Large, Tissue-regulated Domain Diversity of Heparan Sulfates Demonstrated by Phage Display Antibodies

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Heparan sulfates (HS) are long, linear polysaccharides with a high degree of variability. They bind to a vast number of proteins such as growth factors and cytokines, and these interactions are likely to be mediated by specific HS domains. To investigate the structural diversity and topological distribution of HS domains in tissues, we selected a panel of 10 unique anti-HS antibodies using phage display technology. All 10 antibodies recognize a specific HS epitope as demonstrated by enzyme-linked immunosorbent assay using defined synthetic HS oligosaccharides, modified HS/heparin molecules, and HS isolated from a variety of organs. The chemical groups involved in the epitopes could be indicated and the position of sulfate groups is of major importance. All HS epitopes have a defined tissue distribution as shown by immunohistochemistry using rat organs. Taken together, the data show that in vivo, a large number of defined HS epitopes exist that do not occur randomly but are tightly, topologically regulated.

Heparan sulfates (HS), a class of glycosaminoglycans, are long linear complex polysaccharides covalently bound to a protein core. They have a ubiquitous distribution in the extracellular matrix and on cell surfaces and have been implicated in many basic biological phenomena such as cell migration, adhesion, and differentiation. They play a role in such diverse processes as growth factor/cytokine handling, enzyme regulation, lipid metabolism, blood coagulation, and viral entry (1–4). This involvement is mediated by the interactions of HS with a vast number of proteins such as growth factors/cytokines, enzymes, protease inhibitors, extracellular matrix molecules, and viral coat proteins (5, 6). The large number of interactions suggests an extensive structural variation within HS. Chemical analysis of HS-derived disaccharides indeed indicates a large structural diversity (7–9), which is brought about by specific chain modifications during HS biosynthesis. The importance of defined monosaccharide sequences for specific interactions with proteins has been demonstrated for the binding and activation of antithrombin III by HS/heparin (10, 11). In addition, specific structural requirements for binding to basic fibroblast growth factor and hepatocyte growth factor have been defined (12, 13). These observations indicate that HS modifications do not occur randomly but that a controlled expression of specific domains/sequences in HS exists. To investigate whether a large number of different HS domains indeed occur in tissues, we selected a panel of epitope-specific anti-HS antibodies using phage display technology. Using these antibodies, we chemically and topologically characterized the HS epitopes involved. We chose antibody phage display because it allows for the generation of antibodies against poorly immunogenic molecules such as HS.

EXPERIMENTAL PROCEDURES

Materials

A human semisynthetic antibody phage display library (14, now officially named synthetic scFv Library No. 1) was generously provided by Dr G. Winter, Cambridge University, Cambridge, UK. This library contains 50 different V<sub>H</sub> genes with synthetic random complementarity determining region 3 segments, which are 4–12 amino acid residues in length. The heavy chains are combined with a single light chain gene (DPL 16). The library contains >10<sup>14</sup> different clones. All antibodies contain a c-Myc tag.

Heparan sulfate from bovine kidney, chondroitinase ABC (Proteus vulgaris, EC 4.2.2.4), chemically modified heparan sulfate and heparin kits, antichondroitin sulfate "stub" antibody (2B6), and antiheparan sulfate stub antibody (3G10) were from Seikagaku Kogyo Co., Tokyo, Japan. Heparin from porcine intestinal mucosa, chondroitin 4-sulfate from whale cartilage, chondroitin 6-sulfate from shark cartilage, dermatan sulfate from porcine skin, keratan sulfate from bovine cornea, hyaluronate from human umbilical cord, DNA from calf thymus, dextran sulfate, bovine serum albumin (fraction V), heparinase I (from Flavobacterium heparinum, EC 4.2.2.7), heparinase II (from F. heparinum), and heparinase III (heparitinase, from F. heparinum, EC 4.2.2.8) were from Sigma. Alkaline phosphatase-conjugated rabbit anti-mouse IgG was from Dakopatts, Glostrup, Denmark. Mouse anti-c-Myc tag IgG (clone 9E10) was from Roche Molecular Biochemicals. Alexa 488-conjugated goat anti-mouse IgG was from Molecular Probes, Eugene, OR. The PCR kit was from Promega, Madison, WI. ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit was from PE Applied Biosystems, Norwalk, CT. Mowiol (4–88) was from Calbiochem. Restriction enzyme BstNI was from New England Biolabs, Beverly, MA. Polystyrene Maxisorp Immunotubes were from Nunc, Roskilde, Denmark. Various HS preparations were a generous gift from Dr. Keiichi Yoshida, Seikagaku Corp., Japan. Synthetic HS oligosaccharides were all from Sanofi-Synthélabo Recherche, Toulouse, France.

Methods

Selection of Antibodies Against Heparan Sulfate by Library Panning—Phage display-derived antibodies were obtained as described (15) using four rounds of panning against bovine kidney HS, human lung

HS (HS isolated using papaine/alkaline borohydride digestion followed by anion exchange chromatography), and mouse and human skeletal muscle HS (16). Briefly, antibody-expressing phages were added to HS-coated tubes, and bound phages were eluted at high pH to allow for the infection of Escherichia coli TG1 cells. After overnight amplification, phages were rescued by the addition of helper phage and cultured for further rounds of selections. After the last two rounds, single bacterial colonies were picked, and the antibodies expressed were evaluated.

**Screening for Bacteria Expressing Antibodies against Heparan Sulfate**—Screening for bacteria expressing anti-HS antibodies was performed as described (15). Briefly, single colonies picked from the last two rounds of selection were grown in E. coli periplasmic fraction. Plates were centrifuged, and supernatants containing soluble antibodies were applied to wells of polystyrene microtiter plates previously coated with HS. Bound antibodies were detected using a mouse monoclonal antibody (9E10) directed against the c-Myc tag followed by incubation with alkaline phosphatase-conjugated rabbit anti-mouse IgG. Alkaline phosphatase activity was detected using p-nitrophenyl phosphate as a substrate. Absorbance was measured at 405 nm.

**Screening Bacterial Clones for Unique Antibody Inserts Using PCR, Fingerprinting, and Sequencing**—Bacteria expressing anti-HS antibodies were analyzed for the presence of full-length inserts by 25 cycles of PCR on the amplified DNA as template (15). Fingerprinting was performed using BacNII (CC(A/T)GG) as the restriction enzyme. PCR and fingerprinting analysis were performed using 1% (w/v) and 4% (w/v) agarose gels, respectively. To establish the complementarity determining region 3 and the germ line VH gene DNA segments, unique clones were sequenced using the primer (located in the linker region between the VH and the VL gene). For this purpose, double-stranded DNA was isolated using standard procedures.

**Source of Antibodies**—To obtain optimal amounts of soluble anti-HS antibodies, phages were allowed to infect the non-suppressor E. coli strain HB2151. The periplasmic fraction of bacteria was used as the source of antibodies (15). Briefly, bacteria were grown at 37 °C until an optical density of 0.5–0.8 was reached. Induction was obtained by the addition of isopropyl-β-D-thiogalactopyranoside. After incubation at 30 °C for 3 h, the culture was harvested, and the pellet was resuspended in 200 mM sodium borate buffer (pH 8.0) containing 160 mM NaCl and 1 mM EDTA. After centrifugation, the supernatant was filtered and dialyzed versus PBS. The preparation thus obtained is the periplasmic fraction.

**Characterization of Antibodies by ELISA**— Reactivity of the anti-HS antibodies with various molecules was evaluated in ELISA in two ways: (a) by the application of antibodies to wells of polystyrene plates coated with the test molecules and (b) by an inhibition assay in which the antibodies were incubated with the test molecule for 16 h at 22 °C followed by transfer to wells previously coated with HS (bovine kidney, (15)). Test molecules included HS from bovine kidney, aorta, lung, intestine, pig intestine, and whale lung; heparin, dermatan sulfate, chondroitin 4- and 6-sulfate, keratan sulfate, dextran sulfate, hyaluronate, and K5 polysaccharide from E. coli; bovine serum albumin; marvul (the non-fat milk preparation used as a blocking reagent during panning); and DNA. To analyze which chemical groups in HS are involved in antibody binding, a large number of modified HS preparations and synthetic HS-oligosaccharides were evaluated. They included HS from bovine kidney, which was digested with heparinase I, II, or III or with HNO2 at pH 1.5, and chemically modified HS (bovine kidney) and heparin (porcine intestine). The latter included preparations that were completely desulfated and N-acetylated, preparations that were fully O-desulfated and N-sulfated, and preparations that were N-desulfated and N-acetylated. The antibodies were also analyzed using a panel of 12 defined synthetic HS oligosaccharides.

**Selection of Antibodies**—Bacteria expressing anti-heparan sulfate antibodies were selected on the basis of the amino acid sequence of the complementarity determining region 3 and/or VH gene (Table I) (17). Five antibodies were obtained against bovine kidney HS (antibodies HS3A8, HS3B7, HS4A5, HS4D4, and HS4E4), and three antibodies were obtained against human lung HS (antibodies EV3B2, EV3D1, and EV3C3). The antibodies against mouse and human skeletal muscle HS (antibodies AO4B08 and RB4EA12, respectively) have been described previously (16).

**Specificity of Antibodies**—Using ELISA, antibodies were shown to be specific for HS and heparin and not to be reactive with other glycosaminoglycans such as dermatan sulfate, chondroitin sulfates, hyaluronate, and keratan sulfate nor with other polyanionic molecules such as dextran sulfate and DNA. Antibodies show a characteristic reactivity with HS preparations isolated from various sources such as bovine kidney, aorta, lung, and intestine, human aorta, and whale lung (Table II). None of the antibodies is reactive with the bacterial capsular polysaccharide K5, which is similar to the HS precursor polysaccharide. Therefore, all 10 antibodies recognize specific structural modifications.

**Analysis of HS Epitopes Recognized by the Antibodies**—Antibodies were tested for reactivity with modified HS/heparin preparations (Table III) and with various synthetic HS oligosaccharides (Table IV) to identify which modifications in HS are involved in binding. Results show that all antibodies recognize different structures in HS. In all cases, sulfate groups are essential. HS and heparin stripped from their O- and N-sulfate groups are not reactive with the antibodies. Treatment of HS with HNO2, at pH 1.5 (cleavage at N-sulfated glucosamine residues (GlcNS) and the rare N-unsubstituted glucosamine residues) abolishes antibody binding. Treatment of HS

### Table I

| Antibody | DP gene | VH family | VH CDR3 sequence |
|----------|---------|-----------|-----------------|
| HS3A8    | DP-38   | Vh-3      | GMRPL           |
| HS3B7    | DP-3    | Vh-1      | SRTKTRPFMRK     |
| HS4A5    | DP-65   | Vh-4      | WYTP            |
| HS4D4    | DP-58   | Vh-3      | GMRPL           |
| HS4E4    | DP-38   | Vh-3      | HAPLENTFTNT     |
| EV3B2    | DP-38   | Vh-3      | GKMKLR          |
| EV3C3    | DP-42   | Vh-3      | GYPRF           |
| EV3D1    | DP-47   | Vh-3      | SISNMVGVRQI     |
| AO4B08   | DP-47   | Vh-3      | SLIRMNWRAHQ     |
| RB4EA12  | DP-32   | Vh-3      | BRYALD          |

Given are the DP gene number (according to Ref. 17), Vh germ line gene family, and amino acid sequence of the VH CDR3 complementarity determining region 3.
with heparinase I (cleaves at sulfated domain structures) results in the loss of binding activity of eight out of 10 antibodies. For a number of antibodies, structural modifications of HS important for binding could be identified (Tables III and IV). HS3A8 recognizes highly sulfated domains within HS since it reacts only with highly sulfated oligosaccharides. The antibody strongly prefers 6-O- N-sulfated glucosamine (GlcNS6S) and prefers sulfated iduronic acid (IdoUA2S) over non-sulfated iduronic acid (IdoUA). The preferred sequence (starting at the non-reducing end) is GlcNS6S-IdoUA2S-GlcNS6S. Antibodies HS3B7 and RB4EA12 recognize IdoUA-GlcNS6S containing domains, 2-O sulfation of IdoUA impeding binding (compare compounds 10 and 12 and compounds 11 and 12, Table IV). Differences in topological distribution (see below), however, indicate that each antibody recognizes a different epitope. An-
(Table III). This generic sequence may indicate the recognition of a wide variety of structures, but the restricted topological distribution (blood vessel-associated basement membranes only, see below) argues against this (Fig. 1). Antibodies HS4D4 and AO4B08 are reactive with domains containing GlcNS6S-IdoUA2S-GlcNS6S sequences. Non-sulfated IdoUA impedes binding (compare compounds 10 and 12, Table IV). For AO4B08, an additional 3-O-sulfate group on GlcNS6S has no influence (compare compounds 9 and 10, Table IV). HS4D4, however, requires an additional modification, possibly 3-O-sulfation of GlcNS6S (compare the reactivity of HS4D4 and AO4B08 for compounds 5, Table IV). N-sulfation seems of minor importance (Table III). HS epitopes recognized by the two antibodies have a different topological distribution (see below). Antibody HS4E4 is not reactive with any of the synthetic HS oligosaccharides tested. It is the only antibody that reacts with completely desulfated/N-sulfated heparin/HS (Table III), suggesting that N-sulfation is of major importance. It reacts strongly with human aorta HS (Table II), which is largely devoid of 6-O-sulfation (18), and which may indicate that this modification is inhibitory. Taken together, these data suggest that HS4E4 recognizes GlcNS-IdoUA2S-containing domains. EV3B2 recognizes domains containing GlcNS ± 6S – UA – GlcNS ± 6S, where UA represents GlcUA or IdoUA. 2-O-sulfation of IdoUA improves binding (compare compounds 10 and 12, Table IV); 3-O-sulfation of GlcNS seems to be of no importance (compare compounds 9 and 10, Table IV). EV3D1 needs GlcUA in a certain chemical context, preferably flanked by a GlcNS (± 6S) but not by a non-sulfated IdoUA (compare compounds 6 and 7 and compounds 10 and 12, Table IV). The final antibody, EV3C3, recognizes HS structures that could not be clearly identified. All antibodies, except EV3C3, EV3D1, and EV3B2, bind to perlecan, an HS-proteoglycan isolated from the extracellular matrix of the Engelbreth-Holm-Swarm mouse sarcoma (19) (data not shown).

Localization of HS Epitopes Using Immunohistochemistry—Each antibody showed that the HS structure it recognizes has a defined topology. In the kidney, the anti-HS antibodies primarily stain basement membranes (Fig. 1, Table V). Antibodies HS4A5, RB4EA12, and HS4E4/AO4B08 (both have a similar topology) recognize structures with a clearly differently topological distribution as compared with structures recognized by the other antibodies. These antibodies show a more or less similar tissue distribution, although with some difference in staining intensity. Some antibodies (HS3A8 and HS4D4) show a similar topological staining in renal tissue but a differential distribution in testicular tissue (Fig. 1).

Staining of all the antibodies was abolished by pretreatment of sections with heparinase III but not with chondroitinase ABC. Preincubation of antibodies with HS and heparin precluded staining. Incubation with other glycosaminoglycans, with dextran sulfate, or with DNA has no effect.

DISCUSSION

Next to proteins and nucleic acids, polysaccharides may form a third group of information-dense biopolymers. This especially holds for HS, a class of linear polysaccharides involved in many basic cellular phenomena. Theoretically, cells can generate a large potential of different monosaccharide sequences (domains) in HS (see below). To study whether such a potential indeed exists in vivo, we selected 10 anti-HS antibodies by phage display technology. This technology has proven to be very useful for obtaining antibodies against HS, which are almost non-immunogenic (15, 16). All 10 antibodies

![Fig. 1. Immunostaining of rat renal (a–f) and testicular tissue (g and h) with anti-HS antibodies. Cryosections were incubated with antibody HS4E4 (a), HS4A5 (b), RB4EA12 (c), a non-related phage display-derived antibody (d), HS3A8 (e and g), and HS4D4 (f and h). Note the similar staining of HS3A8 and HS4D4 in renal tissue (e and f) and the differential staining of these antibodies in testicular tissue (g and h). Bar, 25 μm (d, g, and h) or 50 μm (a–c, e, and f). Arrow in g and h, smooth muscle cells.](image)

| Antibody | HS3A8 | HS3R7 | HS4A5 | HS4D4 | HS4E4 | EV3B2 | EV3C3 | EV3D1 | AO4B08 | RB4EA12 |
|----------|-------|-------|-------|-------|-------|-------|-------|-------|--------|---------|
| Glomerulus | ++    | +     | ++    | ++    | –     | ++    | ++    | ++    | –      | –       |
| Bowman’s capsule | +     | ++    | –     | ++    | +     | ++    | ++    | ++    | +      | +       |
| Peritubular capillaries | ++    | ++    | ++    | ++    | ++    | ++    | ++    | ++    | +      | +       |
| Cortical tubules | ++    | ++    | –     | ++    | ++    | ++    | ++    | ++    | +      | +       |
| Smooth muscle cells | +     | +     | –     | ++    | +     | ++    | +     | +     | +      | +       |
| Large blood vessel endothelium | +     | +     | ++    | +     | +     | +     | +     | +     | +      | +       |

* Only a subset of tubules is stained.
recognize a unique epitope structure, the position of sulfate groups being of major importance. Together with the five anti-HS antibodies already described (15, 20, 21), which are different with respect to the ones described here, it implies that at least 15 different HS epitopes are present in the kidney, the nature of the epitopes being highly complex. All epitope structures have a defined topological tissue distribution, indicating that in vivo, a tightly controlled biosynthetic apparatus for HS exists. This may also be true for chondroitin sulfates since a number of anti-chondroitin sulfate antibodies have been generated that show a specific distribution in tissues (22, 23).

The biosynthesis of HS allows for a large number of different sequences. The HS precursor polysaccharide chain is made up of repeating disaccharide units that consist of a glucuronic acid and a GlcNAc residue. These disaccharide units, of which there can be up to a 150 in HS, can undergo a number of modifications. Some GlcNAc residues are N-deacetylated and subsequently N-sulfated, and this step serves as a trigger for further modifications such as epimerization of GlcUA to IdoUA, 2-O-sulfation of GlcUA and IdUA, and 6-O- and/or 3-O-sulfation of GlcN. Modification occurs in specific regions of the polysaccharide chain leading to highly modified domains (N- and O-sulfate rich) interspaced by less modified domains (1, 4). The number of disaccharide units and their possible modifications can (theoretically) give rise to a tremendous structural variation. An octasaccharide, for instance, already has over a million possible monosaccharide sequences. The importance of specific modifications in vivo have been demonstrated. Genetic studies in Drosophila, mice, and humans reveal the involvement of HS in cell differentiation and morphogenesis. In Drosophila, mutations in genes encoding enzymes involved in HS biosynthesis (sugarless, sulfateless, and tout-velu) lead to a loss of specific signaling pathways (FGF, Wingless, and Hedgehog), resulting in severe alterations in phenotype. In mice, a mutation in the gene encoding 2-O-sulfotransferase leads to multiple developmental abnormalities, whereas targeted gene disruption in the genes encoding glucosaminy N-deacetylase/N-sulfotransferase-1 and -2 result in lethal respiratory distress and mast cell abnormalities, respectively (24).

In humans, mutations in glycosyltransferases involved in HS biosynthesis (EXT1 and EXT2) lead to hereditary multiple exostoses (25). These findings indicate that specific HS modifications play a crucial role in developmental processes. Our data indicate that indeed a large number of HS epitopes exist in vivo and that these do not occur randomly but are tightly, topologically, regulated. This indicates the existence of a tightly controlled pattern of modifications occurring during the biosynthesis of HS.

Aberrations in HS fine structure have been implied in various pathological conditions including nephropathies, cancer, and bacterial and viral infections (21, 26–30). The antibodies described here, which probably have a Kp of about 0.1 μM (15), can be used in ELISA, immunohistochemistry, and fluorescence-activated cell sorter analysis. They can probably also be used for immunoblotting, although we did not test this.

With the emerging HS sequencing technology (31, 32) and new methods to prepare synthetic HS oligosaccharides (33), the antibody-defined HS structures presented here may be characterized at the monosaccharide level. The epitope defined by anti-HS antibody 10E4 was recently partially characterized (34). Well defined anti-HS antibodies will make it possible to pinpoint defined structural modifications, and perhaps even monosaccharide sequences, to biological processes in health and disease.

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