 was prepared by incubating deacetylated heparosan with unlabeled 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and NST, 2OST and C2-epi in subsequent steps. This compound was [35S]-labeled at the 6-O position using [35S]PAPS and 6OST. The rest of the constructs (2–9) were prepared in a similar fashion using recombinant enzymes, as shown in the synthetic scheme (Figure 2). The structure of each substrate was confirmed by disaccharide analysis as described previously (Peterson and Liu 2010).

A disaccharide analysis of the Sulf-2-treated substrate was also performed for each construct. The HPLC analysis of untreated and Sulf-2 treated construct 1 is shown as an example in Figure 3. The presence of the trisulfated peak, ΔUA2S-GlcNS6S, is nearly completely eliminated by treatment with the Sulf-2 enzyme. The reduction in this peak is balanced by the appearance of a large free sulfate peak in the treated sample. In addition, there is a partial decrease in the ΔUA-GlcNS6S peak that contributed to the free sulfate peak.

To quickly quantify the activity of Sulf-2 against the different substrates, spin column assays were performed. Here, the column separates the analytes based on the size of the molecule. We anticipated that the [35S]sulfate would be trapped in the column while the intact polysaccharides would pass through the column without retardation. Substrates were incubated overnight in the presence of Sulf-2 or water as a control, and the samples...
were purified by QuickSpin column to determine how much $^{35}$S-radioactivity had been converted to smaller $[^35]$Sulfate. The results from the spin column assays are shown in Table I. From these assays, it is clear that the best substrate for Sulf-2 is the polysaccharide containing N-, 2-O- and 6-O-sulfations. Further analysis of the susceptibility of the different sulfated polysaccharides to $[^35]$Sulfate release permitted us to dissect the substrate specificities of Sulf-2.

Effect of other sulfogroups in addition to 6-O-sulfation
Constructs 2–4 were prepared to examine the effect of other sulfogroups on the cleavage of 6-O-sulfation. IdoA-[6-O-$^{35}$S]GlcNS6S (construct 2) showed a 24.3 ± 2.7% decrease in $[^35]$S-labeled groups compared with 8.3 ± 0.1% in the control sample. These two substrates indicate that Sulf-2 is able to desulfate the 6-O-sulfogroup on the GlcNS6S that is flanked by a non-reducing-end GlcA or IdoA residue. Construct 4, GlcA-[6-O-$^{35}$S]GlcNA6S, which lacked N-sulfation, was not a very effective substrate, showing 28.1 ± 1.3% cleavage compared with 14.2 ± 3.5% in the control. Despite the fact that Sulf-2 can hydrolyze both constructs 2 and 3 (containing -IdoA-GlcNS6S- and GlcA-GlcNS6S-, respectively), Sulf-2 most effectively desulfates the 6-O-sulfation that is located in trisulfated disaccharide repeating regions in which N- and 2-O-sulfation are also present.

The reactivity of Sulf-2 toward other sulfation types
To determine whether Sulf-2 could hydrolyze sulfogroups from positions other than the 6-O position, constructs 5–9 were synthesized. Constructs 5 and 6 were $^{35}$S-labeled at the
2-O position and also contained N-sulfation. Unlike 6, construct 5 was synthesized using C5-epi and thus contained IdoA. Both of these substrates showed negligible 35S sulfate release when treated with Sulf-2 (12.6 ± 1.3 and 13.7 ± 3.0% compared with 10.4 ± 2.6 and 6.7 ± 1.9% in the control, respectively). Constructs 7 and 8 were 35S-labeled at the 3-O position, and construct 9 was labeled at the N position (Table I). These substrates also showed no decrease in 35S-labeled groups after Sulf-2 treatment. From these results, we conclude that only 6-O-sulfation can be removed by the Sulf-2 enzyme. We also investigated the effect of epimerization on Sulf-2 recognition. Although GlcA2S-[6-O-35S] GlcNS6S proved difficult to prepare, based on the results of substrates 2 and 3, it appears that Sulf-2 is able to desulfate both GlcA- and IdoA-containing disaccharides.

Fig. 2. Synthetic scheme for the 35S-labeled HS constructs. Heparosan or HS were treated with HS biosynthetic enzymes and PAPS to achieve the desired sulfation groups and epimerization of the uronic acid. In HS, both GlcA and IdoA are present. 35S-labeled groups are shown in bold.

Effect of Sulf-2 treatment on the affinity of HS to AT binding
HS interacts with AT to inhibit the activity of thrombin and factor Xa and regulate the blood coagulation cascade. Thus, we were interested to know whether Sulf-2 treatment would affect the binding of HS to AT. AT-binding HS was prepared by incubating HS from the bovine kidney with 3OST-1 and [35S]PAPS. A Sulf-2-treated fraction was prepared by incubating this material with Sulf-2. Concanavalin A (ConA)-Sepharose beads were incubated with AT and HS, and the AT-bound HS fraction was eluted using a 1-M NaCl solution (Figure 4). When the treated and untreated fractions were incubated with ConA-Sepharose and AT, 51.2% of the untreated fraction and 43.6% of the Sulf-2-treated fraction were recovered, suggesting that Sulf-2 does not remove critical 6-O-sulfo groups from the AT-binding pentasaccharide within the polysaccharide. [N-35S]HS, which does not considerably bind AT, was used as a negative control.

Effect on Sulf-2 treatment on HS-PF4 binding
In addition to AT, the binding of heparin to PF4 has significant clinical relevance. PF4-HS-immunoglobulin G complexes initiate an immune response known as heparin-induced
thrombocytopenia, a dangerous and deadly side effect of heparin (Arepally and Ortel 2010). Thus, we examined whether Sulf-2 could decrease the binding of HS to PF4. For these studies, we used [35S]HS from CHO cells as our untreated material due to its large number of radioactive sulfo groups per unit of HS.

A dot blot membrane binding assay was used to compare the PF4 binding capabilities of Sulf-2-treated and untreated HS (Figure 4A). The wells contained 6000 cpm of Sulf-2-treated or untreated [35S]HS with increasing amounts of PF4. [35S]HS bound to PF4 was captured by the nitrocellulose membrane. The untreated samples reached a maximum binding of 66.3% with 152 nM PF4, but the Sulf-2-treated samples bound only up to 3.1% with 608 nM PF4. From the two binding curves, it is apparent that Sulf-2 treatment can reduce the binding of HS to PF4 by over 10-fold.

For a more quantitative assessment of the effect of Sulf-2 treatment on the binding affinities of HS to AT and PF4, affinity co-electrophoresis was performed. This is an established method for determining the affinity constant of radiolabeled polysaccharide ligands with proteins (Lee and Lander 1991). The mobility of each lane is determined, and the protein concentration in each lane can be used to determine a $K_d$ value based on the Scatchard equation. For AT, gels containing serial dilutions from 0–3.2 µM AT were prepared and 50,000 cpm of the AT-binding fraction of 3OST-1-treated [35S]HS from CHO cells was added. The gel was run for 2 h, dried overnight, imaged and analyzed using ImageQuant TL software. For the untreated and treated samples, a plot of $R/[AT]$ vs $R$ gave linear slopes of $y = -0.0949x + 0.048$ ($R^2 = 0.87$) and $y = -0.094x + 0.44$ ($R^2 = 0.95$), respectively. These correspond to $K_d$ values of 10.53 and 10.59 nM, indicating that the binding affinity of HS to AT is unaffected by treatment with Sulf-2.

For PF4, serial dilutions of 0–1.41 µM PF4 were used, and 50,000 cpm of 3OST-1-treated [35S]HS from CHO cells was added to each gel. The gels were run for 2.5 h and analyzed as above. Linear regression slopes of $y = -0.1627x + 0.1238$ ($R^2 = 0.77$) and $y = -0.0085x + 0.0037$ ($R^2 = 0.74$) were calculated for the untreated and treated substrates, respectively. These correspond to $K_d$ values of 5.47 and 117.6 nM, showing that the binding affinity of HS for PF4 is decreased $\approx 20$-fold by treatment with Sulf-2. Taken together, our data suggest that Sulf-2 treatment decreases the binding affinity of HS to PF4, while the affinity to AT remains intact.
Disaccharide analysis of AT-binding HS with and without Sulf-2 treatment

We compared the disaccharide composition of 3-O-[35S]sulfated HS with and without Sulf-2 treatment (Figure 5). The 3-O-sulfated glucosamine residue is known to be present at the center of the AT-binding site in HS (Liu et al. 1996). The disaccharide analysis revealed the presence of three 3-O-sulfated disaccharides: Glc-AnMan3S, IdoA2S-AnMan3S and GlcA-AnMan3S6S. We observed that the level of the disaccharide GlcA-AnMan3S6S in the Sulf-2-treated sample was decreased to 38.3% from 51.4%, suggesting that Sulf-2 removed a 6-O-sulfo group from the 3-O-sulfated glucosamine residue. However, the removal of this 6-O-sulfo group from the 3-O-sulfated glucosamine residue has no impact on the binding affinity to AT. This observation is consistent with the fact that AT-binding HS is composed of a disaccharide unit of -GlcA-GlcNS3S- (without a 6-O-sulfo group in the disaccharide unit) and -GlcA-GlcNS3S6S- (with a 6-O-sulfo group in the disaccharide unit) (Colliec-Jouault et al. 1994).

Discussion

The Sulfs are interesting enzymes due to their involvement in development and cancer and their potential role in preparing HS structures. Although some initial studies of Sulf-2 activity have been carried out, the full substrate specificity of Sulf-2 had not been elucidated. To address this issue, we synthesized

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<tr>
<td>1</td>
<td>[-IdoA2S-[6-O-[35S]GlcNS6S-]n</td>
<td>1.7</td>
<td>2.7 ± 1.0</td>
<td>97.5 ± 0.8</td>
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<tr>
<td>2</td>
<td>[-IdoA-[6-O-[35S]GlcNS6S-]n</td>
<td>1.9</td>
<td>8.3 ± 0.1</td>
<td>24.3 ± 2.7</td>
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<tr>
<td>3</td>
<td>[-GlcA-[6-O-[35S]GlcNS6S-]n</td>
<td>1.4</td>
<td>6.6 ± 1.6</td>
<td>43.1 ± 2.4</td>
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<tr>
<td>4</td>
<td>[-GlcA-[6-O-[35S]GlcNAc6S-]n</td>
<td>0.1</td>
<td>14.2 ± 3.5</td>
<td>28.1 ± 1.3</td>
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<tr>
<td>5</td>
<td>[-2-O-[35S]IdoA2S-GlcNS-]n</td>
<td>1.1</td>
<td>10.4 ± 2.6</td>
<td>12.6 ± 1.3</td>
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<tr>
<td>6</td>
<td>[-2-O-[35S]GlcA2S-GlcNS-]n</td>
<td>1.3</td>
<td>6.7 ± 1.9</td>
<td>13.7 ± 3.0</td>
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<tr>
<td>7</td>
<td>3-O-[35S]HS (consisting of -GlcA-[3-O-[35S]GlcNS3S ± 6S-domain)</td>
<td>1.1</td>
<td>13.6</td>
<td>12.2</td>
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<tr>
<td>9</td>
<td>N-[35S]sulfated HS</td>
<td>1.6</td>
<td>11.7</td>
<td>13.2</td>
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Synthetic substrates were incubated with or without the Sulf-2 enzyme and isolated from released sulfate groups by QuickSpin column. The remaining [35S] sulfate on the polysaccharide was quantified with a scintillation counter.
Characterization of heparan sulfate 6-O-endosulfatase

99 HS polysaccharides with unique sulfation patterns and 35S-labeled groups at a specific position. Using these polysaccharides, we were able to determine which constructs acted as substrates for Sulf-2 based on their decrease in radioactivity after Sulf-2 treatment. We found that, as previously understood, trisulfated IdoA2S-[6-O-35S]GlcNS6S regions served as an excellent substrate for Sulf-2, showing ~90% removal of the radiolabeled group. In addition, this study found for the first time that disulfated UA-[6-O-35S]GlcNS6S units experienced the hydrolysis of 6-O-sulfo groups. Sulfation at positions other than the 6-O position was unable to be removed by the enzyme. These results were determined by spin column assay and by HPLC analysis.

We also examined how Sulf-2 treatment affected binding to AT and PF4. A major application of synthetic HS is that it can be used to make heparin-like drugs. For heparin to have anticoagulant activity, it is essential that it interact with AT, which occurs through a specific AT-binding pentasaccharide: GlcNAc/NS6S-GlcA-GlcNS3S6S-IdoA2S-GlcNS6S. It appears from our results that Sulf-2 removes 6-O-sulfo groups that are not within the critical AT-binding region, allowing HS to maintain its interaction with AT. It should be noted that the importance of 6-O-sulfation for AT-binding is well documented. Atha et al. (1985), using a series of synthetic pentasaccharides, demonstrated that a particular 6-O-sulfo group on the non-reducing end side of the 3-O-sulfated glucosamine residue contributed the AT binding energy nearly equal to that of the 3-O-sulfo group. At the polysaccharide level, 6-O-sulfation is also important for AT-binding (Zhang et al. 2001; Chen et al. 2007). The unique substrate specificity of Sulf-2 allows the removal of 6-O-sulfo groups that are non-essential for AT binding.

Reducing dangerous side effects is a major goal for new heparin therapies. Currently, heparin-induced thrombocytopenia is one of the most clinically important drug complications, affecting ~3% of patients receiving unfractionated heparin (Arepally and Ortel 2010). Heparin–PF4 complexes are recognized by IgG molecules, which initiate an immune reaction that ultimately leads to platelet degradation and uncontrolled bleeding. For this reason, we were interested to determine whether Sulf-2 treatment could affect the binding of HS to PF4. Based on a ligand binding assay and affinity co-electrophoresis, we found that Sulf-2 can reduce binding to PF4 on the order of 10–20-fold. Heparin exhibits its anticoagulant activity by binding to AT, and the complex inhibits the activity of factors Xa and IIa. We anticipate that Sulf-2 treatment should not affect the anti-Xa activity because anti-Xa activity is directly correlated with AT-binding affinity. However, we do not know whether Sulf-2 treatment affects its anti-IIa activity as the structural requirements for anti-IIa activity are unknown at the present time.

Given that binding to AT is maintained with Sulf-2 treatment, the decrease in PF4 binding could have exciting implications in the preparation of a new generation of heparin drugs that have reduced side effects. In addition, an extended understanding of the substrate specificity of Sulf-2 could be beneficial for the treatment of cancers in which this enzyme is up-regulated.

Materials and methods

Preparation of 35S-labeled polysaccharides

Radiolabeled polysaccharide substrates 1–6 were prepared using ~1 µg heparosan, a capsular polysaccharide isolated from the Escherichia coli K5 strain, as a starting material (Vann et al. 1981). Substrates 7–9 were prepared from bovine kidney HS. The starting materials were modified with 5–10 µg of C5-epi, NST, 6OST-1/3, 2OST, 3OST-1 and 3OST-5 (as indicated in Figure 2) in sequential 200-µL reactions containing approximately 1 × 106 cpm [35S]PAPS and 10 nmol unlabeled PAPS in 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 7) and 0.5% Triton X-100. The enzymatic reactions were incubated at 37°C for 60 min, heat inactivated and purified using a diethylaminoethyl (DEAE) column (Vann et al. 1981).
Expression of recombinant Sulf-2 in CHO cells

A plasmid consisting of a full-length cDNA encoding human Sulf-2 was purchased from Open Biosysmtem (Clone ID #7969293). The gene was cloned into a pcDNA3.1A mammmalian expression vector (from Invitrogen, Carlsbad, CA) using XhoI/HindIII sites to obtain the Sulf-2 expression plasmid designated as pcDNA3.1A-myc/His-HSulf-2. Wild-type CHO cells were transiently transfected with the expression plasmid pcDNA3.1A-myc/His-HSulf-2 or an empty pcDNA3.1A vector according to a standard protocol. Briefly, CHO cells were seeded in 6-well plates in F-12 media supplemented with 10% fetal bovine serum (FBS) and were maintained in a 5% CO2 humidified incubator at 37°C. When the cells reached 90–95% confluence, they were transfected using Lipofectamine 2000 reagent (Invitrogen) and Opti-MEM Reduced Serum Media (Invitrogen) according to the manufacturer’s protocol. After 4-6 h, the medium was replaced with F-12 media containing 10% FBS. The conditioned medium was collected after 48–72 h of incubation and centrifuged at 3300 × g for 15 min to remove cellular debris.

Western blotting

Sulf-2- and EV-transfected cells were collected using trypsin and washed with phosphate-buffered saline. Cells were lysed with 1.5 M sucrose, 1% phenylmethylsulfonyl fluoride. Sulf-2 and EV conditioned medium (CM) and lysates were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and visualized with an anti-myc primary antibody (Cell Signaling Technology) and the SuperSignal detection system (Thermo Scientific), revealing a band of ~132 kDa in the Sulf-2 samples (Morimoto-Tomita et al. 2002).

Sulf-2 enzymatic assay

A 100-µL reaction, containing 35S-labeled substrate, 50 mM MES (pH 6.5), 10 mM CaCl2, 0.1% Triton X-100 and 50 µL of Sulf-2 enzyme, was incubated overnight at 37°C. Sulf-2-treated and untreated polysaccharide substrates were purified using Quick Spin Columns for radiolabeled DNA purification (Roche, Indianapolis, IN). Columns were centrifuged at 1100 × g for 2 min to remove the column buffer, then placed into a fresh collection tube and loaded with 50 µL of reaction mixture. After centrifugation for 4 min at 1100 × g, the eluate was collected and the amount of 35S-labeled polysaccharide was quantified using a scintillation counter.

Analysis of synthetic polysaccharides

To determine the structural compositions of the radiolabeled substrates, disaccharide analyses were performed using heparin lyase digestion. Polysaccharide substrates were incubated overnight with a mixture of heparin lyases I, II and III (0.1 mg/mL each) in 200 µL of 50 mM sodium phosphate (pH 7) at 37°C. The reaction was terminated by boiling at 100°C for 5 min and was loaded onto a Bio-Gel P-2 column (Bio-Rad, Hercules, CA) to isolate disaccharides. These disaccharides were analyzed using reverse-phase ion-pairing HPLC as described previously (Duncan et al. 2006).

Preparation of 35S HS from CHO cells

A T-75 flask of wild-type CHO cells was grown to confluence in F-12 media supplemented with 10% FBS. The cells were then incubated with 1 mL of 1.0 mM sodium [35S]sulfate (Perkin Elmer, Boston, MA) for 6 h at 37°C with 5% CO2. Two hundred microliters of a pronase stock solution containing 1 mg/mL pronase (Sigma, St. Louis, MO), 240 mM NaAcO (pH 6.5) and 1.92 M NaCl was added to the cells, and the flask was incubated overnight at 37°C. The pronase-digested sample was centrifuged at 10,000 rpm for 15 min and filtered using a 0.45-µm filter, then purified using a DEAE-Sepharcel column (Sigma), which was equilibrated using a buffer containing 20 mM NaAcO (pH 5) and 150 mM NaCl. The [35S]HS was eluted from the column with 1 M NaCl in 20 mM NaAcO and was dialyzed overnight against 50 mM ammonium bicarbonate using a 14,000 MWCO membrane, then dried. The sample was reconstituted in 1 mL of water, and 10 µL of a solution containing 10 N NaOH and 0.89 M sodium borohydride was added to break the linkage between the core protein and HS. It was incubated at 46°C for 16 h. The sample was also treated with 20 U/mL chondroitinase ABC to remove chondroitin sulfate before use.

Preparation of 3OST-1-treated [35S]HS

To 3-O-sulfate [35S]HS using unlabeled PAPS for affinity co-electrophoresis, ~100,000 cpm of [35S]HS was added to a 50-µL reaction containing 0.05 ng of 3OST-1, 10 mM MnCl2, 5 mM MgCl2, 75 µg/mL protamine chloride, 0.4 mg/mL chondroitin sulfate, 0.12 mg/mL bovine serum albumin, 1% Triton X-100 and 0.5 mM PAPS. The reaction was incubated at 37°C for 20 min, inactivated at 80°C for 10 min, then diluted with 60 µL of H2O and centrifuged at 2000 × g for 10 min. Substrates used for AT-binding studies were then isolated using a ConA-Sepharose column (Sigma).

Preparation of mPF4

PF4 and tobacco etch virus protease expression plasmid. The full-length murine PF4 cDNA was obtained from Open Biosystems (Clone ID: 582960). The heparin-binding domain of PF4 (Ala33-Ser105) was cloned into a PET32 vector (Novagen) using NcoI and HindIII sites to give a plasmid named as PF4-PET32 (Novagen) using NcoI and HindIII sites to give a plasmid named as PF4-PET32/ tobacco etch virus protease (TEV). In this plasmid, a TEV cleavage hexapeptide sequence, EQLYFQG, was constructed between thioredoxin and PF4. The design permitted cleavage of the thioredoxin–PF4 fusion protein to release PF4. The resultant recombinant PF4 protein has five extra amino acid residues (GSRHG) at the N terminus. A bacterial strain, BL21 (DE3)-RIL/pRK793, expressing TEV protease was a generous gift from Dr Lars Pedersen (National Institute of Environmental Health Sciences).

The PF4-PET32/TEV plasmid was introduced into BL21 cells, and the cells were grown in LB medium containing 50 µg/mL of carbenicillin and induced with isopropyl β-D-thiogalactopyranoside. The cells were pelleted, lysed in 25 mM Tris, 500 mM NaCl and 300 mM imidazole (pH 7.5) and purified using a nickel column. The fractions containing protein were collected and incubated overnight with TEV protease (1:25 w/w ratio of TEV:PF4). After TEV cleavage, the PF4 was dialyzed against 20 mM Tris and 250 mM NaCl.
Characterization of heparan sulfate 6-O-endosulfatase

Dot blot assay for PF4 binding
A dot blot assay was used to determine the binding affinities of Sulf-2-treated and untreated HS to PF4. A 300-μL reaction containing 130 mM NaCl, 50 mM Tris (pH 7) buffer was incubated with [35S]HS from CHO cells and 0–608 nM PF4 for 30 min at 37°C before being blotted on a nitrocellulose membrane (GE Healthcare). The membrane was washed using the sample buffer, and the spot containing bound HS was cut out to quantify using a scintillation counter.

Affinity co-electrophoresis
Affinity co-electrophoresis gels were prepared using a standard protocol (Lee and Lander 1991) with lanes containing 0–1.41 μM PF4 or 0–3.2 μM AT and ~50,000 cpm 3OST-1-labeled [35S]HS per gel. Gels were run for 2 (AT) or 2.5 (PF4) h and the bands were imaged using a Storm 860 phosphorimager (Molecular Dynamics) and ImageQuant TL software (GE Healthcare Life Sciences). The resultant disaccharides were desalted and concentrated.

AT-binding assay
To quantify the binding of Sulf-2-treated and untreated HS to AT, 3OST-1-labeled substrates were incubated with 0.1 mg/mL AT, and the complex of AT and HS was captured using ConA-Sepharose beads (Sigma). For this experiment, the AT-binding [35S]HS was prepared by incubating HS from bovine kidney with the 3OST-1 enzyme and [35S]PAPS.

Disaccharide analysis of 3-O-[35S]sulfated HS
3-O-[35S] sulfated HS, with or without Sulf-2 treatment, were subjected to the nitrous acid degradation to yield 35S-labeled disaccharides. The HS was deacetylated in a hydrazine solution containing 12.5 mg/mL hydrazine sulfate followed by the degradation with nitrous acid at pH 4.5 and 1.5 as well as sodium borohydride reductions described previously (Shively and Conrad 1976). The resultant disaccharides were desalted on a BioGel P-2 column and resolved on a C18-column (Vydac) under the reverse phase ion-pairing HPLC conditions (Liu et al. 1999).

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Conflict of interest
None declared.

Abbreviations
AT, antithrombin; C5-epi, C5-epimerase; ConA, concanavalin A; CHO, Chinese hamster ovary; CM, conditioned medium; DEAE, diethylaminoethyl; FBS, fetal bovine serum; GlcNS6S, N,6-O-sulfo glucosamine; HPLC, high-performance liquid chromatography; HS, heparan sulfate; MES, 2-(N-morpholino)ethanesulfonic acid; NTS, N-sulfotransferase; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PF4, platelet factor 4; 2OST, 2-O-sulfotransferase; 3OST-1, 3-O-sulfotransferase isoform 1; 3OST-5, 3-O-sulfotransferase isoform 5; 6OST-1/3, 6-O-sulfotransferase isoform 1/isoform 3; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Sulf, 6-O-endosulfatase.

References

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