Antibodies against heparan sulfate (HS) are useful tools to study the structural diversity of HS. They demonstrate the large sequence variability within HS and show the distribution of HS oligosaccharide sequences within their natural environment. We analyzed the distribution and the structural characteristics of the oligosaccharide epitope recognized by anti-HS antibody HS4C3. Biosynthetic and synthetic heparin-related oligosaccharide libraries were used in affinity chromatography, immunoprecipitation, and enzyme-linked immunosorbent assay to identify this epitope as a 3-O-sulfated motif with antithrombin binding capacity. The antibody binds weakly to any N-sulfated, 2-O- and 6-O-sulfated hexa-to octasaccharide fragment but strongly to the corresponding oligosaccharide when there is a 3-O-sulfated glucosamine residue present in the sequence. This difference was highlighted by affinity interaction and immunohistochemistry at salt concentrations from 500 mM. At physiological salt conditions the antibody strongly recognizes basal lamina of epithelia and endothelia. At 500 mM salt conditions, when 3-O sulfation is required for binding, antibody recognition was more restricted and selective. Antibody HS4C3 bound similar tissue structures as antithrombin in rat kidney. Furthermore, antithrombin and antibody HS4C3 could compete with one another for binding to heparin. Antibody HS4C3 was also able to inhibit the anti-coagulant activities of heparin and Arixtra as demonstrated using the activated partial thromboplastin time clotting and the anti-factor Xa assays. In summary, antibody HS4C3 selectively detects 3-O-sulfated HS structures and interferes with the coagulation activities of heparin by association with the antithrombin binding pentasaccharide sequence.

Heparan sulfate (HS) proteoglycans consist of a core protein with covalently linked HS side chains, and occur on cell surfaces and in the extracellular matrix. HS polysaccharides consist of up to ~200 repeating disaccharide units (glucosamine α1–4-glucuronic acid β1–4 and glucosamine α1–4-iduronic acid α1–4), which are variably modified by N-acetyl/N-sulfate and O-sulfate groups (1, 2). The HS chains have fundamental roles in embryonic development, homeostasis, and disease, by interaction with regulatory proteins (morphogens, growth factors, enzymes etc.), mediated by specific HS domains (3). HS-protein interactions are believed to be dictated not only by the overall charge of the HS chain but also by the distribution and positioning of the negatively charged carboxyl and sulfate groups within the HS chain (4). The structural diversity within the HS chain arises through the ordered action of sulfotransferases and an epimerase (1, 5, 6) during HS biosynthesis within the Golgi apparatus and may be further affected by the extracellular action of endosulfatases after biosynthesis (7). The biosynthetic HS modification reactions include N-deacetylation/N-sulfation of the glucosamine (GlcN) residues by N-deacetylase/N-sulfotransferases, C5 epimerization of glucuronic acid (GlcA) to form iduronic acid (IdoA) by glucuronyl-C5 epimerase, 2-O-sulfation of uronic acids by 2-O-sulfotransferase (2-OST), and 3-O and 6-O-sulfation of GlcNs by 3-O and 6-O-sulfotransferases (3-OST and 6-OST) (1, 5, 8–13). The 3-O-sulfation of GlcN is the last event during biosynthesis and introduces one of the rarest HS modifications (~0.5% of disaccharide units substituted albeit highly variable) (10). The different 3-OST isoforms act on specific target sequences to create unique oligosaccharide structures that have been implicated with blood anticoagulation (14), viral infection (15, 16), kidney anionic filtration (17), and cancer (by silencing the 3-OST-2 gene through methylation) (18). The availability of an antibody recognizing 3-O-sulfated HS oligosaccharides would facilitate (in situ) studies to analyze the role of this modification in health and disease. Here we demonstrate that the anti-HS antibody HS4C3 recognizes a 3-O-sulfated epitope and has selective neutralization capacity for the heparin AT-pentasaccharide binding sequence as compared with the clinically used nonspecific antidote protamine.

**EXPERIMENTAL PROCEDURES**

**Materials—**All chemicals used were purchased from Merck (Darmstadt, Germany), unless stated otherwise. Bacterial medium (2× TY) was from Invitrogen (Carlsbad, California). Isopropyl β-D-thiogalactopyranoside, the activated partial thromboplastin time (APTT) reagent, normal reference plasma, and HIS-select cobalt affinity gel were from Sigma. Protamine sulfate was from MP Biomedicals (Irvine, CA) and the Coatest heparin was from Chromogenix AB (Lexington, MA). The protease inhibitor mixture was from Roche (Basel, Switzerland). Mowiol (4–88) and AT III from human plasma were obtained from Calbiochem (La Jolla, CA). Microlon 96-well microtiter plates were from Greiner (Frickenhausen, Germany). Protein A-Sepharose beads, Sephadex G-15, a prepacked Superox 30 column (1.6 × 60 cm), prepacked PD-10 columns, and NaBH₄ (50–75 Ci/mmol) were from Amersham Bio-
variable fragment antibodies were eluted with buffer containing 200 mM Na2HPO4, and 5 mM imidazole, pH 8.0) and incubated for 3 h at 4 °C. The antibody concentration was determined at 280 nm and bovine serum albumin (1%) and sodium azide (0.02%) were added for stabilization and preservation. The efficiency of purification was assessed by SDS-PAGE and Western blotting. Purified samples were separated on a Bio-Gel P-10 column (1 × 146 cm in 0.5 M NH4HCO3) and fractions corresponding to octasaccharides were recovered for further separation by anion exchange high performance liquid chromatography. The pooled octasaccharides were applied to a ProPac PA10 column in H2O adjusted to pH 3 with HCl, and eluted using a linear gradient of NaCl (10 mM/min in H2O, pH 3, at a flow rate of 1 ml/min. Peaks corresponding to one, two, or three 2-O-sulfate groups per octamere, respectively, were pooled separately and desalted on PD-10 columns in 0.2 M NH4HCO3.

Oligosaccharides with one, two, or three 2-O-sulfate groups were separately applied to further enzymatic O-sulfation, preferentially at the 6-O-position. Each octamere substrate (0.3 nmol) was incubated with a mouse mastocytoma microsomal fraction (1 mg of protein) (28) in a final incubation volume of 100 µl of 50 mM Hepes, pH 7.4, containing 1 mM PAPS, 10 mM MnCl2, 3.5 µM NaF, 0.3% (v/v) Triton X-100. Incubations were done at 37 °C for 1 h. The reactions were terminated by boiling samples for 2 min at 96 °C and the reaction products were then analyzed on a ProPac PA10 column using the same gradient as described above. Peak fractions were pooled and desalted as described above.

**Isolation of AT-binding Oligosaccharide Sequences—**Bovine lung heparin 8-mers were prepared by partial deamination, reduction of products with NaB3H4, and gel chromatography, as described (29). The end-labeled products (106 cpm; 7.5 × 106 cpm/nmol) were loaded on an AT-Sepharose column (2 ml of gel with 10 mg of AT III/ml of gel) in 2 ml of loading buffer (50 mM Tris/HCl, pH 7.4, 0.15 M NaCl) and followed by a 15-ml wash with the same buffer (30). Fractions with low and high affinity for AT were eluted with 15 ml each of 0.6 and 3 M NaCl, respectively, in 50 mM Tris/HCl, pH 7.4, desalted on PD-10 columns in 0.2 M NH4HCO3 and used for affinity chromatography on antibody HS4C3 columns. Oligosaccharide 16 (Table 1/4/Arixtra and its 3-O-sulfate-free analog (31) were radiolabeled in a similar manner by partial deamination and reduction with NaB3H4, as described above. The products were separated on a column of Sepharose G-15 (1 × 190 cm) in 0.2 M NH4HCO3 and labeled pentasaccharides were isolated after separation from smaller products.

**Isolation of Oligosaccharides by HS4C3 Immunoadfinity Columns—**Purified antibodies (1 mg) were incubated with 0.5 mg of Protein A-Sepharose beads in a final volume of 2 ml of 50 mM Tris/HCl, pH 7.4, overnight at 4 °C. The beads were then transferred to a Bio-Rad column and equilibrated with 50 mM Tris/HCl, pH 7.4. For affinity selection 3H-radiolabeled oligosaccharides were loaded in 0.5 ml of equilibration buffer and allowed to incubate on the column for half an hour at 4 °C. The oligosaccharides were eluted using a stepwise gradient of 5 column volumes each of 0.15, 0.25, 0.35, 0.45, and 2 M NaCl in 50 mM Tris/HCl, pH 7.4.
3-O-Sulfates Recognized by Antibody HS4C3

Evaluation of HS4C3 Synthetic Oligosaccharide Specificity by ELISA—To study the reactivity of synthetic HS oligosaccharides with antibody HS4C3, competition ELISAs were performed as described (21, 22). The periplasmic fraction of antibody HS4C3 was preincubated with various concentrations of synthetic oligosaccharides for 10 min and transferred to 96-well microtiter plates previously coated with HS from bovine kidney. The plates were washed and bound antibodies were detected by anti-VSV tag antibody P5D4 followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG. All assays were performed at least 3 times and representative results are shown. Inhibition was defined as an IC50 (oligosaccharide concentration needed to obtain 50% inhibition of antibody binding) less than 5 μg/ml.

To determine whether antibody HS4C3 and AT bound to similar heparin structures, a competition ELISA was performed. Antibody HS4C3 (0.5 μg/ml) was incubated with increasing concentrations of AT (0–5 μg/ml) or conversely, AT (12.5 μg/ml) was incubated with increasing concentrations of antibody HS4C3 (0–20 μg/ml) in 100 μl and transferred to a 96-well plate previously coated with heparin. Plates were washed 6 times with PBS before measuring antibody HS4C3 or AT by adding anti-VSV tag antibody P5D4 or sheep anti-AT antibodies, respectively, followed by alkaline phosphatase-conjugated anti-mouse or anti-sheep IgGs.

Immunoprecipitation of Oligosaccharides with Antibody HS4C3—Immunoprecipitation was performed with Fraxiparine, Arixtra/oligosaccharide 16, native heparin 6-mer (heparin dp6), N-desulfated/N-acetylated heparin 6-mer (Hep-Ac dp6), and synthetic oligosaccharides (Table 1), using antibody HS4C3 (VSV/His tagged). Oligosaccharides (10 μg) or mixtures of oligosaccharides were incubated with 100 μg of purified antibody in PBS for 1 h. Then, 100 μl of HIS-select cobalt beads were added to the oligosaccharide/antibody mixture and incubated for 1 h at room temperature while shaking. Beads were spun down (10,000 × g), washed 3 times with PBS, mixed with PAGE loading buffer (Tris acetate buffer, pH 7.0, 0.5 M NaCl), and heated for 2 min. Samples were analyzed by PAGE using a 33% gel (32), fixed in Alcian blue (0.8% (w/v) in 2% (v/v) acetic acid), and silver stained (33). Control incubations included either the omission of antibody HS4C3 or heparin oligosaccharides, and the use of an irrelevant single chain antibody. These control incubations revealed no binding of heparin oligosaccharides with the HIS-select cobalt beads or with the irrelevant single chain antibody.

Activated Partial Thromboplastin Time Clotting Assay—To evaluate if antibody HS4C3 could inhibit the effect of heparin on the clotting time, an aPTT clotting assay was performed. Purified antibody HS4C3 at the indicated concentrations was incubated with 50 μl of 10 μg/ml heparin for 60 min. Following addition of 100 μl of human reference plasma, samples were incubated for 1 min at 37 °C, and were then mixed with 100 μl of aPTT reagent and incubated for another 3 min at 37 °C. Finally, 100 μl of 20 mM CaCl2 was added, and the clotting time was recorded according to the manufacturer’s instructions.

Anti-factor Xa Assay—To evaluate if antibody HS4C3 could act as an antidote for heparin and Arixtra, the Coatest heparin assay was performed. Heparin (25 ng) or Arixtra (9 ng) were incubated with purified antibody HS4C3 (0–0.4 nmol/assay) or protamine (0–2.0 nmol/assay) in the presence of human plasma and human antithrombin at 37 °C for 30 s. Factor Xa (in excess) was added and incubated at 37 °C for 30 s. Next, the chromogenic substrate was added and incubated at 37 °C for 3 min and the remaining factor Xa activity was measured at 405 nm according to the manufacturer’s instructions.

Immunohistochemistry—Immunohistochemical analysis was performed as described previously (25, 34). Briefly, tissue sections were incubated with antibody HS4C3 and bound antibodies were visualized using anti-VSV antibody P5D4 followed by Alexa-labeled (488) anti-mouse antibodies. Finally, tissue sections were fixed in ethanol, air-dried, and embedded in Mowiol. As a control, primary antibodies were omitted or substituted by an irrelevant single chain antibody.

To evaluate the HS specificity of the antibody and to locate all HS within a tissue, tissue sections were pretreated with heparinase III (0.02 IU/ml in 50 mM NaAc, 50 mM Ca(AC)2, pH 7.0, 2 h incubation at 37 °C) and stained with antibody 3G10 (35), which recognizes HS stubs generated after HS digestion. As a control, tissue sections were incubated with reaction buffer without enzyme.

Rat kidney sections were incubated with AT (10 μg/ml) and bound AT was detected by sheep anti-AT antibodies and visualized by Alexa-labeled (488) anti-sheep antibodies. Alternatively, tissue sections were incubated with antibody HS4C3, in the presence of 0.4–1.0 M NaCl to increase selectivity for binding sites with higher affinity. Finally, tissue sections were incubated with mixtures of antibody and oligosaccharides to evaluate inhibition of antibody staining by the oligosaccharides. Antibody and oligosaccharides (10 μg/ml) were preincubated for 2 min before being added to the tissue section. Bound antibodies were visualized by anti-VSV antibody P5D4 followed by Alexa-labeled (488) anti-mouse antibodies.

RESULTS

Importance of N-Sulfation for Binding to Antibody HS4C3—We previously demonstrated that antibody HS4C3 strongly reacts with the glomerular basement membrane (GBM), peritubular capillaries, and large blood vessels in the kidney and that it binds heparin and the sulfated structures in HS (21). To identify the epitope recognized by antibody HS4C3 and characterize its structure in detail different approaches were taken. A population of heparin hexasaccharides (Hep dp6) prepared by lyase digestion, with different degrees of sulfation, was applied to precipitation with antibody HS4C3. The 6-mer was precipitated, yet with different degrees of efficiency for different subspecies (Fig. 1A). A completely N-desulfated/N-acetylated heparin 6-mer preparation (Hep Ac dp6) on the other hand was not precipitated at all (Fig. 1B), indicating that antibody HS4C3 recognized N-sulfated motifs in heparin.

Importance of O-Sulfates for Binding to Antibody HS4C3—To evaluate the importance of O-sulfation we prepared a library of radiolabeled, N-sulfated octasaccharides with a variable degree of O-sulfation, by partial deamination of partially O-desulfated heparin. 3H-End group-la-
beled octasaccharide populations with one, two, or three remaining 2-O-sulfate groups were recovered for enzymatic 0-sulfation by mastocytoma microsomal enzymes. The incubation conditions employed favor incorporation of 6-O-sulfate groups (36), and thus yielded products with known degrees of 2-O-sulfation and variable 6-O-sulfation (Fig. 2, A–C). Each of these pools contained several different species with the same number, but differently distributed O-sulfates along the oligosaccharide, manifested in composite elution patterns for each subpopulation on anion exchange high performance liquid chromatography. These charge defined, sequence heterogeneous octasaccharides were analyzed for binding to antibody HS4C3 by affinity chromatography. Oligosaccharides were eluted by stepwise raising the salt concentration of the elution buffer. The results showed that highly sulfated octasaccharides bound more tightly to antibody HS4C3 than octasaccharides with fewer sulfates. Whereas octasaccharides with one 2-O-sulfate and one 6-O-sulfate were eluted from the HS4C3 affinity column with 150 mM NaCl (Fig. 2A'), octasaccharides with three 2-O-sulfates and three 6-O-sulfates were eluted with 350 mM NaCl from the column (Fig. 2C'). No further increase in affinity was achieved with a fully sulfated, native heparin 8-mer containing 8-O-sulfate groups (data not shown). Four O-sulfate groups, independent of their position on the sugar units or along the chain resulted in similar binding behavior (e.g. Fig. 2, A'', B', and C'). Furthermore, the partially separated peaks in e.g. Fig. 2B'' contained the same 8-mer subspecies, as indicated by anion exchange high performance liquid chromatography (data not shown), independently whether they eluted in the early or later part of the double peak, indicating only a subtle if any influence on affinity of the positioning of the O-sulfate groups in these structures.

Identification of High Affinity Binding Sites—To assess the affinity of antibody binding sites in tissue, we stained rat kidney sections with antibody HS4C3 in buffer with increasing NaCl concentrations ranging from 150 to 1000 mM (Fig. 3). Staining of peritubular capillaries started...
3-O-Sulfates Recognized by Antibody HS4C3

The reactivity of antibody HS4C3 with the synthetic oligosaccharides was studied by immunoprecipitation analysis and competition ELISA, as described under "Experimental Procedures" and "Results." Data as illustrated in Fig. 5 and not shown are combined in this table. A strong positive reaction with an oligosaccharide is indicated by +, a weak reaction is indicated by +/−, and no reaction is indicated by −. The oligosaccharides indicated + in competition ELISA are those with an IC50 of <5 μg/ml in the binding of HS4C3 to immobilized HS. All oligosaccharides are derivatives of oligosaccharide number 16, which is commercially available under the name Arixtra. The total number of sulfate groups per oligosaccharide is indicated. The following abbreviations are used in the table: GlcNS, N-sulfated glucosamine; Glc, glucose; 2S, 2-O-sulfated; 3S, 3-O-sulfated; 6S, 6-O-sulfated; Me, methyl; Bu, butyl; Oc, octyl.

| Component name | Monosaccharide units | Number of sulfate groups | Immunoprecipitation with HS4C3 | Reactivity with HS4C3 in competition ELISA |
|----------------|----------------------|--------------------------|-------------------------------|------------------------------------------|
| SR90107 (16)   | GlcNS6S              | 8                        | +                             | +                                        |
| SR80497 (4)    | Glc236triS           | 8                        | +                             | +                                        |
| SR80498 (7)    | Glc236triS           | 8                        | +                             | +                                        |
| SR80588 (10)   | Glc236triS           | 8                        | +                             | +                                        |
| IC892979 (17)  | GlcNS6S              | 7                        | −                             | −                                        |
| SR80028 (20)   | Glc236triS           | 9                        | +                             | +                                        |
| SR80498 (10)   | Glc236triS           | 8                        | +                             | +                                        |
| IC871812 (9)   | GlcNS6S              | 7                        | +/−                           | −                                        |
| IC851669 (8)   | GlcNS6S              | 6                        | −                             | −                                        |
| SR80409 (21)   | Glc236triS           | 7                        | +/−                           | −                                        |
| IC892045 (19)  | GlcNS6S              | 6                        | −                             | −                                        |
| IC881950 (5)   | GlcNS6S              | 4                        | −                             | −                                        |
Antibody staining was inhibited with oligosaccharides 17 and 16/Arixtra, whereas hardly any inhibition was seen with oligosaccharides 6 and 10.

**Distribution of HS4C3 Epitopes in Rat Tissues**—To analyze the distribution pattern of HS4C3 epitopes, in particular 3-O-sulfated HS structures, rat kidney, liver, pancreas, and intestine were stained with antibody HS4C3. Strong staining was observed of the basal lamina of blood vessels and capillaries, sinusoids, and epithelium. Antibody HS4C3 staining was also performed in the presence of 0.5 M NaCl to reveal the high affinity binding sites within HS. Such sites were observed in large blood vessels in the liver and pancreas, in the GBM, and to a minor extent in peritubular capillaries of the kidney. In intestine these sites were found in capillaries and in the basal lamina of the crypts (Fig. 7).

To confirm the presence of AT-binding motifs in tissue we incubated rat kidney tissue sections with AT at physiological ion strength. Essentially the same structures were visualized by this procedure as with antibody HS4C3 (Fig. 8). Both proteins bind in similar fashion to larger blood vessels and capillaries, and both stain the glomerulus, however, antibody HS4C3 stained areas that were not stained by AT.

**Functional Significance of Interaction between Antibody HS4C3 and AT Binding**—A potential functional implication of antibody HS4C3 binding to the AT-binding pentasaccharide was explored in competition studies. Antibody HS4C3 was able to compete with AT for binding to heparin in an ELISA (Fig. 9A) and, conversely, AT inhibited binding of HS4C3 to heparin (Fig. 9B), suggesting that they competed for the same binding site on heparin. Heparin inhibits blood coagulation by binding to and activating AT (38). The capacity of antibody HS4C3 to block the anti-coagulant activity of heparin was assessed using an APTT (Fig. 9C) and anti-factor Xa (Fig. 9, D and E) assay. Antibody HS4C3 was able to block the anti-coagulant activity of heparin presumably by binding to the pentasaccharide sequence and thereby interfering with the AT-heparin interaction. Moreover, antibody HS4C3 was able to block the coagulant activity of Arixtra, making antibody HS4C3 more selective than the nonspecific heparin antidote protamine (Fig. 9E).
DISCUSSION

The goal of our study was to characterize the oligosaccharide epitope recognized by antibody HS4C3. Antibody HS4C3 is one of a number of single chain variable fragment phage display antibodies selected against bovine kidney HS. These antibodies have a large potential for application because of differential reactivity with selective epitopes (24, 25, 39). HS4C3 belongs to the V\textsubscript{H}3 family and dp38 gene segment with the CDR3 amino acid sequence GRRLKD (21). The antibody reacts strongly with HS from bovine kidney, bovine intestine, and heparin (porcine mucosa) but barely with HS from bovine and human aorta, and moderately with HS from other sources (21). It does not react with completely desulfated/N-acetylated heparin, O-desulfated/N-sulfated heparin, and N-desulfated/N-acetylated heparin (21). These observations indicated that antibody HS4C3 binds to sulfated structures in HS or heparin oligosaccharides. The reduced binding of HS4C3 to HS from aorta, which is of a low degree of sulfation as compared with HS from lung, intestine, or liver (40) supports the view that HS4C3 recognizes some highly sulfated sequence. Interaction with intact (Fig. 1A) but not with N-desulfated (Fig. 1B) heparin oligosaccharides consolidated this point.

The structural properties of HS with stretches of low and high degrees of sulfation provide a vast number of protein binding sites with potential for redundancy. Not all of the sulfate groups in such structures may be necessary for interaction with a given protein ligand although they are present (4). On the other hand, proteins may bind to different sequences because of polycationic patches that can bind to several different HS sequences. Such redundancy was indicated using biosynthetically produced oligosaccharide libraries in HS4C3 binding, which
showed an incremental increase in affinity for each additional sulfate group in the oligosaccharide, independent of its location. This is exemplified by the oligosaccharides containing an equal number of O-sulfates, although in different positions (2-O- and 6-O-) and of different sequences (cf. Fig. 2, A′, B′, and C′). All of these sequences were eluted at similar ionic strengths from the HS4C3 affinity column. Yet endogenous HS displays antibody binding sites with apparent affinities higher (Figs. 3 and 7) than those found in the most highly sulfated biosynthetically modified heparin oligosaccharides. There are several possible reasons for this difference. The antibodies could react in multimeric fashion with several epitopes along a single chain or on different, but tightly co-localized, chains in a tissue section. Such a phenomenon would prevail if the antibody, its monomeric binding site accommodating oligosaccharide of 5–6 sugar units, is prone to form oligomers. Alternatively, enhanced affinity could be because of recognition of a stronger binding, different epitope present in only some HS chains and essentially lacking in the oligosaccharide fragments tested. Our findings favor the latter possibility, because HS4C3 was found to bind with high affinity to oligosaccharides containing the AT binding sequence, with its distinctive GlcN-3-O-sulfate group. The pentasaccharide sequence GlcNAc/Sar1-4GlcA/Sar1-4GlcNAc/NS3,6Sar1-4idoA2Sar1-4GlcNS,6S is the minimal motif to mediate strong interaction with the coagulation inhibitor AT (14). Antibody HS4C3 indeed binds to oligosaccharides containing this motif, and removal of the unique 3-O-sulfate group leads to marked loss of affinity (Figs. 4C and 5B, and Table 1). In further proof of this interaction, HS4C3 was capable of neutralizing the blood anticoagulant activity of heparin in an AT-pentasaccharide specific manner as compared with the nonspecific action of protamine, a fish sperm DNA-binding protein that is clinically used as a heparin antidote (Fig. 9, C–E).

Antibody HS4C3 bound strongly to various tissue epitopes, suggesting widely distributed HS chains with AT binding or highly similar sequences. Immunohistochemical studies of the rat kidney, using HS4C3 and AT as probes revealed similar binding sites in capillaries and larger blood vessels (Figs. 7 and 8). Notably, the staining pattern of antibody HS4C3 and AT in the glomerulus was not identical, as antibody HS4C3 stained areas that were not reactive with AT. This observation suggested that antibody HS4C3 recognize not only AT-binding sequences but also other 3-O-sulfated oligosaccharide motifs. The GBM of the kidney contains abundant 3-O-sulfated sequences with an IdoA or a 2-O-sulfated IdoA residue at the nonreducing side of the 3-O-sulfated GlcN. These structures are not recognized by AT (46), but presumably by HS4C3. Occurrence of additional 3-O-sulfated structures in native HS has yet to be demonstrated, but appears highly plausible in view of the substrate specificities defined for various members of the 3-O-sulfotransferase family. 3-O-Sulfation is the last and the rarest modification, although in different positions (2-O- and 6-O-) and of different sequences in native HS has yet to be demonstrated, but appears highly plausible in view of the substrate specificities defined for various members of the 3-O-sulfotransferase family. 3-O-Sulfation is the last and the rarest step in HS biosynthetic modification. To date, six members of the 3-O-sulfotransferase family are known (16, 41, 42). These isoforms have been reported to act on specific target sequences. Whereas 3-OST isoform 1 transfers a sulfate group to the 3-OH position of an N-sulfated GlcN residue with an unsubstituted glucuronic acid on the non-reducing side (GlcA-GlcNS,6S), 3-OST isoform 3A transfers a sulfate group to the

Detection of heparin-bound AT and HS4C3, respectively, was performed as described under "Experimental Procedures." Antibody HS4C3 was tested in an APTT clotting assay as described under "Experimental Procedures." (C). The coagulation time was measured in the presence or absence of heparin (10 μg/ml) and antibody HS4C3 (0–10 μg/ml) as indicated. Antibody HS4C3 and protamine sulfate were tested in an anti-factor Xa assay as described under "Experimental Procedures." (D and E). The effect of antibody HS4C3 (0–4 nmol/assay) and protamine sulfate (0–2.0 nmol/assay) on heparin (2 pmol/assay; D) and Arixtra (6 pmol/assay; E) was determined by measuring the remaining factor Xa activity (A405) as described. Triangles represent the antidote protamine.
3-OH position of an N-unsubstituted GlcN residue, which has a 2-O-sulfated iduronic acid in the same position (IdoA2S-GlcNH2-2-6S) (43). 3-OST-5 demonstrates a broader substrate specificity as it sulfates both N-sulfated and N-unsubstituted GlcNS (44). Specific 3-O-sulfotransferases can therefore create target sequences that can act as receptors for AT (created by 3-OST-1 (45, 46), and 3-OST-5 (47)), or HSV-1 envelope glycoprotein gD (created by 3-OST-3 and -5 (15)). Which of the various potential 3-OST products that are recognized by antibody HS4C3 remain to be investigated.

The potential of anti-HS antibodies in identifying specific HS modifications is again illustrated by HS4C3, which specifically recognizes 3-O-sulfated motifs. The growing panel of characterized anti-HS antibodies (34, 48, 49), and now antibody HS4C3, extends the analysis of the spatial distribution of specific HS epitopes in situ. Still, the precise structures of these epitopes are not readily defined through the use of antibodies. The selection of oligosaccharides used to define the specificity of an antibody (or any HS-binding protein) is crucial to such information. The microsomal HS/heparin oligosaccharide library used in this study served to establish the role of overall sulfate density in HS4C3 binding, but lacked the specific 3-O-sulfated high affinity species. Similar oligosaccharide libraries have been useful for identification of the HS motifs binding diverse growth factors (e.g. fibroblast growth factors (50, 51)). Size-fractioned heparin fragments, or oligosaccharides excised from different domains of authentic HS have been identified to bind sequences of endostatin, fibroblast growth factor 1, 2, and fibroblast growth factor receptor 4 (51–54). Synthetic oligosaccharide libraries that cover an extended sequence space are potent tools in identifying protein-binding motifs; however, the structural diversity and the total number of different oligosaccharides are decisive factors for success.

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