The functions of heparan sulfate (HS) depend on the expression of structural domains that interact with protein partners. Glycosaminoglycans (GAGs) exhibit a high degree of polydispersity in their composition, chain length, sulfation, acetylation, and epimerization patterns. It is essential for the understanding of GAG biochemistry to produce detailed structural information as a function of spatial and temporal factors in biological systems. Toward this end, we developed a set of procedures to extract GAGs from various rat organ tissues and examined and compared HS expression levels using liquid chromatography/mass spectrometry. Here we demonstrate detailed variations in HS GAG chains as a function of organ location. These studies shed new light on the structural variation of GAG chains with respect to average length, disaccharide composition, and expression of low abundance structural epitopes, including unsubstituted amino groups and lyase-resistant oligosaccharides. The data show the presence of a disaccharide with an unsubstituted amino group and lyase-resistant oligosaccharides. From the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

Xiaofeng Shi and Joseph Zaia
From the Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

The elucidation of structural details as a function of developmental stage, disease state, mutation, and cellular phenotype has led to an evolving understanding of GAG structure-function relationships (14–17). GAGs, like other carbohydrates, are synthesized and modified without templates. Biosynthetic modifications, including epimerization of uronic acid residues, deacetylation, and sulfation of the amino group of the hexosamine residues and sulfation on the hydroxyl groups of both residues, result in tremendous polydispersity of the 15–150 monosaccharide GAG chains (10–12, 18). Further, cells dynamically alter their responses to growth factor stimuli by modulating the structures of expressed GAG chains (10–12, 18). The variety of chemical properties of GAG substructures makes it difficult to produce a comprehensive structural measurement with any given technique. As a result, understanding of GAG structural biochemistry is far from complete.

It has been demonstrated that the formation of unsubstituted glucosamine amino groups in HS is related to limiting availability of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) during enzymatic N-deacetylase/N-sulfotransferase reactions (19). In the absence of PAPS, N-deacetylase/N-sulfotransferase produces deacetylation of GlcNAc residues of the nascent chain without subsequent N-sulfation. Unsubstituted glucosamine has been detected by using amine-reactive fluorescence labeling (20, 21) and deaminative cleavage at pH 3.9 (22–24). A tetrasaccharide containing a GlcA-GlcNH2 unit was identified by nuclear magnetic resonance spectroscopy following chromatographic purification of lyase-depolymerized heparan sulfates (20). Treatment of HS with recombinant 3-O-sulfotransferase created tetrasaccharides containing an IdoA2S-GlcNS3S±6S unit (25–27). Free amino-containing disaccharides in glypican-1 HS chains have been found to be susceptible to nitric oxide (NO)-mediated cleavage as part of the regulation of polyanamine uptake (28, 29).

Access to PAPS is likely to vary in different tissue environments. Therefore, it should be possible to directly detect disaccharide units with unsubstituted glucosamine using disaccharide analysis. Lawrence et al. (30) detected an abundant unsubstituted N-unsubstituted disaccharide in H. vulgaris and D. melanogaster. N-Unsubstituted disaccharides were of very low abundances in cultured Chinese hamster ovary cells and mutants thereof and were not reported in porcine intestinal mucosa heparin and mouse embryos. Wei et al. (31) determined N-unsubstituted disaccharides in commercial samples to comprise 12% of the total (each ranging from 1.9 to 2.2%) from bovine kidney HS and 1.1% of porcine intestinal mucosa HS. Despite these two recent reports, disaccharides with N-un-
substituted disaccharides have often not been reported from endogenous HS isolated from animal tissues in previous studies. We hypothesize that the chemical properties of the amino group on these glucosamine-containing saccharides limit their detection. Glucosamine amino groups will not be detected by deaminative cleavage following deacetylation of heparin or HS chains. Lyase-generated disaccharides with free amino groups are likely to be less acidic than either N-sulfated or N-acetylated disaccharides, and likely to have a zwitterionic character. Such chemical properties may make free amino-containing disaccharides difficult to detect by traditional chromatographic and electrophoretic approaches to disaccharide analysis. We therefore applied a new system based on size exclusion chromatography (SEC) with mass spectrometry (MS) detection to the problem of detection of free amino-containing disaccharides and other products of depolymerized HS to show their distributions in different organ tissues. Due to the nonadsorptive chromatographic mechanism, SEC has great potential for non-selective separation of GAG disaccharides (32).

The Leary group has developed an electrospray ionization-multistage MS platform to profile and quantify HS disaccharides by directly infusing lyase depolymerized HS (33, 34). Our laboratory has developed an LC/MS/MS platform to analyze oligosaccharides derived from chondroitin sulfate from connective tissue (35, 36). Analysis of GAGs from animal organs, however, is more challenging due to higher fat content and variability in fibrous structure. We therefore developed a general, MS-compatible tissue extraction procedure to enable analysis of organ tissues using SEC LC/MS.

The results demonstrate the presence of ΔHexA-GlcNH₂6S disaccharides that have not been detected previously in studies of HS isolated from mammalian organ tissues or embryos. The levels of this disaccharide depends on the tissue of expression, consistent with the hypothesis that conditions whereby limiting PAPS occurs during HS biosynthesis exist in a variety of tissue contexts.

**EXPERIMENTAL PROCEDURES**

**Materials**—Heparin lyase I, II, and III from *Flavobacterium heparinum* were purchased from Ibex Pharmaceuticals (Montreal, Canada). Pronase was purchased from Roche Applied Science, and benzonase was from Sigma. Heparan sulfate disaccharides were from Sigma and V-Labs (Covington, LA). The structure of HS 4,5-(Δ)-disaccharides, together with the nomenclature in this study are shown in Fig. S1. DEAE material packed micro-/macrospin columns were purchased from Harvard Apparatus (Holliston, MA). The rat organs were obtained from the laboratory of Professor Louis Gerstenfeld (Boston University School of Medicine).

**GAG Extraction from Tissues**—Rat organs were isolated and cut into pieces of less than 2 mm². Tissue samples were then suspended in distilled water and homogenized. Powder of tissue samples was obtained after lyophilization. Dry tissue (~20 mg) of each organ, in triplicate, was suspended in 0.5 ml of protein digest buffer (50 mM Tris/HCl, pH 8, 1 mM CaCl₂, 1% Triton X-100) containing 0.5 mg of Pronase and incubated for 48 h at 55 °C with end-over-end mixing. A second aliquot of Pronase was added after 20 h. After heat inactivation of the Pronase, the buffer was adjusted to 2 mM MgCl₂, and benzonase (300 milliunits) was added. The sample was incubated for 2 h at 37 °C, heat-inactivated, adjusted to a final sodium chloride concentration of 0.1 M, and centrifuged at 13,000 × g for 10 min.

For purification of GAGs from tissue digests, macrospin columns (15–30 μg of binding capacity/column) with DEAE resins were used. The columns were saturated by washing with 300 μl of elution buffer (2 mM NH₄HCO₃) and then 3 × 300 μl of loading buffer, pH 8 (50 mM Tris/HCl, pH 8, 0.1 M NaCl, 0.1% Triton X-100). The supernatants of the tissue extracts were applied and allowed to adhere to the column for 10 min. The sample solutions were repipetted through the column once to ensure binding. The columns were washed successively with 6 × 300 μl of loading buffer, 6 × 300 μl of wash buffer, pH 4 (50 mM NaOAc, pH 4, 0.1 M NaCl, 0.1% Triton X-100), a 300-μl volume of H₂O, and 5 × 300 μl of 0.2 M NH₄HCO₃. The elution of GAGs was achieved with 6 × 300 μl of 2 M NH₄HCO₃. The GAG fraction was collected in 2-ml Eppendorf tubes and lyophilized until no visible salt remained (24–36 h).

HS disaccharides may be analyzed in the presence of bulk intact chondroitin sulfate by SEC LC/MS, because these long-chain GAG polysaccharides are well separated from lyase digestion products in this chromatographic mode. Therefore, the GAG pools with both chondroitin sulfate and HS were digested directly with enzymes targeted to the GAG of interest. For the digestion of HS, 20% of the GAG pool was digested with 3.2 milliunits of heparin lyase I, 3.2 milliunits of heparin lyase II, and 1.6 milliunits of heparin lyase III in a final volume of 100 μl of 50 mM Tris/HCl buffer, pH 7.45, in the presence of 1 mM CaCl₂. The HS digestion was incubated at 37 °C for 16 h. The digests were analyzed by SEC LC/MS without further treatment.

**Liquid Chromatography/Mass Spectrometry**—The Size Exclusion Chromatography column (Superdex™ peptide PC 3.2/30) was purchased from GE Healthcare. For disaccharide analysis, the mobile phase (12.5 mM formate acid, pH adjusted to 4.4 by ammonia, in 10% acetonitrile) was delivered isocratically by a Waters Acquity UltraPerformance LC system at 0.015 ml/min. The LC system was plumbed with 0.025-inch ID Peek tubing. A 2.3-μm filter was placed before the column, as well as a 15 cm × 75 μm inner diameter silica capillary, which generates a reasonable high back pressure to avoid possible generation of bubbles. The high pressure liquid chromatography fraction was monitored simultaneously by a UV (2400 nl, 10-mm light path) detector at 232 nm after the SEC column and before the mass spectrometer. A switch valve was placed before the MS ion source to divert unwanted fractions away from the mass spectrometer. Disaccharides and oligosaccharides eluting from the SEC column were analyzed using an Applied Biosystems QSTAR Pulsar-I (Q-ToF) mass spectrometer operating in negative ion mode. The ionization of the LC flow was accomplished by a TurboIonSpray source with nebulizer gas set at 50, curtain gas at 25, turbo gas at 0, and the sprayer position optimized to give least sulfate loss for ΔHexA2S-GlcNS6S standard (<15%).

**RESULTS**

**GAG Extraction and Purification**—The methods developed in the present study were aimed to illustrate and characterize...
FIGURE 1. Extracted HS dp2 ion chromatogram from rat brain tissues analyzed by SEC LC/MS. Top, 4,5-ununsaturated dp2; bottom, saturated dp2. 1, ΔHexA-GlcNAc; 2, ΔHexA-GlcNS; 3, ΔHexA(2S)-GlcNAc (6S); 4, ΔHexA(2S)-GlcNS (6S); 5, ΔHexA2S-GlcNAc6S; 6, ΔHexA2S-Ns6S; 1′, HexA-GlcNAc; 2′, HexA-GlcNS; 3′, HexA(2S)-GlcNAc (6S); 4′, HexA(2S)-GlcNS (6S); 5′, HexA2S-GlcNAc6S; 6′, HexA2S-GlcNS6S.

The structural diversities of GAGs in mammalian organ tissue samples with increased depth of coverage of rare but functionally important substructures. We used Pronase digestion to cleave the protein backbone and at the same time isolate the GAG chain from the protein core of the proteoglycans. This procedure combines protein digestion and GAG isolation in one pot and is therefore time-saving. These studies targeted di- and oligosaccharides generated by exhaustive lyase digestion. In future work, a β-elimination step via alkaline sodium borohydride will be added to release the linker oligosaccharide from the peptide backbone to enable the detection of the linker domain. In the pivotal DEAE weak ion exchange steps, commercially available spin columns were adopted in place of the traditional gravity columns used in previous studies. This modification greatly facilitates ion exchange chromatography and increases the throughput by utilizing a centrifuge that can run up to 16 samples at one time. The inclusion of detergent (Triton X-100) in the Pronase digestion (1%) and DEAE workup step (0.1%) eliminated the need for organic solvent extraction without causing interference by fat and lipids in the mass spectrometry experiments. This is echoed by the miniscale GAG extraction protocol of Ledin et al. (16). As part of an MS-compatible procedure, it is important to remove ammonium salts by thorough lyophilization as an improvement to the repetitive drying in a centrifugal vacuum concentrator in the original procedure. The lyase digest solutions were analyzed by SEC with direct electrospray ionization-MS detection.

SEC LC/MS Analysis of Native HS Disaccharides—SEC has advantages for disaccharide analysis over adsorption chromatographic modes (32, 35, 38). The disaccharides are separated based on hydrodynamic volume, roughly proportional to the number of sulfate groups present. Because SEC is a non-adsorptive chromatography mode, there are no concerns about losses of weakly absorbed disaccharide compositions. For the same reason, oligosaccharides are not bound to the column, and extensive column cleaning and maintenance steps are not necessary. There is no mobile phase gradient in SEC mode; hence, the electrospray conditions are constant for all di- and oligosaccharide chains eluting from the column. In addition, the mobile phase conditions do not have to be optimized for different sizes or compositions of oligosaccharides.

The pH value of the mobile phase was adjusted to 4.4 to allow the ionization of the GAGs but not for weaker acids. The salt of the buffer was ammonium formate, which is volatile and widely used in LC/MS. The concentration was selected to be relatively low at 12.5 mM so that it can provide minimal background MS signals but does not affect the chromatographic separations significantly. The SEC resolution is maximized at 15 μl/min according to a van Deemter curve that has been measured for GAGs (32). This flow rate produces acceptable analysis times for HS lyase digestion products.

Extracted ion chromatograms for Δ-ununsaturated and saturated disaccharide digestion products are shown in Fig. 1. An example mass spectrum (shown in Fig. S2) summed from 96 to 105 min of the SEC LC/MS total ion chromatogram from HS digest of rat brain tissues shows a series of Δ-ununsaturated and saturated disaccharides. The relative quantities of eight chromato graphically separated HS disaccharides extracted from the LC-mass spectral data are shown in Fig. 2. The plot compares HS Δ-disaccharide profiles corresponding to nonsulfated ΔHexA-GlcNAc, singly sulfated ΔHexA(2S)-GlcNAc(6S), doubly sulfated ΔHexA2S-GlcNAc6S, singly sulfated ΔHexA-GlcNS, doubly sulfated ΔHexA(2S)-GlcNS(6S), and triply sulfated ΔHexA2S-GlcNS6S among seven rat tissues: brain, muscle, liver, heart, kidney, spleen, and lung. The relatively small error bars from triplicate experiments enable significant differences in disaccharide abundances to be observed among the different tissues. In addition, these data allow calculation of HS N-acetylation and N-sulfation (Fig. S3) and the average number of sulfate groups per 100 disaccharide units (Fig. 3). These characteristics reflect the overall HS chain composition as expressed in the tissue sample. Such values have potential as important indicators of aging, disease, or other biological transformations. Similar profiling of HS disaccharides from different mammalian tissues by other detection platforms, such as reverse phase ion pairing and strong anion exchange, coupled with radioactivity and fluorescence have been reported (16, 20, 39, 40). The SEC LC/MS data overall parallel the disaccharide profiles of previously reported HS from murine organs and...
embryos. However, a detailed comparison based on these cross-platform data that were not calibrated and normalized to absolute quantities was not attempted. In addition, the animals used in these studies were of different species (rats versus mice) and of different or unspecified genders, ages, or development stages.

The SEC LC/MS data show the presence of both $\triangle$-unsaturated disaccharides from the internal portion of the HS chain and saturated disaccharides from the nonreducing end (Fig. 2). The abundances of the saturated and $\triangle$-unsaturated disaccharide are very useful, since they provide a way to calculate the average chain lengths by dividing the total numbers of disaccharides with the numbers of saturated disaccharides (41). The calculated values, shown in Fig. 4, indicate significant variations in chain length among the different tissues. The HS chain length is likely to result from factors including expression of biosynthetic enzymes and availability of nucleotide sugar precursors transported through the Golgi apparatus (42). In addition, heparanases may degrade HS in a tissue-specific manner, shortening the observed chain lengths (43). Therefore, the differences of the average chain lengths among different tissues may be a reflection of how the expression of the genes of these enzymes differs in different organ tissues. The average

**FIGURE 2.** HS disaccharide profiles in rat tissues analyzed by SEC LC/MS. The percentage of disaccharides of total digest abundance was as follows: heart, 95.7%; kidney, 92.5%; lung, 96.2%; spleen, 91.4%; brain, 84.9%; liver, 95.3%; muscle, 96.1%.

**FIGURE 3.** The average number of sulfations per 100 dp2s among rat tissues.

**FIGURE 4.** Average chain lengths of HS in rat tissues calculated by unsaturated and saturated disaccharides.
chain lengths in the seven organ tissues range from 30 to 60 disaccharide units, which corresponds to a molecular mass of 12–24 kDa.

Another interesting value that may be calculated is the percentage of each of the saturated disaccharides of the total number of the saturated disaccharides. This percentage is also the probability that a particular disaccharide appears at the nonreducing end. This distribution is shown in Fig. 5. Comparing Figs. 2 and 5, it can be seen that HexA-GlcNS and HexA (2S)-GlcNS (6S) have higher probabilities of locating at the nonreducing end than in the whole chain. HexA-GlcNS, and to a lesser degree, HexA (2S)-GlcNS (6S) (except for brain and muscle) are overall most likely to be the nonreducing end disaccharides. This finding is consistent with the previous report that the nonreducing end of bovine kidney HS are heavily sulfated and especially N-sulfated (44). However, there is no observable relationship of the occurrence of one particular dp2 relating to the chain length. When the total chain sulfation degree versus the chain lengths and the nonreducing end sulfation degree versus the chain lengths were plotted, there is a pattern that more sulfated HS tends to be shorter (Fig. S4, A and B).

When the percentages in Fig. 5 are divided by percentages in Fig. 3, the resulting value indicates whether a certain disaccharide is more or less likely to be at the reducing end, as shown in Fig. 6. A positive value on the logarithmic scale indicates that the particular disaccharide has a higher tendency to be at the nonreducing end compared with its probability in the entire chain. This plot highlights the organ-specific differences in the most probable nonreducing end disaccharides.

Unsubstituted Glucosamine Amino Groups in Organ-specific HS—The SEC LC/MS data also show the presence of a monosulfated disaccharide containing a free amino group. Disaccharide units with an unsubstituted glucosamine amino group are the result of incomplete N-deacetylase/N-sulfotransferase enzymatic activity after the initial chain polymerization during the biosynthesis of the GAGs (45, 46). Monosulfated N-unsubstituted disaccharide ΔHexA2S-GlcNH2 and ΔHexA-GlcNH2,6S have been detected in commercial HS (31), invertebrate HS, and Chinese hamster ovary cell HS (30). Commercially available disaccharides ΔHexA-GlcNH2,6S and ΔHexA2S-GlcNH2 are products of lyase digestion of chemically de-N-sulfated heparin. These two disaccha-
rides are isomers of $\Delta$HexA-GlcNS, sharing the same molecular weight (417.06). For several of the rat tissue HS samples, we observed three peaks for the extracted ion chromatograms of $m/z$ 416, with the peak at 103 min identified as $\Delta$HexA-GlcNS and a less abundant peak at 95 min identified as the loss of sulfate peak of $\Delta$HexA (2S)-GlcNS (6S), both by comparison with known individual standards (Fig. 7A and Fig. S5). The minor peak at 110 min is clearly discrete from the two peaks identified above. Commercial standards of $\Delta$HexA-GlcNH$_2$6S and $\Delta$HexA2S-GlcNH$_2$ under the same conditions had the same retention time of 110 min. Tandem mass spectrometric experiments on the disaccharides eluting from the SEC column identified the 110 min peak as $\Delta$HexA-GlcNH$_2$6S. Fig. 7, B and C, shows that the tandem mass spectrum of the 110 min peak from lung tissue closely resembles that of the commercial $\Delta$HexA-GlcNH$_2$6S standard. The fragmentations in Fig. 7C were according to the Costello-Domon nomenclature (47). The commercial standards of $\Delta$HexA-GlcNS and $\Delta$HexA2S-GlcNH$_2$ produced substantially different tandem mass spectra, as shown in Fig. S5, A and B, respectively. The three isomers have also been shown to produce distinct tandem mass spectral profiles by others (48, 49). The percentage of $\Delta$HexA-GlcNH$_2$6S relative to the total amount of disaccharides is shown in Fig. 8. It is evident that the lung has the most abundant $\Delta$HexA-GlcNH$_2$6S, whereas it is not detected in kidney, liver, and muscle tissues.
Organ-specific, Lyase-resistant Oligosaccharides—The data by SEC LC/MS analysis also show the presence of oligosaccharides despite the stringent lyase conditions used. A range from 85 to 96% of the digest products are in dp2 form, leaving 4–15% of the HS chains lyase-resistant. Forty-one ion species (representing 36 oligosaccharides, five of which are observed as both singly and doubly charged ions) were identified using the mass accuracy (less than 20 ppm compared with theoretical values) and retention time as estimated by their molecular sizes (as a function of the numbers of sugar units, sulfates, and acetates). The compositions and abundances of these oligosaccharides are shown in Fig. 9 (15 ion species with abundances greater than 0.1%) and Fig. S6 (complete set of data). From Fig. 9, four oligosaccharides are relatively more abundant compared with others. They are monosaccharides (00111), and oligosaccharide (11202), (11211), and (11222), as named by numbers of ΔHexA, HexA, GlcN, sulfates, and acetates, respectively. These saccharides range from 0.1 to 0.6% relative to the total abundances of all digestion products. The coefficients of variation for the oligosaccharides are higher than those of disaccharides due to the relatively lower ion abundances. Despite this, the information about the lyase-resistant oligosaccharides from different rat tissues provides a potentially useful indicator of how the expression of the GAG biosynthesis enzymes and the biosynthesis process itself differ in different tissues. Tetrasaccharides (11240 and 11250) have the same compositions as those identified as products generated by recombinant 3-O-sulfotransferase isofrom 3A enzyme that contains an IdoA2S-GlcNH23S unit (25). These tetrasaccharides were not abundant enough for tandem mass spectrometric analysis, and thus the sulfation positions were not determined.

DISCUSSION

The chemical reactivity of the free glucosamine amino group toward nitrous acid was first used to identify their presence in endogenous HS (22, 23, 46). The levels of amino groups in HS were found to range from 0.7 to 4% of disaccharide units from a variety of organ tissues using amine-reactive fluorescence analysis (21) and radioactive labeling (24). The recombinant 3-O-sulfotransferase isofrom 3A enzyme was observed to generate IdoA2S-GlcNH23S units (25, 50). The presence of these structures implies the existence of Ido2S-GlcNH23S units in endogenous HS. The IdoA2S-GlcNH23S unit generated using recombinant 3-O-sulfotransferase isofrom 3A has been identified as part of an HS octasaccharide that binds specifically to herpes simplex type I glycoprotein D (26, 27, 51).

Degradation of glypican-1 in endosomal compartments has been observed to occur by both heparanase enzyme- and NO-catalyzed deaminative cleavage at glucosamine residues containing a free amino group (52, 53). Such residues have been detected in glypican-1 and appear to be present near the site of protein attachment (54). Similar free amino-containing glucosamine residues are present near the linker region in intestinal HS (24). Cleavage of HS chains by endogenous NO follows a similar mechanism as for in vitro nitrous acid cleavage (55, 56) and generates oligosaccharides with anhydromannose residues at the reducing end. Cysteine residues of glypican-1 become S-nitrosylated in endosomal compartments in cultured cells and subsequently release NO when exposed to a reducing agent, such as ascorbate. The release of NO in the locality of the glypican HS chain results in deaminative cleavage as part of the regulation of polyamine uptake in mammalian cells through glypican-1 recycling (28, 29). The level of glucosamine residues with...
free amino groups has been found to increase when polyamine synthesis is inhibited (57).

Our results show the presence of a monosulfated N-unsubstituted disaccharide, the tandem mass spectrometric profile of which closely resembles disaccharide ΔHexA-GlcNH26S. The LC/MS data rule out the presence of the two isomers ΔHexA-GlcNS and ΔHexA2S-GlcNH2. These results indicate the presence of the two isomers of HS disaccharides corresponding to GlcA-GlcNH26S that were apparently often not detectable using other methods. We have not observed ΔHexA-GlcNH26S using CE-LIF analysis (58). It is likely that disaccharides with free amino groups are difficult to detect using other disaccharide analysis methods, because their zwitterion nature causes poor retention on ion exchange resin or weak pairing with ion-pairing agents. The SEC LC/MS method, by virtue of nonadsorptive SEC chromatography, provides a means of detecting such disaccharides as well as oligosaccharides that resist lyase depolymerization. Disaccharides of type ΔHexA-GlcNH2 were not detected, although they were known to be present in HS preparations (20). It is likely that the low hydrodynamic volume of this unsulfated disaccharide resulted in its co-elution with buffer salts in the SEC profile and thus its nondetection using MS.

The presence of disaccharide units containing free glucosamine amino groups in mature HS chains seems to be related to the mechanisms of action of N-deacetylase/N-sulfotransferase enzymes and the availability of PAPS (19, 46). Our work shows that disaccharide units containing GlcA-GlcNH26S are widely expressed in organ tissue. HS chains containing such disaccharide units would be susceptible to NO-mediated cleavage, as has been described for glypican-1 cycling (28). Conditions for NO-mediated cleavage may exist in other biological processes. Glucosamine amino groups of HS are also known to react rapidly with oxidizing agents, such as HOCl, under conditions that are likely to be present at inflammation sites (21, 37, 59–61). It is clear that the GlcA-GlcNH26S disaccharide is a widely expressed and functionally relevant HS substructure.

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