Characterization of Anti-heparan Sulfate Phage Display Antibodies AO4B08 and HS4E4*

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Heparan sulfates (HS) are linear carbohydrate chains, covalently attached to proteins, that occur on essentially all cell surfaces and in extracellular matrices. HS chains show extensive structural heterogeneity and are functionally important during embryogenesis and in homeostasis due to their interactions with various proteins. Phage display antibodies have been developed to probe HS structures, assess the availability of protein-binding sites, and monitor structural changes during development and disease. Here we have characterized two such antibodies, AO4B08 and HS4E4, previously noted for partly differential tissue staining. AO4B08 recognized both HS and heparin, and was found to interact with an ubiquitous, N-, 2-O-, and 6-O-sulfated saccharide motif, including an internal 2-O-sulfate group. HS4E4 turned out to preferentially recognize low-sulfated HS motifs containing iduronic acid, and N-sulfated as well as N-acetylated glucosamine residues. Contrary to AO4B08, HS4E4 did not bind highly O-sulfated structures such as found in heparin.

Heparan sulfates (HS) are linear, sulfated polysaccharides that occur covalently bound to core proteins in proteoglycans structures at cell surfaces and in extracellular matrices. Due to their negative charge, HS chains interact with a variety of proteins, and thus modulate important processes in embryogenesis and tissue homeostasis (1, 2). Biosynthesis of HS involves formation of a precursor polysaccharide composed of alternating N-acetylgalactosamine (GlcNAc) and glucuronic acid (GlcA) residues, which is subsequently modified through a series of enzymatic reactions. The modifications include N-deacetylation and N-sulfation of GlcNAc residues, C5-epimerization of GlcA to iduronic acid (IdoA) units, and finally O-sulfation at C2 of hexuronic acid and C6, more rarely C3, of glucosamine units. The reactions are generally incomplete, yielding products of extensive structural heterogeneity (3). The modification process is not under template control, yet tightly regulated, such that differences in structure of HS generated in different tissues are strikingly conserved (4, 5). Although aspects of specificity of HS-protein interactions remain unclear, these differences are presumably of functional significance (6). Elucidation of these problems is hampered by the lack of high-throughput tools for detailed structural characterization of HS, which remains a tedious task.

Phage display antibodies have been developed to overcome the limited immunogenicity of HS, thereby making it possible to highlight tissue-specific HS structures and to follow changes in development and disease. Using such antibodies, the differential expression of HS motifs in various tissues has been demonstrated (7–9). Yet, few of these antibodies have so far been characterized for their epitope specificity, which hampers further use of these tools and interpretation of results. We have selected two of these phage display antibodies, AO4B08 and HS4E4, based on their tissue staining properties (8, 9) and their selective interaction with HS from different sources (10). The epitopes recognized by these two antibodies were further defined in the present study. AO4B08 thus preferentially binds a N-sulfated octasaccharide epitope with three consecutive 6-O-sulfates and an internally located 2-O-sulfate group, and interacts efficiently with fully sulfated heparin. HS4E4 binds preferentially to different structures present in HS with a moderate degree of sulfation and N-acetylated in addition to N-sulfated glucosamine residues and epimerized iduronic acid.

EXPERIMENTAL PROCEDURES

Materials—Protein A-Sepharose and Sephadex G-15 beads, a prepacked Superdex 30 (1.6 × 60 cm) and prepacked PD-10 columns were purchased from GE Healthcare. A Propac PA1 column was purchased from Dionex (Surrey, United Kingdom), a Partisil-10 SAX column from Whatman Inc., and Bio-Gel P-10, fine, was obtained from Bio-Rad. Molecular porous membrane dialysis tubing was from Spectrum (Gardena, CA). NaB3H4 (50–75 Ci/mmol) and [3H]acetic anhydride (500 mCi/ml) were purchased from GE Healthcare. Carrier-free

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3 The abbreviations used are: HS, heparan sulfate; GlcNAc, N-acetyl-D-glucosamine; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; RPIP-HPLC, reversed phase-ion pair high performance liquid chromatography; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; VSV, vesicular stomatitis virus; dpc, days post coitus.
Bovine mucosal heparin was used for kidney cortex (14) and heparin preparations from bovine intestinal derivatives are listed in Table 1. HSs from porcine liver and mucosa HS (used in ELISA) was purchased from Celsus, Cincarin, were from Neoparin, Alameda, CA. Porcine intestinal heparin were from Seikagaku, Tokyo, Japan. Carboxyl-reduced heparinizations were: 50%, 80%, and 98% conversion) were incubated with microsomal heparinases from a heparin producing, murine mast-cell tumor, in the presence of NaB₃H₄, and separation by gel chromatography as described earlier (18). 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 as described earlier (18).

Radiolabeled Polysaccharides—Heparin from intestinal mucosa, HS (from porcine kidney and liver), and variously N-deacetylated/N-sulfated K5 preparations were N-[³H]acetylated as described previously (19), targeting residual N-unsubstituted glucosamine residues. Polysaccharide (200 µg) was dissolved in 100 µl of 0.5% Na₂CO₃ in 10% methanol, and 3.5 mCi of [³H]acetic anhydride was added in aliquots over a period of 1.5 h. The labeled material was separated from free radioactivity on PD-10 columns equilibrated with 1 M NaCl in 0.5 M sodium acetate. Labeled samples excluded from the gel were collected and desalted by extensive dialysis against deionized water using dialysis tubing with a 3500 Da molecular weight cut-off. The specific ³H activities for the various preparations were: 50% N-sulfated K5, 3,500 cpm/µg; 80% N-sulfated K5, 156,000 cpm/µg; epimerized 98% N-sulfated K5, 24,000 cpm/µg; two samples of HS from porcine liver, 7,000 and 31,000 cpm/µg, respectively; HS from porcine kidney cortex, 48,000 cpm/µg; and heparin from bovine intestinal mucosa, 26,000 cpm/µg.

Partially N-acetylated/N-sulfated K5 polysaccharide samples were enzymatically modified along with ³⁵S labeling, as follows. Samples of chemically N-deacetylated and N-sulfated K5 (50, 80, and 98% conversion) were incubated with microsomal enzymes from a heparin producing, murine mast-cell tumor, in the presence of 35SO₄ (1500 Ci/mmol) was purchased from PerkinElmer Life Sciences. Ham’s F-12 medium was from Invitrogen and fetal calf serum from PAA Laboratories (Pasching, Austria). The mouse anti-VSV tag IgG antibody P5D4 was obtained from Roche Applied Science and the rabbit anti-VSV tag IgG antibody was from Sigma. The Alexa 488-conjugated goat anti-rabbit IgG and Alexa 488-conjugated goat anti-mouse IgG were from Molecular Probes (Eugene, OR). Alkaline phosphatase-conjugated rabbit anti-mouse IgG was from Dakopatts (Glostrup, Denmark). The phosphatase substrate p-nitrophenyl phosphate was from ICN (Aurora, OH). Mowiol was from Calbiochem (La Jolla, CA).

CHO K1 and pgSF-17 cell lines (11) were kindly provided by J. Esko, La Jolla, CA, and were cultured in Ham’s F-12 medium supplemented with 10% fetal calf serum. Hsepi⁺/− and Hs2st⁺/− mouse embryos (17.5 dpc) (13) were obtained from C. Merry, Manchester, UK.

Capsular polysaccharide from Escherichia coli K5 (abbreviated K5), N-deacetylated/N-sulfated K5, partially epimerized N-deacetylated/N-sulfated K5 (~40% of total hexuronic acid being IdoA), chemically O-sulfated K5, and N-deacetylated/N-sulfated/O-sulfated K5 (with low or high degrees of O-sulfation) were kindly given by P. Oreste, Glycogenes, Milan, Italy. Heparin from porcine intestinal mucosa (used in ELISA), N-desulfated/N-acetylated heparin, 2-O-desulfated heparin, 6-O-desulfated heparin, and variously N-sulfated K5 (N-sulfation degree 50, 80, and 98%) were kindly given by A. Naggi, G. Ronzoni Institute, Milan, Italy. Completely desulfated/N-acetylated heparin and completely desulfated/N-sulfated heparin were from Seikagaku, Tokyo, Japan. Carboxyl-reduced heparin was from Neoparin, Alameda, CA. Porcine intestinal mucosa HS (used in ELISA) was purchased from Celsus, Cincinnati, OH. The characteristics of HS, various K5, and heparin derivatives are listed in Table 1. HSs from porcine liver and kidney cortex (14) and heparin preparations from bovine intestinal mucosa and bovine lung (15) were isolated as described. Bovine mucosal heparin was used for N-[³H]acetylation (as described below). The exoenzyme iduronate-2-O-sulfatase was a kind gift by John Hopwood, Women’s and Children’s Hospital, North Adelaide, Australia. PAPS, ATP-sulfurylase, ATP, and inorganic pyrophosphatase were purchased from Sigma. APS-kinase was kindly given by M. Kusche Gullberg, Bergen, Norway. [³⁵S]PAPS was generated by incubating ATP-sulfurylase, APS-kinase, ATP, and inorganic pyrophosphatase with carrier-free Na₂⁷³⁵SO₄ (16). The resultant [³⁵S]PAPS was purified by anion-exchange chromatography on 20 ml of DEAE-Sephatcel using a linear gradient (total volume, 200 ml) from 10 to 1000 mM NH₄HCO₃. All other reagents were of best grade available.

Production and Purification of Phage Display Antibodies—Phage display antibodies HS4E4 (selected against kidney HS (7)) and AO4B08 (selected against mouse skeletal muscle HS (17)) belong to the same Vᵥ Ig gene family (Vᵥ-Vᵥ) and have the Vᵥ complementarity determining region 3 sequences HAPLRN-TRTN and SLRMNGWRAHVQ, respectively. The DNA sequences encoding for the antibodies were subcloned into vector pUC119-His-VSV (J. M. H. Raats, Department of Biochemistry, Faculty of Sciences, Radboud University Nijmegen). To produce antibodies, periplasmic fractions of infected bacteria were isolated as described (18). Briefly, bacteria expressing HS4E4 or AO4B08 single chain variable fragment antibodies were grown and induced by isopropyl β-D-thiogalactopyranoside to produce antibodies. The bacterial periplasmic fraction containing antibody was isolated, dialyzed against PBS, and stored at −20 °C. Antibodies AO4B08 and HS4E4 were purified using Protein A-agarose or HIS-cobalt affinity-agarose beads (18). 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 as described earlier (18).

Oligosaccharides—Oligosaccharides from bovine lung heparin were generated by partial deaminative cleavage at N-sulfated glucosamine residues, followed by reduction of products with NaB₃H₄ and separation by gel chromatography as described (20). The end-labeled products had a specific activity of ~7.5 × 10⁶ cpm/nmol. An octasaccharide fraction was sep-
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arated further by affinity chromatography on immobilized antithrombin, as described (18). Octasaccharide libraries were generated from bovine lung heparin as described (18). Briefly, heparin was chemically O-desulfated to eliminate all 6-O-sulfate and about half of the 2-O-sulfate groups, and was then subjected to partial deaminative cleavage followed by NaB₃H₄ reduction. Labeled 8-mer (1.4 × 10⁶ cpm/nmol) was recovered and separated into subfractions with different numbers of O-sulfate groups. Each subfraction was separately incubated with mastocytoma microsomal enzymes in the presence of NaCl in 50 mM Tris/HCl, pH 7.4. Radiolabeled polysaccharide samples were eluted with 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. All polysaccharide samples compared for antibody binding were applied in similar amounts, based on specific radioactivity determined by colorimetric analysis (21).

For ELISA, wells of microtiter plates were coated overnight with K5 polysaccharide derivatives and (modified) heparin preparations (Table 1) as described before (7) by passive adsorption. After blocking with PBS, 3% (w/v) BSA, 1% (v/v) Tween 20, for 1 h, periplasmic fractions containing AO4B08 or HS4E4 in PBS, 1% (w/v) BSA, 0.1% (v/v) Tween 20 were added and incubated for 1.5 h. Bound antibodies were detected using mouse anti-VSV tag IgG antibody P5D4 (1:10), followed by incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG (1:2000), both for 1 h. Enzyme activity was detected using 100 μl of 1 mg/ml p-nitrophenyl phosphate in 1 M diethanolamine, pH 9.8, 0.5 mM MgCl₂, as a substrate. Absorbance was read at 405 nm.

Immuno-staining—Cryosections of 4 μm were obtained from Hs2st+/+ and Hs2st−/− mouse embryos (17.5 dpc), Hs6st1+/+ and Hs6st1−−/− mouse embryos (15.5 dpc), and Hs6st1−−/− mouse embryos (18.5 dpc). Nonspecific binding sites were blocked for 20 min with PBS, 2% (w/v) BSA, 0.05% (w/v) Tween 20 (2% BSA-PBST). Sections were then incubated with anti-HS antibodies AO4B08 or HS4E4 in 2% BSA-PBST for 1 h. Bound antibodies were detected by incubation with rabbit anti-VSV tag IgG antibody (1:500), followed by Alexa 488-conjugated goat anti-rabbit IgG (1:200), incubated for 45 min each. Cryosections were fixed in 100% ethanol for ~10 s, air-dried, and embedded in 10% (w/v) mowiol. Cryosections of rat kidney were incubated with antibodies AO4B08 or HS4E4 in the presence of 0.15–1 M NaCl to determine the relative affinities of antibody binding to HS (18). Bound antibodies were detected by incubation with mouse anti-VSV tag IgG antibody P5D4 (1:10), followed by Alexa 488-conjugated goat anti-mouse IgG (1:200). Immunohistochemistry on CHO K1 (wild type) and pgsF-17 (2-O-sulfotransferase-deficient) cell lines was performed by adding antibodies AO4B08 or HS4E4 in culture medium to the cells for 1 h. Bound antibodies were detected essentially as above.

Characterization of Epitopes—For sequencing of epitope motifs, selected library octasaccharides were preparatively affinity purified on an AO4B08 column. The binding and non-binding fragments were separately pooled, desalted, dried in a centrifugal evaporator, and sequenced through a combination of chemical and enzymatic degradation procedures as described (22). Samples were first subjected to partial HNO₂ cleavage by treatment with 2 mM NaNO₂ in 20 mM HCl at room temperature. Aliquots were incubated for 5, 10, 15, 20, and 25 min, and then the reaction was stopped by addition of 200 mM sodium acetate, pH 6. The aliquots were pooled, and a fraction of the pool analyzed directly by anion-exchange chromatography on a Propac column as described (22). Separate aliquots of the deamination pool were subjected to enzymatic digestion using 1 milliunit of recombinant iduronate-2-O-sulfatase, in a final volume of 25 μl of 50 mM sodium acetate, pH 5, 0.1 mg/ml BSA. After incubation at 37 °C for 2 h digests were analyzed on the Propac column. Sequence information was obtained by detecting shifts in the elution positions of the enzyme-treated fragments.

Affinity-fractionated liver HS was subjected to either exhaustive deamination at pH 1.5 (23) or to N-deacetylation by hydrazinolysis at room temperature (24) followed by deamination at pH 3.9. The products were separately applied to repeated affinity chromatography on immobilized HS4E4 antibody.

For disaccharide analysis K5 derivatives and HS were treated with 0.4 milliunits each of heparin lyase I, II, and III in 15 μl of heparin lyase buffer (5 mM Hepes, pH 7.0, 50 mM NaCl, 1 mM CaCl₂, 0.7 mg/ml bovine serum albumin) and incubated for 16 h at 37 °C. Heat-inactivated digests were dried and resuspended in 45 μl of H₂O for analysis by reverse phase-ion pair high performance liquid chromatography (RP-IP-HPLC) as described (5). To determine the degree of epimerization, disaccharides were obtained by deamination of the chains at pH 1.5 (25), followed by end labeling using NaB₃H₄ and isolation by gel filtration on a Sephadex G-15 column (20). The labeled disaccharides were then analyzed by strong ion-exchange chromatography on a Partisil-10 SAX column as described (20) and the non-sulfated species by paper chromatography on Whatman No. 1 paper in ethyl acetate/acetic acid/water (3:3:1, v:v) (26).

Glycosaminoglycans were quantified by colorimetric analysis of hexuronic acid using the meta-hydroxydiphenyl method (21) with GlcA as a standard. An arbitrary factor of 3 was employed to convert values to saccharide mass.

RESULTS

Binding of Heparin and HS to Antibodies AO4B08 and HS4E4—The two antibodies, AO4B08 and HS4E4, had been selected by panning to mouse skeletal muscle glycosaminoglycan and kidney HS, respectively, and showed overlapping as well as differential staining of rat spleen tissue sections (8). Differences in staining patterns were also manifest when human kidney sections were incubated with AO4B08 and HS4E4 (9). Here, we incubated rat kidney sections with antibodies at varied ionic strength to highlight differences in affinity for different epitopes in the tissues. Both antibodies stained Bowman’s capsules, renal tubules, and smooth muscle cells at physiologic ionic conditions, whereas no staining of the glomerular tuft and the mesangium was seen (Fig. 1). Increasing the salt concentra-
tion resulted in a decreased AO4B08 staining of Bowman’s capsule as well as renal tubules and smooth muscle cells. For HS4E4, increasing the salt concentration resulted first in decreased staining of the renal tubules. Only at a higher concentration of salt was staining of Bowman’s capsule and smooth muscle cells also decreased. These findings confirmed that the two antibodies preferentially bind to different epitopes.

We therefore compared the antibodies regarding the ability to bind various defined 3H-labeled polysaccharides in affinity chromatography. Antibody AO4B08 retained heparin (Fig. 2A) and liver HS (Fig. 2E) to similar extents, both types of polysaccharides being eluted up to 0.45 M NaCl concentration. HS from kidney cortex bound poorly compared with liver HS or heparin and was largely unbound at 0.15 M NaCl (Fig. 2C). The ligand preference of antibody HS4E4 differed from that of AO4B08, in that both heparin and kidney HS were poor ligands (Fig. 2, B and D), whereas major portions of the liver HS sample required relatively high ionic strength for elution (Fig. 2F).

**Evaluation of Antibody Specificity by ELISA**—The two antibodies showed more clear cut distinctions in their interactions against a panel of heparin/HS analogues (Table 1) in direct ELISA (Fig. 3). AO4B08 reacted with heparin and, to a similar extent, with HS from porcine intestinal mucosa (Fig. 3, upper panel). Removal of N-sulfate, 2-O-sulfate, or 6-O-sulfate groups from heparin drastically decreased antibody binding, indicating that all types of sulfate groups contribute to interaction. Sulfation alone would seem not to be the only key determinant, however, as indicated by the avid binding of intestinal HS (in average 1 sulfate group per disaccharide unit, compared with 2.4 for heparin). Moreover, none of the modified (epimerized, sulfated) K5 derivatives tested showed any significant AO4B08 binding (Fig. 3), despite overall sulfation levels approaching 3.8 residues per disaccharide unit (Table 1). Notably, carboxyl reduction of heparin also abolished interaction, thus implicating intact hexuronic acid residues in epitope function.

HS4E4 showed a different interaction pattern (Fig. 3, lower panel). Contrary to AO4B08, which was unable to bind O-desulfated heparin, HS4E4 showed a strikingly augmented
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### TABLE 1

Preparations of heparin and K5 polysaccharide used in ELISA

| Preparation* | Abbreviation | Source | Sulfate groups/disaccharide unita | Distribution of sulfate groupsb | Major disaccharide unitc |
|--------------|--------------|--------|---------------------------------|-------------------------------|--------------------------|
| E. coli K5 polysaccharide | K5 | Glycores | 0 0 0 0 | - | |
| N-Deacetylated/N-sulfated K5 | NdAc/NS K5 | Glycores | 1 100 0 0 | - | |
| N-Deacetylated/N-sulfated K5, epimerizedd | EpiNdAc/NS K5 | Glycores | 1 100 0 0 | - | |
| O-Sulfated K5 (low) | OS K5 (low) | Glycores | 0.9 0 <10 90 | - | |
| N-deacetylated/N-sulfated/O-sulfated K5 (low) | NdAc/NS/OS K5 (low) | Glycores | 1.9 100 <10 90 | - | |
| N-Deacetylated/N-sulfated/O-sulfated K5 (high) | NdAc/NS/OS K5 (high) | Glycores | 3.8 100 100 100 | - | |
| Heparan sulfate (intestinal mucosa) | HS | Celsus | 0.8 44 12 25 | - | |
| Heparin | Heparin | G. Ronzoni | 2.4 89 69 79 | - | |
| N-Desulfated/N-acetylated | NdS/NAc heparin | G. Ronzoni | 1.5 0 69 79 | - | |
| 2-O-Desulfated | 2-O-dS heparin | G. Ronzoni | 1.7 87 0 79 | - | |
| Pref. 6-O-desulfated | 6-O-dS heparin | G. Ronzoni | 1.8 87 67 23 | - | |
| Completely desulfated/N-sulfated heparin | CdS/NS heparin | Seikagaku | -0.9 -0.9 0 0 | - | |
| Completely desulfated/N-acetylated heparin | CdS/NAc heparin | Seikagaku | <0.1 - - - | - | |
| Carboxyl-reduced heparin | Carboxyl-reduced heparin | Neoparin | ~2.4 + + + | - | |

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* Modified polysaccharides of porcine intestinal mucosa heparin, HS and E. coli K5 polysaccharides used in.

b Average number of sulfate groups per disaccharide unit as calculated from composition data (see below).

c Data provided by the suppliers. The proportions of disaccharide units carrying the indicated sulfate groups are expressed as percent of total disaccharide units where supplied or as non-existing (−) and existing (+) for other preparations.

d Contains ~40% IdoA.

e HS contains different types of disaccharide species in a heterogeneous sequence.

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ability to bind 2-0- as well as 6-0-desulfated heparin compared with the fully sulfated parent compound. Removal of both types of O-sulfate substituents again reduced binding toward the level of intact heparin, similar to that of intestinal HS. Selective loss of all N-sulfate groups in the N-desulfated/N-acetylated (NdSNAc) heparin sample prevented binding completely, indicating that N-sulfate groups are crucial for antibody binding. Conversely, introduction of N-sulfate groups in K5 polysaccharide induced HS4E4 recognition, but only provided that IdoA units were also present. Reduction of the carboxyl groups in heparin in turn abolished binding. Collectively, these results indicated that HS4E4 recognizes an epitope containing N-sulfate and IdoA residues, and that excessive O-sulfation may inhibit antibody binding.

**Immunostaining of Tissues Defective in HS Biosynthesis**—Mice lacking functional genes encoding one of the HS biosynthetic enzymes, C5-glucuronyl epimerase (Hsepi−/−), 2-O-sulfotransferase (Hs2st−/−), or 6-O-sulfotransferase 1 (Hs6st1−/−) have been shown to generate HSs with distinct structural aberrations (12, 13).4,5 AO4B08 was unable to stain tissue sections from any of the mutated animals (Fig. 4A), in accordance with the ELISA evidence, suggesting that both 2-O- and 6-O-sulfate groups were needed for antibody recognition (Fig. 3, upper panel). The failure to stain tissues from the epimerase knock-out embryos could reflect the fact that such tissues also lack 2-O-sulfate groups, but would also be compatible with a requirement for IdoA in antibody recognition. The need for 2-O-sulfate groups was directly confirmed by the failure of antibody AO4B08 to recognize also CHO cells deficient in 2-O-sulfo-

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Antibody HS4E4 was capable of staining tissues from Hs2st−/− as well as Hs6st1−/− animals (Fig. 4A). It also bound to 2-O-sulfotransferase-deficient cells (Fig. 4B), but did not stain tissues from the epimerase knock-out embryos (Fig. 4A). These findings reinforce the conclusion that IdoA residues are crucial for HS4E4 recognition, whereas 2-O-sulfate groups are not.

**Specific O-Sulfate Groups in AO4B08 Binding**—The importance of type and positioning of O-sulfate groups for AO4B08 binding was evaluated further by affinity chromatography, using a library of heparin-based, fully N-sulfated octasaccharides carrying defined numbers of O-sulfate groups in different combinations (18). The preparation strategy was designed such

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4 Habuchi, H., Nagai, N., Sugaya, N., Atsumi, F., Stevens, R. L., Kimata, K. (2007) J. Biol. Chem. 282, 15578–15588.

5 T. J. M. Wijnhoven, G. J. Jenniskens, and T. H. van Kuppevelt, unpublished observations.
that octasaccharide subpopulations identical in charge but heterogeneous with respect to the positioning of O-sulfate groups could be individually tested for antibody binding. Octasaccharides containing three or fewer O-sulfate groups were essentially unable to bind AO4B08 at physiological ionic strength (Fig. 5A). A fully (N- and 2,6-O-sulfated heparin octamer was eluted from the antibody column with 0.25 m NaCl, similar to an octasaccharide fraction selected for affinity to antithrombin, suggesting that 3-O-sulfation did not selectively contribute to AO4B08 binding (Fig. 5B). However, the total number of O-sulfate groups was not the only factor determining affinity, because an 8-mer with three 2-O-sulfates and one 6-O-sulfate (designated 3 + 1 in Fig. 5A) was unable to bind to the antibody, whereas the converse (1 + 3) pattern was, in part, compatible with interaction (Fig. 5A). This latter 8-mer library fraction was consistently separated into bound and non-bound species on the AO4B08 column. The proportion of bound fraction increased for tri-6-O-sulfated octasaccharides with additional 2-O-sulfate groups.

**Sequence Analysis of AO4B08-binding and Non-binding Oligosaccharides—**The occurrence of two distinct affinity fractions within the 1 + 3 8-mer population was interpreted in terms of positional differences of importance for antibody binding. Because these 8-mers were all fully 6-O-sulfated (except for the reducing terminal [3H]anhydromannitol residues, that were not recognized as substrate by microsomal 6-O-sulfotransferase), the problem was limited to defining the position(s) of the single, critical IdoA-2-O-sulfate group. To this end, we applied the 1 + 3 fraction to preparative affinity separation on the AO4B08 column, followed by sequence analysis of binders and non-binders (22). The 3H-end-labeled 8-mers were subjected to partial deaminative cleavage, yielding mixtures of shorter even-numbered, labeled fragments all representing the reducing terminus of the initial octasaccharide. Anion exchange HPLC of fragments thus derived from the AO4B08-binding 8-mer (Fig. 6A, upper panel) showed two major and three minor peaks that could be assigned, by comparison with previously identified structures (22, 27), to a non-sulfated disaccharide (elution at 5 ml; 2a, 2b), a tetramer with one O-sulfate (elution at 28 ml; 4a), a tetramer with two O-sulfate groups (elution at 47 ml; 4b), a hexamer with three O-sulfate groups (elution at 75 ml; 6a), and the original octamer with four O-sulfate groups (elution at 93 ml; 8a, 8b). To elucidate the site(s) of 2-O-sulfation, the mixture of deamination products

**FIGURE 3. Evaluation of reactivity to various glycosaminoglycan species by ELISA.** Periplasmic fractions containing AO4B08 (upper panel) or HS4E4 (lower panel) were applied to various glycosaminoglycan species (see Table 1) coated on microtiter plates. Bound antibodies were detected using mouse anti-VSV tag IgG antibody P5D4, followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG. Enzymatic activity was measured using anti-VSV tag IgG antibody, followed by Alexa 488-conjugated goat anti-rabbit IgG (B). Bars represent the mean reactivity ± S.E. (n = 4) of the antibodies in percent relative to the reactivity with heparin.

**FIGURE 4. Immunostaining of mouse embryonic tissue sections and cells deficient for HS biosynthetic enzymes.** Cryosections of control and knock-out embryos for CS-epimerase (HsepiHsepi), 2-O-sulfotransferase (Hs2stHs2st), and 6-O-sulfotransferase (Hs6stHs6st) were stained with anti-HS antibodies AO4B08 and HS4E4 (see “Experimental Procedures”), which were subsequently detected using rabbit anti-VSV tag IgG antibody, followed by Alexa 488-conjugated goat anti-rabbit IgG (A). Results apply to all organs examined, only liver, lung, and heart, respectively, being shown here. CHO K1 (wild type) and pgsF-17 (Hs2stHs2st) cells were similarly incubated with AO4B08 and HS4E4, and subsequently stained (B). Bar, 50 μm; magnification was identical for each specimen.
was digested with IdoA-2-O-sulfatase, and the digest was again subjected to anion exchange chromatography (Fig. 6A, lower panel). Shifts in elution position of individual fragments induced by IdoA-2-O-sulfatase digestion provide a readout for positions of 2-O-sulfate groups in the initial octasaccharide sample. The intact octasaccharide was not affected by IdoA-2-O-sulfatase digestion, indicating a nonreducing terminal IdoA residue (see schematic representation of structures in Fig. 6C). The tri-O-sulfated hexamer was largely shifted downward in the gradient, due to loss of one 2-O-sulfate group (new elution position at 57 ml; 2c). Similar experiments were conducted with unfractionated heparin octasaccharides (UF) or with heparin octasaccharides selected for high affinity to antithrombin (AT) (B).

Alternative approaches using intact polysaccharide chains were therefore applied. Because affinity chromatography on HS4E4 columns had shown that fractions of porcine liver HS bound well to the antibodies (Fig. 2F), such material was used to study the N-substituent requirement of binding epitopes in authentic HS. Again, the starting material was $^{3}$H-acetylated by reacting unsubstituted amino groups in the HS with $^{3}$H-acetic anhydride. The labeled product was subjected to preparative affinity chromatography on immobilized HS4E4, yielding a non-bound pool emerging with 0.15 M NaCl, and two bound pools eluted with 0.25 and 0.35 M salt, respectively. Rechromatography of each fraction showed the expected elution patterns (Fig. 7, A, C, and F). Aliquots of each fraction were subjected to exhaustive deaminative cleavage at pH 1.5, thus eliminating all N-sulfated disaccharide units but leaving N-acetylated sequences intact (28). The radiolabeled oligosaccharide products were all unable to bind the antibody (data not shown), confirming a requirement for one or more N-sulfate groups, or for components (IdoA, O-sulfate groups) associated with N-sulfation (3). Conversely, samples were N-deacetylated by hydrazinolysis, followed by deamination at pH 3.9, a process leaving N-sulfated structures intact (28). To replace the label lost in N-deacetylation these cleavage products were $^{3}$H-end-labeled by reduction with NaB$_{3}$H$_{4}$. Reapplication of the labeled N-sulfated fragments to the HS4E4 column again failed to produce any retained material (Fig. 7, B, D, and F). These results strengthen the notion that epitopes recognized by HS4E4 contain both N-acetylated and N-sulfated structural elements.

To further clarify the importance of chain modification in HS4E4 recognition, we conducted a series of experiments using...
Epitopes of Anti-HS Antibodies AO4B08 and HS4E4

FIGURE 6. Sequence analysis of library octasaccharides separated with regard to affinity for AO4B08. End group \(^{3}H\)-labeled (1 + 3)-octasaccharides were fractionated on immobilized antibody, as shown in Fig. 5A. A, the bound fraction was subjected to partial deaminative cleavage, and the products were fractionated by anion exchange HPLC on a Propac column (upper panel), using a linear salt gradient for elution (- - -). The mixture of deamination products was digested with iduronate-2-O-sulfatase and analyzed by reverse phase HPLC (lower panel). The letters \(a-d\) associated with the various oligosaccharides relate to the variants present in the 8-mer species (for further details see the text). C, scheme of structures deduced for AO4B08-bound (sequences \(a\) and \(b\)) and AO4B08-unbound (sequences \(c\) and \(d\)) library octasaccharides. Each graph is representative for at least two reproducible runs.

FIGURE 7. Effects of selective deaminative cleavage on HS4E4 affinity of porcine liver HS. Porcine liver N\(^{3}H\)-acetyl-labeled HS was fractionated on the HS4E4 column, as described in the text, and fractions of low (A), intermediate (C), and high (D) affinity were rerun to ascertain specificity of binding. Each fraction (low affinity, B; intermediate affinity, D; high affinity, F) was N-deacetylated by hydrazinolysis, cleaved by exhaustive deamination at pH 3.9, and the products were relabeled by reduction with NaB\(^{3}H\)\(_{4}\) (see “Experimental Procedures”). Fractions of disaccharides or larger size were recovered and reapplied to affinity chromatography. Alternatively, fractions were subjected to exhaustive deamination with nitrous acid at pH 1.5. The results (not shown) were indistinguishable from those in panels B, D, and F. The stepwise salt gradient is indicated (- - -). Effluent fractions were analyzed for radioactivity.

various chemically N-sulfated K5 polysaccharide preparations as substrates for mastocytoma microsomal enzymes (N-deacetylases/N-sulfotransferases, GlcA C5-epimerase and O-sulfotransferases), in the presence of \(^{35}S\)PAPS. The native K5 polysaccharide has the same (GlcA-GlcNAc)\(_{n}\) structure as the initial polymer precursor in heparin/HS biosynthesis, and is enzymatically modified through N-deacetylation/N-sulfation, C5-epimerization of GlcA to IdoA units, and O-sulfation in various positions (29). We applied three K5 samples that had been chemically modified, such that \(\sim 50, \sim 80\), and \(\sim 98\)% of the glucosamine residues were N-sulfated, part of the remaining N-acetyl groups being \(^{3}H\)-labeled (see “Experimental Procedures”). These samples were all unretarded by immobilized HS4E4 (Fig. 8). By contrast, \(\sim 60\)% of the total \(^{35}S\)-radiolabel incorporated into 50% N-sulfated K5 was retained on the antibody column following enzymatic epimerization/sulfation, and required \(>0.5\) M NaCl for complete elution (Fig. 8A). Also the 80% N-sulfated K5 derivative yielded some HS4E4-binding material upon enzymatic modification (Fig. 8B), whereas the 98% N-sulfated K5 was a poor precursor to create HS4E4-binding epitopes (Fig. 8C).

The non-bound (eluted at 0.15 M NaCl) and retarded (eluted at \(\geq 0.35\) M NaCl) fractions of the K5 polysaccharides were analyzed after lyase digestion for disaccharide composition by RPIP-HPLC (Fig. 9). Interestingly, the non-bound fractions obtained from modified 50 and 80% N-sulfated K5 differed markedly in N-substitution, whereas the retarded fractions were quite similar, \(\sim 80\)% of the total glucosamine residues being N-sulfated. Furthermore, the antibody-bound fractions from both types of precursor K5 showed slightly increased, albeit overall modest, levels of O-sulfation compared with their non-bound counterparts. These results clearly support the notion of a complex epitope structure recognized by HS4E4, the minimal essential structural features including both N-acetyl and N-sulfate groups as well as O-sulfate substituents. The poor yield of binding species produced by \(\sim 98\)% N-sulfated K5 (Fig. 8C) probably reflects the relative lack of N-acetylated disaccharide units. In accordance with these conclusions, fully N-sulfated heparin-derived octasaccharides, irrespective of the
degree of O-sulfation, were unable to interact with the antibody, nor was the high affinity binding motif for antithrombin recognized (data not shown).

All K5-derived samples were C5-epimerized to some extent after microsomal modification (Table 2), as revealed by disaccharide analysis of the N-sulfated domains following deaminative cleavage. The IdoA content ranged from 20 to 30% of the total hexuronic acid in these fractions. However, we have no information regarding the degree of epimerization of the HS4E4-bound and -unbound fractions. Remarkably, ~95% N-sulfated [3H]K5 with an epimerization degree of roughly 40%, but no O-sulfation showed significant binding to the HS4E4 column (Fig. 8D), contrary to a non-epimerized but similar sample (data not shown). This observation supports the notion of IdoA contributing to HS4E4 recognition.

**DISCUSSION**

Here we describe the characterization of epitopes recognized by two different phage display anti-HS antibodies, AO4B08 and HS4E4. These antibodies belong to a large family of single-chain antibodies, selected for binding to HS to provide tools for analyzing HS structural changes in development and disease (7). Our goal was to characterize AO4B08 and HS4E4, which show distinct differences in epitope recognition. According to current notion, HS function involves binding of proteins to the various sulfated domains of the polysaccharide chain. Such domains are of essentially two types, NS-domains composed of alternating N-acetylated and N-sulfated units. The NA/NS-domains contain IdoA as well as 6-O-sulfate groups, but differ from NS-domains in lacking 2-O-sulfate residues (4). The third major structural component, NA-domains, are fully N-acetylated and essentially lack IdoA and sulfate groups.

Antibody AO4B08 was selected against mouse skeletal muscle glycosaminoglycan (17) and reacts with both heparin and HS from various sources (Fig. 2, and data not shown). It stains a variety of tissue structures (Fig. 1) as it reacts with a relatively short N-sulfated motif containing both 6-O-sulfated glucosamine units and 2-O-sulfated hexuronic acid. N-, 2-O-, and 6-O-sulfate groups are all essential for antibody recognition as evident from binding experiments with variously sulfated polysaccharides (Fig. 3) and chemoenzymatically modified oligosaccharides (Fig. 5), as well as from immunostaining of embryonic or cells deficient in selected enzymes involved in HS biosynthesis (Fig. 4). Sequence analysis of minimally O-sulfated oligosaccharides capable of AO4B08 binding revealed a requirement for three consecutive 2-O-sulfated disaccharide units along with an essential, internally positioned 2-O-sulfated hexuronic acid (Figs. 5 and 6). Authentic NS-domains usually contain 2-O-sulfate groups in excess of 6-O-sulfation (30, 31), and the presence of 2-O-sulfates in addition to the single essential 2-O-sulfated group did not preclude epitope recognition. It therefore seems likely that the broad epitope distribution in tissues revealed by staining with AO4B08 reflects the occurrence of heavily O-sulfated NS-domains composed of at least three disaccharide units. Notably, longer NS-domains appear to be relatively rare in HS (32, 33).

Interestingly, Hs6st-1−/− embryonic mouse heart sections were not stained by antibody AO4B08 (Fig. 4A), indicating that the epitope was not generated through the combined action of Hs6st-2 and Hs6st-3. This deficiency could reflect the differential expression patterns of the Hs6st isoforms (34), alternatively a specific requirement for Hs6st-1 in generating consecutive 6-O-sulfated disaccharide units.

HS4E4, selected by panning to bovine kidney HS, showed more restricted tissue staining (8). Several lines of evidence point to an HS epitope structure more complex than the simple NS-domain recognized by AO4B08. Contrary to AO4B08, HS4E4 was thus able to bind to tissue sections derived from either Hs2st or Hs6st-1 knock-out mice (Fig. 4). Yet binding to completely O-desulfated heparin chains is weak as seen in the
Cleavage of HS chains at fucosyl groups from heparin promoted HS4E4 binding (Fig. 3). Mains intact, abolished antibody binding, suggesting that the epitope for HS4E4 recognition is indicated by the fact that HS4E4 bind to HS4E4 either (data not shown). Another important feature, unlikely, because heparin oligosaccharides up to 18-mer did not recover in this way from HS are too short appears for chemoenzymatically modified K5 polysaccharide (Figs. 8 and 9). The disaccharide products, containing non-reducing terminal 4,5-unsaturated hexuronic acid residues (ΔHexA) were analyzed by RPIP-HPLC, as described under “Experimental Procedures.” The disaccharide composition is indicated for 50% N-sulfated K5, non-retained (white bar) and retained (fraction eluted at ≥0.35 M NaCl; dark-gray bar) fractions, and for 80% N-sulfated K5, non-retained (light-gray bar) and retained (fraction eluted at ≥0.35 M NaCl; black bar) fractions. The inset shows overall sulfate contents (Total sulfation), specified according to type of substituent as total N-sulfated disaccharides (NS), total 6-O-sulfated disaccharides (6S), and total 2-O-sulfated disaccharides (2S).

TABLE 2
Microsomal preparation modified K5 polysaccharides used in affinity assays

| Preparation | Sulfate groups/ disaccharide unit | Distribution of sulfate groups | Epimerization degree |
|-------------|---------------------------------|------------------------------|---------------------|
| K5 50% N-sulfated | 0.7                             | 52 7 12 22                    |
| K5 80% N-sulfated | 1.2                             | 87 12 19 17                   |
| K5 98% N-sulfated | 1.1                             | 93 6 8 26                     |

* E. coli K5 polysaccharides of the indicated level of N-sulfation were modified by incubation with microsomal enzymes as described under “Experimental Procedures” and used in affinity chromatography.

* Average number of sulfate groups per disaccharide unit as calculated from composition data (see below).

* Composition of chains determined by RPIP-HPLC of enzymatically released disaccharide units.

* Degree of epimerization of N-sulfated disaccharides determined by HNO2/NaB3H4 treatment and analysis of labeled disaccharides by SAX-HPLC and paper chromatography.

direct ELISA (Fig. 3, lower panel), indicating a contribution from O-sulfate groups in the antibody binding epitope. A role for O-sulfates was also inferred from the binding experiments with chemoenzymatically modified K5 polysaccharide (Figs. 8 and 9). However, HS4E4 did not bind to any of the disaccharide library fragments, fully N-sulfated with defined numbers of O-sulfate. In fact, selective removal of either 2-O- or 6-O-sulfate groups from heparin promoted HS4E4 binding (Fig. 3). Cleavage of HS chains at N-acetylated sites, leaving NS-domains intact, abolished antibody binding, suggesting that the motif may contain one or more essential N-acetyl group. The alternative interpretation, that the purely N-sulfated oligosaccharides recovered in this way from HS are too short appears unlikely, because heparin oligosaccharides up to 18-mer did not bind to HS4E4 either (data not shown). Another important feature for HS4E4 recognition is indicated by the fact that HS4E4 does not bind to tissue derived from the Hsepi−/− embryos (Fig. 4A), indicating a requirement of either GlcA C5-epimerization, i.e. IdoA residues, or 2-O-sulfation. Consequent to the epitope loss, HS from such embryos largely lack 2-O-sulfate groups along with the absence of IdoA (12). However, because HS4E4 does recognize epitopes in Hs2st−/− embryos (Fig. 4A) and in Hs2st−/− cells (Fig. 4B), 2-O-sulfation appears not to be critical, but rather the IdoA per se. Taken together, these observations point to a complex HS4E4-binding epitope involving N-acetylated and N-sulfated glucosamine residues, IdoA and GlcA units (all antibody-binding polysaccharides most likely contain a -GlcNAc-GlcA sequence), and some unspecified O-sulfated group. Partially O-sulfated structures are better ligands than fully O-sulfated ones.

The various HS-binding phage display antibodies differ from each other regarding the dp genes and their complementarity determining region 3 domains, which encompass at most a 12-mer amino acid sequence (10). Yet these domains are sufficient to account for the widely differing HS-recognition properties, not only of the two antibodies described in this report, but also of antibody HS4C3 that preferentially binds 3-O-sulfated motifs (18). Thus, even short structural motifs in otherwise similar proteins may impart distinct HS binding properties.

Aspects of specificity in HS-protein interactions attract much current interest. HS biosynthesis is strictly regulated, and we must assume that the different types of epitopes/domains in a chain are generated for distinct functional purposes. HS chains, as part of proteoglycans at the cell surfaces or in the extracellular matrix, serve as extracellular storage sites for growth factors/morphogens or chemokines, stabilize growth factor/morphogen gradients in embryonic development, position bioactive proteins for interactions with their receptors, and participate in actual formation of signaling complexes. Recent biochemical and genetic information has shed new light on the nature of HS-protein binding. Whereas there are a few examples of highly specific interactions, others appear to depend more on the organization of HS domains, or on the overall level of sulfation of such domains, rather than on their fine structure (6). The AO4B08 epitope represents a “heparin-like” domain of a type likely to interact with a variety of proteins. The functional properties of such domains are presumably dictated by variations in the levels of sulfation. The structural features recognized by antibody HS4E4 rather depend on the mode of domain organization within the HS chain. We predict that characterization of additional antibodies of both types will provide important tools to study HS structure-function relations in development, homeostasis, and disease.

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