Characterization of Heparan Sulfate Oligosaccharides That Bind to Hepatocyte Growth Factor*

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Proteoglycans from rat liver had the ability to bind hepatocyte growth factor (HGF). Digestion of the proteoglycans with heparitinase resulted in the complete loss of the activity, while digestion with chondroitinase ABC had no effect. Heparan sulfate (HS)-conjugated gel also bound HGF, and the binding was competitively inhibited by heparin and bovine liver HS, but not by Engelbreth-Holm-Swarm sarcoma HS, pig aorta HS, or other glycosaminoglycans, suggesting the specific structural domain in HS for the binding of HGF.

Among limited digests with heparitinase I of bovine liver HS, octasaccharide is the minimal size to bind HGF. Comparison of the disaccharide unit compositions revealed a marked difference in the IDa(2SO4)2-GlcNSO3(6SO4) unit between the bound and unbound octasaccharides. The contents of this disaccharide unit were calculated to be 2 mol/mol for the bound octasaccharide but 1 mol/mol for the unbound one. Considering both the substrate specificity and properties of heparitinase I, the above results suggest that the bound octasaccharide should contain two units of IDa(2SO4)2-GlcNSO3(6SO4) contiguously or alternately in the vicinity of the reducing end. The bound decasaccharide was more than 20 times as active as the unbound one with regard to the ability to release HGF bound to rat liver HS proteoglycan. The ability was comparable to the one-fourth of that of heparin.

HS has been shown to have activities to bind to various molecules (1). Of those, heparin-binding growth factors are particularly important, considering the physiological significance of potential ligands of HS (1). bFGF is such a typical molecule and was detected as a complex with HSPG in the extracellular matrix such as basement membranes of the kidney glomerulus (2). In addition, the low affinity receptor for bFGF on the cell surface was identified to be a cell-surface HSPG (3, 4). Recent studies (5–8) have shown that the binding of bFGF to the cell-surface and/or extracellular matrix HSPG is essential for the interaction of bFGF with its high affinity receptor. Heparin or HS may also be involved in protecting bFGF from protease digestion or heat/acid inactivation (9). It is of note here that the binding of bFGF to HS requires the domain structure composed of a cluster of IDa(2SO4)2-GlcNS units (10–13).

HGF was identified initially as a mitogen for hepatocytes (14, 15). Subsequently, HGF was found to be identical not only with a scatter factor (16) but also with a tumor cytotoxic factor (17). Thus, HGF promotes the dissociation of epithelial cells and vascular endothelial cells in vitro and stimulates angiogenesis in vivo (18, 19). In addition, HGF is considered to be a unique pleiotropic factor that acts as a mitogen, a tumor suppressor, a motogen, and a morphogen. Further, HGF may mediate epithelial and mesenchymal interactions during embryogenesis, organ repair, and neoplasia (20).

HGF is known to have the ability to bind to heparin, and there are two classes of receptors for HGF with different affinities (16, 21–24). The high affinity receptor (Kd, 4.6 pM) (21) on rat hepatocytes was identified as the c-met proto-oncogene product, a transmembrane tyrosine kinase that is expressed predominantly on epithelial cells (16, 22, 25). The low affinity receptor (Kd, 275 pM) (21) was found to be a HSPG at the cell surface. Possible functional consequences after binding are as follows; stabilization of HGF (26, 27), induction of conformational changes to fit HGF to the high affinity receptor (28, 29), or, conversely, blocking of the biological activity due to ligand sequestration (30). HSPGs in rat liver are identified as perlecain, syndecan, and fibroglycan (31–34). However, it remains to be determined which is likely for a low affinity receptor. A mutant HGF without the affinity for heparin showed neither the affinity for c-met protein nor the biological activity (35–39). However, exogenous addition of heparin reduced the interaction of HGF with c-met protein (23, 28) and, consequently, reduced the mitogenic (40, 41) and motogenic (42) responses of cells to HGF. This was explained by the observation that a HGF-exogenous heparin complex could not be bound to c-met protein (28), which suggests, interestingly, that exogenous heparin does not function as the cell-surface HSPG. Certain molecular structures and/or spatial localization of endogenous HSPG may be important in regulating the binding of HGF to c-met protein (28).

Therefore, the significance of interaction between cell-surface HSPG and HGF may be the same as that of bFGF, but the mechanism appears to be different and complex. To understand it, the precise analysis for the interaction between HSPG and HGF is needed.

In this study, fractionated HS oligosaccharides prepared from the HS digested with heparitinase I, in accordance with the different affinities to HGF, and characterized a possible
to dishes coated with HSPG could release bound HGF from the structure involved in the HGF binding. In addition, we showed that the addition of oligosaccharides with HGF binding activity to dishes coated with HSPG could release bound HGF from the HSPG.

**EXPERIMENTAL PROCEDURES**

**Materials—**Heparin was purchased from Sigma. HSs from pig aorta, pig liver, bovine liver, and EHS sarcoma were gifts of K. Yoshida and T. Harada, Seikagaku Corp. Chondroitin 4-sulfate from whale cartilage, chondroitin sulfate E from squid cartilage, dermatan sulfate from pig skin, hyaluronic acid, heparitinase I (Flavobacterium heparinum, EC 4.2.2.8), heparitinase II (F. heparinum, EC 4.2.2.7), and heparitinase ABC (F. heparinum, EC 4.2.2.8), and heparitinase II (F. heparinum, EC 4.2.2.8), heparitinase ABC (Flavobacterium heparinum, EC 4.2.2.8, Flavobacterium heparinum, EC 4.2.2.8) were purchased from Seikagaku Corp. (Tokyo, Japan). Recombinant human HGF was a gift of Mitsubishi Kasei Co. (Yokohama, Japan). Sephadex G-50, CNBr-activated Sepharose 4B, and epoxy-activated Sepharose 6B were purchased from Pharmacia (Uppsala, Sweden). Anti-digoxigenin-AP, Fab fragments, 5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt, and nitro blue tetrazolium chloride were purchased from Boehringer Mannheim Biochemica (Mannheim, Germany).

**Preparation of Proteoglycans from Rat Liver—**Liver was quickly excised. Livers from five rats (total wet weight, approximately 65 g) were minced into small pieces and homogenized in 50 mM guanidine HCl extraction solution containing 50 mM sodium acetate, 10 mM EDTA, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM 6-aminohexanoic acid, 20 mM benzamidine HCl, 2% (v/v) Triton X-100. The homogenate (approximately 360 ml) was stirred at 4°C for 48 h. Insoluble residues were removed by centrifugation at 12,000 x g for 30 min at 4°C. The supernatant was recovered. Twenty ml of the supernatant solution were diluted with 19 volumes of 7 M urea buffer (7 M urea, 20 mM Tris-HCl, pH 7.2, 10 mM EDTA, 5 mM N-ethylmaleimide, 0.5 mM phenylmethylsulfonyl fluoride, 2% (v/v) Triton X-100). The solution was applied to DEAE-Sephadex (2 ml) equilibrated with 7 M urea buffer at 4°C. The column was washed with 10 ml of 0.2 M NaCl in 7 M urea buffer. Proteoglycan fraction (equivalent to 0.2 g of rat liver) was subjected to HGF binding in the solution containing 0.2 mg/ml digoxigenin-HGF, 0.2 mg/ml chondroitin 4-sulfate, 0.9 mw CaCl2. After 1 h at room temperature, unbound digoxigenin-HGF was removed by washes with TBS as described above. Membranes were then treated with anti-digoxigenin-AP, Fab fragments (1:500 dilution) for 1 h. Unbound antibodies were washed out as described above, and membranes were soaked in 5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt (1:200 dilution) and nitro blue tetrazolium chloride for 1 h. Membranes were then treated with anti-digoxigenin-AP, Fab fragments (1:500 dilution) for 1 h. Unbound antibodies were washed out as described above, and membranes were soaked in 5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt (1:200 dilution) and nitro blue tetrazolium chloride for 1 h.

**Preparation of Digoxigenin-conjugated HGF and 125I-HGF—**Digoxigenin-conjugated and 125I-labeled HGF was prepared according to the method recommended by the manufacturer. Briefly, 10 μg of HGF in 200 μl of 0.2 mM phosphate buffer, pH 8.5, were added into N-acetylated heparan sulfate and then mixed with 8.75 nmol of digoxigenin in dimethyl sulfoxide followed by 2 h incubation at room temperature. The HGF solution was applied to 0.5 ml of heparin-Sepharose gel equilibrated with 7 M urea buffer containing 0.02% (v/v) Triton X-100 and 1 mg/ml BSA (solution A). Heparin-Sepharose gels were equilibrated with phosphate-buffered saline (PBS; 0.1 M sodium phosphate, 1.37 M NaCl, 2.7 mM KCl, pH 7.2) containing 0.02% (v/v) Triton X-100, and 2% (v/v) dimethyl sulfoxide followed by 2 h incubation at room temperature. The precipitate was dissolved in 300 μl of NaOH and kept for 4 h at room temperature, unbound digoxigenin-HGF was removed by washes with TBS and then treated with anti-digoxigenin-AP, Fab fragments (1:500 dilution) for 1 h. Unbound antibodies were washed out as described above, and membranes were soaked in 5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt (1:200 dilution) and nitro blue tetrazolium chloride for 1 h. Membranes were then treated with anti-digoxigenin-AP, Fab fragments (1:500 dilution) for 1 h. Unbound antibodies were washed out as described above, and membranes were soaked in 5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt (1:200 dilution) and nitro blue tetrazolium chloride for 1 h. Membranes were then treated with anti-digoxigenin-AP, Fab fragments (1:500 dilution) for 1 h. Unbound antibodies were washed out as described above, and membranes were soaked in 5-bromo-4-chloro-3-indolyl phosphate, 4-toluidine salt (1:200 dilution) and nitro blue tetrazolium chloride for 1 h.
with 1 μg of HS or HS oligosaccharides was digested with a mixture of 1 milliunit of heparitinase I, 0.1 milliunit of heparinase II, and 1 milliunit of heparinase in 50 μl of 50 mM Tris-HCl, pH 7.2, 1 mM CaCl₂, 5 μg of BSA at 37°C for 1 h. Unsaturated disaccharides products were analyzed by HPLC using a polyamino-bound silica PAM column (YMC). The elution was performed with a linear gradient elution from 0 to 2.0 mM NaCl in 50 mM Tris-HCl, pH 7.2.

Composition Analysis of HS and Its Oligosaccharides—About 1 μg of HS or HS oligosaccharides was digested with a mixture of 1 milliunit of heparitinase I, 0.1 milliunit of heparinase I, and 1 milliunit of heparinase II in 50 μl of 50 mM Tris-HCl, pH 7.2, 1 mM CaCl₂, 5 μg of BSA at 37°C for 1 h. Unsaturated disaccharides products were analyzed by HPLC using a polyamino-bound silica PAM column (YMC). The elution was performed with a linear gradient elution from 0 to 2.0 mM NaCl in 50 mM Tris-HCl, pH 7.2.

Degradation of about 1 μg of HS or HS oligosaccharides with nitrous acid at pH 1.5 and reduction of degradation products with [3H]NaBH₄ was carried out as described by Shively and Conrad (45). The products were desalted using Fast desalting columns. The fractions containing disaccharides were collected and analyzed by HPLC on a Partisil-10 SAX column (Whatman, Clifton, NJ) as described by Bienkowski and Conrad (47). The elution was monitored by measuring the radioactivity in a liquid scintillation counter.

HGF-Releasing Activity of HS Oligosaccharides and Heparin—The releasing activity was measured by ELISA by the method recommended by the manufacturer with a minor modification. A 96-well Nunc-Immuno Plate MaxiSorp (A/S Nunc, Roskilde, Denmark) was coated with 0.1 nmol (as hexuronic acid) of rat liver proteoglycans overnight at 4°C. Wells were washed three times with 200 μl of PBS and then blocked with 200 μl of PBS containing 10 mg/ml BSA (solution C) for 1 h. Then, 200 μl of the solution containing 0.2 μg/ml digoxigenin-HGF, 0.2 mg/ml chondroitin 4-sulfate, 0.9 mM CaCl₂, and 100 μl of PBS was added into each well. After 1 h at room temperature, unbound digoxigenin-HGF was removed by washes as described above. Then, 100 μl of PBS containing 1 ng to 10 μg of heparin or 1 pmol to 1 nmol as hexuronic acid of HS oligosaccharides were added into wells. After 1 h at room temperature, wells were washed as above, and then alkaline phosphatase-conjugated Fab fragments of anti-digoxigenin antibody (1:50 dilution) were added. After 1 h at room temperature, unbound Fab fragments were removed by washing, and the alkaline phosphatase substrate (1 mM/ml of pNPP in 1 ml/diliter diethanolamine, pH 9.8, containing 0.5 mM/mmliter) was added into each well. The enzyme activity in each well was measured by a MTP-100 microplate reader (Corona Electric Co., Ibargi, Japan).

RESULTS

Binding of HGF to Rat Liver Proteoglycans—PG preparations from whole rat liver were subjected to SDS-PAGE. PGs separated on the gel were transferred to a membrane for the blot analysis of HGF binding using digoxigenin-conjugated HGF. At least three species of PGs showed the affinity for HGF, of which molecular masses were 220, 180, and 120 kDa (Fig. 1, lane 1). When these PGs on the membrane were digested with a mixture of heparitinase I and II and heparinase (the HSase mixture) before exposing to HGF, none of them could bind HGF (Fig. 1, lane 2). However, the digestion of the PGs with chondroitinase ABC had no effect on the HGF binding (Fig. 1, lane 3). The results, therefore, suggested that HGF appeared to bind to proteoglycans only with HS chains, but not with chondroitin sulfate or dermatan sulfate chains.

HGF Binding Activities of Various Glycosaminoglycans—The activities of various GAGs were assessed by their capacities to inhibit 125I-HGF binding to pig liver HS-conjugated Sepharose gel as described under “Experimental Procedures.”
CaCl₂, 0.2 mg/ml chondroitin 4-sulfate). After a wash with PBS, the gel was washed with PBS containing 0, 0.01, 0.1, 1, 10 µg/ml of GAG and 1 × 10⁴ dpm (0.2 ng) of [125I]HGF and was fractionated by Dowex1 column chromatography and DEAE-Sepharose column chromatography.

Each [125I]HGF-conjugated Sepharose was equilibrated with solution B (10 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 0.9 mM CaCl₂, 0.2 mg/ml chondroitin 4-sulfate). After a wash with solution B, the bound [125I]HGF-conjugated oligosaccharides were eluted with 2 M NaCl in 10 mM Tris-HCl, pH 7.2. The percent proportion of the bound radioactivity to the applied radioactivity for each fraction is shown in Fig. 3A. The proportion increased as the molecular size increased. However, a sharp increase in the proportion was observed between HS-I and HS-IV (4 and 17%, respectively). The results suggest that HS-IV is the smallest size of the structures required for HGF binding, which was estimated to be HS octasaccharide judging from its molecular weight and disaccharide composition as described below (see Table II). The chain size dependence of the heparin-binding to HGF was also determined using [3H]labeled heparin oligosaccharides (Fig. 3B). The octasaccharide (Hep-8) was also the smallest fraction to show a sharp increase in the binding proportion, although the proportions tended to increase as the size of oligosaccharides increased.

The results suggest that the sizes of HS/heparin saccharides are one of the structural factors required for the binding of HS/heparin to HGF and the octasaccharides are the minimal.

Characterization of HGF-bound and -unbound Oligosaccharides—Bound and unbound oligosaccharides of HS-I were prepared as described under "Experimental Procedures." Rechromatography of the HS-I-unbound fraction showed that more than 95% of the radioactivity passed through the HGF column reproducibly (data not shown), indicating no significant contamination of HGF-bound species. Both bound and unbound fractions of HS-I were further fractionated in accordance with their negative charges by ion-exchange chromatography on a Mono-Q column (Fig. 4). Most of HS-I-bound fraction was eluted at the NaCl concentration of above 0.88 M (fractions 43–50; designated IV-B in Fig. 4A). On the other hand, the HS-I-unbound fraction was eluted with a broad distribution pattern. But 16% of the HS-I-unbound fraction was recovered in the subfraction similar in the elution position to HS-I-bound fraction (fractions 44–50; designated IV-UB in Fig. 4A). Therefore, the difference in HGF affinity between IV-B and IV-UB may be due to structural factors other than their net negative charges.

Both nonlabeled IV-B and IV-UB, after the extensive digestion with the HSase mixture, were subjected to the compositional analysis by HPLC on a polyaniline silica column as described under "Experimental Procedures" (Table II). Comparison of the unsaturated disaccharide compositions between them showed a marked difference: 47% of the disaccharides obtained from IV-B were D[di-(N6,6U)]trIS, whereas only 26% were in those obtained from IV-UB. Considering the molecular weights of IV-B and IV-UB, these composition data suggested that IV-B and IV-UB corresponded to the octasaccharide (4 disaccharide units) containing at least 2 Hexa(2SO₄)⁻GlcNSO₃(6SO₄) units and a mixture of the octa- and decasaccharides containing only 1 above unit, respectively. Moreover, considering both the substrate specificities and catalytic properties of enzymes used for the preparation of these HS oligosaccharides, nonreducing ends of the HS oligosaccharides are supposed to have nonsulfated unsaturated HexA. Hence, 2 Hexa(2SO₄)⁻GlcNSO₃(6SO₄) units in HGF-bound octasaccharides should be localized contiguously or alternately at or near the reducing ends.

HS-V fraction was also fractionated into HGF-bound and -unbound fractions by HGF affinity chromatography. Both V-B and V-UB were fractionated on a Mono-Q column (Fig. 4B), and the resulting fractions (V-B and V-UB) were subjected to the compositional analysis. V-B that was estimated to be a decasaccharide contained more than 50% Hexa(2SO₄)⁻GlcNSO₃(6SO₄) but V-UB contained only 12% (Table II). Thus, the composition analysis gave similar results to those obtained with IV-B and IV-UB fractions.

To identify the hexuronic acid residues participating in HGF binding, IV-B was treated with nitric acid at pH 1.5 and then washed with 0.15 M NaCl, 0.9 mM CaCl₂, 0.2 mg/ml chondroitin 4-sulfate). After a wash with solution B, the bound [125I]HGF-conjugated oligosaccharides were eluted with 2 M NaCl in 10 mM Tris-HCl, pH 7.2. The percent proportion of the bound radioactivity to the applied radioactivity for each fraction is shown in Fig. 3A. The proportion increased as the molecular size increased. However, a sharp increase in the proportion was observed between HS-I and HS-IV (4 and 17%, respectively). The results suggest that HS-IV is the smallest size of the structures required for HGF binding, which was estimated to be HS octasaccharide judging from its molecular weight and disaccharide composition as described below (see Table II). The chain size dependence of the heparin-binding to HGF was also determined using [3H]labeled heparin oligosaccharides (Fig. 3B). The octasaccharide (Hep-8) was also the smallest fraction to show a sharp increase in the binding proportion, although the proportions tended to increase as the size of oligosaccharides increased.

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HS-V fraction was also fractionated into HGF-bound and -unbound fractions by HGF affinity chromatography. Both V-B and V-UB were fractionated on a Mono-Q column (Fig. 4B), and the resulting fractions (V-B and V-UB) were subjected to the compositional analysis. V-B that was estimated to be a decasaccharide contained more than 50% Hexa(2SO₄)⁻GlcNSO₃(6SO₄) but V-UB contained only 12% (Table II). Thus, the composition analysis gave similar results to those obtained with IV-B and IV-UB fractions.

To identify the hexuronic acid residues participating in HGF binding, IV-B was treated with nitric acid at pH 1.5 and then

### Table I

| Materials                        | IC₅₀ (µg/ml) | Sulfate degree |
|----------------------------------|-------------|----------------|
| Bovine liver                     |             |                |
| Heparan sulfate fraction 1       | 45          | 0.77           |
| Heparan sulfate fraction 2       | 3.4         | 1.21           |
| Heparan sulfate fraction 3       | 0.75        | 2.12           |
| Pig liver heparan sulfate        | 38          | 0.80           |
| Pig aorta heparan sulfate        | ND*         | 0.42           |
| EHS sarcoma                      |             |                |
| bHGF-bound heparan sulfate       | ND*         | 0.72           |
| bHGF-unbound heparan sulfate     | ND*         | 0.71           |
| Heparin                          | 0.15        | 2.59           |
| Chondroitin 4-sulfate            | ND*         | 1.00           |
| Chondroitin sulfate E            | ND*         | 1.43           |
| Dermatan sulfate                 | ND*         | 1.03           |
| Chemically sulfated dermatan sulfate | ND*     | 1.31           |
| Hyaluronic acid                  | ND*         | 0               |

ND, not detected.
reduced with $[^3]$HNaBH$_4$ according to the method of Shively and Conrad (45). 85% of the total labeled saccharides were recovered in the disaccharide fraction (data not shown). The disaccharides were identified by HPLC on a SAX column. Of these disaccharides, 52% were IdoA(2SO$_4$)-GlcNSO$_3$(6SO$_4$), and only 2% were GlcA(2SO$_4$)AManR(6SO$_4$). Therefore, HexA(2SO$_4$)-GlcNSO$_3$(6SO$_4$), which was a major disaccharide component of IV-B, was an IdoA-type. The identification of hexuronic acid residues was also performed with the other HGF-bound fraction, V-B. Molar ratios of disaccharides per mol of IV-B or V-B estimated from both the results of Table II and the above identification of hexuronic acid residues are shown in Table III. In both IV-B and V-B, IdoA(2SO$_4$)-GlcNSO$_3$(6SO$_4$) was the only component with the content close to or exceeding 1 mol/mol, suggesting an essential involvement of this disaccharide unit in the HGF binding. Other disaccharide components were present in less than 1 mol/mol. However, contents of N-sulfated disaccharides such as IdaA-GlcNSO$_3$ and GlcA-GlcNSO$_3$(6SO$_4$) were relatively high, compared to those of N-acetylated disaccharides, and the sum of these N-sulfated disaccharide contents was more than 1 mol/mol. The results suggest that clustering of 2 IdoA(2SO$_4$)-GlcNSO$_3$(6SO$_4$) units and one N-sulfated component (HexA-GlcNSO$_3$ or HexA-GlcNSO$_3$(6SO$_4$)) may form the binding site for HGF.

HGF Releasing Activities of HS Oligosaccharides and Heparin from the Complex of HGF and HSPGs—Affinities to HGF of HS-bound and -unbound oligosaccharides and heparin were assessed by their releasing activities of HGF from the complex of HGF and HSPGs. The HSPG preparation from rat liver were used to coat ELISA plates. Digoxigenin-HGF was bound on the plate via coated HSPGs. After 1 h of incubation with oligosaccharides at various concentrations on the plate, digoxigenin-HGF yet bound on the plate was determined using anti-digoxigenin Fab fragment as described under "Experimental Procedures." The HGF-releasing activity was compared among HGF-bound HS oligosaccharide (V-B), HGF-unbound HS oligosaccharide (V-UB), and heparin (Fig. 5). The concentrations to give a 50% release of bound HGF were 1.3, 3, and 110 ng/ml for heparin, V-B, and V-UB, respectively. The releasing activity of V-B was 20 times more active than V-UB and only one fourth less than heparin.

DISCUSSION

Our present study has shown that HGF bound only to heparin and some species of HS, suggesting possible involvements of some unique structures on the chains in the binding (Table I). HGF affinity gel chromatography of HS oligosaccharides prepared by a limited digestion of bovine liver heparan sulfate with heparitinase I has shown that minimal sizes of the chains for HGF binding are octasaccharide (Fig. 3). Bound and unbound octasaccharides thus obtained were subjected to structural analyses. HS-bound octasaccharides (IV-B) characteristically comprised 2 mol of IdoA(2SO$_4$)-GlcNSO$_3$(6SO$_4$) per molecule (Table III). These results, considering the fact that their nonreducing ends were nonsulfated, unsaturated hexuronic acid, suggest that at least two IdoA(2SO$_4$)-GlcNSO$_3$(6SO$_4$) units are present contiguously or alternately each other at or near the reducing ends (see Fig. 6). The presence of this structural unit was also detected in the HS-bound decasaccharide fraction (V-B) (Table III).
Lyon et al. (48) have also suggested that heparan sulfate with a high affinity to HGF apparently has a sequence rich in IdoA and GlcNSO 3(6SO 4) residues. However, according to their results, no contiguous sequence of two or more IdoA(2SO 4)-containing disaccharides appeared to be absolutely necessary for the interaction with HGF, because most of fragments prepared from fetal skin fibroblast HS by digestion with heparinase I which specifically attacks N-sulfated disaccharides containing IdoA(2SO 4) residue still retained a HGF affinity. It is in question in our present study whether HexA(2SO 4)-GlcNSO 3 units are involved in the binding of HGF to HS directly, since these HexA(2SO 4)-GlcNSO 3 units comprised only 3.2% of the starting material, bovine liver HS fraction 2.

**TABLE III**

| Disaccharide | Content (mol/mol) |
|--------------|------------------|
|              | IV-B             | V-B              |
| IdoA(2SO 4)GlcNSO 3(6SO 4) | 1.88  | 2.57  |
| IdoAGlcNSO 3(6SO 4) | 0.49  | 0.45  |
| GlcAGlcNSO 3(6SO 4) | 0.40  | 0.20  |
| HexAGlcNSO 3 | 0.66  | 0.69  |
| IdoA(2SO 4)GlcNSO 3 | 0.11  | 0.14  |
| HexAGlcNAc(6SO 4) | 0.31  | 0.95  |
| Unknown &<.01  | 0.15  | 0.01  |

**FIG. 4. Mono Q FPLC of heparan sulfate oligosaccharides fractions.** A, HGF column-unbound (●) and bound (□) fractions of [3H]HS-IV (2 × 10^5 and 4 × 10^5 dpm, respectively) were desalted, freed from chondroitin 4-sulfate, concentrated, and applied to mono Q column. B, HGF column-unbound (●) and bound (□) fraction of [3H]HS-V (7.5 × 10^5 and 2 × 10^5 dpm, respectively) were treated as described above. The elution was performed with the indicated NaCl gradient in 50 mM Tris-HCl, pH 7.2, and fractions (1 ml) were served for the measurement of the radioactivity. Fractions were pooled as shown by closed and open bars and designated as indicated above. In a separate experiment for the compositional analysis, nonlabeled fractions corresponding to HGF-labeled fractions as described above (unbound HS-IV, 10 nmol; bound HS-IV, 2 nmol; unbound HS-V, 4.4 nmol; bound HS-V, 2.6 nmol) were also applied to the same mono Q column as above. Fractions corresponding to labeled fractions shown by closed and open bars were pooled and desalted for analysis.

**FIG. 5. HGF releasing activity of V-B, V-UB, and heparin from the complex with HSPG.** Releasing activity was detected by ELISA as described under "Experimental Procedures." Digoxigenin-HGF was added into wells coated with rat liver proteoglycans (0.1 nmol as hexuronate). After 1 h, unbound digoxigenin-HGF was removed, and then V-B (●), V-UB (○), and heparin (▲) at various concentrations were added. After 1 h, the wells were washed, then anti-digoxigenin-AP, Fab fragments were added to yield color. Nonspecific binding was determined using 100 ng/ml heparin.
However, HexA(2SO₄)-GlcNSO₃ units were not condensed into the HGF-bound fractions such as IV-B (Table II). In addition, bFGF-bound HS from EHS tumor, which has been shown to be composed of some of the HS octasaccharide were nonsulfated, unsaturated hexuronic acid. Structural variants in the HGF binding region are indicated by R. One R is the 6-O-sulfated group, the other R is hydrogen. N-Sulfate groups are not less than three groups in the molecule. Two IdoA(2SO₄)-GlcNSO₃ units (within the shaded boxes) are present contiguously (A and B) or alternately (C) at the reducing side or at the internal side.

![Diagram of minimal structures on heparan sulfate for HGF binding](https://www.3dmodeling.com/diagram/hgf-binding-heparan-sulfate-oligosaccharides.png)

**Fig. 6. Minimal structures on heparan sulfate for HGF binding.** Nonreducing ends of these HS octasaccharides were indicated by boxes. There may be some different types of HSs with respect to their affinity to HGF. As shown in Table I, bovine liver HS had a notably high affinity, which was almost comparable to that of human, and pig liver HS also showed a significant affinity. However, it is of note that pig aorta HS and EHS sarcoma HS showed no detectable affinity. In relation to this difference, rat liver has been found to contain at least three species of PGs with HGF affinity. Indeed, it has been shown that some clusters of IdoA(2SO₄)-GlcNSO₃ units (≥6SO₄) units are present in rat liver HS (49). We have also characterized the presence of highly sulfated HS in lung with a HGF affinity (data not shown). It is now known that lung acts as an endocrine organ with respect to HGF production, and HGF is active in the organogenesis and development of lung (50). The results that HSs derived from some organs have some activities to bind HGF may suggest that HS may be important in regulating functional HGF activity.

Exogenous heparin reduced the HGF/c-met protein interaction (23, 28) and mitogenic (40, 41) and motogenic (42) responses, and does not simply function as a soluble form of the HGF for this experiment, and Drs. K. Yoshida and T. Harada for preparing heparan sulfates from various animal species.

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