The Binding Properties of Minimal Oligosaccharides Reveal a Common Heparan Sulfate/Dermatan Sulfate-binding Site in Hepatocyte Growth Factor/Scatter Factor That Can Accommodate a Wide Variety of Sulfation Patterns*

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Jon A. Deakin, Bärbel S. Blaum, John T. Gallagher, Dušan Uhrín, and Malcolm Lyon

From the Cancer Research UK Glyco-Oncology Group, School of Cancer and Imaging Sciences, Paterson Institute for Cancer Research, University of Manchester, Wilmslow Rd., Manchester M20 4BX, United Kingdom and the Edinburgh Biomolecular NMR Unit, School of Chemistry, University of Edinburgh, West Mains Rd., Edinburgh EH9 3JJ, United Kingdom

Hepatocyte growth factor/scatter factor (HGF/SF) is a mesenchymal factor that provides growth, motility, and morphogenic stimuli to epithelial, endothelial, and neural cells via exclusive activation of the tyrosine kinase receptor MET (for review, see Ref. 1). There is increasing clinical interest in the potential therapeutic use of HGF/SF. Its natural role in wound healing and organ regeneration after injury provides opportunities for enhancement of its efficacy in tissue and organ repair (2–3). Conversely, its elevated or dysregulated activity in many solid tumors and lymphomas strongly correlates with invasiveness, metastasis, and poor disease prognosis and has highlighted its potential as a prominent target for therapeutic inhibition (4–5).

Although the affinity of HGF/SF for MET is very high (KD of ~0.2 nM) (6), subsequent activation of the receptor leading to sustained and effective downstream signaling is highly dependent upon glycosaminoglycan (GAG) co-factors (7–11). Heparan sulfate (HS), heparin, and dermatan sulfate (DS), but not chondroitin sulfate, interact with HGF/SF with KD values in the range of 1–20 nM and function as co-factors for HGF/SF in vitro (12–15). The in vitro activity of covalently cross-linked HGF/SF-GAG or oligosaccharide conjugates (8) together with the affinity of heparin/HS for MET (8–9, 16–17) suggests that a HGF/SF-GAG-MET ternary complex may be the effective signaling unit.

HGF/SF interacts with relatively short GAG oligosaccharides; the minimum even-numbered HS/heparin fragment that displays high affinity binding in vitro is a tetrasaccharide (dp4), and this also corresponds to the smallest, bioactive, repeating disaccharide structure (18, 19). The major HS/heparin-binding site resides in the N-terminal domain of HGF/SF. Indeed, a heparin tetradecasaccharide has been co-crystallized with the truncated NK1 splice variant of HGF/SF, which only comprises the N-terminal domain, N, and the first Kringle domain, K1; the crystal structure identified the major GAG contacts as being in the N-domain, although in some crystals additional contacts...
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were made with K1 (16). Subsequently, all the individually expressed domains of HGF/SF (i.e. the N-domain and the four Kringles, K1–K4, that comprise the α-chain and the serine proteinase homology domain, SP, corresponding to the β-chain) were compared by heparin affinity chromatography and gel mobility shifts assays, and these confirmed the existence of a weaker GAG-binding site in K1 as well as revealing an additional low affinity site in the SP domain (20). However, there are also other reports using domain-deleted (21) and point-mutated (22) forms of HGF/SF that suggest that K2 may contribute to GAG binding by native, full-length HGF/SF even though this was not confirmed by the behavior of isolated K2 (20).

NK1 also binds DS (19), probably mediated by the N-domain (20); neither the isolated K1 domain nor the β-chain from HGF/SF bind independently to DS even though they possess weak heparin affinity (20). Thus, joint heparin/HS and DS affinity appears to be restricted to the N-domain. This begs the question as to whether both GAG types bind with high affinity to a single, dual-specificity binding site or whether the N-domain contains two distinct sites with differential GAG specificities.

A recent investigation of the binding and activating properties of a range of native and specifically desulfated GAGs suggested that GAG binding to NK1 was of relatively low specificity (23). Binding could be sustained by a low level of overall sulfation (as displayed by DS) as long as, critically, the GAG contained iduronate (IdoA), although increased sulfation enhanced affinity (as with heparin) and ultimately could overcome the need for IdoA. The greater conformational flexibility of IdoA is assumed to provide increased overall chain flexibility that may optimize the spatial disposition of the necessary contact groups within the GAG. A low sulfate density in the presence purely of β-D-glucuronic acid, as in mammalian chondroitin sulfate, was inadequate for binding NK1. The results of selective desulfations suggested that there may not be a strict requirement for any specific sulfation position in either HS/heparin or DS. This is compatible with the possibility of the N-domain containing a single low specificity binding site for both HS/heparin and DS. However, interpretations of such studies using polymeric GAGs are complicated because desulfation methods are rarely quantitative, and minor amounts of specific sulfate groups may be sufficient to support binding especially if the protein recognizes short sequences.

The GAG co-factor requirement of HGF/SF-mediated activation of MET provides an opportunity for potential clinical inhibition of HGF/SF activity via disruption of the necessary component co-factor interaction (for review, see Ref. 24). The ability of exogenous GAG species as small as dp4s to engage in productive complex formation opens up possibilities for small-molecule inhibition either based upon potentially inhibitory, modified GAG oligosaccharides or novel synthetic mimetics. Our recent demonstration of the inhibition of HGF/SF-mediated ERK-MAPK signaling and cell migration in vitro by synthetic, sulfated, non-sugar GAG mimics (25) demonstrated proof of principle and opens the way for improved inhibition by better-designed agents.

It is difficult to elucidate the GAG binding specificity of HGF/SF using intact, structurally heterogeneous GAGs. Thus, the aims of the present study were to utilize minimal GAG oligosaccharides of defined structure (see supplemental Tables I and II) to (i) provide an improved description of the structural requirements for GAG-HGF/SF interaction and also (ii) to resolve the question of whether HS/heparin and DS occupy identical or distinct binding sites within the N-domain of HGF/SF. Such information may aid the future rational design of small-molecule inhibitors of HGF/SF that target its GAG co-factor requirement.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human HGF/SF was obtained from R&D Systems (Abingdon, UK). Bovine lung heparin, porcine intestinal mucosal heparin, chondroitinases ABC (Proteus vulgaris; EC 4.2.2.4), and chondroitinase AC-I (Pedobacter heparinus; EC 4.2.2.5) were obtained from Sigma. Low molecular weight heparin (Innohep®) was from Leo Pharma (Princnes Risborough, UK). Porcine intestinal mucosal DS was from Celsus Laboratories Inc. (Cincinnati, OH). Completely de-sulfated/re-N-sulfated heparin was obtained from Seikagaku Corp. (Tokyo, Japan). Heparinases I (P. heparinus; EC 4.2.2.7), II (P. heparinus; no EC number assigned), and III (P. heparinus; EC 4.2.2.8) were purchased from Grampian Enzymes (Harray, Orkney, UK). Bio-Gel P10 was from Bio-Rad Laboratories. Pre-packed PD-10 desalting columns were purchased from GE Healthcare. The CHO pgpA-745 mutant cell line was a generous gift from Prof. Jeffrey Esko (University of California at San Diego, CA).

Preparation of NK1—Human NK1 was purified from the culture medium of a Pichia pastoris recombinant expression system provided by Dr. Ermanno Gherardi (MRC Centre, Cambridge, UK) as previously described (26). 15N-Labeled NK1 for NMR titrations was produced by growth of the P. pastoris clone in 0.8-liter cultures in a Bioflow 3000 fermentor (New Brunswick Scientific, St. Albans, UK) using [15N]ammonium sulfate (>98%; Sigma) as the sole nitrogen source.

Preparation of Hexasulfated Heparin Tetrasaccharide—Heparin dp4s were prepared by the fractionation of low molecular weight heparin by gel filtration. To increase the yield of dp4s, the heparin was partially digested with heparinase I (0.1 unit added to a 60-ml solution of 5 mg/ml heparin in 0.1 M sodium acetate, 0.1 mM calcium acetate, pH 7.0, at 37 °C for 6 h). Small aliquots were removed at regular intervals and rapidly chromatographed on a Superdex Peptide HR 10/30 HPLC column (10 × 300 mm; GE Healthcare Life) eluted with 0.2 M NH4HCO3 at 0.5 ml/min and monitored by UV absorbance at 232 nm to assess the degree of depolymerization. At an appropriate level of breakdown, the digest was terminated by boiling, concentrated by lyophilization, and then subjected to gel filtration chromatography on a Bio-Gel P-10 column (2.5 × 115 cm) eluted with 0.2 M NH4HCO3 at a flow rate of 10 ml/h. Fractions of 2 ml were collected and monitored for UV absorbance at 232 nm. The peak corresponding to total dp4s was pooled and freeze-dried twice to remove NH4HCO3. This was then sub-fractionated by application in pH 3.5 water (adjusted with HCl) to a semipreparative ProPac PA1 strong anion-exchange (SAX) HPLC column (4.6 × 250 mm; Dionex, Camberley, UK) eluted with a gradient of 0–1.5 M NaCl, pH 3.5, over 90 min at a flow
rate of 4 ml/min with in-line monitoring of UV absorbance at 232 nm. The latest eluting dp4 peak, corresponding to the hexa-sulfated species, was collected and desalted on a PD10 column run in water. Quantitation was by absorbance at 232 nm. Composition was confirmed by disaccharide analysis.

Preparation of DS Tetrasaccharides—Porcine intestinal mucosal DS (1 g) in 50 ml of 75 mM NaCl, 10 mM sodium phosphate, pH 7.0, was fully digested with 1 IU of chondroitinase AC-1 followed by partial digestion with 1 IU of chondroitinase ABC. Small aliquots were removed at regular intervals and checked for the degree of breakdown as described earlier. The final digest was fractionated on a Bio-Gel P-10 column (as described for heparin oligosaccharides above), and the dp4 peak was pooled and freeze-dried twice to remove NH₄HCO₃.

The mixed DS dp4 pool was applied in water to a Sphere-clone 5-µm SAX-HPLC column (4.6 × 250 mm; Phenomenex, Macclesfield, UK) and eluted with a linear gradient of 0–1.0 M NaCl over 60 min at a flow rate of 1 ml/min. UV absorbance at 232 nm was monitored on-line. Individual dp4 peaks were separately pooled, desalted on PD-10 columns eluted with distilled water, and then freeze-dried. Quantitation was by absorbance at 232 nm. Compositions and, thus, likely structures were confirmed by disaccharide analyses.

Preparation of Heparin and DS Trisaccharides—Trisaccharides (dp3s) were prepared by chemical removal of the non-reducing unsaturated uronate residue from heparin, and DS dp4s by the method of Ludwigs et al. (27). dp4S (1 mg) were dissolved in 75 µl of 10 mM mercucric acetate, pH 5.0, and incubated at 20 °C for 40 min. The sample was adjusted to 1 ml with 5 M NaCl and applied to a Superdex Peptide HR 10/30 HPLC column equilibrated in 0.2 M NH₄HCO₃. The column was run at a flow rate of 0.5 ml/min with on-line UV monitoring at 206 nm. Fractions of 0.25 ml were collected, and the peak corresponding to the dp3 was collected and freeze-dried twice to remove NH₄HCO₃.

Preparation of Heparin Tetrasaccharides Desulfated at a Single Specific Ring Position—Hexasulfated heparin dp4 was subjected to specific desulfations at either 2-O, 6-O, or N positions using the procedures previously described for intact heparin (23). The specifically desulfated dp4s were separated from partially and over-desulfated species on a semi preparative ProPac PA1 SAX-HPLC column as described earlier. Individual species were desalted on PD10 columns, and their compositions were checked by disaccharide analysis.

Preparation of N-Sulfated Heparin Tetrasaccharide—Completely de-N/O-sulfated/re-N-sulfated heparin in 50 mM sodium acetate, 0.5 mM calcium acetate, pH 7.0, was digested with 0.2 IU/ml each of heparinases I, II, and III. Digestion was allowed to proceed at room temperature until the resulting dp2 and dp4 species constituted more than 50% of the total products (as assessed by chromatography of an aliquot upon a Superdex Peptide column, as described earlier). The dp4s, recovered by Superdex Peptide gel filtration, were freeze-dried and subjected to disaccharide compositional analysis.

Preparation of 2-O- or 6-O-Sulfated Heparin Tetrasaccharides—Porcine intestinal heparin was subjected to either (i) de-6-O sulfation, with concomitant de-N sulfation, followed by re-N acetylation or (ii) de-2-O sulfation followed by sequen-
tial de-N sulfation and re-N acetylation, essentially as described in Catlow et al. (23). Each product was partially digested with heparinas, and the dp4 pool was collected by gel filtration and then subfractionated by SAX-HPLC as described for singly desulfated heparin dp4s (above). Compositions were checked by disaccharide analyses.

Disaccharide Analysis of GAGs and Oligosaccharides—DS and heparin species were completely digested to disaccharides using chondroitinase ABC or a mixture of heparinases I, II, and III. The resulting disaccharides were separated on ThermoQuest Hypersil SAX-HPLC (4.6 × 240 mm; ThermooQuest Hypersil Division, Runcorn, UK) or ProPac PA1 SAX-HPLC (4 × 250 mm; Dionex) columns as previously described (23). Columns were calibrated with known disaccharide standards.

Fluorescent Labeling of Oligosaccharides and Gel Mobility Shift Assays—Oligosaccharides were tagged at their reducing ends with 2-aminoacridone (AMAC), as described in Lyon et al. (19). For gel mobility shift assays (GMSA), AMAC-tagged oligosaccharides were preincubated with NK1 protein in a 1:1 ratio for 30 min in a total volume of 10 µl of phosphate-buffered saline. Samples were electrophoresed on either agarose gels (1% (w/v)) or polyacrylamide gels (6% (w/v)) for basic GMSA or 10% (w/v) for selection GMSA) at a constant 100 V for between 5 and 20 min (shorter times for basic GMSA but often longer for selection GMSA) and then imaged under UV light (19). In selection GMSA experiments, NK1 is titrated against a fixed concentration of a mixture of two or more oligosaccharides (of equivalent molar concentrations); the molar concentration of NK1 is expressed relative to the total ligand concentration (i.e. a NK1/ligand ratio of 0.33 for a mixture of three ligands indicates that the NK1 molar concentration is equal to that of any single ligand species in the mixture).

Reverse-phase Chromatography of AMAC-labeled Oligosaccharides—Bands of free AMAC oligosaccharides were excised from 1% agarose GMSA gels with a scalpel blade under UV visualization. Gel fragments were mixed end-over-end for 2 h in 1 ml of distilled water in a sealed tube to extract the bulk of the oligosaccharides. The supernatant was then freeze-dried, redissolved in 20 µl of water, and then applied to an Eclipse XDB C-18 reverse-phase HPLC column (4.6 × 150 mm; Agilent Technologies, Stockport, UK) pre-equilibrated in 0.1 M ammonium acetate (Solution A). After a short and steep 2-ml gradient of 0–10% solution B (100% methanol), the oligosaccharide components were resolved over a 50-ml linear gradient of 10–30% solution B at a flow rate of 1 ml/min, with in-line monitoring of AMAC fluorescence (excitation at 425 nm, emission at 520 nm).

Assay for Activation of ERK-MAPK in GAG-deficient Cells—GAG-deficient CHO pgsA-745 mutant cells were cultured as previously described (8). Cells were serum-starved for 2 h before treatment with HGF/SF with or without oligosaccharides (when combined, they were preincubated for 30 min before the addition to the cells). Downstream signaling was assayed 20 min after reagent addition. Cell lysis preparation, SDS-PAGE, and Western blotting using a mouse monoclonal antibody against dual-phosphorylated (Thr183/202/Tyr185/204)
ERK-1/2 MAPKs (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were performed as previously described (8).

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ERK-1/2 MAPKs (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were performed as previously described (8).

NMR Titrations—Concentrations of 15N-labeled NK1 used in the GAG titrations were 50 and 44 μM when titrated with heparin dp4 and DS dp6, respectively. Titration points were recorded at protein-oligosaccharide ratios of 1:1 and 1:2 for heparin dp4, whereas for the DS dp6 the ratios used were 1:0.5, 1:1, and 1:2.5. All spectra were recorded in 20 mM sodium acetate, 100 mM sodium chloride, pH 6.0, at 30 °C on an 800-MHz Avance NMR spectrometer. Spectra were processed using AZARA (Version 2.7, Wayne Boucher and Dept. of Biochemistry, University of Cambridge), and the chemical shift changes were quantified using ANALYSIS (28).

RESULTS

NK1 Binding to Heparin and DS Tetrasaccharides—Previous studies had reported that a dp4 was the smallest even-numbered heparin oligosaccharide with affinity for HGF/SF and NK1 (18, 19). By contrast, a dp6 appeared to be the minimal DS oligosaccharide (19). This size difference in the recognition of these two GAGs has now been revisited.

dp4s were generated from heparin by limited heparinase I digestion followed by gel filtration chromatography (Fig. 1A). The major dp4 species, corresponding to the hexasulfated ΔHexA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS(6S), was further purified by SAX-HPLC (Fig. 1C), and its composition and sequence were confirmed by disaccharide analysis and NMR (not shown). A porcine intestinal mucosal DS preparation with a high IdoA content (indicated by its substantial resistance to chondroitinase ABC scission) at 100 °C. The dp4 pool was recovered by gel filtration chromatography (Fig. 1B). Fractionation of this pool by SAX-HPLC (Fig. 1D) yielded a major disulfated species (~75% total) that disaccharide analysis revealed to be comprised predominantly of 4-O-sulfated disaccharides (92.5% 4-O-sulfates and 7.5% 6-O-sulfates, with no detectable 2-O-sulfates), i.e. corresponding primarily to the sequence ΔHexA-GalNAc(4S)-IdoA-GalNAc(4S).

Both heparin and DS dp4s were labeled with AMAC, and their interaction with NK1 was assessed by GMSA. As previously demonstrated (19), the heparin dp4 bound strongly to NK1 and was retained in the well (Fig. 2A). The DS dp4 also bound but required higher concentrations of NK1 than the heparin (Fig. 2B) and higher than used in the previous study (19). Thus, an equivalent minimal size of heparin/HS and DS support binding to NK1, although affinity is appreciably higher for a heparin-derived species.

The original unfractionated DS dp4 pool also contains a small proportion (~7%) of a trisulfated species (Fig. 1D) that, from disaccharide analysis, is comprised of equal proportions of ΔHexA-GalNAc(4S) and a disulfated disaccharide that is primarily ΔHexA-GalNAc(4S,6S) (much lesser amounts of ΔHexA(2S)-GalNAc(4S) and ΔHexA(2S)-GalNAc(6S) were also present). Based on its complete resistance to chondroitinase ACI (not shown), the predominant sequence of this trisulfated dp4 is likely to be ΔHexA-GalNAc(4S,6S)-IdoA-GalNAc(4S).

To directly compare the relative affinities of NK1 for dp4s that differ in sulfate densities and/or polymer backbones, it is possible to perform a GMSA experiment in a new mode, which we refer to as a “selection” mode. In this new variant the protein is incubated simultaneously with a mixture of equimolar amounts of the different AMAC-tagged dp4s. If the latter differ in sulfate density they will, as free species, resolve into separate bands on the GMSA gel. Thus, if for example the species differ very markedly in protein affinity, then there is sufficient protein present to selectively and almost quantitatively bind and deplete the highest affinity species while leaving the other, low...
affinity species relatively unaffected (though the latter will progressively bind if the ratio of protein is increased). Alternatively, if all species possess very similar protein affinities, then all bands should become partially depleted in parallel as the protein content is increased. The overall pattern of selective or non-selective band depletion can, therefore, be interpreted as a reflection of relative NK1 affinities.

Comparison of the disulfated and trisulfated DS dp4s by selection GMSA revealed a clear preferential affinity for the latter, more highly sulfated species (Fig. 3A). When the experiment is extended to also include the hexasulfated heparin dp4, then it is clear that the latter is greatly preferred over both DS species (Fig. 3B). Thus, the order of relative dp4 affinities is hexasulfated heparin \( \gg \) trisulfated DS > disulfated DS.

**NK1 Binding to Heparin and DS Trisaccharides**—Previously, only even-numbered oligosaccharides, generated by enzymatic scission of polymers with repeating disaccharide structures, have been tested for NK1 affinity. Odd-numbered oligosaccharides can be generated by mercier ion-catalyzed removal of the unsaturated hexuronate from the non-reducing end of such even-numbered species. There is no corresponding procedure for alternative monosaccharide removal at the reducing end. Truncation at the non-reducing end will at most result in the loss of only one sulfate group (i.e. a 2-O-sulfate) and often none at all. Thus, we decided to prepare and test the affinity of the respective pentasulfated and disulfated dp3s, i.e. GlcNS(6S)-IdoA(2S)-GlcNS(6S) and GalNAc(4S)-IdoA-GalNAc(4S), which could be generated from the major hexasulfated heparin and disulfated DS dp4s described earlier. In addition, the tetrasulfated heparin dp3, GlcNS(6S)-IdoA(2S)-GlcNS, was prepared from the pentasulfated dp4, \( \Delta \)HexA(2S)-GlcNS(6S)-IdoA(2S)-GlcNS, a less common species in the heparin dp4 pool (Fig. 1B) whose composition and sequence was previously determined by disaccharide analysis and NMR (29). In all cases removal of the unsaturated hexuronate residue from the non-reducing end could be confirmed by any of a variety of techniques, i.e. smaller size and, thus, later elution on a Superdex Peptide column (with concomitant loss of absorption at 232 nm, so requiring monitoring of the dp3 at 206 nm), an altered size and charge density that affects electrophoretic mobility on a polyacrylamide gel, or changed elution properties on a C18 reverse-phase HPLC column (not shown).

Direct GMSA assays to test individually the two heparin dp3s showed that they also both bind to NK1 (Fig. 4A). Binding of the DS dp3 (that has lost a hexuronate from the non-reducing end, but no sulfation) required a higher ratio of NK1-oligosaccharide, as with the parent dp4, and their affinities appear to be broadly similar (not shown).

In the latter case both dp3 and dp4 species are disulfated; consequently, their migration on PAGE is almost identical, and thus, direct comparison of affinities by selection GMSA could not be performed at that time. However, selection GMSA to compare the hexasulfated heparin dp4 *versus* its slightly faster-moving pentasulfated dp3 derivative (i.e. lost a 2-O-sulfated hexuronate from the non-reducing end) shows a clear preference for NK1 for the dp4 species (Fig. 4B). In contrast, selection GMSA in which the pentasulfated dp3 from heparin is compared with both the di- and tri-sulfated dp4s from DS reveals that NK1 still retains a preference for the heparin ligands even though the DS ones are longer (Fig. 4C). Affinity is, thus, not primarily dependent upon ligand length but also involves aspects of sulfate density and/or GAG backbone. A previous study had revealed the importance of IdoA for GAG binding (23), and the binding of dp3s indicates that only a single internal IdoA is actually required.

**Effects of Single and Double, Site-specific Desulfations of Heparin Tetrasaccharides upon NK1 Binding**—Heparin oligosaccharides have a higher NK1 affinity than same-sized DS oligo-
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<table>
<thead>
<tr>
<th>Dp4</th>
<th>2S / 6S</th>
<th>NS / 6S</th>
<th>NS / 2S</th>
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<tbody>
<tr>
<td>NK1</td>
<td>-</td>
<td>+</td>
<td>-</td>
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![Figure 5](image)

**FIGURE 5.** Binding of NK1 to heparin tetrasaccharides lacking sulfation at a specific position. Binding of NK1 (20 μg) by direct GMSA on a 6% polyacrylamide gel to tetrasulfated heparin dp4s (0.25 μg) that specifically lack N-sulfates, 2-O-sulfates, or 6-O-sulfates.

Oligosaccharides, which may reflect the higher sulfate densities present in the former. Heparin oligosaccharides can be potentially N-, 2-O-, and 6-O-sulfated. To test whether the position of sulfation has any significant influence upon NK1 binding, we specifically desulfated the hexasulfated heparin dp4 to yield the three alternative tetrasulfated dp4s (i.e. 2-O/-6-O-, N-/2-O-, and N-/6-O-sulfated) that completely lack sulfation at one of the three specific positions. The advantage of working with dp4s is that subsequent SAX-HPLC separation (with confirmation of composition by disaccharide analysis) allows the desired, homogeneous, fully modified species to be selectively purified from any non- or partially desulfated species or from species that may have experienced additional, nonspecific changes. This contrasts with the experience of working with desulfated GAG chains where complete specific desulfation is rarely achieved, and nonspecific effects can also accumulate to varying degrees, leading to chain heterogeneities that potentially complicate affinity interpretations (23).

GMSA analysis of NK1 binding to each of these three tetrasulfated heparin dp4s showed that they all bind with high affinity to NK1 (Fig. 5). Thus, importantly, heparin/HS binding to NK1 is not critically dependent upon the presence of sulfation at any one specific position within the repeating disaccharide unit, at least if both other major positions are fully sulfated.

Clearly, only two sulfate groups are sufficient to elicit relatively weak binding of a DS dp3 or dp4 to NK1, although heparin species that naturally possess higher levels of sulfation (and variable positioning) bind NK1 with greater affinity. Would a heparin-derived dp4 that possesses an equivalent sulfate density to a DS dp4 also possess a similar NK1 affinity?

To address this issue we specifically desulfated heparin at all three combinations of two specific positions, *i.e.* leaving sulfation at just one remaining, but specific position (i.e. an N-, 2-O-, or 6-O-sulfate) in each disaccharide repeat, as are often found in HS sequences. These were then partially fragmented using heparinases, and the dp4 pools were recovered from the digests by gel filtration and then fractionated by SAX-HPLC. The three distinct, homogeneous desulfated dp4s were, thus, recovered as pure species, their compositions were confirmed by disaccharide analyses, and their NK1 affinities were tested by GMSA.

All three desulfated heparin dp4s bound NK1 by direct GMSA, broadly similar to the behavior of a desulfated DS dp4 (Fig. 6A and Table 1). Note that the affinity for the DS dp4 appears to be greater here than was originally revealed in Fig. 2. This is probably because a relatively old batch of NK1 was used for the latter, whereas all subsequent experiments used a fresh batch whose concentration of effective, monomeric protein was likely to be higher.

![Figure 6](image)

**FIGURE 6.** Binding of NK1 to heparin tetrasaccharides sulfated solely at a specific position. A, binding of NK1 (20 μg) by direct GMSA on a 1% agarose gel to three desulfated heparin dp4s that contain sulfates at only one position within the disaccharide repeat (i.e. either N-sulfates, 2-O-sulfates, or 6-O-sulfates) and also the di-4-O-sulfated DS dp4. On the right-hand side of the gel the GMSA is performed with an equimolar mix of the four dp4s above (1 μg each) titrated against an increasing molar ratio of NK1. B, the bands in A cor- responding to the mixtures of non-complexed AMAC-labeled dp4s (labeled in the figures as free dp4s) from the titration with NK1 were excised, and the dp4 species were eluted and then resolved by reverse-phase HPLC (the elu- tion gradient is only shown on the bottom panel for clarity) as described under "Experimental Procedures." Fluorescence peak areas were quantified, and the relative proportions of each dp4 species present are presented in Table 1. Note that equimolar amounts of the various dp4 species were used though the efficiency of AMAC labeling, and thus, the fluorescence yield, varied between individual species as can be seen from the relative band intensities in A and peak areas in the top panel of B.
To detect any preferential NK1 binding affinity within these dp4s it would be useful to perform GMSA in the selection mode as used earlier. Unfortunately, all three heparin dp4s as well as the DS dp4 are disulfated and, thus, have the same charge/mass ratio and consequently do not separate from each other on GMSA gels. When NK1 is incubated with a mixture of equimolar amounts of all four dp4s and run on a GMSA gel, a single combined band of free dp4s is seen which becomes increasingly depleted as the ratio of NK1 is increased, confirming that all four species can bind (Fig. 6A). However, in such a scenario it is possible to excise the fluorescent band of free dp4s from the gel, elute them by diffusion, and subsequently separate them into their four constituent dp4s by reverse-phase HPLC with fluorescent detection (as recently developed by us for disaccharide analysis of GAGs (30)). It is clear from such an analysis that as NK1 is increased, leading to the recruitment of a greater proportion of the total dp4s into complexes, the increasingly depleted pool of free dp4s still retains an approximate equimolar ratio of the four constituent dp4s (Fig. 6B). A more simplified selection GMSA in which only the di-N-sulfated heparin dp4 was compared with the di-4-O-sulfated DS dp4 confirmed this finding (not shown). As the free dp4 pool was progressively reduced (from 100 to 53, 36, and finally, 18% of total pool) by an increasing amount of NK1, the ratio of heparin dp4 to DS dp4 remained almost constant throughout (ratios of 1.05, 1.11, and 1.05, respectively). Not only is there no discrimination between the three different HS/heparin dp4s but NK1 does not now select preferentially for the HS/heparin over the DS species. Thus, similar NK1 affinities for these disulfated dp4s are essentially dictated by their equivalent sulfate densities and are independent of both GAG backbone and sulfate positioning.

Stimulation of ERK-MAPK Phosphorylation by HGF/SF in Response to Tri- and Tetrasaccharides—Various dp3 and dp4 species were tested for their ability to elicit HGF/SF activity in the CHO pgsA-745 mutant cell line, as measured by dual phosphorylation of ERK-MAPK (25 ng/ml) with or without various dp3 and dp4 species (1 μg/ml) derived from heparin and DS. The heparin oligosaccharides are hexasulfated dp4 (4a), pentasulfated dp3 (3a), di-N-sulfated dp4 (N), di-6-O-sulfated dp4 (6), di-2-O-sulfated dp4 (2). The DS oligosaccharides are trisulfated dp4 (4b), disulfated dp4 (4a), disulfated dp3 (3a). Quantitation of relative phosphorylation levels by densitometry (normalized to a value of 1.0 for the control of HGF/SF alone) gave values of 3.7, 3.0, 5.8, 6.2, 5.9, 8.8, 4.8, and 1.6 for the eight oligosaccharides in the order given above.

**TABLE 1**

Relative abundances of the four different dp4 species present in the free dp4 pool

<table>
<thead>
<tr>
<th>GAG</th>
<th>Heparin</th>
<th>DS</th>
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<tr>
<td>4a</td>
<td>3b</td>
<td>N</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>4b</td>
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<td>4a</td>
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The band corresponding to the free dp4 pool was extracted from a selection GMSA gel (Fig. 6A), and the four constituent disulfated dp4s were resolved by reverse phase HPLC (Fig. 6B). The peak areas were calculated for each NK1/GAG ratio and then expressed as % of that originally present in the absence of NK1.

**FIGURE 6.** Stimulation of ERK-MAPK phosphorylation by HGF/SF in response to tri- and tetrasaccharides. Downstream activation (dual phosphorylation) of ERK-MAPK in GAG-deficient CHO pgsA-745 cells in response to HGF/SF (25 ng/ml) with or without various dp3 and dp4 species (1 μg/ml) derived from heparin and DS. The peak areas were calculated for each NK1/GAG ratio and then expressed as % of that originally present in the absence of NK1.

**FIGURE 7.** Activation of HGF/SF-MET signaling by tri- and tetrasaccharides. Downstream activation (dual phosphorylation) of ERK-MAPK in GAG-deficient CHO pgsA-745 cells in response to HGF/SF (25 ng/ml) with or without various dp3 and dp4 species (1 μg/ml) derived from heparin and DS. The peak areas were calculated for each NK1/GAG ratio and then expressed as % of that originally present in the absence of NK1.
GAG Oligosaccharides Binding to HGF/SF

FIGURE 8. NMR chemical shift titrations of NK1 with oligosaccharides. A, a selected portion of the more extensive $^1$H/$^15$N heteronuclear single quantum correlation spectra (supplemental Fig. 1) showing the chemical shifts of $^1$H/NH cross-peaks in NK1 (black) induced by 1:1 titrations with either heparin dp4 (blue) or DS dp6 (orange). Three identified residues, Cys-70, Arg-73, and Leu-80 demonstrate shifts (connected by a line) elicited by both oligosaccharides but consistently greater with the heparin dp4 than with the DS dp6. B, comparative histogram of combined chemical shift changes. Shift changes observed upon the titrations with heparin dp4 (blue) or DS dp6 (orange) were calculated using

$$\Delta \delta = \sqrt{\delta_{1H}^2 + \left( \frac{\delta_{15N}}{5} \right)^2},$$

(Eq. 1)

where $\delta_{1H}$ and $\delta_{15N}$ are the chemical shift changes in the $^1$H and $^15$N dimensions, respectively. Changes are ranked in the order of declining magnitude of $\Delta \delta$ observed for different NH cross-peaks in the titration with heparin dp4 (supplemental Fig. 1). The shift changes observed with the DS dp6 are then superimposed in the same order of NH cross-peaks.

Although tracking the chemical shift changes of residues during titrations was hindered by overlap and peak broadening in some regions, it was nevertheless possible to perform a qualitative comparison of the heparin dp4 and DS dp6 interactions. Where possible, the combined $^1$H and $^15$N chemical shift changes in NK1 elicited by heparin dp4 were calculated and then sorted in decreasing order of magnitude. The corresponding changes observed upon titration with the highest ratio of DS dp6 were then overlaid in the same order of cross-peaks (Fig. 8B). This comparison reveals major similarities between the two titrations, consolidating the evidence that HS/heparin- and DS-binding sites in NK1 overlap. For the majority of the cross-peaks, the absolute changes were larger for the heparin dp4 than for the DS dp6 at the same protein/ligand ratio, and indeed the NMR confirms the relative order of binding affinity for NK1 as heparin dp4 $>$ DS dp6 $>$ DS dp4. However, a fraction of the shifting cross-peaks observed in the DS dp6 titration exceeded the corresponding changes caused by the heparin dp4, as maybe would be expected for the different nature and/or sizes of the two oligosaccharide ligands.

DISCUSSION

The known ability of both heparin/HS and DS to interact strongly with HGF/SF and to act with similar potencies as cofactors in its activity is a relatively unusual property among “heparin-binding” proteins. Such proteins generally show greater GAG selectivity, i.e. relatively weak affinities for DS compared with heparin/HS.

Proteins with a less strict GAG specificity potentially pose greater problems in elucidating their recognition properties than might be faced when dealing with a highly specific protein like for example antithrombin-III, which recognizes an unusual heparin/HS dp5 sequence and tolerates relatively little variation (32). Working with structurally heterogeneous, intact GAGs or their incompletely desulfated variants often provides relatively limited information, especially if only short sequences are sufficient for protein recognition. Ultimately, definitive information can only emerge from the use of rigorously defined structures; hence, our present study of the minimal sizes of GAG oligosaccharides required for NK1 binding and the consequent effects of precise positional changes in their sulfate patterning.

GMSA analyses revealed that dp3s/dp4s are the minimal odd- and even-numbered species of both heparin/HS and DS that possess affinity, albeit weak, for NK1. Application of the GMSA technique in a newly developed selection mode has allowed us to make simultaneous assessments of the relative affinities for NK1 of differently sulfated oligosaccharides. The results clearly indicate that affinity is primarily influenced by sulfate density, even across different GAGs and ligand sizes and is not dependent upon the presence of sulfation at any specific ring position (consolidating previously suggestions from the behavior of differently sulfated GAG chains (23)). Importantly, NK1 does not discriminate between HS/heparin and DS dp4s of equivalent sulfate densities; affinity is, thus, essentially independent of hexosamine identity and glycosidic linkage within IdoA-containing GAGs.

A major, unresolved issue had been whether the dual GAG binding properties of NK1 and HGF/SF are due to the existence of two distinct, non-overlapping binding sites with exclusive GAG preferences or of a single binding site that accommodates both GAGs non-selectively. The former situation, although not common, occurs for example in TSG-6, where there are separate binding sites for hyaluronan and HS/heparin (33) and also in HARE, where hyaluronan/chondroitin sulfate and heparin bind independently to different sites (34). Previously we showed that HS and DS could compete for binding to NK1 in a GMSA assay (19). However, in the case of TSG-6 it is known that binding of GAG to one site can indirectly influence GAG binding to the other site even though they are non-overlapping and cannot be occupied simultaneously (33, 35).
To further investigate the situation in NK1, we compared NMR titrations of [15N]NK1 with heparin and DS oligosaccharides. NMR provides residue-specific information and, by monitoring the protein rather than the GAG, thereby complements GMSA. The broadly similar cross-peak shifts elicited by the DS dp6 and heparin dp4 species indicate similar protein contacts and, thus, overlapping positioning, which is consistent with a single binding site accommodating either GAG. However, in the absence of the complete resonance assignments of the NH cross-peaks of NK1, it was mostly not possible to identify which specific amino acid residues were principally affected in these titrations. Nevertheless, using more limited sets of published data from NMR-monitored titrations of low molecular weight heparin and sucrose octasulfate with the N-domain and/or NK1 (36), a few amino acid residues that were affected in both titrations, namely Asp-68, Cys-70, Arg-73, and Leu-80, could be tentatively assigned. It is notable that Arg-73 is implicated, as it had been previously identified as a pivotal residue in the NK1-heparin dp14 crystal complex (16).

The existence of a smaller cohort of NH cross-peaks that shift more strongly in the presence of DS dp6 than with heparin dp4, may signify subtle differences in the contacts made by different GAGs within the binding site. However, it may also reflect additional contacts made by the extra disaccharide unit present in the dp6. In this context it is notable that even though protein contacts are seen primarily between the N-domain (Lys-58, Lys-60, Thr-61, Lys-62, Lys-63, Arg-73, and Gly-79) and a core dp4 segment of the dp14 in the crystal complex (16), a fifth monosaccharide (a GlicNS residue) contiguous with the non-reducing residue of this dp4 sequence can make an additional ionic contact with a further N-domain residue, Arg-76. In addition, some crystals also showed evidence of the interaction of a dp2 extension on the reducing side of the dp4 sequence, with a secondary cluster of basic residues (Lys-132, Arg-134, and Arg-181) on the face of the K1 domain. However, in the crystal this particular K1 domain belongs to the adjacent protomer in a head-to-tail NK1 dimer. Interestingly, the isolated K1 domain does exhibit independent heparin affinity in vitro, although it is significantly weaker than the isolated N domain (20). NMR data suggests, however, that NK1 in solution, unlike in the crystal, exists as a monomer. Thus, depending upon the interdomain configuration in solution, it is possible that oligosaccharides longer than dp4 could make further contacts in the N-domain outside the core binding site and additionally may bridge across to the contiguous K1-domain, thereby eliciting additional cross-peak movements in NMR.

Further useful comparisons may also be made between our observations and the contacts evident in the NK1-dp14 crystal structure. In the latter the IdoA situated at the non-reducing end of the core dp4 segment makes just one protein contact via a hydrogen bond between its 2-O-sulfate group and the key Arg-73 residue (that is also engaged in three other contacts elsewhere, one of which is with a 6-O-sulfate). It might, therefore, be expected that this 2-O-sulfated IdoA would not be essential for interaction, and indeed when such a residue was removed from the hexasulfated heparin dp4 to generate the pentasulfated dp3 it had only slightly reduced NK1 affinity. Also, the disulfated DS dp3 and dp4 species lack such 2-O-sulfation but possess affinity.

It is also notable that two of the sulfates in the core dp4 segment (i.e., the central 2-O- and N-sulfates) make no observable contacts with NK1 in the co-crystal. This suggests that tetrasulfated dp4s specifically lacking these two sulfates should bind without compromising affinity. Although it is not possible to selectively remove these two specific sulfates, our GMSA analyses demonstrate that tetrasulfated dp4s continue to bind well irrespective of whether they lack both copies of the N-, 2-O-, or 6-O-sulfates. Furthermore, on the basis of the co-crystal it would be expected that a disulfated HS or DS dp4 would sustain a significant loss of specific contacts, which could explain the reduced affinities we observe. However, the equivalent affinities of the four disulfated dp4s with radically different sulfate patterns suggest that there may be sufficient conformational flexibility within the GAG sequence to allow alternative contacts to be made than those evident purely in this particular crystal complex. For example, the latter would suggest that interaction with a purely di-2-O-sulfated HS dp4 would be maintained by a salt bridge to only one of its two sulfates (at the non-reducing end), which seems unlikely.

A contributory factor in this flexibility may be the known conformational plasticity of IdoA (for reviews, see Refs. 37 and 38), and indeed the presence of IdoA is primarily what distinguishes a binding DS from a non-binding chondroitin sulfate. In addition, it is known from FGF2-heparin interactions that a kink in the overall helical structure of the chain can occur in certain trisaccharide segments, influenced in part by the conformation of the central IdoA, and such a kink can optimize protein contacts (39). The specific topology of the binding site in FGF2 also allows heparin to dock in either of its chain orientations (39). It is conceivable that similar structural accomodations may also operate to allow sequences of very variable sulfate patterns to make sufficient stable contacts with NK1 and HGF/SF. Consequently, it would be of great interest if future crystallography or NMR studies were to investigate how lesser sulfated sequences than fully sulfated heparin fragments are able to dock with NK1.

Overall, our observations with small oligosaccharides have conclusively revealed the operation of a single, high affinity but inherently low specificity GAG co-factor binding site in the NK1 region of HGF/SF. Affinity is dictated by the need for a minimum of an IdoA residue and two sulfates, which can be positioned in a variety of ways, located within a dp3/dp4 segment of either a HS/heparin or DS backbone. Separation of these minimal two sulfates by an intervening non-sulfated monosaccharide may be important, as an equally or higher sulfated dp2 does not bind NK1 (19). Thus, in vivo, initial interaction (and consequent co-factor activity) could be initiated almost anywhere within the IdoA-containing, sulfated domains of HS and DS, although highest activity will result from subsequent migration of the protein along the chain until it locates the most sulfated sequences of highest affinity. In HS these would correspond to the heparin-like cores of the sulfated domains, where N-, 2-O-, and 6-O-sulfates co-exist, whereas in

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DS they would be primarily where 2-O-sulfated IdoA residues are clustered.

The above characteristics of the GAG-binding site, as elucidated with minimal oligosaccharides, help to explain various disparate data in the literature relating to the binding of intact GAGs, suggesting that the behavior of small ligands does accurately reflect that of the natural polymers. The absence of any requirement for specific positional sulfation in HS/heparin mirrors what was previously observed in DS, where both mammalian and *Ascidia nigra* DS species bind and activate HGF/SF, although they possess either 4-O or 6-O sulfation of GalNAc, respectively (23). Also, the phenotype of the knock-out of the HS-2-O-sulfotransferase (the only HS sulfotransferase that exists as a single isozyme) (40) does not resemble that of either the HGF/SF or MET knock-outs (41–43), as would be expected if there was no specific requirement for 2-O sulfation. Indeed, HS derived from the knock-out animal continues to bind and activate HGF/SF similarly in vitro (44). This may be a consequence of the increased N and 6-O sulfations of HS that occur in this knock-out and appear to compensate in overall charge for the loss of 2-O sulfation (44), thereby sustaining HGF/SF affinity and activity.

Conversely, the noted reduction in HGF/SF-mediated cell signaling, proliferation, and migration of carcinoma cells elicited by elevated expression of Sulf-1 (45, 46), an extracellular HS 6-O-sulfatase, can be explained not by any specific requirement for 6-O sulfation but because 6-O-sulfates within the trisulfated, heparin-like disaccharides of HS are a major target of the Sulf enzymes (47, 48). By reducing the overall sulfate density of these highest affinity sites, the effectiveness of the endogenous HS as a HGF/SF ligand and co-factor is likely to be diminished.

The activity of small oligosaccharides also has implications for the mechanism of MET activation by HGF/SF. Heparin binding to NK1 does not induce any conformational change in the latter (16), although it is reported to dimerize NK1 in solution (49), consistent with the crystal complex (16). HGF/SF predominantly forms a 1:1 complex with the full-length MET ectodomain independently of GAGs (17); however, heparin dramatically enhances MET ectodomain dimerization in the presence of NK1 (49). A truncated MET ectodomain can also form dimeric 2:2 complexes with HGF/SF (50) probably via the same NK1-NK1 dimer interface seen in the heparin-NK1 crystal (16). Thus GAG binding and resultant dimerization may drive the formation of an active, dimeric, 2:2 complex of HGF/SF-MET.

In contrast to the above, it is unlikely that an oligosaccharide as small as a dp3/dp4 will effect dimerization of NK1 or HGF/SF. Indeed, during the NMR titrations with small oligosaccharides described in this study, the NK1 appeared to remain monomeric. This concurs with our previous observation that a monomeric HGF/SF, which has been cross-linked to an oligosaccharide and is, thus, physically unable to form a dimer upon the same GAG molecule, is also active (8). This implies that activation may be possible through two distinct mechanisms, dependent upon the size of the GAG, which may be analogous to the situation recently described with FGF2 (51). Small oligosaccharides may promote a 1:1:1 complex of GAG-HGF/SF-MET that can then more readily dimerize to form an active 2:2:2 complex. The latter may, however, be less stable and effective in signaling than a 1:2:2 complex that can form upon longer GAG species.

A notable difference from the situation seen with FGF2 is in the apparent correlation between GAG structure and signaling. For FGF2, specific losses of sulfation within a heparin dp4/dp6 species may have little effect upon FGF2 binding, but can abolish activity, which was interpreted as possibly being due to a requirement for additional, receptor binding determinants within the oligosaccharide (51). With HGF/SF, activity appears to remain inextricably linked to GAG binding across a wide spectrum of sulfate densities and patterns. By comparison, this may suggest a lesser role, if any, for a putative GAG-MET interaction in the ternary complex, and indeed we have only previously detected a relatively weak intrinsic affinity of extracted, cellular MET for intact heparin (8).

In conclusion, a novel strategy of employing minimal oligosaccharides of variable, but well defined sulfation patterns coupled with new methodological developments in GMSA application has enabled us to refine the molecular parameters of GAG-HGF/SF interaction and MET activation. The data obtained should help in the future design of GAG mimics for HGF/SF inhibition. This approach should also have general applicability in elucidating the detailed recognition properties of many other GAG-binding proteins.

**REFERENCES**
