Structural Recognition by Recombinant Human Heparanase That Plays Critical Roles in Tumor Metastasis

HIERARCHICAL SULFATE GROUPS WITH DIFFERENTIAL EFFECTS AND THE ESSENTIAL TARGET DISULFATED TRISACCHARIDE SEQUENCE

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Human heparanase is an endo-β-D-glucuronidase that degrades heparan sulfate/heparin and has been implicated in a variety of biological processes, such as inflammation, tumor angiogenesis, and metastasis. Although the cloned enzyme has been demonstrated to have a critical role in tumor metastasis, the substrate specificity has been poorly understood. In the present study, the specificity of the purified recombinant human heparanase was investigated for the first time using a series of structurally defined oligosaccharides isolated from heparin/heparan sulfates. The best substrates were ΔHexUA(±2S)-GlcN(±3S,6S)-GlcUA-GlcN(±NS,6S)-GlcUA-GlcN(±NS,6S) and ΔHexUA(±2S)-GlcN(±3S,6S)-GlcUA-GlcN(±NS,6S) (where ΔHexUA, GlcN, GlcUA, NS, 2S, and 6S represent unsaturated hexuronic acid, D-glucosamine, D-glucuronic acid, 2-N-sulfate, 2-O-sulfate, and 6-O-disulfate, respectively). Based on the percentage conversion of the substrates to products under identical assay conditions, several aspects of the recognition structures were revealed. 1) The minimum recognition backbone is the trisaccharide GlcN-GlcUA-GlcN. 2) The target GlcUA residues are in the sulfated region. 3) The -GlcN(±6S)-GlcUA-GlcN(±NS)-sequence is essential but not sufficient as the cleavage site. 4) The IdUA(±2S) residue, located two saccharides away from the target GlcUA residue, claimed previously to be essential, is not indispensable. 5) The 3-O-sulfate group on the GlcN is dispensable and even has an inhibitory effect when located in a highly sulfated region. 6) Based on these and previous results, HexUA(±2S)-GlcN(±3S,6S)-IdoUA-GlcNa(±6S)-GlcUA-GlcN(±NS,±6S)-IdoUA(±2S)-GlcN(±NS,±6S) (where HexUA represents hexuronic acid) has been proposed as a probable physiological target octasaccharide sequence. These findings will aid establishing a quantitative assay method using the above tetrasaccharide and designing heparan sulfate-based specific inhibitors of the heparanase for new therapeutic strategies.

Interactions between adherent cells and the extracellular environment influence maintenance of cellular functions such as proliferation, differentiation, and migration. Heparan sulfate proteoglycans (HS-PGs) are covalently linked protein-HS glycosaminoglycan (GAG) conjugates found in extracellular matrix (ECM) and on the cell surface of most cells and have been demonstrated to be key components of the cell-cell and cell-ECM interactions (for reviews, see Refs. 1–4). Most of the biological properties of HS-PGs are conferred by the HS moiety, which is a sulfated polysaccharide polymer of alternating GlcN and HexUA (GlcUA or IdoUA) residues (for reviews, see Refs. 5 and 6). HS-GAG binds to and co-localizes with structural proteins, such as fibronectin and collagen in the ECM, providing a framework for matrix organization (for reviews, see Refs. 7–9). Both cell surface and ECM HS-PGs also tether various growth/differentiation factors and cytokines as storage depots of bioactive signaling molecules. There is clear evidence that an association of a ligand with HS-GAGs can activate or stabilize the ligand and also facilitate signal transduction via its high affinity receptor (for reviews, see Refs. 10–12).

The interaction of a ligand with ECM or cell surface HS-PGs is regulated by biosynthesis of specific sulfation sequences, which bind to the ligand (for reviews, see Refs. 5, 13, and 14). Indeed, fine sulfation structures of HS-GAGs change during development, aging, or tumor progression to malignancy, and the abilities of specific ligands to bind HS-PGs are switched (15–18). Another way to alter the functional state of HS-PGs is to degrade and release HS-GAGs from the core proteins, which is achieved by the specific action of an endoglycosidase, heparanase (for reviews, see Refs. 19 and 20). Heparanase is the name of mammalian endoglucuronidase capable of specifically cleaving HS-GAGs and differs from bacterial eliminases such as heparinase and heparitinase (21). The extracellular heparanase has been implicated in basement membrane remodeling after injury or at inflammation sites by destroying HS-GAG chains and in the regulation of cell growth and differentiation by releasing growth factors that are bound to extracellular HS-PGs. There is also evidence that the heparanase activity correlates with the metastatic potential of tumor cells in animal models and increases in the sera of human patients with metastatic cancers (22–24).

The heparanase research had been hampered by the limited...
abundance and unstable enzyme activity as well as the lack of a simple assay method. Recently, several laboratories have developed a variety of assay methods, purified human heparanase, and isolated the cDNA (25–29). It became clear that heparanases previously purified from various sources are identical and that the heparanase is initially synthesized as an inactive 65-kDa glycoprotein and then processed into the active 50-kDa enzyme by cleavage of the N terminus peptide (30). A direct role of the heparanase in tumor cell invasion was confirmed by the transfection of the sense and antisense heparanase cDNA into cells, which acquired highly and poorly metastatic phenotypes, respectively (26, 30). High expression of the heparanase mRNA was also observed in advanced stage tumors and metastatic cell lines derived from various tissues (27, 28, 31, 32).

The aim of the present study was to explore the substrate recognition property of the human recombinant heparanase. Previously, several attempts were made to define the substrate specificity of the heparanase partially purified from different animal sources (for reviews, see Refs. 19 and 20). Most approaches have involved the structural analysis of the fragments generated by enzymatic cleavage of polymer HS from various tissues and heparin (Hep) polysaccharides derived from the lung and intestine. Specificity studies on human heparanase using structurally defined oligosaccharide substrates have been limited to those performed using partially purified enzyme preparations (33, 34). In the present study, we systematically investigated the specificity of the purified recombinant human heparanase using a number of structurally defined oligosaccharides as substrates and revealed the hitherto unreported specificity of the enzyme, which will aid establishing the quantitative assay methods and designing inhibitors for new therapeutic strategies in highly metastatic cancer and other heparanase-related diseases, notably inflammatory or cardiovascular diseases.

**EXPERIMENTAL PROCEDURES**

**Materials—** HS (sodium salt) from bovine kidney and concanavalin A-agarose (Invitrogen), TSK-GEL G3000SW_{2} was from TOSOH Corp. (Tokyo, Japan). Sephacryl S-300HS and Hep-Sepharose CL-6B columns as well as a prepacked disposable PD-10 column containing Sephadex G-25 medium were purchased from Amersham Biosciences. Fluorescein isothiocyanate (FITC) was from Sigma-Aldrich.

**Preparation of Human Heparanase—** Structurally defined oligosaccharides were isolated from porcine intestinal Hep or bovine kidney HS and used to investigate the substrate specificity of the heparanase. The enzyme was purified by capillary electrophoresis as described previously (35–41). Enzyme assays were carried out in 100 μl of 0.1 M sodium acetate buffer, pH 4.2, containing 1 μg of FITC-Hep, at 37 °C for 3 h and terminated by the addition of 100 μg of nontagged hep hep intestinal Hep and subsequent heating at 100 °C for 5 min. The digests were centrifuged at 15,000 rpm for 5 min to precipitate the insoluble materials. The supernatant fluids were analyzed by gel filtration HPLC on a TSK-GEL G3000SW_{2} column (0.78 × 30 cm) preequilibrated with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.05%/v/v Na2EDTA, at a flow rate of 1 ml/min.

**Enzymatic Digestion of Structurally Defined Oligosaccharides with Heparanase—** Structural and tetra- or hexasaccharide isolated from porcine intestinal Hep or bovine kidney Hep were digested with 0.88 or 0.44 units of the purified human heparanase in a total volume of 100 μl of 0.1 M sodium acetate buffer, pH 4.2, at 37 °C for 21 h or the indicated periods. The enzymatic reactions were terminated as described above. As control experiments, each oligosaccharide was incubated with the heat-inactivated heparanase under the same conditions. Each enzyme digest was analyzed by HPLC on an amine-bound silica column as reported previously (42). Eluates were monitored at 232 nm. The sensitivity of each oligosaccharide to the enzyme was judged by the peak shift to earlier elution positions. The area of the individual peaks was compared before and after heparanase treatment, and the amount of the degraded oligosaccharide was calculated from the decrease in the intact peak area using an integrator.

**RESULTS**

**Expression and Purification of the Recombinant Human Heparanase—** The pcDNA3/Hygro vector containing the full-length human heparanase cDNA clone was expressed in human melanoma A375M cells. The cell lysate prepared from the stable transfectant cells was subjected to affinity chromatographies using the matrices Hep-Sepharose, concanavalin A-agarose, and anti-heparanase antibody-immobilized Affi-Gel 10 to purify the recombinant enzyme. Aliquots of fractions 1–17 eluted from the anti-heparanase antibody column were subjected to the heparanase assay and SDS-PAGE followed by silver staining (Fig. 1). The strong catalytic activity toward FITC-HS was detected in fractions 6–8. Heparanase is initially synthesized as an inactive 65-kDa glycoprotein that is cleaved at the N terminus to generate an active 50-kDa enzyme (for reviews, see Refs. 19 and 20). Being consistent with this, a 50-kDa band was detected coincidently on SDS-PAGE in the fractions containing HS-degrading activity. Silver staining showed a 30-kDa protein band in fractions 6–8 and the faint 65-kDa protein band, which corresponded to the IgG light chain, probably leaking from the antibody-immobilized column and the enzyme precursor, respectively. Fractions 6–8 were combined and used to investigate the substrate specificity of the heparanase. The protein concentration and the heparanase activity of
the pooled fractions were measured. The specific activity was 1.6 units/μg of protein.

Detection of the Human Heparanase Recognition Sites in Porcine Intestinal Hep and Bovine Kidney HS—The FITC-labeled Hep was incubated with the purified recombinant heparanase to confirm the enzyme activity and also the presence of the heparanase recognition sequences in bovine kidney HS and porcine intestinal Hep chains. The digest was analyzed by gel filtration HPLC on a column of TSK-GEL G3000SW_{x} as shown in Fig. 2. Although both FITC-HS and -Hep were degraded by the enzyme, the latter appeared to be more susceptible to the heparanase-catalyzed cleavage than the former. Since porcine intestinal Hep contains the heparanase cleavable sites, experiments were undertaken to elucidate the substrate recognition property of the heparanase using a series of structurally defined sulfated oligosaccharides, which were isolated from the repeating disaccharide region of porcine intestinal Hep and bovine kidney HS (41, 45) and included 1 tri-, 28 tetra-, 1 penta-, 9 hexa-, and 3 octasaccharides.

Development of the Assay Conditions and Analytical HPLC Assays of the Recombinant Human Heparanase Using Structurally Defined Oligosaccharides—The substrate specificity of the human heparanase was investigated using 8 hexa- and 12 tetrasaccharides derived from Hep/HS (Tables I and II). Their fine structures have been established by 500-MHz NMR analysis (35–41). Eighteen contained GlcUA residue(s) at the internal position of each oligosaccharide sequence, whereas the other two contained only ΔHexUA and IdoUA but not GlcUA residues as uronic acid components. These 20 oligosaccharides (0.3 nmol each) were individually incubated with the purified recombinant heparanase under identical conditions (using 0.88 units of the enzyme, at 37 °C for 21 h), and the reaction products from each digestion were analyzed by HPLC on an amine-bound silica column for identification and quantification to compare the relative susceptibilities of the oligosaccharide substrates with the enzyme. Since the elution profile was monitored by absorbance at 232 nm of the ΔHexUA residue at the nonreducing terminus, only the intact substrate and the product derived from the nonreducing side of each substrate were detected, but the product from the reducing side was not detected due to the lack of appreciation UV absorbance. It is most likely that the tetrasulfated trisaccharide product GlcN(NS,6S)-GlcUA-GlcN(NS,6S)-GlcUA, produced by the cleavage at the glucuronic linkage on the reducing side, and may represent a minor digestion product. The remaining peaks at the elution position of the intact hexasaccharide substrates in Fig. 3, A and B, may suggest the incompletion of digestion or be derived from minor contaminants in the original samples.

Hexa-1, -8, -15, and -16 showed partial degradation, and Hexa-13S was resistant to the enzyme action under the reaction conditions used (Tables I and II). As representative chromatograms, the HPLC profiles of Hexa-1, -15, and -16 are depicted in Fig. 4. The closed or open arrow in each panel suggests the elution position of the intact hexasaccharide substrates before digestion or the main peak of the digestion products, respectively. About 26, 59, and 30% of each hexasaccharide was converted to the corresponding product, respectively, under the reaction conditions used. The peak, which remained at the original position, being intact in each panel, was not a resistant component, because these substrates were almost completely degraded with a higher concentration of the enzyme (data not shown). The percentage of the substrate conversion to products should reflect the enzyme preference for the substrate recognition (46), although it is conceiv-
able that the enzyme may become thermally inactivated before completion of the reaction.

Tetrasaccharides were also used to profile the substrate specificity of the recombinant heparanase. Some of them were sensitive to the enzyme despite their small sizes, suggesting that the minimum size for the heparanase recognition is most likely a trisaccharide with the -GlcN-GlcUA-GlcN sequence backbone. As shown in Fig. 5, the heparanase digestion resulted in nearly complete degradation of Tetra-1, ΔHexUA(2S)-GlcN(6S) and Hexa-7S, and Tetra-23, ΔHexUA-GlcN(6S)-IdoUA-GlcN(6S) with 2-sulfated. The structure of human heparanase-resistant oligosaccharides

| Fraction No. | Structure | Percentage cleavage<sup><sup>c</sup></sup> | Reference<sup><sup>c</sup></sup> |
|--------------|-----------|---------------------------------|-------------------|
| Hexa-1       | ΔHexUA(2S)-GlcN(6S)-IdoUA-GlcNAc(6S)-GlcUA-GlcN(3S,6S) | 26                  | Fractions 6–34 in Ref. 40 |
| Hexa-4       | ΔHexUA(2S)-GlcN(6S)-IdoUA-GlcNAc(6S)-GlcUA-GlcN(6S) | 85                  | Fractions 6–27 in Ref. 40 |
| Hexa-7       | ΔHexUA(2S)-GlcN(6S)-GlcUA-GlcN(6S)-GlcUA-GlcN(6S) | >95                 | Fractions 6–31 in Ref. 40 |
| Hexa-8       | ΔHexUA(2S)-GlcN(6S)-IdoUA-GlcNAc(6S)-GlcUA-GlcN(3S) | 60                  | Fractions 6–32 in Ref. 40 |
| Hexa-15      | ΔHexUA(2S)-GlcN(6S)-IdoUA-GlcNAc(6S)-GlcUA-GlcN(6S) | 59                  | Fractions 6–26 in Ref. 40 |
| Hexa-16      | ΔHexUA(2S)-GlcN(6S)-IdoUA(2S)-GlcNAc-GlcUA-GlcN(6S) | 30                  | Fractions 6–30 in Ref. 40 |
| Tetra-1      | ΔHexUA(2S)-GlcN(6S)-GlcUA-GlcN(6S) | >95                 | Fraction VIII in Ref. 38 |
| Tetra-6      | ΔHexUA(2S)-GlcN(6S)-GlcUA-GlcN(6S) | 44                  | Fraction IV in Ref. 38 |
| Tetra-10     | ΔHexUA-GlcN(6S)-GlcUA-GlcN(6S) | 40                  | Fraction II in Ref. 38 |
| Tetra-28     | ΔHexUA-GlcN(6S)-GlcUA-GlcN(6S) | 56                  | Fraction V in Ref. 38 |

<sup>a</sup>The fraction numbers refer to those cited in Ref. 45.
<sup>b</sup>Calculated based on the proportion of the starting oligosaccharide remaining in the digest.
<sup>c</sup>The fraction numbers refer to those designated in the original studies.
<sup>d</sup>The arrows indicate the presumable cleavage sites of human heparanase in the oligosaccharide substrates.

This trisulfated hexasaccharide was prepared by the digestion of ΔHexUA(2S)-GlcN(6S)-GlcUA-GlcN(6S)-GlcNAc-GlcN(6S) with 2-sulfatase.

| Fraction No. | Structure | Reference<sup><sup>c</sup></sup> |
|--------------|-----------|-------------------|
| Hexa-13S<sup>2</sup> | ΔHexUA-GlcN(6S)-IdoUA(2S)-GlcNAc-GlcUA-GlcN(6S) | Fractions 6–22S in Ref. 40 |
| Tetra-3      | ΔHexUA-GlcN(6S)-IdoUA(2S)-GlcNAc(6S) | Fraction XIII in Ref. 38 |
| Tetra-21     | ΔHexUA-GlcN(6S)-GlcUA-GlcNAc | Fraction 6,7–8 in Ref. 37 |
| Tetra-22     | ΔHexUA-GlcN(6S)-GlcUA-GlcN(6S) | Fraction 6,7–8 in Ref. 37 |
| Tetra-23     | ΔHexUA-GlcN(6S)-GlcUA-GlcN(6S) | Fraction 6,7–8 in Ref. 37 |
| Tetra-25     | ΔHexUA-GlcN(6S)-GlcUA-GlcNAc(6S) | Fraction 6,7–8 in Ref. 37 |
| Tetra-26     | ΔHexUA-GlcN(6S)-GlcUA-GlcNAc(6S) | Fraction 6,7–8 in Ref. 37 |
| Tetra-27     | ΔHexUA-GlcN(6S)-IdoUA(2S)-GlcNAc(6S) | Fraction 6,7–8 in Ref. 37 |

<sup>a</sup>The fraction numbers refer to those cited in Ref. 45.
<sup>b</sup>The fraction numbers refer to those designated in the original studies.
<sup>c</sup>This trisulfated hexasaccharide was prepared by the digestion of ΔHexUA(2S)-GlcN(6S)-GlcUA-GlcN(6S)-GlcNAc-GlcUA-GlcN(6S) with 2-O-sulfatase.

Endo-β-D-glucuronidase Activity of the Heparanase Is Dependent on the Size and High Sulfation of the Oligosaccharide Substrates—Heparanase cleavage occurred only at glucuronic linkages but not iduronic. The glucuronic linkages, confirming the endo-β-D-glucuronidase nature of this enzyme as reported previously (19–21, 47). The percentages of the heparanase-catalyzed conversion of the individual oligosaccharides to products can be used to make a reasonable estimate of the sensitivity of the target glucuronic linkages. At a cursory glance at Tables I and II, it was noticed that the recombinant human heparanase acted preferentially on the glucuronic linkages in the highly sulfated regions. The effects of chain lengths, sulfation degrees, and sulfation positions on the heparanase action were evaluated below.

First, we noticed the size dependence of the heparanase cleavage of the substrate oligosaccharides. The following four pairs of hexa- and tetrasaccharides were compared in the susceptibility to the enzyme; Hexa-4 and Tetra-29, Hexa-7 and Tetra-1, Hexa-7S and Tetra-28, or Hexa-15 and Tetra-25 share, around the heparanase cleavage site, the identical tri- or tet-
respectively. The peaks eluted at around 5 and 10 min in each panel were derived from the incubation buffer and the enzyme preparation.

The peaks eluted at around 5 and 10 min in each panel were derived from the incubation buffer and the enzyme preparation.

Heparanase digests of Hexa-4 (A), -15 (B), and -16 (C) (0.3 nmol each) were analyzed by HPLC on an amine-bound silica column using a linear gradient of NaH$_2$PO$_4$ from 213 to 803 mM over 60 min as indicated by the dashed lines. The open arrow in each lower panel indicates the main peak of the heparanase digestion products. The peaks eluted at around 5 and 10 min in each panel were derived from the incubation buffer and the enzyme preparation.

that the high sulfation density confers on the oligosaccharides the preferential recognition by the enzyme.

Since Hexa-4, -7, and -7S and Tetra-1 were almost completely degraded under the digestion conditions used, they were next digested under milder conditions to make a better comparison of their digestibility to the enzyme; the incubation time was shortened to 1, 5, 10, or 23 h, and the amount of the recombinant enzyme was reduced to 50% (0.44 units). The results on the percentage of cleavage of individual substrates during each incubation period are tabulated in Table III. After 10 h of incubation, Hexa-7 was almost completely converted to products, whereas Hexa-4, Hexa-7S, and Tetra-1 were degraded by 21, 85, and 44%, respectively. The relative rates of the degradation of these oligosaccharides suggest that the order of the substrate preference by the human recombinant heparanase is as follows: Hexa-7 > Hexa-7S > Tetra-1 > Hexa-4.

The 6-O-Sulfated Group on the Nonreducing Side GlcN Residue of the Target GlcUA Is an Important but Not Absolute Requirement—Some oligosaccharides that have the same degree of sulfation but distinct sequences were degraded with different efficiencies, suggesting that sulfation patterns on GlcN/GlcNAc residues adjacent to the target GlcUA residue also significantly influence the heparanase action. Therefore, the hierarchy of different sulfate groups in the human heparanase recognition was inspected. When the trisaccharide structures around the cleavage sites in Hexa-15, -GlcNAc(6S*)-GlcUA-GlcN(NS), and Hexa-16, -GlcNAc-GlcUA-GlcN(NS,6S*), are compared, they are different only in the location of the 6-O-sulfate group suggested by an asterisk. Since the human heparanase cleaved Hexa-15 more efficiently than Hexa-16 (59 versus 30%) (Table I), the 6-O-sulfate group of the GlcN residue on the nonreducing side of the cleavage site appears to be preferable for the enzyme recognition over that on the reducing side. When the digestion efficiencies of Tetra-6 and -29 were compared, the latter was more efficiently degraded than the former (44 versus 56%) (Table II). The structural difference between the two was the sulfation positions on the GlcN residue on the nonreducing side of the heparanase cleavage site; Tetra-6, $\Delta$HexUA(2S)-GlcN(6S*)-GlcUA-GlcN(NS,6S*), has an N-sulfate group on the GlcN residue, and Tetra-29, $\Delta$HexUA(2S)-GlcNAc(6S*)-GlcUA-GlcN(NS,6S*), con-
tains a 6-O-sulfate group. However, the GlcNAc(6S) structure on the nonreducing side of the GlcUA is not an absolute requirement for the cleavage, since Hexa-16 and Tetra-6, which lack this structure, were also susceptible to the enzyme action. It appears that the 6-O-sulfation on the GlcNAc residue on the nonreducing side of the cleavage site can be replaced by sulfate groups on other positions of the flanking GlcN residues or those on other residues in the adjacent sequence (see below).

The GlcN(NS) Residue on Reducing Side of the Target GlcUA Is an Essential but Not Sufficient Requirement—Notably, the oligosaccharides sensitive to the human heparanase always contained a N-sulfated GlcN residue on the reducing side of the targeted glucuronic linkages, and hence this structure appears to be essential for the heparanase action. However, oligosaccharides that contain a GlcUA-GlcNAc-sequence and highly sulfated GlcN residues adjacent to the GlcUA residue have never been isolated (12, 45, 48, 49), chemical synthesis of oligosaccharides that contain a unique sequence, such as -GlcN(9S,6S)-GlcUA-GlcNAc(6S)-, will be desirable to define whether the GlcN(NS) structure on the reducing side per se is an absolute requirement for the substrate recognition by the enzyme. However, the glucosamine residue on the immediate reducing side of the target GlcUA in native HS and Hep chains is assumed to be always N-sulfated, as will be discussed below. The enzyme failed to act on Hexa-13S and Tetra-22, which contains only this N-sulfate group on GlcN residues flanking the target GlcUA residue (Table II), suggesting that this GlcN(NS) structure is not sufficient for the heparanase cleavage.

The GlcN(3S) Residue on the Reducing Side of the Target GlcUA Exhibits a Promoting Effect in a Relatively Low Sulfated Sequence but an Inhibitory Effect in a Highly Sulfated Sequence—It has been reported that heparanase catalyzed the cleavage of the glucuronic linkage in the antithrombin III-binding heparin octasaccharide, IdoUA-GlcNac(6S)-GlcUA-GlcN(3S,6S)-IdoUA(2S)-GlcN(3S,6S)-IdoUA(2S)-2,5-anhydromannitol(6S) (33, 34). However, the susceptibility of the oligosaccharides lacking the GlcN(3S) structure demonstrated in the present study suggests that 3-O-sulfation is not obligatory. Hexa-1, Hexa-8, or Tetra-10 comprises a portion of the antithrombin III-binding pentasaccharide sequence containing the unique 3-O-sulfate group (35, 39, 40). The structures in Hexa-4, Hexa-15, and Tetra-25 lack this particular 3-O-sulfate residing on the reducing terminal GlcN residue found in the corresponding structures of Hexa-1, Hexa-8, and Tetra-10, respectively. Comparison of the data from Tetra-10 and -25 suggested a promoting effect of the 3-O-sulfate group (Tables I and II). Yet, there was no significant difference in susceptibility to the heparanase action between Hexa-8 and -15 (Table I). In contrast, when the data from Hexa-1 and -4 were compared, the inhibitory effect of the 3-O-sulfate group was evident. In summary, a 3-O-sulfate group appears to have a dual effect on the heparanase action, exhibiting a promoting effect when it resides in a relatively low sulfated sequence but an inhibitory effect when located in a highly sulfated sequence. The observation that the unique GlcN(3S)-containing oligosaccharides served as poorer substrates is consistent with the concept that the Hep-GAG fragments stored in cytoplasmic granules of mast cells have been generated by the postbiosynthetic degradation of Hep-PG by the heparanase action but still contain antithrombin III-binding sites (50–52). Thus, it is reasonable to assume that the glucuronidic linkage in the antithrombin III-binding pentasaccharide does not serve as a good substrate for the heparanase under physiological conditions.

A IdoUA(2S) Residue Located Two Sugar Residues away from the Target GlcUA Residue toward the Reducing Side Is Not an Absolute Requirement—Pikas et al. (34) suggested the involvement of the 2-O-sulfate group on a hexuronic acid residue located two monosaccharide units away from the cleavage site toward the reducing end in the heparanase action and proposed a minimally O-sulfated hexasaccharide sequence required for the recognition by the heparanases purified from human hepatoma and platelets (Fig. 6A). The panel of the oligosaccharides used in the present study contained no such 2-O-sulfated IdoUA residue on the reducing side of the cleavage site, suggesting that the IdoUA(2S) structure per se is not an absolute requirement for the heparanase action.

We addressed the question of whether the ΔHexUA(2S) at the nonreducing terminus might substitute for such an IdoUA(2S) residue and be recognized by the enzyme, suspecting the similarity of the steric configuration between IdoUA(2S) and ΔHexUA(2S) structures based on our observation that the 2-O-sulfate group of ΔHexUA(2S) of a disaccharide ΔHexUA(2S)-GlcN(6S,6S) can be removed by the human

### Table III

**Comparison of the heparanase digestibility of highly sensitive oligosaccharides**

| Fraction No. | Incubation time | % | % | % | % |
|--------------|-----------------|---|---|---|---|
|              | 1 h             | 5 h | 10 h | 23 h |
| Hexa-4       | <10             | <10 | 21  | 42  |
| Hexa-7       | 30              | 77  | >95 | >95 |
| Hexa-15      | 11              | 54  | 85  | >95 |
| Tetra-1      | <10             | 16  | 44  | 68  |

**Fig. 6.** Structures of the human heparanase cleavage site in Hep/HS. A, the human heparanase cleavage site proposed by Pikas et al. (34). B, the minimum trisaccharide structure of the human heparanase cleavage site in Hep/HS. The arrow indicates the glucuronic linkage cleaved by human heparanase. It appears that the highly sulfated structure is critical for the enzyme action. The GlcN(2-N-sulfate) structure on the reducing side and GlcN(6-O-sulfate) structure on the nonreducing side of the cleavage site are considerably important for the substrate recognition by the enzyme. These requisite groups are shown in the largest font size. The additional 2-N-sulfate group on the nonreducing GlcN or 6-O-sulfate group on the reducing GlcN appears to have a promoting effect on the heparanase action. These effectual sulfate groups are shown in the middle font size. The GlcN 3-O-sulfate structure, which is enclosed in the rectangle, causes inhibition of the enzyme in principle, although it also has promoting effects through its negative charge. C, the putative octasaccharide recognition sequence by the human heparanase in native Hep chains deduced from the results obtained by the heparanase digestion experiments of structurally defined oligosaccharides in the present study and the mono- and oligosaccharides previously isolated by bacterial heparanase/heparitinase digests of porcine intestinal Hep (36, 40, 53) and bovine lung and intestinal Hep (54).
Substrate Specificity of Human Recombinant Heparanase

recombinant iduronate-2-O-sulfatase (see also Ref. 55). Therefore, we investigated the susceptibilities to the heparanase of the oligosaccharides with or without a 2-O-sulfated ΔHexUA residue. Tetra-28 has the same structure as Tetra-1 but lacks the 2-O-sulfate group on the nonreducing ΔHexUA residue. When these tetrasaccharides were compared, Tetra-28 was more susceptible than Tetra-1 (Table I), supporting the promoting effect of the 2-O-sulfate group. Heparanase digestion experiments on a pair of hexasaccharides, Hexa-7 and Hexa-7S, that was prepared by glycuronate-2-O-sulfatase digestion of Hexa-7 (Tables I and III) also suggest that the sulfate group on the ΔHexUA residue augments the susceptibility of a substrate to the heparanase. Since, however, neither Hexa-7 nor Hexa-7S contains IdoUA(2S) at the position two saccharides away from the enzyme cleavage site toward the reducing side, the IdoUA(2S) structure is not an absolute requirement for the cleavage by the human recombinant heparanase. However, the possibility cannot be excluded that the IdoUA(2S) residue has a promoting effect on the heparanase action. Since the heparanase preparations that Pikas et al. (34) used were partially purified from human hepatoma and platelets, the contradictory specificities may be attributable to possible differences in the enzyme sources, glycoforms, and/or purities as discussed below. Alternatively, the possibility that the IdoUA(2S) residue in question may be critical for polymer HS and Hep chains to be cleaved by the heparanase in the specific conformational orientation under physiological conditions.

The Structure of the Predominant Cleavage Site of the Heparanase—Based on the results obtained in the present study, we suggest that the minimum trisaccharide cleavage site structure in Hep/HS recognized by the human recombinant heparanase is as illustrated in Fig. 6B. It appears that the highly sulfated structure in the immediate vicinity of the targeted GlcUA residue is critical for the enzyme action. The preference of the heparanase for the GlcN(NS) structure on the reducing side and the GlcN(6S) structure on the nonreducing side was demonstrated. However, the GlcN(NS) on the reducing side and the GlcN(6S) on the nonreducing side are not sufficient, since Tetra-25 was not cleaved by the enzyme. An additional 2-N-sulfate group on the nonreducing GlcN, 6-O-sulfate group on the reducing GlcN, or an additional sulfate group on the adjacent sequence (see Hexa-15) appears to have a promoting effect on heparanase action. The GlcN 3-O-sulfate structure has dual effects as discussed above.

DISCUSSION

In the present study, the substrate recognition by the homogenous recombinant human heparanase was examined in detail for the first time using a series of structurally defined oligosaccharides. The findings of the present study demonstrated the importance of the highly sulfated structure, which is located on both sides of the targeted GlcUA residue. Notably, the preferred sulfation positions by the heparanase were revealed; the enzyme cleaves in principle the glucuronidic linkage in the -GlcNAc(6S)-GlcUA-GlcN(NS)- sequence, although this sequence is not sufficient, and an additional sulfate group on this or adjacent sequence (see Hexa-15) appears to be required.

The elucidated sequence requirement was in good agreement with the cleavage sequence, suggested based on the structures of the remnant cleavage sites found in the penta- and trisaccharides isolated from porcine intestinal native Hep-GAG after digestion with bacterial heparanase/heparitinases (36, 40, 53). Among a series of sulfated oligosaccharides generated by bacterial lyases (45), the unsaturated pentasaccharide, ΔHexUA(2S)-GlcN(NS,6S)-IdoUA-GlcNAc(6S)-GlcUA (fraction 6–23 in Ref. 40 and fraction VI in Ref. 53), and the saturated trisaccharide, GlcN(NS)-IdoUA(2S)-GlcN(NS,6S) (fraction III-5b in Ref. 36), are of particular interest in view of the recognition sequence by porcine or mammalian heparanase. They are odd-numbered unlike other even-numbered oligosaccharides and appear to be derived from the reducing and nonreducing ends of the parent Hep-GAG chains as discussed previously (36, 40), since they do not possess GlcN at the reducing terminus or ΔHexUA at the nonreducing terminus, respectively. The above mentioned oligosaccharides were most likely generated from the sequence of -HexUA(2S)-GlcN(NS,6S)-IdoUA-GlcNAc(6S)-GlcUA*-GlcN(NS)-IdoUA(2S)-GlcN(NS,6S)- by the action of endogenous heparanase on the glucuronic bond indicated by an asterisk. Although the unsaturated pentasaccharide is a major digestion product (40, 53), the above mentioned trisaccharide was a minor product. In this context, it is noteworthy that Nader et al. (54) identified free GlcN(NS,6S) as one of the major products among other unsaturated di- and tetrasaccharides in the Flavobacterium heparinum heparanase digest of bovine lung and intestine Hep preparations. Based on these findings, the structural characteristics of the predominant saccharide sequence required for the substrate recognition by the heparanase in native Hep polysaccharides could be summarized as shown in Fig. 6C. It is conceivable that human, porcine, and bovine heparanases are similar in their specificity. The proposed structure is consistent with the previous finding by Oldberg et al. (47) for human platelet heparanase and the recent finding by Podyма-Inoue et al. (56) for rat parathyroid heparanase that the predominant GlcN residue on the nonreducing side of the cleavage site GlcUA of was N-acetylated and also overlaps with the structure proposed for human hepatoma and platelet heparanases by Pikas et al. (34) (Fig. 6A), although the present results are apparently contradictory to the previously claimed indispensability of the IdoUA(2S) and GlcN(3S) structures (33, 34, 57). It should also be noted that rat parathyroid heparanase is strikingly different from our enzyme in that it works at neutral and slightly acidic pH values and does not cleave heparin (56).

The presence of a 2-O-sulfate group on a HexUA residue, which is located two residues away from the cleavage site toward the reducing end, turned out to be not obligatory for the enzyme action, which is incompatible with the previous proposal that 2-O-sulfated IdoUA residues in HS are essential for the heparanase action (34, 57). In the previous studies, which demonstrated the importance of IdoUA(2S), partially purified enzymes from human hepatoma and platelets (34) and cultured CHO cells were used (57). Therefore, the partially purified enzyme preparations might contain unidentified regulatory factors that interact with the 2-O-sulfate group and modulate the enzyme activity. The purified platelet heparanase was reported a 137-kDa protein (58), which may suggest aggregation or complex formation with other protein(s). The 2-O-sulfate group in question appears to be nonessential for the enzyme action, as suggested previously (59), but may facilitate the enzyme action. Although the formation of intermediate HS breakdown products characteristic of the heparanase action was prevented in the HS 2-O-sulfotransferase-deficient CHO mutant cells, the heparanase transcript was not detected in the purified CHO cell mRNAs (19). Therefore, the major enzyme responsible for degrading HS in CHO cells may be a distinct heparanase(s) (60, 61).

Structural changes in HS in animal models of malignancy have been reported, showing a transformation-associated reduction in charge density of HS chains (for a review, see Ref. 62). In metastasizing tumor cells, heparanase is highly ex-

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2 S. Yamada, H. Maeda, and K. Sugahara, unpublished data.
pressed and degraded the ECM and vascular basement membranes (22–26), whereas less sulfated HS chains on the tumor cell surface may evade the attack of the heparanase secreted by the tumor cells themselves. Alternatively, heparanase plays critical roles in tumor growth and metastasis by releasing bioactive HS fragments from the tumor cell surface HS-PGs, which can serve as potent promoters of tumor progression. Being consistent with this concept, HS fragments generated from tumor cell surface HS-PGs by the treatment with bacterial heparanase promoted tumor cell growth mediated through basic fibroblast growth factor signaling pathways (63). In the transition to malignancy in human colon adenoma cells, the overall molecular organization of HS is preserved but distinctly modified in the sulfated domains, and it may contribute to the aberrant behavior of the cancer cells (15).

Tetra-1,1\DeltaHexUA2S-GlcNS2S-GlcUA-GlcNS2S, was one of the best substrates among the oligosaccharides tested in the present study (Table 1). This is the major tetrasaccharide product generated from commercial Hep preparations by the treatment with the bacterial heparanase (3.6 μmol from 100 mg of heparin) (38, 64). Previous heparanase assay methods used heterogeneous Hep/HS polysaccharides as substrates (19, 20). Since the number of the heparanase cleavage sites varies among different Hep/HS preparations, it has been difficult to make a precise comparison of the activities. The use of structurally defined substrate Tetra-1 as a substrate makes it possible to develop a simple and quantitative assay to measure the heparanase activity, which will provide a powerful diagnostic method to assess the invasive state of the cancer.

The findings of the present study also provided clues for developing specific inhibitors for the heparanase. The minimum heparanase recognition sequence contained neither GlcNS2S nor IdoUA2S, which are imperative elements for Hep/HS to interact with antithrombin III or basic fibroblast growth factor, respectively (for reviews, see Refs. 5 and 12). Since the number of the heparanase cleavage sites varies among different Hep/HS preparations, it has been difficult to make a precise comparison of the activities. The use of structurally defined substrate Tetra-1 as a substrate makes it possible to develop a simple and quantitative assay to measure the heparanase activity, which will provide a powerful diagnostic method to assess the invasive state of the cancer.

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