Hepatocyte Growth Factor/Scatter Factor Binds with High Affinity to Dermatan Sulfate*

(Received for publication, July 28, 1997, and in revised form, October 9, 1997)

Malcolm Lyon‡§, Jon A. Deakin‡, Hassan Rahmouné, David G. Fernig, Toshikazu Nakamura**, and John T. Gallagher‡

From the ‡Cancer Research Campaign & University of Manchester, Department of Medical Oncology, Christie Hospital National Health Service Trust, Manchester M20 4BX, United Kingdom, the §School of Biological Sciences, University of Liverpool, Liverpool L69 7ZB, United Kingdom, and the **Division of Biochemistry, Biomedical Research Center, Osaka University Medical School, Osaka 565, Japan

We have demonstrated by affinity chromatography that hepatocyte growth factor/scatter factor (HGF/SF) binds strongly to dermatan sulfate (DS), with a similar ionic strength dependence to that previously seen with heparan sulfate (HS). Analysis of binding kinetics on a biosensor yields an equilibrium dissociation constant, $K_D$ of 19.7 nM. This corresponds to a 10–100-fold weaker interaction than that with HS, primarily due to a faster dissociation rate of the complex. The smallest DS oligosaccharide with significant affinity for HGF/SF by affinity chromatography appears to be an octasaccharide. A sequence comprising unsulfated idurionate residues in combination with 4-O-sulfated N-acetylgalactosamine is sufficient for high affinity binding. The presence of 2-O-sulfation on the idurionate residues does not appear to be inhibitory. These observations concur with our previous suggestions, from analyses of HS binding (Lyon, M., Deakin, J. A., Mizuno, K., Nakamura, T., and Gallagher, J.T. (1994) J. Biol. Chem. 269, 11216–11223), that N-sulfation of hexosamines and 2-O-sulfation of iduronates are not absolute requirements for glycosaminoglycan binding to HGF/SF. This is the first described example of a high affinity interaction between a growth factor and DS, and is likely to have significant implications for the biological activity of this paracrine-acting factor.

Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic factor with the ability to influence the growth, motility, differentiation, and morphogenesis of its target cells (for a recent review, see Ref. 1). It acts in a paracrine manner, with the major secretors being fibroblasts, vascular smooth muscle cells, nonparenchymal liver cells, etc., whereas those cells that possess the requisite tyrosine kinase receptor (Met) are primarily epithelial and endothelial cells. Recent evidence suggests that multipotent and erythroid hemopoietic progenitor cells are also responsive to HGF/SF. The HGF/SF-Met system appears to operate primarily during the morphogenetic and differentiation events occurring in organogenesis, as well as in the repair of organ damage in the adult (reviewed in Ref. 1). Aberrant expression of HGF/SF and/or Met has been strongly implicated in tumor progression, particularly in the acquisition of an invasive malignant phenotype (2–5). This presumably results from its ability to directly stimulate the growth and motility of tumor cells, as well as increasing the secretion of matrix-degrading proteases (6), thereby facilitating invasion of the surrounding stroma. Additionally, its potent angiogenic action (7, 8) may contribute to the development of a tumor vasculature, which is essential for sustaining an expanding tumor mass.

In addition to Met, HGF/SF also interacts in vitro with the heparan sulfate (HS) chains of heparan sulfate proteoglycans (HSPGs) (9). The latter probably constitute the more abundant, but relatively lower affinity, HGF/SF-binding sites present on most cells (10). The interaction of HGF/SF with cell surface HSPGs may facilitate its binding to Met and the subsequent activation of its tyrosine kinase activity (11–15). The HGF/SF-binding site is present within the idurionate- and sulfate-rich domains of HS, and its structure has been partially elucidated (9, 16). Additionally, the affinity of the HGF/SF-HS interaction is known to be high with a $K_D$ of 0.2–3 nM.

Dermatan sulfate (DS), although biosynthetically unrelated and compositionally distinct from HS, nevertheless does possess some similar organizational features (for a review, see Ref. 17). DS contains N-acetylgalactosamine rather than N-acetylglucosamine, and the glycosidic linkages are alternating $\beta$1–3 and $\beta$1–4, rather than all $\alpha$1–4. However, both glycosaminoglycans (GAGs) possess idurionate-rich domains of variable length, formed by epimerization of glucurononates to iduronates, although in DS the adjacent hexosamines are N-acetylated rather than being specifically N-sulfated (a prerequisite for epimerization in HS). Whereas, in DS, the idurionate-linked hexosamines are relatively uniformly O-sulfated on C-4, in HS, they can become O-sulfated, but to a lesser extent. In both GAGs, additional sulfation can also occur at C-2 on the iduronates, although generally less frequently in DS. Overall, DS experiences much less additional sulfation within the idurionate-rich domains, compared with the glucurononate-rich se-

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* This work was supported by grants from the Cancer Research Campaign (to M. L., J. A. D., and J. T. G.), the North West Cancer Research Fund (to H. R. and D. G. F.), and the Mizutani Foundation for Glycoscience. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Medical Oncology, Christie CRC Research Centre, Christie Hospital NHS Trust, Wilmotow Rd., Manchester M20 4BX, United Kingdom. Tel: 44-161-446-3202; Fax: 44-161-446-3269; E-mail: MLyon@pirc.man.ac.uk.

§ The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; GAG, glycosaminoglycan; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; DS, dermatan sulfate; DSF, dermatan sulfate proteoglycan; CS, chondroitin sulfate; FGF, fibroblast growth factor; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; dp, degree of polymerization (i.e. number of monosaccharides); GlcA, $\beta$-n-glucurionate; IdoA, $\alpha$-l-iduronate; HexA, un-sulfated hexuronate; $\Delta$HexA, $\Delta^2$-unsaturated hexuronate; IdoA(2-/3-SO$_3$), $\alpha$-l-idurionate 2-sulfate; GalNAc(2-/3-O-SO$_3$), $\beta$-N-acetylgalactosamine 2-sulfate; GalNAc(4-O-SO$_3$), $\beta$-N-acetylgalactosamine 4-sulfate; GalNAc(6-O-SO$_3$), $\beta$-N-acetylgalactosamine 6-sulfate; GalNAc(4,6-O-SO$_3$), $\beta$-N-acetylgalactosamine 4,6-disulfate; MDCK, Madin-Darby canine kidney.

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quencies, than does HS, in which the great majority of the overall chain sulfation is concentrated in such domains.

The parallel occurrence of contiguous sequences of iduronate and adjacent sulfated hexosamines in both types of GAG may help to explain why DS, unlike the iduronate-lacking chondroitin sulfates (CS), often interacts relatively weakly with many HS/heparin-binding proteins, e.g., basic fibroblast growth factor (FGF) (18), platelet factor 4 (19), fibronectin (20), interleukin-7 (21), and protein C inhibitor (22). Only in the specific case of heparin cofactor II has a comparable high affinity interaction with DS been demonstrated, and this is dependent upon the presence of a specific, highly sulfated DS oligosaccharide sequence (23, 24).

In the present study, we demonstrate that HGF/SF also binds to DS with high affinity. This gives further insight into the specific structural requirements for oligosaccharide binding to HGF/SF. The existence of this interaction is likely to have significant implications for the biological activity of this paracrine-acting, mesenchymal factor.

**EXPERIMENTAL PROCEDURES**

**Materials—**Recombinant human HGF/SF was purified as described by Nakamura et al. (25). Porcine mucosal heparin, bovine mucosal dermatan sulfate, bovine kidney heparan sulfate, and shark cartilage chondroitin sulfate were purchased from Sigma (Poole, United Kingdom). Low molecular weight heparin (Innohep) was obtained from Leo Laboratories Ltd. (Princes Risborough, UK). Decorin proteoglycan, purified from bovine skin (26), was a generous gift from Dr. C. H. Pearson (University of Alberta, Edmonton, Alberta, Canada). Affi-Gel 10 and Bio-Gels P-2 and P-10 were obtained from Bio-Rad (Hemel Hempstead, UK). PD-10 desalting columns were from Pharmacia Biotech (Uppsala, Sweden). d-[6-3H]Glucosamine hydrochloride (20–45 Ci/mol) was from NEN Life Science Products (Stovengaen, UK). Chondroitin AC I lyase (Flavobacterium heparinum; EC 4.2.2.5), chondroitin AC II lyase (Arthrobacter aurescens; EC 4.2.2.5), chondroitin ABC lyase (Proteus vulgaris; EC 4.2.2.4), and chondroitin B lyase (F. heparinum; no EC number assigned) were from Seikagaku Kogyo Co. (Tokyo, Japan). Mouse Balb/c 3T3 cells were from the American Type Culture Collection (ATCC). The FS8 fibroblast cell line was derived from fetal calf skin (27). Madin-Darby canine kidney (MDCK) cells were kindly provided by Dr. E. Gherardi (Cambridge University Medical School, Cambridge, UK).

**Preparation and Operation of HGF/SF Affinity Columns—**HGF/SF affinity columns were prepared by coupling recombinant human HGF/SF to Affi-Gel 10, in the presence of an excess of heparin to protect the GAG-binding site, as described by Lyon et al. (9). Radiolabeled poly- or oligosaccharides were applied to the HGF/SF column in anionic strength of 0.15 m at room temperature, and then recirculated at least 10 times to desalinate. After washing with PBS, the column was eluted with an increasing stepwise gradient of NaCl in 20 mM sodium phosphate, pH 7.4. Fractions were collected and monitored for radioactivity.

In a competition experiment, the binding of d-[6-3H]labeled DS tetrasaccharides (2 x 10⁶ cpm) to a small HGF/SF column was compared in the presence and absence of 1 μg of low molecular weight heparin (average Mₐ of 3000; approximately 6-fold molar excess relative to HGF/SF). The sample was circulated through the column 20 times in 50 mM NaCl, 20 mM phosphate, pH 7.0, with an excess of 0.2 mM NaCl, 20 mM phosphate, pH 7.0, and the bound oligosaccharides were step-eluted with 0.6 mM NaCl, 20 mM phosphate, pH 7.0.

**Partial Depolymerization of Chondroitin/Dermatan Sulfate Chains—**Confluent cultures of either FS8 fibroblasts or Balb/c 3T3 cells, grown in minimum Eagle’s medium containing 10% fetal calf serum, were washed five times with cold binding solution (no HGF/SF or Tris-HCl). The cells were then chromatographed on a Bio-Gel P-10 (fine grade) column (1 x 133 cm) eluted with 0.2 mM NH₄HCO₃ at a flow rate of 5 ml/h. Fractions of 1 ml were collected and aliquots were counted for radioactivity. Peaks corresponding to individual size fractions of enzyme-resistant oligosaccharides were individually collected, exhaustively freeze-dried, and then redissolved in distilled water. Samples of each size fraction, from hexa- to dodecasaccharides, were then applied to a BioPac PA1 (Dionex; 0.4 x 25 cm) strong anion-exchange HPLC column equilibrated with distilled water adjusted to pH 3.5 with HCl. After a wash with pH 3.5 water, the column was eluted with a linear gradient of 0–1 mM NaCl, pH 3.5, at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected and aliquots counted for radioactivity. Fractions corresponding to individual oligosaccharide peaks were concentrated by centrifugal vacuum evaporation, desalted on PD-10 columns eluted with distilled water, and then freeze-dried.

**Disaccharide Composition Analysis—**Oligosaccharide species were completely digested to disaccharides using a mixture of 40 μIU/ml each of chondroitin ABC lyase and chondroitin B lyase in 50 mM NaCl, 50 mM Tris-HCl, pH 8.0, at 37 °C for 24 h. The digests were then resolved by chromatography on a Bio-Gel P-2 (superfine) column (1 x 47 cm) eluted with 0.1 M NH₄HCO₃ at a flow rate of 4 ml/h. Fractions corresponding to the disaccharide peaks were collected, freeze-dried, and then redisolved in 1 ml of distilled water adjusted to pH 3.5 with HCl. Samples were applied either to a BioRad PA1 (0.4 x 25 cm; Dionex) or a 5 μm Spherisorb (0.4 x 25 cm; Technicul, Stockport, UK) strong anion-exchange HPLC column equilibrated in pH 3.5 water. After a wash with pH 3.5 water, the column was eluted with a linear gradient of 0–1 mM NaCl, pH 3.5, at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected and counted for radioactivity. Disaccharide peaks were identified by comparison with the elution positions of known CS/DS disaccharide standards (Seikagaku Kogyo Co., Tokyo, Japan).

**Binding of HGF/SF to MDCK Cells and Competition with Various GAGs—**MDCK cells were seeded into the wells of a 96-well plate at a density of 10⁴ cells/well in minimum Eagle’s medium containing 5% fetal calf serum, 1% (v/v) bovine serum albumin, 10 mM HEPES, pH 7.4, at 4 °C. The cells were then incubated with 0.2 mM/well of the above solution containing 5 nM [125I]-HGF/SF (prepared as described by Lyon et al. in Ref. 9) in the presence of a range of concentrations (0–100 μg/ml) of heparin, HS, DS, or CS. After incubation at 4 °C for 4 h, the cells were washed five times with cold binding solution (no HGF/SF or GAG). Cells were then solubilized in 1 ml NaOH, and the released [125I] radioactivity was measured in a γ counter.

**Kinetic Analysis of HGF/SF Binding to Dermatan Sulfate—**Bovine skin decorin (250 μg of decorin in 100 μl of distilled water) was biotinylated on its core protein by addition of three 10-μl aliquots of a 50 nm solution of succinimidyl-6-(biotinamido)hexanoate (Pierce & Warriner, Chester, UK) in dimethyl sulfoxide over a 24-h period. Biotinylated decorin was separated from unreacted biotin reagent, by passage over a PD-10 desalting column eluted with distilled water, and then lyophilized.

**Binding Reactions were performed in an IAsys resonant mirror biosensor (Affinity Sensors, Cambridge, UK) using three-dimensional carboxymethylcellulose surfaces.** The surfaces were derivatized with streptavidin, according to the manufacturer’s instructions, and then loaded with the biotinylated decorin proteoglycan. All HGF/SF binding reactions were performed in PBS, pH 7.2, containing 0.02% (v/v) Tween 20 at 20 °C, and data were collected three times a second. HGF/SF, at a known concentration, was introduced into the cuvette in 100 μl of phosphate, pH 7.4. The late-eluting peak, corresponding primarily to CS/DS chains, was recovered, dialyzed against distilled water, and concentrated to approximately 1 ml. The sample was treated with pH 1.5 nitrous acid (28) to degrade any contaminating HS, neutralized, and purified by chromatography on a Sepharose CL2B column (1.5 x 55 cm) eluted with 0.2 mM NH₄HCO₃ at a flow rate of 5 ml/h. The high molecular weight CS/DS chains were collected and freeze-dried. The heterogeneity of the major oligosaccharide fractions was further analyzed by electrophoresis on a 25–35% (v/v) gradient polyacrylamide gel, with a 5% (v/v) polyacrylamide stacking gel, followed by semidry electrotransfer to a positively charged nylon membrane and then fluorographic detection.

**Partial Depolymerization of Chondroitin/Dermatan Sulfate Chains and Purification of Iduronate-Rich Oligosaccharides Species—**Purified 3H-labeled CS/DS chains were dissolved in 1 ml of 50 mM sodium acetate, pH 6.5, and digested with a mixture of 0.1 units/ml each of chondroitin AC I and ACII lyases at 37 °C for 24 h. A further addition of enzymes was made, and the incubation continued for 4 h. The digest was then chromatographed on a Bio-Gel P-10 (fine grade) column (1 x 133 cm) eluted with 0.2 mM NH₄HCO₃ at a flow rate of 5 ml/h. Fractions of 1 ml were collected, and aliquots were counted for radioactivity. Peaks corresponding to individual size fractions of enzyme-resistant oligosaccharides were individually collected, exhaustively freeze-dried, and then redissolved in distilled water. Samples of each size fraction, from hexa- to dodecasaccharides, were then applied to a ProPac PA1 (Dionex; 0.4 x 25 cm) strong anion-exchange HPLC column equilibrated with distilled water adjusted to pH 3.5 with HCl. After a wash with pH 3.5 water, the column was eluted with a linear gradient of 0–1 mM NaCl, pH 3.5, at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected and aliquots counted for radioactivity. Fractions corresponding to individual oligosaccharide peaks were concentrated by centrifugal vacuum evaporation, desalted on PD-10 columns eluted with distilled water, and then freeze-dried.
binding solution and its association to the immobilized decorin was monitored until a plateau was reached. The cuvette was then rapidly washed twice with PBS, 0.02% (v/v) Tween 20, pH 7.2, and the dissociation of HGF/SF from the immobilized decorin was followed. The decorin surface was regenerated by washing twice with 20 μl of 2 M NaCl, 10 mM sodium phosphate, pH 7.2, followed by re-equilibration with PBS, 0.02% (v/v) Tween 20, pH 7.2. Three independent sets of binding reactions at five different HGF/SF concentrations were carried out. The association and dissociation rate constants, $k_a$ and $k_d$, respectively, were calculated from each set of association and dissociation curves, using the nonlinear curve-fitting FastFit software (Affinity Sensors) supplied with the instrument. The individual values of $k_a$ and $k_d$, together with their associated errors, were then combined. HGF/SF did not itself bind to streptavidin-derivatized carboxymethyl dextran surfaces.

RESULTS

Interaction of HGF/SF with CS/DS Chains and Oligosaccharides—$^3$H-Labeled CS/DS chains purified from cultures of FS8 fetal human skin fibroblasts bound to an HGF/SF affinity column, but not a control column, at physiological pH and ionic strength. The bound CS/DS fraction was eluted over the range of 0.4–1.0 M NaCl with the majority being released by 0.8 M NaCl (Fig. 1). This is very similar behavior to that reported previously for the HS chains derived from the same cell line (9). CS/DS chains prepared from cultures of Balb/c 3T3 cells also bound and eluted with similar characteristics (data not shown).

In light of the previously determined requirements for iduronate residues, in combination with a specific pattern of O-sulfation, for the binding of HS to HGF/SF (9), together with the lack of affinity for pure CS (9), it was considered likely that interaction of HGF/SF with CS/DS would also occur specifically within the iduronate-containing sequences of these polysaccharides. Thus, a large scale preparation of $^3$H-labeled CS/DS chains purified from Balb/c 3T3 cells (disaccharide composition: 6.0% nonsulfated, 79.7% mono-4-O-sulfated, 10.2% mono-6-O-sulfated, 1.5% di-2,4-O-sulfated, 1.4% di-2,6-O-sulfated, and 1.2% di-4,6-O-sulfated disaccharides) was exhaustively digested with a mixture washing of chondroitin ACI and ACII lyases, to degrade specifically within the glucuronate-containing regions and thereby excise intact iduronate-rich sequences. A combination of enzymes was employed as chondroitin ACII lyase is more efficient at cleaving adjacent to any oversulfated glucuronate-containing disaccharides, whereas the more endolytic action of chondroitin ACI lyase results in better cleavage at isolated internal glucuronate residues (29). Bio-Gel P-10 gel filtration chromatography of the resulting digest (Fig. 2) revealed a predominant disaccharide peak, together with a series of discrete, well resolved peaks corresponding to tetra- to dodecasaccharides. A population of larger, but poorly resolved fragments, extending into the void volume of the column, were also present. The proportion of resistant glycosidic linkages, as calculated from the relative content of differently sized oligosaccharide products (Fig. 2), suggests an iduronate content of at least 33%. The series of oligosaccharides larger than disaccharides should predominantly correspond to the general structure, $\Delta$HexA-GalNAc(OSO$_3$)$_n$-[HexA($\geq$2-OSO$_3$)$_n$]-GalNAc(OSO$_3$)$_{n-1}$, where the $\Delta$HexA would have derived from an original GlcA residue, the internal HexA residues should be predominately, if not exclusively, IdoA (a proportion of which could be 2-O-sulfated), and the GalNAc residues would be sulfated predominantly at the C-4 position. Because of uncertainty as to the ability of the combined chondroitinases ACI and ACII to cleave at all GlcA linkages, it is possible that a minor proportion of the HexA residues may be GlcA.

Aliquots of the individual oligosaccharide peaks corresponding to di- to dodecasaccharides, as well as a combined pool of those oligosaccharides larger than dodecasaccharides, were assayed for binding to the HGF/SF column. Di- and tetrasaccharides did not bind, and only a very small proportion (2.1%) of hexasaccharides bound (data not shown). A minor fraction (6.6%) of octasaccharides bound to the HGF/SF column, requiring 0.4 M NaCl for elution (Fig. 3A). As oligosaccharides increased further, that proportion of material which bound progressively increased (i.e. the majority of dp12 and nearly all the dp > 12), as did the NaCl concentration required for elution of the bound components (Figs. 3, B–D). Although the majority of the largest oligosaccharides required 0.8 M NaCl for elution (Fig. 3D), this was still lower than the 0.8 M NaCl required to elute intact CS/DS chains (compare Fig. 1).

In a competition experiment, the presence of 1 μg of low molecular weight heparin (average length of dp 10–12) was able to inhibit by 67% the binding of $2 \times 10^5$ cps of a DS tetradesaccharide to a small HGF/SF affinity column. This suggests that both DS and HS oligosaccharides are likely to be binding to the same site on the HGF/SF molecule.

Correlation between DS Oligosaccharide Structure and Affinity for HGF/SF—The individual hexa-, octa-, deca-, and dodecasaccharide fractions in Fig. 2 were further resolved by preparative strong anion-exchange HPLC into molecular spe-
cies with differing sulfation patterns. Comparisons of both their disaccharide compositions and their respective HGF/SF affinities, as determined by the concentration of NaCl required to elute them from a HGF/SF affinity column, may help to elucidate the minimum specific structural requirements for this interaction. All four oligosaccharide size populations yielded surprisingly similar profiles upon strong anion-exchange HPLC. A single major species (labeled peak B in all cases), comprising 70–83% of the total population, was always present, together with a substantially less abundant (13–19%), later-eluting species (labeled peak C) (Fig. 4 and Table I). A minor, earlier-eluting species (labeled peak A) was also present in all four size populations, and the content of this species appeared to generally increase with oligosaccharide size (Table I). A fourth, highly charged, minor species (labeled peak D) was found only in the dodecasaccharide population (Fig. 4).

Analysis of the disaccharide compositions (Table I) of each of the resolved HPLC peaks (peaks A–D) revealed the apparent structural basis for the similarities in HPLC profiles across the oligosaccharide size series. With one exception (dp10), the major component (peak B) in each population appeared, from its composition, to correspond to a uniformly 4-sulfated oligosaccharide, probably \( \Delta \text{HexA-GalNAc}(4-\text{OSO}_3)\) \(\text{IdoA-GalNAc}(4-\text{OSO}_3)\) \(\text{GalNAc}(4-\text{OSO}_3)\) \(\text{GalNAc}(4-\text{OSO}_3)\). In which 2-sulfates were absent. High resolution gradient polyacrylamide gel electrophoresis appeared to confirm this by revealing only a single oligosaccharide band in each size fraction (data not shown). Surprisingly, peak B from the decasaccharides appeared, from its disaccharide composition (Table I), to be more complicated, and indeed electrophoretic analysis revealed the presence of an additional minor species (data not shown). The latter may correspond to a species with the same overall charge density, but possibly containing both a nonsulfated and a sulfated disaccharide (Table I). The compositions of the earlier-eluting and lesser-charged minor species (peak A) were consistent with one of the disaccharides in the structure above (possibly the nonreducing terminal one in which the uronate was originally a glucuronate) lacking the O-sulfate group at C-4 of GalNAc. The later-eluting, more highly charged minor species (peak C) appeared to be more structurally heterogeneous (Table I). Their broader elution on some HPLC runs (e.g. Fig. 4, dp6 and dp10) suggested the likelihood of molecular heterogeneity, and indeed electrophoretic analysis resolved two major species, although the material became more homogeneous with increasing oligosaccharide size (data not shown). In general, the composition of the peak C material was consistent with the presence of a single sulfated disaccharide (with the additional sulfate being on C-2 of iduronate) within an otherwise monosulfated disaccharide size (data not shown). In general, the composition of the peak C material was consistent with the presence of a single sulfated disaccharide (with the additional sulfate being on C-2 of iduronate) within an otherwise monosulfated disaccharide size (data not shown).

The highly charged minor species (peak D), positively identified only in the dodecasaccharide fraction, proved to be highly unusual in its structure. Approximately half of its constituent disaccharides were trisulfated (i.e., \(\text{IdoA}(2-\text{OSO}_3)\)-GalNAc(4,6-\text{OSO}_3)\)), with the remainder being 4-O-monosulfated. This suggests a likely dodecasaccharide with differing sulfation patterns. Comparisons of both their disaccharide compositions and their respective HGF/SF affinities, as determined by the concentration of NaCl required to elute them from a HGF/SF affinity column, may help to elucidate the minimum specific structural requirements for this interaction. All four oligosaccharide size populations yielded surprisingly similar profiles upon strong anion-exchange HPLC. A single major species (labeled peak B in all cases), comprising 70–83% of the total population, was always present, together with a substantially less abundant (13–19%), later-eluting species (labeled peak C) (Fig. 4 and Table I). A minor, earlier-eluting species (labeled peak A) was also present in all four size populations, and the content of this species appeared to generally increase with oligosaccharide size (Table I). A fourth, highly charged, minor species (labeled peak D) was found only in the dodecasaccharide population (Fig. 4).

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Dermatan Sulfate Binding to HGF/SF

**TABLE I**

| Oligosaccharide species* | Disaccharide composition (%) | HGF binding |
|-------------------------|-----------------------------|-------------|
|                         | Nonsulfated | Mono-4-sulfated | Mono-6-sulfated | Di-2,4-sulfated | Di-2,6-sulfated | Tri-2,4,6-sulfated |
| 6A                      | 1.9         | ND             | ND             | ND             | ND             | ND             | —             |
| 6B                      | 83.4        | 2.5            | 95.1           | 2.4            | —              | —              | —             |
| 6C                      | 14.7        | 29.9           | 21.0           | —              | 22.5           | 26.6           | —             |
| 8A                      | 3.7         | 16.7           | 83.3           | 2.1            | —              | —              | —/—           |
| 8B                      | 83.2        | 1.3            | 96.6           | 4.6            | 7.0            | 9.5            | +             |
| 8C                      | 13.1        | 9.9            | 69.0           | —              | 4.6            | 7.0            | —             |
| 10A                     | 7.5         | 19.5           | 80.5           | —              | —              | —              | —/—           |
| 10B                     | 76.0        | 9.6            | 72.2           | 6.7            | 5.0            | 6.5            | +             |
| 10C                     | 16.5        | 10.3           | 60.1           | 4.2            | 11.5           | 13.9           | +             |
| 12A                      | 6.6         | 5.4            | 90.3           | 4.3            | —              | —              | —             |
| 12B                     | 70.1        | 4.4            | 91.6           | 4.0            | —              | —              | —             |
| 12C                     | 19.1        | 5.6            | 81.8           | 3.8            | 4.4            | 4.3            | —             |
| 12D                     | 4.2         | —              | 41.5           | —              | —              | —              | 58.5          |

* A dash indicates that the specific disaccharide species was not detected.

**Fig. 5.** Binding of HGF/SF to MDCK cells and competition by various GAGs. Binding of 125I-HGF/SF to MDCK cells was determined in the presence of porcine mucosal heparin, bovine kidney HS, bovine mucosal DS and shark cartilage CS over a concentration range of 1 ng/ml to 0.1 mg/ml GAG, as described under "Experimental Procedures." The results are presented as % binding relative to the control (i.e. binding in the absence of exogenous GAG set as 100%) with each point representing the mean ± S.E. of duplicate determinations.
association rate constant was 1.58 (± 0.29) × 10^6 M⁻¹ s⁻¹. The dissociation phase was extremely fast, with nearly two-thirds of the dissociation occurring during the initial saline washes (Fig. 6, top panel), and the mean dissociation rate constant was 0.031 (± 0.005) s⁻¹. Both the association and dissociation kinetics fitted a one-site model with no evidence for a two-site model. The standard errors quoted above are therefore derived from the deviation of the data from a one-site model, and the HGF/SF binding sites in this DS preparation are considered to be kinetically homogeneous. The combined data yields a calculated equilibrium dissociation constant, \( K_D \) (equivalent to the ratio \( k_d/k_a \), of 19.7 (± 5.0) nM.

**DISCUSSION**

We have demonstrated by affinity chromatography that HGF/SF interacts with DS. Kinetic analysis on immobilized decorin DSPG in a biosensor reveals a strong interaction with a \( K_D \) of 19.7 nM. Although lower than its affinity for HS, this remains a physiologically relevant, high affinity interaction with likely consequences for the biology of HGF/SF. Comparisons between the present data with DS and that previously obtained with HS (9) reveal further insights into the oligosaccharide-binding specificities of HGF/SF.

The smallest DS oligosaccharide with a noticeable affinity for HGF/SF corresponds to an octasaccharide, although higher apparent affinity is observed with dodecasaccharides, or larger. These elute from the HGF/SF affinity column with 0.6 M NaCl, whereas 0.8 M NaCl is required to elute the intact DS chain. This size-dependent behavior is reminiscent of the previously described interaction with HS, although with the latter hexasaccharides were the smallest oligosaccharides with significant affinity. This may reflect true differences in HGF/SF affinity for oligosaccharides excised from the two GAGs. However, for both HS and DS, specific enzymic scission techniques were used which produce even-numbered oligosaccharides. These may only closely approximate the minimal binding sequences, as they could still contain irrelevant terminal monosaccharides. The apparently higher affinity (by chromatography) seen with intact DS and HS, compared with excised oligosaccharides, may be a consequence of the inherent polyvalency of long chains for HGF/SF.

Previous studies demonstrated that HGF/SF binds specifically within the iduronate-rich, N-/O-sulfated domains of HS (9, 16). Interaction did not, however, appear to directly require the characteristic N-sulfates of these domains, as their specific removal failed to abrogate binding (9). It was also suggested that high affinity binding was more closely correlated with the presence of 6-O-sulfates than of 2-O-sulfates, and that the latter may make, at most, only a small contribution (9). In contrast, Ashikari et al. (16) suggested a definite requirement for clusters of iduronate-2-O-sulfates in combination with adjacent 6-O-sulfated hexosamines. The present study of the interaction with DS both complements and extends our understanding of HGF/SF's binding specificity. It immediately confirms that N-sulfates are nonessential, while iduronate residues are an absolute requirement (as CS does not bind to HGF/SF; Fig. 5). Importantly, there is no apparent requirement for 2-O-sulfation of these iduronates. HGF/SF-binding octa-, deca-, and dodecasaccharide fractions included molecular species that did not possess 2-O-sulfates (i.e., HPLC peaks A and B), as well as those which did (i.e., HPLC peaks C and D) (Fig. 4 and Table I). Indeed, the great majority of the dodecasaccharide fraction is able to bind to HGF/SF (Fig. 3) when 77% of its constituent species lack 2-O-sulfates (Fig. 4 and Table I). Likewise, the presence of 2-O-sulfates is clearly not inhibitory and might make a small additional contribution to binding affinity. The minimum structural requirement for the binding of DS to HGF/SF may thus be satisfied by the repeating sequence [IdoA-GalNAc(4-OSO₃)]₃ as found in a minimal octasaccharide (although whether all the IdoA residues are strictly necessary and whether a limited GlcA presence at certain positions could be permissible is at present not known).

In the hexa- to dodecasaccharides excised by chondroitin ACU/ACII lyases, the above repeating disaccharide is by far the major component of the iduronate-rich domains in the Balb/c 3T3 DS. Indeed only 2.9% of the hexuronates are 2-O-sulfated compared with an estimated iduronate content of 33%. Likewise, the corresponding 2-O-sulfate contents of the bovine mu-
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such as decorin and biglycan. This has to be traversed in order for HGF/SF to act as a paracrine factor at the basolateral surface of epithelial and endothelial cells, where the Met receptor is preferentially expressed. An increasing gradient of affinity from the DSPG-rich environment of the stromal cells to the HSPG-rich basement membrane supporting the epithelial/endothelial cells may help to guide the delivery of HGF/SF to the epithelium/endothelium. The ability of DSPGs to rapidly capture HGF/SF in the extracellular matrix may also prevent uncontrollable diffusion as well as protecting it from proteolytic inactivation. A high capacity for binding of HGF/SF in the matrix may also create a potential reservoir of latent factor. This could be rapidly mobilized under the influence of matrix-degrading proteases during tissue remodeling and wound healing, as well as during tumor invasion. The potential release of HGF/SF at the edge of an expanding carcinoma could stimulate further tumor growth by promoting increased tumor cell proliferation, motility, and invasion, as well as providing a pro-angiogenic stimulus that could contribute to tumor vascularization. Functionally, it will be of particular interest to ascertain whether DS is also capable of facilitating the binding of HGF/SF to its receptor, in a similar way to that described for HS.

Acknowledgment—We thank Suzanne Bridge for secretarial assistance.

REFERENCES

1. Matsumoto, K., and Nakamura, T. (1996) J. Biochem. (Tokyo) 119, 591–600
2. Di Renzo, M. F., Olivero, M. Ferro, S., Prat, M., Bongarzone, I., Pilotti, S., Belluoi, A., Constantino, A., Vigneri, R., Pierotti, M. A., and Comoglio, P. M. (1992) Oncogene 7, 2549–2555
3. Natali, P. G., Nicotra, M. R., Di Renzo, M. F., Prat, M., Bigotti, A., Cavaliere, R., and Comoglio, P. M. (1993) Br. J. Cancer 68, 746–750
4. Rong, S., Jeffers, M., Rezaa, J. H., Tarsfayt, I., Oskarsson, M., and Vande Woude, G. F. (1993) Cancer Res. 53, 5355–5360
5. Bellusi, S., Moens, G., Gaudio, G., Comoglio, P. M., Nakamura, T., Thierot, P.-F., and Joannon, J. (1994) Oncogene 9, 1091–1099
6. Jeffers, M., Rong, S., and Vande Woude, G. F. (1996) Mol. Cell. Biol. 16, 1115–1125
7. Bussolino, F., Di Renzo, M. F., Ziche, M., Bochietto, E., Olivero, M., Naldini, L., Gaudio, G., Tamagnone, L., Coffee, A., and Comoglio, P. M. (1992) Cell Biol. 119, 629–641
8. Grant, D. S., Kleinman, H. K., Goldberg, I. D., Bhargava, M. M., Nickoloff, B. J., Kinsella, J. J., Polverini, P., and Rosen, E. M. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 10747–10751
9. Lyon, M., Deakin, J. A., Mizuno, K., Nakamura, T., and Gallager, J. T. (1994) J. Biol. Chem. 269, 11216–11223
10. Tajima, H., Matsumoto, K., and Nakamura, T. (1992) Exp. Cell Res. 202, 423–431
11. Naka, D., Ishii, T., Shimomura, T., Hishida, T., and Har, H. (1993) Exp. Cell Res. 209, 317–324
12. Lyon, M., and Gallager, J. T. (1994) Biochem. Soc. Trans. 22, 365–370
13. Zianchev, T. F., Richardson, L., Liu, J., Chang, L., King, K. L., Bennett, G. L., Fugedi, P., Chamow, S. M., Schwall, R. H., and Stack, R. J. (1995) J. Biol. Chem. 270, 16871–16878
14. Schwall, R. H., Chang, L. Y., Godowski, P. J., Kahn, D. W., Hillan, K. J., Bauer, K. D., and Zianchev, T. F. (1996) J. Cell Biol. 135, 709–718
15. Sakata, H., Stahl, S. J., Taylor, W. G., Rosenberg, J. M., Sakaguchi, K., Wingfield, P. T., and Rubin, J. S. (1997) J. Biol. Chem. 272, 9457–9463
16. Ashikari, S., Hanbuchi, H., and Kimata, K. (1995) J. Biol. Chem. 270, 29586–29593
17. Scott, J. E. (ed) (1993) Dermatan Sulfate Proteoglycans: Chemistry, Biology, Chemical Pathology, Portland Press, London
18. Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C., and Gallager, J. T. (1995) J. Biol. Chem. 270, 16793–16799
19. Celli, G., Boeri, G., Saggioratta, G., Paolino, R., Lazzotto, G., Teribile, V. I. (1992) Angiology 43, 59–62
20. Walker, A and Gallager, J. T. (1996) Biochem. J. 317, 871–877
21. Clarke, D., Katoh, O., Gibbs, R. V., Griffiths, S. D., and Gordon, M. Y. (1992) J. Biol. Chem. 267, 16871–16879
22. Maimone, M. M., and Tollesen, D. M. (1990) J. Biol. Chem. 265, 16823–16827
23. Maselli, G., Liverani, L., Bianchini, P., Parma, B., Torri, G., Biasio, A., Guerzoni, M., and Casu, B. (1993) Biochemistry 32, 648–654
24. Nakamura, T., Nishizawa, T., Hayagi, M., Seki, T., Shimomishii, M., Sugiura, A., Tashiro, K., and Shimizu, S. (1998) Nature 344, 440–443
25. Pearson, C. H., Winterbottom, N., Packe, D. S., Scott, P. G., and Carpenter, M. R. (1985) J. Biol. Chem. 259, 15101–15104
26. Schor, S. L., Schor, A. M., Rushston, G., and Smith, J. (1985) J. Cell Sci. 73, 221–234
27. Shively, J. E., and Conrad, H. E. (1976) Biochemistry 15, 3943–3950
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29. Yoshida, K., Arai, M., Kohno, Y., Maejima, K.-I., Miyazono, H., Kikuchi, H., Morikawa, K., Tawada, A., and Suzuki, S., (1993) in Dermatan Sulfate Proteoglycans: Chemistry, Biology, Chemical Pathology (Scott, J. E., ed) pp. 55–70, Portland Press, London

30. Svennevig, K., Prydz, K., and Kolset, S. O. (1995) Biochem. J. 311, 881–888

31. Ferro, D. R., Provasoli, A., Ragazzi, M., Torri, G., Casu, B., Gatti, G., Jacquinet, J.-C., Sinay, P., Petitou, M., and Choay, J. (1986) J. Am. Chem. Soc. 108, 6773–6778

32. Van Boeckel, C. A. A., van Aelst, S. F., Waagenaars, G. N., Mellema, J.-R., Paulsen, H., Peters, T., Pollex, A., and Sinowell, V. (1987) Rec. Trav. Chim. Pays-Bas Belg. 106, 19–29

33. Scott, J. E., Heatley, F., and Wood, B. (1995) Biochemistry 34, 15467–15474

34. Venkataraman, G., Sasisekharan, V., Cooney, C. L., Langer, R., and Sasisekharan, R. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6171–6175

35. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1996) Science 271, 1116–1120

36. Sambandam, T., Baker, J. R., Christner, J. E., and Ekborg, S. L. (1991) Arterioscler Thromb 11, 561–568

37. Gigli, M., Ghuselli, G., Torri, G., Naggi, A., and Rizzo, V. (1993) Biochim. Biophys. Acta 1167, 211–217

38. Maaroufi, R. M., Jozefowicz, M., Tapon-Bretaudiere, J., Jozefonvicz, J., and Fischer, A. M. (1997) Biomaterials 18, 359–366