Presence of N-Unsubstituted Glucosamine Units in Native Heparan Sulfate Revealed by a Monoclonal Antibody*

Jacob van den Born‡§, Kerstin Gunnarsson†, Marinka A. H. Bakker‡, Lena Kjellén‡, Marion Kusche-Gullberg‡, Marco Maccarana‡, Jo H. M. Berden‡, and Ulf Lindahl‡

From the ‡Division of Nephrology, University Hospital St. Radboud, 6500 HB Nijmegen, The Netherlands, †Department of Medical and Physiological Chemistry, University of Uppsala, S-75123 Uppsala, Sweden, and §Swedish University of Agricultural Sciences, Department of Veterinary Medical Chemistry, University of Uppsala, S-75123 Uppsala, Sweden

Immunohistochemical application of antibodies against heparan sulfate proteoglycan core protein and heparitinase-digested heparan sulfate stubs showed the presence of heparan sulfate proteoglycan in all basement membranes of the rat kidney. However, a monoclonal antibody (JM-403) against native heparan sulfate (van den Born, J., van den Heuvel, L. P. W. J., Bakker, M. A. H., Veerkamp, J. H., Assmann, K. J. M., and Berden, J. H. M. (1992) Kidney Int. 41, 115-123) largely failed to stain tubular basement membranes, suggesting the presence of heparan sulfate chains lacking the specific JM-403 epitope. Heparan sulfate preparations from various sources differed markedly with regard to JM-403 binding, as demonstrated by liquid phase inhibition in enzyme-linked immunosorbent assay, the interaction decreasing with increasing sulfate contents of the polysaccharide. Mapping of the JM-403 epitope indicated that it was dominated by one or more N-unsubstituted glucosamine units(s), since treatments that destroyed or altered the structure of such units in heparan sulfate preparations (deacetylation of N-unsubstituted glucosamine units with HNO₃ at pH 3.9 and acetylation with acetic anhydride, respectively), abolished antibody binding. Conversely, immunoreactivity could be induced in a (N-glucuronyl-1,4-N-acetylglucosaminyl-1,4) polysaccharide by the generation of N-unsubstituted glucosamine units by chemical N-deacetylation. The presence of N-unsubstituted glucosamine in a JM-403-binding heparan sulfate (preparation HIS from human aorta) was demonstrated by an 3-fold reduction in molecular size following HNO₃ (pH 3.9) treatment. Further characterization of the epitope recognized by JM-403, based on enzyme-linked immunosorbent assay inhibition tests with chemically/ enzymatically modified polysaccharides, indicated that one or more N-sulfated glucosamine units are invariably present, whereas iduronic acid and O-sulfate residues appear to inhibit JM-403 reactivity. It is concluded that the epitope contains one or more N-unsubstituted glucosamine and N-glucuronic acid units and is located in a region of the heparan sulfate chain composed of mixed N-sulfated and N-acetylated disaccharide units.

Proteoglycans consist of one or more glycosaminoglycan side chains covalently bound to a core protein, and the heparan sulfate proteoglycan (HSPG)† constitute a major class of proteoglycans that are found in the extracellular matrix, especially in basement membranes, and at the cell surface, associated with the cell membrane (3-5). Many biological activities of HSPGs are due to interactions between the heparan sulfate (HS) polysaccharide side chains and a variety of proteins, which include extracellular matrix molecules, enzymes, enzyme inhibitors, growth factors and other cytokines (2, 5-7). These interactions can be specific, dependent on defined sulfation patterns within given sequences of sugar residues, as described for antithrombin (8), basic fibroblast growth factor (9, 10), and hepatocyte growth factor (11); others appear to be mainly based on relatively nonspecific electrostatic interactions, and involve proteins such as lipoprotein lipase (12), platelet factor 4 (13) and mast cell protease I (14) (for a general discussion, see Ref. 15).

The biosynthesis of HS involves the formation of a nonsulfated (GlcAβ1,4-GlcNAcβ1,4), precursor polysaccharide, which subsequently undergoes a series of polymer-modification reactions. These reactions start with N-deacetylation/N-sulfation of GlcNAc residues, which is followed by C-5 epimerization of GlcA to iduronic acid (IdoA) units, and finally by O-sulfation at various positions (5). The GlcA C-5 epimerization and O-sulfation reactions occur in the close vicinity of N-sulfate groups, pointing to a key role for the glucosaminyl N-deacetylasel/ N-sulfotransferase enzyme in determining the overall extent of modification of the HS chain. Structural analysis of HS preparations have revealed that the modifications tend to colocalize in block sequences, separated by relatively unmodified domains (16-19). The extent of biosynthetic modification, affecting the number, length, and substitution patterns of the modified domains as well as their position along the HS chain, may differ among cell types (20), alter during proliferation (21), and change as a result of cell transformation (22, 23). Structural analysis of HS is complicated by the fact that even highly purified and uniform preparations consist of mixtures of polysaccharide chains that have reached different levels of modification. Monoclonal antibodies (mAbs) that specifically recognize well defined epitopes in HS could be major tools in such analysis. We recently described the production of such an an...
ti-HS mAb, JM-403 (24), which has now been partially characterized with regard to the epitope requirement. In the present report, evidence is provided that the epitope is dominated by the presence of an unsubstituted glucosamine residue(s). Moreover, immunohistochemical application of the mAb demonstrates that the N-unsubstituted GlcN units occur in situ, hence in native HS, and is not introduced as an artifact during the isolation procedure. The possible biological significance of these findings is discussed.

MATERIALS AND METHODS

Glycosaminoglycans—HS (preparation HS-I), isolated from human aorta essentially according to Iversen (28), was provided by W. Murphy (University of Monash, Melbourne, Australia). Five HS preparations, isolated from bovine aorta, lung, intestine, and kidney (two different preparations), were provided by K. Yoshida (Seikagaku Corp., Tokyo, Japan). HS from bovine kidney (used to coat the ELISA plates) was purchased from Seikagaku. HS from bovine liver was as described previously (26). HS from pig intestine, the Escherichia coli K5 capsular polysaccharide, with the same (GlcA-GlcNAc) structure as the nonsulfated HS/heparin precursor polysaccharide (27), chemically O-sulfated K5 polysaccharide, and N-desulfated heparin were given by Dr. G. van Dam (Organon Teknika, Turnhout, Belgium). Intact heparin (stage 14) from pig intestinal mucosa was obtained from Inolex Pharmaceutical Division (Park Forest South, IL) and purified by repeated precipitation with cetylpyridinium chloride from 1.2 M NaCl (28).

Chemical/Enzymatic Modifications of Polysaccharides—Glycosaminoglycans were cleaved by deamination with HNO3 under two sets of conditions. Reaction at pH 1.5 (10 min) was performed as described previously (29) and results in selective attack of N-sulfated GlcN units, with cleavage of the corresponding glucosaminidic linkages (30). Alternatively, deamination at pH 3.9 (29) cleaves the polysaccharide chain at N-unsubstituted GlcN residues (30). Following reduction with NaBH4, the deamination products (essentially \( \text{\textit{zeta}}\)-sasasasasaccharides) were recovered by chromatography on Sephadex G-15. For N-deacetylation by hydrazinolysis (31), samples (1 mg) of polysaccharides were dissolved in 1 mL of hydrazine hydrate (Fluka; H2O content, 36%) containing 1% (w/v) hydrazine sulfate and heated in sealed glass tubes at 96 °C. After 4 h, the samples were repeatedly evaporated to dryness and desalted on PD-10 columns (Pharmacia Biotech Inc.) according to the instructions of the manufacturer. N-Acetylation of primary amino groups was achieved by treatment with acetic anhydride (32).

Heparitinase Digestion—Heparitinase (Seikagaku, enzyme also known as heparinase III, EC 3.2.1.1) was provided by W. Murphy (University of Monash, Melbourne, Australia). Five HS preparations, isolated from different sources, were digested under standard conditions as described previously (40). Samples of HS preparations were treated with heparitinase (Seikagaku) at a concentration of 100 units in 0.5 mL at 37 °C for 16 h. The superantants obtained after centrifugation at 13,000 rpm for 2 min were recovered.

Hydrolysis—The HS preparations were hydrolyzed by treatment with HCl in glycerol at 80 °C for 3 h. The hydrolysates were dried in vacuo and the resulting glucosamine residues were determined by GLC-MS (33).

Immunofluorescence with anti-HS mAb JM-403 on cryostat sections showed the presence of the epitope in a variety of basement membranes, as demonstrated in Fig. 1A for the rat kidney. The glomerular basement membrane stained most intensely, along with Bowman’s capsule and the basal lamina surrounding vascular smooth muscle cells. By contrast, the tubular basement membranes were largely negative. On the other hand, all renal basement membranes were stained with an antibody against the core protein of a HSPG derived from human glomerular basement membrane (Fig. 1B). They were likewise positive for mAb 3G10, which reacts with the residual HS stubs remaining after enzymatic cleavage by heparitinase (Fig. 1C). The resultant 3G10 epitope contains an essential, terminal, 4-sulfated uronate residue and thus can serve as a general HS marker (39). These results indicate the presence of HSPG molecules in all renal basement membranes, only some of which contain the JM-403 epitope. To strengthen this conclusion, we performed double-staining experiments on rat kidney cryostat sections, using biotinylated mAb JM-403 and another anti-HS mAb (JM-13), which also shows a selective basement membrane staining (37). We thus found HS positive tubular basement membranes with anti-HS mAb JM-13 (Fig. 2A), which are negative (or below the detection limit of the method) for JM-403 (Fig. 2B).

The heterogeneity of HS preparations with regard to JM-403 binding was furthermore demonstrated by testing the inhibitory capacity of eight HS preparations, isolated from different sources, in the JM-403 ELISA. The results (Table I) clearly demonstrate a great variability in antibody binding to these HS preparations. Interestingly, the sulfate content of these polysaccharides correlated with the IgG (\( p < 0.0011 \)) in the JM-403 ELISA, indicating that the JM-403 epitope is predominantly found in low sulfated (<1.0 sulfate group/disaccharide unit) HS species.

The JM-403 Epitope Is Dependent on the Presence of an N-Unsubstituted Glucosamine Residue(s)—In order to further define the JM-403 epitope, HS from human aorta (HS-I) was selectively degraded so as to allow the separate isolation of N-sulfated and N-acetylated block structures. N-Sulfated oligosaccharides were isolated after deacetylation of GlcNAc residues using hydrazinolysis followed by exhaustive digestion at pH 3.9, which results in cleavage of the polysaccharide at the N-unsubstituted units (see "Materials and Methods"). N-Acetylated oligosaccharides were recovered following exhaust-
N-Substituted \( \text{\textit{\textalpha-}} \) Glucosamine Units in Heparan Sulfates

Fig. 1. Indirect immunofluorescence staining of rat kidney cryostat sections. A, anti-HS mAb JM-403; B, anti-HSPG-core protein antibodies; C, anti-HS-stub mAb 3G10. Magnification, \( \times 350 \).

Fig. 2. Double-staining of heparan sulfate in rat kidney cryostat sections by indirect immunofluorescence. A, anti-HS mAb JM-13; B, anti-HS mAb JM-403. Arrows indicate tubular basement membranes positive for JM-13 but negative for JM-403. Magnification, \( \times 350 \).

Fig. 3A demonstrates loss of JM-403 binding for the \( \text{\textit{\textalpha-}} \) sulfated as well as the \( \text{\textit{\textalpha-}} \) acetylated oligosaccharides derived from HS-II. Unexpectedly, pH 3.9 deamination alone, i.e. not preceded by \( \text{\textit{\textalpha-}} \) deacetylation, also abolished antibody reactivity, pointing to the presence of \( \text{\textit{\textalpha-}} \)-unsubstituted GlcN in HS-II and to a role for a \( \text{\textit{\textalpha-}} \)-unsubstituted GlcN residue(s) in antibody recognition. Conversely, generation of \( \text{\textit{\textalpha-}} \)-unsubstitated GlcN by \( \text{\textit{\textalpha-}} \)-deacetylation not followed by deamination resulted in increased JM-403 binding. Further evidence for an essential role of \( \text{\textit{\textalpha-}} \)-unsubstituted GlcN derived from the complete loss of reactivity following chemical \( \text{\textit{\textalpha-}} \)-acylation of HS-II using acetic anhydride (Fig. 3A). In addition, however, \( \text{\textit{\textalpha-}} \)-sulfated GlcN residues would seem to be important, as indicated by the loss of antibody binding after deamination at pH 1.5.

Presence of \( \text{\textit{\textalpha-}} \)-Unsubstituted Glucosamine Units in Native HS—The presence of \( \text{\textit{\textalpha-}} \)-unsubstituted GlcN units in native HS-II was investigated by gel chromatography on Sephacryl
N-Unsubstituted D-Glucosamine Units in Heparan Sulfates

<table>
<thead>
<tr>
<th>HS preparation</th>
<th>Sulfate groups/di saccharide</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; ng HS/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human aorta&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.60</td>
<td>300</td>
</tr>
<tr>
<td>Bovine aorta&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56</td>
<td>320</td>
</tr>
<tr>
<td>Bovine kidney (1.1)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.84</td>
<td>392</td>
</tr>
<tr>
<td>Bovine lung&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.91</td>
<td>331</td>
</tr>
<tr>
<td>Bovine intestine&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.98</td>
<td>1113</td>
</tr>
<tr>
<td>Bovine kidney (1.25)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.01</td>
<td>1872</td>
</tr>
<tr>
<td>Bovine liver&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.50</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Pig intestine&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.64</td>
<td>&gt;5000</td>
</tr>
</tbody>
</table>

<sup>a</sup> See Ref. 25.
<sup>b</sup> M. Maccarana, Y. Sakura, A. Tawada, K. Yoshida, and U. Lindahl, submitted for publication. The two preparations of kidney HS differed by the concentrations of the NaCl solutions (1.1 and 1.25 m) required to elute the polysaccharides from Dowex 1-X2.
<sup>c</sup> See Ref. 26.
<sup>d</sup> As determined by Organon.

---

Fig. 3. Fluid phase inhibition of JM-403 binding to kidney heparan sulfate by chemically modified HS-II, K5, and heparin preparations. Dose-response inhibition curves were generated as described under "Materials and Methods." A, native HS-II (●); N-deacetylated HS-II (+); HS-II treated with HNO<sub>3</sub> at pH 3.9; N-deacetylated HS-II treated with HNO<sub>3</sub> at pH 3.9, or N-acetylated HS-II (○; all samples showing lack of reactivity). B, N-deacetylated K5 polysaccharide (●); N,O-desulfated, N-deacetylated heparin (+); N-deacetylated, O-sulfated K5 polysaccharide (●); native K5 polysaccharide, native heparin, N-deacetylated heparin, N-desulfated heparin (○).

---

S,300, before and after exhaustive deamination of the polysaccharide at pH 3.9. A shift of the peak elution position, corresponding to a decrease in M<sub>r</sub> from ~45 × 10<sup>3</sup> to 12–15 × 10<sup>3</sup>, was observed (Fig. 4), suggesting the presence of, on the average, two or three N-unsubstituted GlcN units/chain. By contrast, HS-II that had been previously N-acetylated by reaction with acetic anhydride resisted deamination under these conditions. This control experiment ascertained that deaminative cleavage at pH 3.9 is indeed restricted to N-unsubstituted GlcN residues.

---

Fig. 4. Effect of high-pH deamination on HS-II. Samples of HS-II were subjected to gel chromatography on Sephacryl S-300, as described under "Materials and Methods," before (O) and after (●) treatment with HNO<sub>3</sub> at pH 3.9. Effluent fractions were analyzed for hexuronic acid by the carbazole reaction. The elution pattern of a sample of HS-II that had been subjected to chemical N-acetylation followed by deamination at pH 3.9 was essentially unchangeable compared with that of native, untreated HS-II (data not shown).

---

Recruitment of Chemically Modified Heparin and E. coli K5 Capsular Polysaccharides by mAb JM-403—Neither the non-sulfated E. coli K5 capsular polysaccharide, which has the same (GlcA-GlcNac),, structure as the unmodified biosynthetic precursor of heparin/HS (27), nor native heparin, which is extensively modified and carries ~2.5 sulfate groups/di saccharide unit, showed any binding to JM-403 (Fig. 3B). N-Deacetylation of kidney cryostat sections with acetic anhydride abolished all staining due to mAb JM-403 (data not shown). This treatment simultaneously precluded staining of the section with fluorescamine, as expected for acetylation of primary amino groups. By contrast, the staining intensities of three other anti-HS mAbs (JM-13, 10E4, and HepSSI) as well as of the anti-HSPG core antibody B131, were only marginally affected by the treatment with acetic anhydride (data not shown). These findings indicate that unsubstituted amino groups do indeed occur in native HS, as present in tissue sections that have not been treated or fixed in any way (see Fig. 1A) and confirm our conclusion that N-unsubstituted GlcN units are essential for recognition by mAb JM-403.

---

N-Deacetylation of kidney cryostat sections with acetic anhydride abolished all staining due to mAb JM-403 (data not shown). This treatment simultaneously precluded staining of the section with fluorescamine, as expected for acetylation of primary amino groups. By contrast, the staining intensities of three other anti-HS mAbs (JM-13, 10E4, and HepSSI) as well as of the anti-HSPG core antibody B131, were only marginally affected by the treatment with acetic anhydride (data not shown). These findings indicate that unsubstituted amino groups do indeed occur in native HS, as present in tissue sections that have not been treated or fixed in any way (see Fig. 1A) and confirm our conclusion that N-unsubstituted GlcN units are essential for recognition by mAb JM-403.

---

Recruitment of Chemically Modified Heparin and E. coli K5 Capsular Polysaccharides by mAb JM-403—Neither the non-sulfated E. coli K5 capsular polysaccharide, which has the same (GlcA-GlcNac),, structure as the unmodified biosynthetic precursor of heparin/HS (27), nor native heparin, which is extensively modified and carries ~2.5 sulfate groups/di saccharide unit, showed any binding to JM-403 (Fig. 3B). N-Deacetylation of kidney cryostat sections with acetic anhydride abolished all staining due to mAb JM-403 (data not shown). This treatment simultaneously precluded staining of the section with fluorescamine, as expected for acetylation of primary amino groups. By contrast, the staining intensities of three other anti-HS mAbs (JM-13, 10E4, and HepSSI) as well as of the anti-HSPG core antibody B131, were only marginally affected by the treatment with acetic anhydride (data not shown). These findings indicate that unsubstituted amino groups do indeed occur in native HS, as present in tissue sections that have not been treated or fixed in any way (see Fig. 1A) and confirm our conclusion that N-unsubstituted GlcN units are essential for recognition by mAb JM-403.

---

…
The inhibitory activity of naked heparin was ~100-fold lower than that of the N-deacetylated K5 polysaccharide (Fig. 3B). Since the naked heparin differs from the N-deacetylated K5 polysaccharide merely by the presence of IdoA, it is inferred that such units interfere with antibody binding; the residual activity is probably associated with the limited number of GlcA residues present in the polymer (~20% of the total hexuronic acid contents (41)). Contrary to the native heparin, N-deacetylated heparin and N-desulfated heparin, the naked heparin was reactive (Fig. 3B), indicating that removal of O-sulfate groups exposes epitope structures. To confirm the negative effect of O-sulfate groups on JM-403 binding, we compared in the ELISA the inhibition induced by N-deacetylated K5 polysaccharide with that of the N-deacetylated, O-sulfated analog. This latter K5 derivative contains an average of 1.2 O-sulfate (but no N-sulfate) groups/disaccharide unit. The locations of the O-sulfate groups were not defined but would presumably primarily involve C-6 of the GlcN units, along with C-2 and/or C-3 of the GlcA units. The results in Fig. 3B clearly show that O-sulfation of N-deacetylated K5 polysaccharide strongly reduces JM-403 binding.

Taken together, these data explain the lack of JM-403 activity toward native, N-deacetylated, or N-desulfated heparin, and, moreover, conform to the observed decrease in antibody binding to HS preparations with increasing degree of sulfation (hence, increasing IdoA contents (42)). Furthermore, the experiments demonstrate that neither (N- or O-) sulfate groups nor N-acetyl groups are required for JM-403 binding, since N-deacetylated K5 (and naked heparin) lack such groups yet are recognized by the antibody.

Location of JM-403 Epitopes in the HS Chain—In order to define the location of the JM-403 epitope along the HS chain, samples of HS-II were enzymatically or chemically degraded using reagents/conditions with well defined cleavage specificity. The resultant oligosaccharides were then tested in the JM-403 ELISA (Table II). As indicated above, cleavage of HS-II at N-sulfated GlcN residues by the HNO₃/pH 1.5 procedure abolished all binding to JM-403 (Fig. 3A). This finding excludes an epitope consisting of merely N-unsubstituted and JV-acetylated disaccharide units. On the other hand, N-sulfate groups are not required for JM-403 binding (as shown by the N-deacetylated K5 sample; Fig. 3B). These results therefore indicate that N-sulfate groups, while not essential for reactivity, are tolerated, and, in addition, are invariably present in the HS epitope. In fact, complete loss of reactivity occurred on low pH deamination of six different HS preparations from different sources (the inhibitory samples from Table I). Digestion of HS-II by heparitinase (heparinase III) also abolished all reactivity. This enzyme cleaves glucosaminidic linkages between either N-acetylated or N-sulfated GlcN residues and nonsulfated hexuronic acid units. By contrast, HS-II binding to JM-403 was found to be essentially insensitive to heparinase I, which cleaves glucosaminidic linkages between N-sulfated GlcN and 2-O-sulfated IdoA units. The epitope is thus in all probability located outside the heavily modified, heparin-like, N-sulfated block sequences of the HS chain. The most likely site for such a structure is in regions composed of mixed N-acetylated and N-sulfated disaccharide units, with a relative high GlcA content, at the boundaries between unmodified and highly sulfated blocks of HS (Table II).

### Table II

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Effect on heparan sulfate</th>
<th>JM-403 reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>IC₅₀ (ng HS/ml)</td>
</tr>
<tr>
<td>Heparitinase</td>
<td></td>
<td>&gt;5000</td>
</tr>
<tr>
<td>HNO₃, pH 1.5</td>
<td></td>
<td>&gt;5000</td>
</tr>
</tbody>
</table>

*a* The scheme illustrates the predicted effects of the various treatments on different regions of the HS chain. The symbols define N-acetylated, unmodified regions (□); N-sulfated, heavily modified (O-sulfate and IdoA-rich) regions (■); and mixed N-acetylated and N-sulfated regions (○).

DISCUSSION

The present study deals with the saccharide recognition properties of anti-HS mAb JM-403, the generation and basic characteristics of which were described previously (24). Applying the antibody to immunofluorescence studies on rat kidney sections revealed that the carbohydrate epitope is not evenly distributed among the HS subspecies of this tissue. All basement membranes, as expected, were found to contain HSGPCs, as evidenced using other antibodies. However, the polysaccharide present in tubular basement membranes stained poorly with JM-403, suggesting the existence of HS isoforms lacking the corresponding epitope. It thus seemed important to characterize this epitope with regard to saccharide structure.

The results of such studies demonstrated a critical role for an N-unsubstituted GlcN unit(s) within the epitope. The occurrence of these residues in appropriate linkage to GlcA units suffices to induce strong antibody binding. However, the naturally occurring epitope(s) in native HS chains apparently have a more complex structure. The results of chemical and enzymatic degradation experiments suggest that the HS epitopes contain not only GlcA units but also N-sulfated GlcN residues, whereas IdoA units and O-sulfate groups appear to impede antibody recognition. Such structures are likely to be found in regions of the HS chains that contain mixed N-acetylated and N-sulfated sequences. It seems probable that N-unsubstituted GlcN units in a highly N- and O-sulfated, IdoA-rich surrounding, as presumably occurring also in heparin (43), will escape detection by JM-403. Indeed, we cannot exclude that the epitope represents only a minor proportion of the N-unsubstituted GlcN units in HS and that such units occur in multiple structural contexts.

Considering possible mechanisms for the generation of GlcN units with unsubstituted amino groups, there are two major alternatives (disregarding the theoretical but unlikely possibility of a UDP-GlcN sugar nucleotide precursor containing an N-unsubstituted GlcN unit). The normal conversion of N-acetylated to N-sulfated GlcN units in heparin/HIS biosynthesis is catalyzed by a single enzyme protein, which is capable of promoting both the N-deacetylation and the N-sulfation reactions. Two related yet distinct glucosaminyl N-deacetylasen/N-sulfotransferase enzymes have been described, one from rat liver (44) and the other from mouse mastocytoma (45), that differ with regard to size of mRNA transcript, amino-acid sequence, cofactor requirement, and kinetic properties (46–48). While the interrelationship between these enzymes with regard to the biosynthesis of heparin and HS is unclear, both enzymes express both catalytic activities, which normally appear to be tightly coupled. However, under the appropriate experimental conditions the two activities are readily dissociated. Thus, formation of a heparin precursor polysaccharide by incubating a mouse mastocytoma microsomal fraction with UDP-GlcA and UDP-GlcNAc, but in the absence of adenosine 3’-phosphate 5’-phosphosulfate (PAPS), yields a product in which about one-third of the GlcN units are N-deacetylated and have an unsubstituted amino group (49). Indeed, recent exper-
N-Unsubstituted d-Glucosamine Units in Heparan Sulfates

Immunizations showed that incubation of the intact K5 polysaccharide with microsomal enzymes, or with the purified N-deacetylase/N-sulfotransferase from mouse mastocytoma, in the absence of PAPS, will result in the formation of N-unsubstituted GlnN units that are recognized by mAb JM-403. A similar effect can be achieved by treating intact cells with chlorate, which will block the formation of PAPs (50). It is possible that limited formation of N-unsubstituted GlnN units may occur during the normal biosynthesis of HS, by N-deacetylation of selected (?) target GlnNAc residues that is not followed by N-sulfation, or after completion of the biosynthesis, maybe in the extracellular space.

An alternative mechanism for the formation of N-unsubstituted GlnN units would involve the action of an endosulfamidase (N-sulfatase). While sulfamidases have been implicated in HS metabolism, these enzymes seem to act in an exoctic fashion, at the nonreducing terminus of the chain (51). No endoenzyme of the type required to generate the JM-403 epitope has yet been described. Notably, however, Dawes and Pepper (52) proposed that heparin and HS may be extensively desulfated or secreted into the medium/extracellular matrix, was identified through interactions with HS (58-61) and, furthermore, are known to be involved in various forms of glomerulonephritis (62, 63).

The demonstration of N-unsubstituted GlnN units in native HS preparations raises intriguing questions regarding the biological significance of these constituents. It has been reported that endothelial heparin-related molecules can bind to L-selectin (53). Recently, this ligand, associated with the cultured cells or secreted into the medium/extracellular matrix, was identified as HS (54). Surprisingly, these L-selectin-binding HS chains were found to be enriched in N-unsubstituted GlnN units, suggesting a role for the free amino groups in the specific manner with the interactions between HS and other extracellular matrix.

The possible influence of the N-unsubstituted GlnN units on the interaction between HS and other extracellular matrix molecules should be investigated. The expression of the JM-403 epitope by glomerular basement membrane HS is decreased in glomerular diseases that are characterized by mesangial matrix expansion and/or proteinuria (57). This finding could be explained by (a) masking of HS by cationic molecules or immune complexes; (b) decreased synthesis and/or increased degradation of HS; or (c) an altered structure of HS resulting in loss of the JM-403 epitope, for instance, due to loss of N-unsubstituted GlnN units and/or HS oversulfation. The latter alternative has potential implications with regard to the activities of growth factors that are modulated through interactions with HS (58-61) and, furthermore, are known to be involved in various forms of glomerulonephritis (62, 63).

REFERENCES


* I. Eriksson, J. van den Born, and L. Kjellen, unpublished observation.
N-Unsubstituted d-Glucosamine Units in Heparan Sulfates