Detailed analysis of various heparan sulfate (HS) species is seriously hampered by a lack of appropriate tools, such as antibodies. We adopted phage display technology to generate anti-HS antibodies. A “single pot” semi-synthetic human antibody phage display library was subjected to four rounds of selection on HS from bovine kidney using panning methodology. Three different phage clones expressing anti-HS single chain variable fragment antibodies (HS4C3, HS-D10, and HS3G8) were isolated, with an amino acid sequence of the complementarity-determining region 3 of GRRKLD (VH3 gene, DP-38), SLRGNGCATIH (VH3 gene, DP-42), and YY-HYKYN (VH1 gene, DP-8), respectively. The antibodies react with HS and heparin, but not with DNA or other glycosaminoglycans. $K_v$ values for HS are about 0.1 $\mu$m. The three antibodies react differently toward various HS preparations and show different staining patterns on rat kidney sections, indicating recognition of different HS molecules. This also holds for two described mouse anti-HS IgMs (JM403 and 10E4; both generated by conventional hybridoma technique) and indicates the presence of at least 5 different HS species in the kidney. $O$- and $N$-sulfation are important for binding of HS to HS4C3 and HS3G8. The three single chain antibodies, but not JM403, block a basic fibroblast growth factor binding site of HS. It is concluded that phage display technology presents a powerful technique to generate antibodies specific for HS epitopes. This is the first time this technique has been successfully applied to obtain directly antibodies to (poly)saccharides.

Heparan sulfate (HS) represents a heterogeneous class of molecules within the group of glycosaminoglycans. It has been implicated in many basic cellular phenomena, such as cell growth, migration, and differentiation (1–4). HS binds and modulates various proteins, including growth factors and cytokines, enzymes, protease inhibitors, and extracellular matrix proteins. Studies involving specific enzymatic or chemical cleavage and subsequent analysis of the resulting oligosaccharides indicate the existence of many HS species and the presence of domain structures within the HS molecule (5–15). There are clues that specific monosaccharide sequences within the molecule dictate the specific features of a given species, e.g., a pentasaccharide for the binding of HS/heparin to anti-thrombin III and a preferential sequence for the binding of HS to bFGF (4, 16–19). The appreciation of the structural diversity of HS species and its role in pathological conditions is strongly hampered by the lack of appropriate methodologies. Sequence strategies are not at hand, and specific antibodies, obvious tools for studying diversity, are difficult to raise. HS, and glycosaminoglycans in general, are almost nonimmunogenic, and consequently, only a few specific antibodies have been described (20, 21). To circumvent this, we adopted antibody phage display technology because this system allows one to generate antibodies against “self” antigens. We report here on the generation and application of three specific antibodies against HS species using this technique. We compared these antibodies with two described mouse monoclonal antibodies, with regard to immunostaining on sections of rat kidney, immunoreactivity toward various HS preparations, and reactivity with bFGF sites on HS.

**EXPERIMENTAL PROCEDURES**

**Materials**

A “single pot” human semisynthetic phage library (22) (now officially named synthetic scFv library 1) was generously provided by Dr G. Winter, Cambridge University, Cambridge, United Kingdom. This library contains 50 different VH 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 $> 10^7$ different clones. Two Escherichia coli strains were used: the suppressor strain TG1 (K12, D(lac-pro), supE, thi, lactose-negative, proA<sup>B</sup><sup>-</sup>, lacI<sup>q</sup>, lacZDM15), and the nonsuppressors strain HB2151 (K12, ara, D(lac-pro), thi, proA<sup>B</sup><sup>-</sup>, lacI<sup>q</sup>ZDM15). Helper phages VCS-M13 were from Stratagene (La Jolla, CA).

Heparin sulfate from bovine kidney, chondroitinase ABC (Proteus vulgaris, EC 4.2.2.4), chemically modified heparan sulfate and heparin kits, and mouse anti-heparan sulfate antibody (clone 10E4) 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), FITC-conjugated goat anti-mouse IgM, FITC-conjugated goat anti-mouse IgG, 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. Horseradish peroxidase-conjugated, and alkaline phosphatase-conjugated rabbit anti-mouse IgG were from Dakopatts (Glostrup, Denmark). Recombinant

**FURTHER EVIDENCE FOR HEPARAN SULFATE HETEROGENEITY IN THE KIDNEY**

**Generation and Application of Type-specific Anti-Heparan Sulfate Antibodies Using Phage Display Technology**

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bovine bFGF (146 amino acids) and mouse anti-c-Myc tag IgG (clone 9E10) were from Boehringer Mannheim. PCR kit was from Promega (Madison, WI), and the DNA sequencing kit (Sequenase version 2.0) and [α-32P]dATP were from Amersham Pharmacia Biotech. Restriction enzyme BstNI was from New England Biolabs (Beverly, MA). Polyethylene glycol 6000 and 2.5M NaCl were from Flow (Rockville, MD). Various HS preparations were a generous gift from Dr. U. Lindahl, University of Uppsala (Uppsala, Sweden).

### Growth of Library

5.10⁷ bacteria, corresponding to about 10⁸ phage clones, were inoculated into 50 ml 2xTY medium containing 1% (w/v) glucose and 100 μg ampicillin/ml (2xTY/glua/amp), and grown at 37 °C while shaking until an absorbance of 0.6–0.8 was reached. To 1 ml of this culture, 5.10⁷ VCS-M13 helper phages were added, and after 30 min at 37 °C (no shaking) cells were spun down for 10 min at 3000 × g at 37 °C. Bacteria were resuspended in 10 ml of 2xTY and added to 300 ml of preheated (30 °C) 2xTY/vamp containing 25 μg of kanamycin/ml. Incubation was for 16 h at 30 °C with shaking.

### Isolation of Phages

After spinning the culture for 10 min at 10,000 × g (37 °C), 20% (v/v) of a polyethylene glycol/NaCl solution containing 20% (w/v) polyethylene glycol 6000 and 2.5 μg NaCl was added to the supernatant. After mixing, the solution was kept on ice for 1 h, spun (10,000 × g for 30 min at 4 °C), and resuspended in 40 ml of ice-cold Milli Q water followed by addition of 8 ml of the polyethylene glycol/NaCl solution. After 20 min on ice and spinning (15 min at 3000 × g), the supernatant was carefully removed, and the pellet was resuspended in 2.5 ml of ice-cold PBS (4 °C), spun down (10 min at 3000 × g), and filtered through a 0.45-μm filter to remove any bacterial debris. This solution, containing the phages, was immediately used for selection.

### Selection of Heparan Sulfate Binders by Library Panning

The library was subjected to four rounds of panning. Polystyrene tubes were coated with HS from bovine kidney using a solution of 20 μg/ml for 16 h, 4 °C. Tubes were rinsed three times with PBS, blocked by incubation with 2% (w/v) Marvel (nonfat milk powder) in PBS for 2 h (22 °C), and rinsed again three times with PBS. To the tubes, 2 ml of phage suspension and 2 ml of 4% (w/v) PBS with 2% (w/v) Marvel were added, and incubation was for 30 min under continuous rotating, followed by standing by 90 min (22 °C). Tubes were washed 20 times with PBS containing 0.05% (w/v) Tween-20 and 20 times with PBS. Bound phages were eluted by addition of 1 ml 100 mM triethylenamine, which was neutralized with 0.5 ml of 1x Tris/HCl, pH 7.4. One ml of this phage suspension was added to 9 ml of a suspension of TG1 bacteria (λcoo sac1, 0.6–0.8) and incubated for 30 min at 37 °C in order to allow for infection. Bacteria were spun down, taken into 1 ml of 2xTY, and grown for 16 h at 37 °C on a 24 × 24-cm TYE plate (TY with bacto-agar) containing 1% (w/v) glucose and 100 μg of ampicillin/ml. Bacteria were scraped from the plate using a glass spreader and 5 ml of ice-cold 2xTY/15% (v/v) glycerol. Of this suspension, 50 μl was inoculated into 50 ml of 2xTY/glua/amp and grown at 37 °C until an absorbance at 600 nm of 0.6–0.8 was reached. Phages were rescued after addition of helper phages as described and used for further rounds of selections. A total of four selections were carried out. In one case, an additional negative selection step was introduced. In this case, after the second round of selection, 1 ml of phages was added to a tube containing 3 ml of PBS containing 30 μg of derrmanat sulfate and 30 μg of chondroitin 4-sulfate and incubated for 1 h at 22 °C under continuous rotation. Two ml of this suspension was then subjected to two additional rounds of panning on HS.

### Screening for Phages Expressing Heparan Sulfate Binding Antibodies

Bacteria picked from single colonies after the last round of selection were grown in 200-μl 2xTY/0.1% (v/v) glu/amp in 96-well polystyrene round bottom plates for about 3 h at 37 °C until bacterial growth was visible. Antibody production was induced by adding 25 μl of 2xTY containing 0.6–0.8 of HS acetylated. Next, 100 μl of the bacterial culture was transferred to a corresponding well previously coated with HS and incubated for 1.5 h at 22 °C. Bound antibodies were detected using anti-c-Myc tag mouse monoclonal antibody 9E10 and alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody. Enzyme activity was detected using p-nitrophenyl phosphate as the substrate, and absorbance was read at 405 nm.

### Determination of Affinity

Kd values of the antibodies were determined using an indirect competition ELISA, essentially according to Fritquet et al. (25). Briefly, bacterial culture supernatants containing 0.1 μg of antibodies/ml were incubated for 16 h at 22 °C in wells of microtiter plates with various concentrations of HS (bovine kidney). Next, 100 μl of the antibody to a corresponding well previously coated with HS and incubated for 1.5 h at 22 °C. Bound antibodies were detected as described for ELISA. By plotting the reciprocal of the fraction of antibodies bound versus the reciprocal of the concentration HS used, a Klotz plot can be generated in which the slope represents the Kd. For HS, a molecular mass of 20 kDa was taken.
Antibodies to Heparan Sulfate Using Phage Display

Culture media from bacteria expressing anti-HS antibody HS4C3, HS4D10, and HS3G8 were incubated with various molecules immobilized on the wells of microtiter plates. Bound antibodies were visualized using mouse anti-c-myc IgG, followed by phosphatase-conjugated rabbit anti-mouse IgG. In addition, the mouse anti-HS IgM antibodies JM403 and 10E4 were evaluated and visualized using alkaline phosphatase-conjugated goat anti-mouse IgM. The ratio of sulfate groups to disaccharide is indicated in parentheses. Values (in % of reactivity with HS (bovine kidney) represent mean ± S.D. (n = 4).

### Table I

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS4C3</td>
<td>HS4D10</td>
</tr>
<tr>
<td>HS, bovine kidney (used for panning)</td>
<td>100 ± 1 100 ± 5</td>
</tr>
<tr>
<td>K5, capsular polysaccharide</td>
<td>0 ± 0 0 ± 0</td>
</tr>
<tr>
<td>HS, bovine aorta (0.50)</td>
<td>2 ± 0 3 ± 1</td>
</tr>
<tr>
<td>HS, human aorta (0.60)</td>
<td>1 ± 0 0 ± 1</td>
</tr>
<tr>
<td>HS, bovine kidney (0.84) (fraction A)</td>
<td>28 ± 3 16 ± 2</td>
</tr>
<tr>
<td>HS, bovine lung (0.91)</td>
<td>28 ± 2 6 ± 2</td>
</tr>
<tr>
<td>HS, bovine intestine (0.98)</td>
<td>112 ± 1 11 ± 0</td>
</tr>
<tr>
<td>HS, bovine kidney (1.01) (fraction B)</td>
<td>79 ± 1 77 ± 3</td>
</tr>
<tr>
<td>HS, whale lung (&gt;1)</td>
<td>16 ± 0 56 ± 2</td>
</tr>
<tr>
<td>HS, porcine mucosa (1.64)</td>
<td>26 ± 1 35 ± 1</td>
</tr>
<tr>
<td>Heparin, porcine mucosa</td>
<td>26 ± 5 19 ± 2</td>
</tr>
</tbody>
</table>

* Similar to the HS precursor polysaccharide.
* Fraction A elutes from an anion exchange column between 0.7 and 1.1 M NaCl; fraction B between 1.1 and 1.25 M (14).

### Immunohistochemistry

**Specimens from rat kidney (Wistar, male, 3 months old) were snap-frozen in liquid isopentane cooled with liquid nitrogen and stored at −70 °C. Cryosections (6 μm) were rehydrated for 10 min in PBS, blocked with PBS containing 1% (w/v) BSA (PBS/BSA), and incubated with anti-HS antibodies for 90 min at 22 °C. As a control, antibodies (single chain variable fragments) against filaggrin were used. Filaggrin is a nonrelated protein not present in kidney (26).** Phage display antibodies were detected by incubation with mouse anti-c-Myc tag antibodies (9E10, culture supernatant), followed by FITC-labeled goat anti-mouse IgG (1:100 in PBS/BSA), both for 90 min at 22 °C. After each incubation, sections were washed in PBS (3 × 10 min). Additional controls were the omission of primary or conjugated antibody. In addition to the phage display-derived antibodies, two mouse anti-HS IgMs, JM403 (21) and 10E4 (20), were used, both obtained by conventional hybridoma techniques. They were detected using FITC-conjugated goat anti-mouse IgM antibodies (1:100 in PBS/BSA).

**Enzyme Immunohistochemistry—**Samples from human kidney and uterine myometrium (rich in heparin-containing mast cells) were fixed in 4% (w/v) paraformaldehyde in PBS and embedded in paraffin. Sections were deparaffinized and treated for 30 min with 1% (v/v) hydrogen peroxide in methanol to remove intrinsic peroxidase activity. After rehydration, sections were incubated with anti-HS antibodies, which were visualized by incubation with mouse anti-c-Myc tag antibodies (9E10, culture supernantant), followed by peroxidase-conjugated rabbit anti-mouse IgG (1:100 in PBS containing 1% (w/v) BSA) for 90 min. Sites of peroxidase activity were revealed using diaminobenzidine as a substrate. Sections were counterstained with Harris's hematoxylin. The same controls were used as described under immunofluorescence.

**Evaluation of Specificity by Immunofluorescence**

Cryosections were pretreated with heparinase III (digests HS) and with chondroitinase ABC (digests dermatan and chondroitin sulfate; 1 unit/ml; 25 mM Tris/HCl, pH 8.0) for 30 min at 37 °C. As control, sections were incubated in the reaction buffer without enzyme. After rinsing three times with PBS and blocking with PBS/1% BSA, sections were incubated with antibodies and processed for immunofluorescence as described. The efficacy of chondroitinase ABC treatment was evaluated by incubation of sections with antibodies against chondroitin sulfate "stubs," generated by chondroitinase ABC (Ab 2B6 from Seikagaku Kogyo Co., Tokyo, Japan).

Specificity of the anti-HS antibodies was further tested by preincubation of the antibodies with various molecules, viz. HS (bovine kidney), heparin, dermatan sulfate, chondroitin 4- and 6-sulfate, keratan sulfate, dextran sulfate, hyaluronate, and DNA. Incubation was for 16 h at 22 °C at concentrations of 0.5–1.0 mg/ml periplasmatic fraction. Proc-}

**Inhibition of Antibody Binding by bFGF**

To study whether the HS epitope recognized by the antibodies is involved in the binding of bFGF, 100 μl of a solution containing bFGF (1.7 μg/ml TBS containing 0.1% (w/v) Tween-20 and 1% (w/v) BSA) was prepared for 8 h at 22 °C to wells of polystyrene microtiter plates previ-
lated. The 19 clones included 3 unique clones expressing antibody HS4C3, HS4D10, and HS3G8. The clone expressing antibody HS3G8 was obtained after additional negative selection on chondroitin sulfate and dermatan sulfate. Sequencing analysis revealed an amino acid sequence of the VH complementarity-determining region 3 of GRRLKD for antibody HS4C3, of SLRMNGCGAHQ for HS4D10, and of YYHYKVN for HS3G8. The VH family and germ line segments are V_H3 and DP-38 for HS4C3, V_H3 and DP-42 for HS4D10, and V_H1 and DP-8 for HS3G8 (nomenclature according to Ref. 27).

Characterization of Anti-heparan Sulfate Antibodies

Evaluation of Specificity and Affinity Using ELISA—Antibodies from all three clones reacted with HS from bovine kidney (the “antigen”) (Table I). No reactivity was observed with chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, dextran sulfate, hyaluronate, DNA, BSA, Marvel, or polystyrene (data not shown). Major cross-reactivity with heparin was observed for HS4C3 and HS3G8. All three antibodies reacted differently with HS preparations isolated from different organs, each displaying its own pattern (Table I). Antibody HS4C3 preferentially reacted with HS isolated from bovine intestine and with fraction B of HS from bovine kidney, which eluted from an anion-exchange column between 1.1 and 1.25 M NaCl. HS4D10 preferentially reacted with HS isolated from whale lung and with fraction B of HS from bovine kidney. HS3G8 has a reactivity toward different HS preparations that is comparable, but not identical, to that of HS4C3. There appears to be no correlation between antibody reactivity and the ratio of sulfate groups/disaccharide (Table I).

To elucidate some chemical aspects of the epitope, reactivity of the antibodies was evaluated with chemically/enzymatically treated HS and heparin preparations using an inhibition ELISA. Reactivity with antibodies HS4C3 and HS3G8 was abolished when HS or heparin was completely desulfated and N-acetylated, completely desulfated and N-sulfated, or N-desulfated and N-acetylated (Fig. 1). Treatment of HS from bovine kidney with heparinase I, II, or III or with nitrous acid at pH 1.5 also destroys reactivity with these antibodies (data not shown). Antibody HS4D10 could not be analyzed in the inhibition ELISA, probably due to the low amount of HS species containing the epitope in the kidney HS preparation used.

Mouse monoclonal IgM JM403 reacted primarily with HS from bovine and human aorta and from bovine lung. Chemical modification of HS or heparin destroyed immunoreactivity, as did heparinase II and III. Heparinase I, however, did not eliminate the reactivity of HS with JM403. These data are in accordance with published data (28). Mouse monoclonal 10E4 reacted strongly with HS from human aorta and bovine intestine.

The dissociation constants ($K_d$) of antibody HS4C3 and HS3G8 for bovine kidney HS, as deduced from Klotz plots, are 0.12 and 0.15 μM, respectively. No value could be obtained for HS4D10.

Evaluation of Specificity Using Immunohistochemistry—Anti-HS antibodies stained basement membranes in rat kidney (the “antigen”) (Fig. 2, Table II). Antibody HS4C3 predominantly stained basement membranes of the glomerulus and of peritubular capillaries, whereas HS4D10 reacted mainly with HS present in basement membranes of tubules and of smooth muscle cells. HS3G8 had a rather promiscuous staining behavior: it reacted with most basement membranes in kidney.

Mouse monoclonal antibody JM403 preferentially stained basement membranes of the glomerulus and of smooth muscle cells, whereas mouse monoclonal antibody 10E4 reacted mainly with Bowman’s capsule and basement membranes of smooth muscle.
cells. Staining with HS3G8 (Fig. 3), HS4C3, and HS4D10 (not shown) was abolished by treatment of sections with heparitinase III but not with chondroitinase ABC. Staining could be precluded by preincubation of antibodies with HS and heparin but not with hyaluronate, dermatan sulfate, chondroitin 4- and 6-sulfate, keratan sulfate, dextran sulfate, and DNA. Antibodies could also be used on paraffin sections (Fig. 4). Antibody HS4C3 reacted strongly with heparin-containing granules in mast cells (Fig. 4b).

Inhibition of Antibody Binding by bFGF

Preincubation of HS-coated wells with bFGF partially inhibits the binding of all anti-HS antibodies obtained by phage display. Binding of antibody HS4C3 was blocked by 62 ± 2% (mean and range) \((n = 2)\), of antibody HS3G8 by 64 ± 1%, and of HS4D10 by 92 ± 2%. bFGF had no influence on the binding of mouse JM403 antibody to HS (0 ± 2%). Preincubation of rat kidney sections with bFGF largely precluded binding to HS by the three antibodies obtained by phage display (Fig. 5). No effect was observed for mouse monoclonal antibody JM403.

DISCUSSION

We describe the generation of three anti-HS antibodies from a semisynthetic phage display library. To our knowledge, this is the first time this technique has been successfully used for the direct generation of antibodies against (poly)saccharides.

**Table II**

<table>
<thead>
<tr>
<th>Basement membrane</th>
<th>HS4C3</th>
<th>HS4D10</th>
<th>HS3G8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerulus</td>
<td>++</td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>Bowman’s capsule</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Peritubular capillaries</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Proximal tubules</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Distal tubules</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Collecting tubules</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Large blood vessel endothelium</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**FIG. 3. Specificity of anti-HS antibody HS3G8.** Prior to incubation with HS3G8 (periplasmatic fraction), rat kidney cryosections were treated with heparitinase (a), heparitinase incubation buffer (b), chondroitinase ABC (c), and chondroitinase ABC incubation buffer (d). Bound antibodies were visualized using FITC-conjugated goat anti-mouse IgG. Bar, 50 μm.

**FIG. 4. Application of anti-HS antibodies on paraffin sections.** a, section of human kidney incubated with antibody HS4D10 (periplasmatic fraction) (bar, 100 μm). b, section of human uterine myometrium incubated with antibody HS4C3 (periplasmatic fraction) (bar, 50 μm). Note the strong immunoreactivity of mast cells due to the cross reactivity of the antibody with heparin. Bound antibodies were visualized using mouse anti-c-Myc IgG, followed by peroxidase-conjugated rabbit anti-mouse IgG.

**TABLE II**

Immunofluorescence pattern of rat kidney by anti-HS antibodies

Periplasmatic fractions of bacteria expressing anti-HS antibodies HS4C3, HS4D10, and HS3G8 were applied to cryosections of rat kidney. Bound antibodies were visualized by incubation with mouse anti-c-myc IgG followed by FITC-conjugated goat anti-mouse IgG. Staining: ++, strong; +, moderate; +/-, weak; -, absent.

All three antibodies recognize different types of HS molecules, as indicated by their different staining pattern and different reactivity toward various HS preparations. HS biosynthesis starts with the formation of a precursor polysaccharide heparosan, consisting of a glucuronic acid-N-acetylglucosamine polymer, which is then subjected to a number of modifications (4). All three antibodies generated by phage display technology did not react with the bacterial K5 polysaccharide, which has the same structure as heparosan. This indicates that the epitopes involved represent additional modifications. Modification of the HS precursor polysaccharide starts with N-deacetylation and N-sulfation of N-acetylgalactosamine residues, followed by epimerization of glucuronic acid to L-iduronic acid (IdoA) residues and O-sulfation at various positions, including C-2 of the IdoA residues and C6 of GlcN residues (4). The modification reactions are incomplete, so not all disaccharides are similarly modified. In addition, less frequent modifications have been reported, including sulfation at C2 and C3 of glucuronic acid residues, sulfation of C3 of GlcN residues, and the presence of an unsubstituted amino group on GlcN. At least 19 different hexuronic acid-GlcN and 12 GlcN-hexuronic acid disaccharides have been identified in HS/heparin (29). Because an HS chain may be composed of over a 100 disaccharides, a large potential of different HS species can be generated. This may form the
bation with periplasmatic fractions of bacteria expressing anti-HS antibodies. The loss of epitope caused by sulfation is also indicated by the loss of epitope in HS preparations treated with heparinase III, which cleaves HS at sites of glucuronic acid residues, not easily detected by other means. Antibody HS4D10 is unreactive toward HS4C3. This indicates that the epitopes for the antibody JM403 (a–c) and the sequence GRRLKD of antibody HS4C3 fits the binding region 3. The sequence GRRRLKD of antibody HS4C3 fits into the glycosaminoglycan binding site XBXBXX (B, basic amino acid residue; X, any amino acid residue), which has been found in a subset of heparin-binding proteins (30). The sequence SLRMNGCQAHQ for HS4D10 resembles another motif observed in a number of heparin-binding proteins, in which two basic amino acid residues are at the extreme end of the motif (31). However, the sequence YYYHYKVN for HS3G8 does not fit into any of the proposed sequences for heparin binding, and clearly, a linear motif is not a prerequisite for heparin binding.

All three antibodies reacted differently from the described monoclonal IgM antibodies JM403 and 10E4. All five antibodies have their own reactivity toward various HS preparations and display an unique staining pattern in the kidney. This indicates the presence of at least five different HS species in kidney.

Binding and modulation of bFGF is a well defined characteristic of HS, including kidney HS (4, 32–34). On the part of HS, 2-O-sulfated IdoA residues promote binding, whereas 6-O-sulfation of the GlcN residue seems inhibitory. This would be in line with the almost complete blockage by bFGF of the binding between HS and antibody HS4D10, which reacts poorly with heparin in which 6-O-sulfated GlcN residues are prominent. The other two phage display antibodies are much more reactive toward heparin and are less efficiently blocked by bFGF. The epitope recognized by mouse monoclonal antibody JM403, obtained by conventional hybridoma techniques, does not bind bFGF. All epitopes recognized by the phage display antibodies, however, do bind bFGF. Because binding of bFGF to HS seems to be a general characteristic of HS, this indicates that the antibody phage display technique may generate antibodies directed to more common epitopes on the HS molecule, whereas those obtained by hybridoma techniques may be directed to “rare” epitopes. The more restricted staining of kidney basement membranes by the mouse monoclonal antibodies JM403 and 10E4 support this idea.

In conclusion, phage display technology presents a powerful technique to generate antibodies specific for HS epitopes. The availability of specific antibodies and their coding DNAs may be instrumental to further explore the realm of HS and of glycosaminoglycans in general, as is indicated for kidney in this study.

Acknowledgments—We express our gratitude to Dr G. Winter, Cambridge University (Cambridge, United Kingdom), for providing the phage display library; Dr. U. Lindahl, University of Upsala (Uppsala, Sweden), for providing HS preparations and for stimulating discussions; Drs. J. van den Born and J. Berden, Dept. of Nephrology, University of Nijmegen, The Netherlands, for providing the JM403 antibody and sections of human kidney; and Dr. A. Benders for helpful suggestions for affinity measurements.

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Antibodies to Heparan Sulfate Using Phage Display

Generation and Application of Type-specific Anti-Heparan Sulfate Antibodies Using Phage Display Technology: FURTHER EVIDENCE FOR HEPARAN SULFATE HETEROGENEITY IN THE KIDNEY

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