The Mode of Action of Heparan and Dermatan Sulfates in the Regulation of Hepatocyte Growth Factor/Scatter Factor*

Malcolm Lyon‡, Jon A. Deakin, and John T. Gallagher

From the Cancer Research Campaign & University of Manchester Department of Medical Oncology, Christie NHS Trust, Manchester M20 4BX, United Kingdom

Hepatocyte growth factor/scatter factor, in addition to binding to its specific signal-transducing receptor, Met, also interacts with both heparan and dermatan sulfates with high affinity. We have investigated the comparative role of these two glycosaminoglycans in the activation of Met by hepatocyte growth factor/scatter factor. Using glycosaminoglycan-deficient CHO pgSA-745 cells we have shown that growth factor activity is critically dependent upon glycosaminoglycans, and that heparan sulfate and dermatan sulfate are equally potent as coreceptors. Cross-linked 1:1 conjugates of growth factor and either heparan or dermatan sulfate do not dimerize under physiological conditions and are bioactive. This implies that a ternary signaling complex with Met forms in vitro. Native Met isolated from CHO pgSA-745 cells shows only very weak intrinsic affinity for heparin in vitro. Also, a heparin-derived hexasaccharide, which is the minimal size for high affinity binding to the growth factor alone, is sufficient to induce biological activity. Together these observations imply that the role of these glycosaminoglycans may be primarily to effect a conformational change in hepatocyte growth factor/scatter factor, rather than to induce a necessary growth factor dimerization, or to stabilize a ternary complex by additionally interacting with Met.

Hepatocyte growth factor (HGF/SF) is a plasminogen-related growth factor secreted by stromal cells that acts primarily by a paracrine mechanism upon epithelial, endothelial, and hemopoietic progenitor cells (for review, see Ref. 1). Some stromal cells are also now known to be responsive to HGF/SF (2). Activation of its specific tyrosine kinase receptor, Met, elicits a diverse range of cellular activities, including proliferation, motility, morphogenesis, and protection from apoptosis (for review, see Ref. 1). HGF/SF is considered to be an important mediator of mesenchymal-epithelial interactions during organogenesis, as well as in any subsequent organ repair. Developmental studies have identified the essential role of the HGF/SF-Met system in the formation of the liver (3) and placenta (4), as well as in the migration of both motor neurons (5) and myogenic precursors (6). There is also increasing evidence of an involvement of HGF/SF-Met in the growth, invasiveness, and metastasis of both carcinomas and sarcomas (for review, see Ref. 7). Overexpression of wild-type Met and/or HGF/SF, sometimes involving the induction of an autocrine stimulatory loop, is common in cancer tissues. In a minority of cases there is evidence for mutations of Met leading to a dysregulated activity, although these often still remain ligand-dependent (8). Inhibition of Met activity may thus be beneficial in cancer treatment (9). Moreover, HGF/SF is itself considered to have considerable therapeutic potential in wound healing and specific organ regeneration after disease, damage, or surgery (10).

There is increasing evidence of a role for glycosaminoglycans (GAGs)/proteoglycans in HGF/SF activity, as suggested to varying degrees for a number of growth factors/cytokines (for review, see Ref. 11). In vitro, HGF/SF interacts with heparan sulfate (HS) (12–14) and dermatan sulfate (DS) (15) with sufficient high affinities ($K_d$ values of 0.2–20 nM (14, 15)) to support a physiological interaction. The apparently similar affinities of HGF/SF for these two structurally distinctive GAGs is rather unusual, as most HS-binding proteins display relatively weak affinities for DS. It is partly explained by the lack of requirement for either N-sulfate or 2-O-sulfate groups as major binding determinants (12, 15, 16). This raises the question as to whether the binding of HS or DS has the same functional consequences for HGF/SF activity? Indeed, the degree of involvement, and putative role, of GAGs in HGF/SF activity has been somewhat confused. We have previously reported that Madin-Darby canine kidney (MDCK) cells become completely unresponsive to HGF/SF when treated with chlorate, which metabolically inhibits the sulfation of endogenous GAGs (17). Responsiveness to HGF/SF can be restored by exogenous HS, although it has to be presented in the form of an immobilized heparan sulfate proteoglycan substratum, and, interestingly, HS does not work as a soluble ligand in MDCK cells (17). This suggests an obligate requirement for GAGs/proteoglycans for HGF/SF activity. An identical pattern of behavior was subsequently demonstrated with human mammary myoepithelial-like cells (18). Namalwa Burkitt’s lymphoma cells co-transfected with Met and a HS-bearing form of CD44 respond to HGF/SF, but not when a HS-lacking CD44 isoform is used (19). In contrast, Met-transfected BaF3 lymphoblastoid cells, which are reputedly completely deficient in HS, are reported to still respond to HGF/SF, although activity is significantly enhanced by the addition of exogenous heparin (20), a commonly used GAG analogue of the sulfated domains of HS.

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‡ To whom correspondence should be addressed: Dept. of Medical Oncology, Christie CRC Research Center, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, UK. Tel.: 44-0-161-446-3202; Fax: 44-0-161-446-3202; E-mail: MLyon@picr.man.ac.uk.

The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; GAG, glycosaminoglycan; HS, heparan sulfate; DS, dermatan sulfate; MDCK, Madin-Darby canine kidney; CHO, Chinese hamster ovary; FGF-1, fibroblast growth factor-1; FGF-2, fibroblast growth factor-2; PBS, phosphate-buffered saline; dp, degree of polymerization (i.e. number of monosaccharide units in the oligosaccharide); ERK, extracellular signal-regulated kinase; EDC, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride; sulfo-NHS, N-hydroxysulfosuccinimide; HPLC, high performance liquid chromatography; MES, 4-morpholineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
These same cells are completely refractory to the truncated NK1 and NK2 isoforms of HGF/SF (which are partial agonists of Met) unless exogenous heparin is present. The xyloseyl transferase-deficient CHO pgsA-745 mutant cells are similarly unresponsive to NK1 in the absence of heparin (21). It has also been reported that a double arginine reverse charge HGF/SF mutant with a consequent 50-fold reduction in heparin affinity had unimpaired biological activity (22), although it was without activity in the Met/CD44 co-transfected lymphoma cells (19). It is thus unclear as to the true role of GAGs, and whether some of these conflicting observations primarily reflect differences between the properties of full-length HGF/SF and its truncated variants. Indeed, it has been suggested that the various forms of HGF/SF may engage the Met receptor in different ways (23–25).

The putative mechanism(s) by which GAGs can modulate HGF/SF activity is also unclear. HGF/SF can apparently bind with high affinity to a purified Met-Ig fusion protein in vitro, in the absence of GAG (22, 26, 27). This suggests that GAGs are not required for at least the initial binding of HGF/SF to Met (although in the fusion protein constructs the Met is already dimerized). GAGs may, however, still be needed for a subsequent Met activation step. However, the predominant view, mostly based on experiments with the NK1 variant, is that the role of GAGs may be primarily to induce dimerization of the protein ligand (20, 21, 27, 28), thereby facilitating the subsequent dimerization and activation of Met.

To try and further clarify these issues we have specifically investigated the GAG dependence of HGF/SF activity in the CHO pgsA-745 mutant cells. We wished to address experimentally the following: (i) is the activity of full-length HGF/SF essentially dependent upon, or enhanced by, the presence of appropriate GAGs; (ii) do HS and DS differ in their functional properties; (iii) is GAG-induced HGF/SF dimerization essential for activity; and (iv) is there evidence for an additional interaction between Met and HGF/SF-binding GAGs?

**EXPERIMENTAL PROCEDURES**

**Materials—**CHO pgsA-745 cells were provided by Dr. J. Esko (University of California at San Diego, CA), and were routinely cultured in RPMI medium supplemented with 10% (v/v) fetal bovine serum. MDCK cells were provided by Dr. E. Gherardi (MRC Center, Cambridge, UK), and were routinely cultured in Eagle’s modified minimal essential medium with Earle’s salts containing 5% (v/v) heat-inactivated donor calf serum. All cell cultures were supplemented with 1% (v/v) glutamine, 100 IU/ml of penicillin, and 100 μg/ml streptomycin sulfate, and maintained in a humidified atmosphere of 5% CO₂ in air at 37 °C.

**Recombinant human HGF/SF** was obtained from R&D Systems (Abingdon, UK). Heparanase I (Flavobacterium heparinum; EC 4.2.2.7), chondroitinase ABC (Proteus vulgaris; EC 4.2.2.4), and chondroitinase ACI (F. heparinum; EC number 4.2.2.5) were from Seikagaku Kogyo Co. (Tokyo, Japan). Heparinase II (F. heparinum; no EC number assigned) and heparinase III (F. heparinum; EC 4.2.2.8) were from Grampian Enzymes (Orkney, UK). Porcine mucosal heparin, heparin-agarose, and azure A were from Sigma (Poole, UK). Porcine mucosal HS was a gift of NV Organon (Oss, Dassel, Germany). Blots were blocked for 1 h in PBS, 10% (w/v) nonfat dried milk powder, 0.1% (v/v) Tween 20) of a mouse monoclonal antibody to dually-phosphorylated Thr183/Tyr185) (Upstate Biotechnology Inc., Lake Placid, NY).

**Bio-Gel P10 column (1.5 × 163 cm) eluted with NH₄HCO₃ at a flow rate of 12 ml/h. Fractions (1.4 ml) were collected and monitored on a spectrophotometer at 232 nm. Individual size populations of oligosaccharides were pooled, desalted on a PD-10 column eluted with distilled water, and then dried on a centrifugal evaporator.**

**Murine skin DS (10 mg) was dissolved in 1 ml of 50 mM sodium acetate, pH 6.5. Chondroitinase ABC was added to a final concentration of 50 μIU/ml and incubated at 37 °C for 2 h, before the addition of a second fresh batch of enzyme and digestion for a further 18 h. The resulting oligosaccharide mixture was resolved on a ProPac PA-1 strong anion-exchange HPLC column (0.4 × 25 cm; Dionex) using a 0–1.5 M NaCl, pH 3.5, gradient at a flow rate of 1 ml/min and collection of 1-ml fractions. Elution was monitored by on-line UV absorbance at 232 nm, and fractions were pooled and processed as described above.**

**ERK Activation Assay—**Freshly trypsinized CHO pgsA-745 cells were seeded at high density in 1 ml of RPMI, 10% (v/v) fetal bovine serum in a 24-well plate and incubated at 37 °C for 24 h. Medium was removed and replaced with serum-free RPMI for 2 h, before addition of fresh serum-free RPMI containing known concentrations of HGF/SF, cross-linked HGF/SF-GAG conjugates, or GAGs. After 20 min at 20 °C, the supernatants were removed and cells were solubilized in 70 μl M/2W of boiling, nonreducing Laemmli SDS sample buffer. Equivalent loadings of each sample were electrophoresed on a nonreducing 15% (w/v) SDS-polyacrylamide gel with a 5% (w/v) polyacrylamide stacking gel, and then electrophoretically to nitrocellulose (Schleicher & Schuell GmbH, Keene, NH). Blots were blocked for 1 h in PBS, 10% (w/v) nonfat dried milk powder before probing with 1:1000 dilution (in PBS, 3% (w/v) nonfat dried milk powder, 0.1% (v/v) Tween 20) of a mouse monoclonal antibody to dually-phosphorylated Thr183/Tyr185/204) (ERK-1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). After thorough washing with PBS, a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG was added for 30 min. Bands were visualized by enhanced chemiluminescence (ECL; Amersham Bioscience Inc.).

**Transwell Migration Assay—**Freshly trypsinized CHO pgsA-745-745 cells in 1 ml of culture medium were seeded at high density into the top chamber of a 24-well Transwell plate with 12-μm pore polycarbonate membranes (Costar, High Wycombe, UK). Bottom chambers received 1 ml of medium containing known concentrations of HGF/SF, cross-linked HGF/SF-GAG conjugates, or GAG alone. After 4–5 h incubation at 37 °C, wells were emptied and cells were fixed with cold (−20 °C) methanol, air dried, and then stained with 1% (w/v) aqueous crystal violet. Cells on the upper surface of the membrane were removed using a cotton bud. Membranes were excised using a scalpel blade, and the number of migrated cells on the underside of the membrane was counted under the microscope. Representative fields were counted for each replicate membrane. The motility of MDCK cells was also assayed by the colony scatter assay, performed as described in Ref. 17.

**Zero-length Cross-linking to Form HGF/SF-GAG Conjugates—**Zero-length cross-linking of HGF/SF to intact GAGs or oligosaccharides was performed essentially as originally described for protein-protein cross-linking (Grabarek and Gergely, 1989). Cross-linking was performed by incubating HGF/SF (10 μg/ml) with defined oligosaccharide size fractions (10 μg), were dissolved in 0.1 ml of 1 M MES, 0.1 M NaCl, pH 6.0. Sufficient EDTC and sulfo-NHS were added to give 6 and 15 mM concentrations, respectively, and the mixture was incubated at 25 °C for 15 min. Excess reagents were rapidly removed by passage through a 1.5-ml Sephadex G-50 column eluted with 0.1 M MES, 0.1 M NaCl, pH 6.0. Recovered activated GAGs/oligosaccharides were combined with 1 μg of HGF/SF and incubated at 25 °C for at least 2 h. Free HGF/SF was removed by adsorption to 50 μl of heparinagarase beads for 1 h at room temperature. Cross-linked conjugates were stored at 4 °C until further use.

**Aliquots of cross-linked conjugates were made up to 0.1 ml with water and 25 μg of bovine serum albumin was added. Proteins were precipitated with 10% (v/v) trichloroacetic acid at 4 °C for 15 min. Precipitates were pelleted by centrifugation, washed with ice-cold acetone, and then centrifuged. The final pellet was re-dissolved in either: (i) water (for subsequent degradation with pH 1.5 nitric acid, according to the method of Shively and Conrad (1989); or ii) 50 mM sodium acetate, 0.5 mM calcium acetate, pH 7.0 (for digestion with heparinase III); (iii) 50 mM Tris-HCl, pH 7.5 (for digestion with chondroitinase ABC).**

**Samples of HGF/SF, or cross-linked HGF/SF-GAG conjugates, were electrophoresed on a nonreducing 7.5% (w/v) SDS-polyacrylamide gel with a 5% (w/v) polyacrylamide stacking gel. Western blots were probed for 1 h with a goat polyclonal antiserum (4 μg/ml) against human HGF (R & D Systems), followed by a horseradish peroxidase-conjugated rabbit anti-goat IgG (1:5000 dilution) and enhanced chemiluminescent detection.**
Molecular Size of HGF/SF-GAG Conjugates—A TSK G4000PW XL (300 × 7.5 mm; Tosoh Corp., Tokyo, Japan) size exclusion chromatography HPLC column was equilibrated in 0.15 M NaCl, 20 mM phosphate, pH 7.0, at a flow rate of 0.3 ml/min. The void (V0) and total (Vt) volumes were determined with dextran blue and sodium dichromate, respectively. The column was calibrated for molecular mass using ovalbumin (45 kDa), hemoglobin (64.5 kDa), transferrin (80 kDa), and collagenase type 3 (110 kDa), which were monitored by on-line UV absorption at 280 nm. A plot of log V0 versus Mw (on a log scale) was constructed. The elution positions of HGF/SF (500 ng) and a purified cross-linked HGF/SF-heparin conjugate (400 ng), applied in a 0.05-ml volume, were determined by collecting fractions of 0.5 ml, followed by dot blotting to nitrocellulose and probing with an antiseraum against HGF/SF as described earlier. The elution position of free heparin was determined by dot blottng of 0.3-ml fractions to a cellulose acetate membrane followed by staining with 0.08% (w/v) aqueous azure A and subsequent destaining in water.

Extraction of Native Cellular Met—CHO pgsA-745 cell monolayers were washed with PBS and then scraped into 100 µl of extraction solution, comprising 0.15 M NaCl, 25 mM HEPES, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1% (v/v) Nonidet P-40, 0.05% (w/v) SDS, 0.2% (w/v) sodium deoxycholate, 5 mM EDTA, 2 mM EGTA, 1 µg/ml soybean trypsin inhibitor, 0.05% (w/v) sodium orthovanadate, 1 mM NaF, 0.1 mM ammonium molybdate, 1 mM MgCl2, 0.2 mM phenylmethylsulfonyl fluoride, 10 µM calpain inhibitor II, 10 µM aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin A. pH 8.0. Cell extracts were mixed end-over-end for 1 h at 4°C and then centrifuged (12,000 rpm for 10 min) to remove insoluble residues. Met receptor was partially purified by adsorption of the soluble cell extracts onto a suspension (packed bed volume of 300 µl) of wheat germ agglutinin-agarose. After washing with extraction solution, the bound glycoproteins (including Met) were eluted with either nonreducing Laemmli sample buffer, or a 1 µg/ml dilution of a goat antiserum against the ectodomain of murine Met (R & D Systems). These were followed by 1:5000 dilutions of horseradish peroxidase-conjugated goat anti-mouse IgG or rabbit anti-goat IgG, respectively, and enhanced chemiluminescent detection.

Heparin Affinity Chromatography of Native Met—Partially purified Met was applied to a heparin-agarose affinity column (0.5 ml volume) equilibrated with PBS, and re-circulated three times. After extensive washing with 0.5 mM CHAPS in PBS, pH 7.4, any bound material was eluted using 1-mI stepwise additions of 0.15–1 M NaCl in 20 mM phosphate, 0.5 mM CHAPS, pH 7.4. The agaroze-based Sepharose CL-4B was used as a parallel control column. Met was recovered and concentrated from the collected fractions by re-adsorption to wheat germ agglutinin-agarose (30 µl of gel suspension), and then released by treatment with nonreducing SDS sample buffer at 100°C. Samples were electrophoresed on a 7.5% (w/v) SDS-polyacrylamide gel with a 5% polyacrylamide stacking gel. Western blots were probed with either a 1:1000 dilution of the DQ-13-chain (Upstate Biotechnology Inc., Lake Placid, NY), or a 1 µg/ml dilution of a goat antiserum against the intracellular C terminus of the Met gene, resulting in a failure to transfer xylose to targeted serine residues. Consequently these cells are deficient in both HS and DS (31), and also acquire a migratory phenotype (Fig. 1A), although they do not incorporate [3H]thymidine (data not shown). Heparin, alone, in the absence of HGF/SF, had no effect (Fig. 1, A and B).

RESULTS

HGF/SF Activity Is Dependent Upon the Presence of Sulfated GAGs: Stimulation by Both HS and DS—The CHO pgsA-745 mutant cells are functionally mutated in the xylosyltransferase gene, resulting in a failure to transfer xylose to targeted serine residues and thus to initiate the synthesis of the sulfated GAGs. Consequently these cells are deficient in both HS and DS (31), although they do possess Met (21). However, they fail to respond to HGF/SF by either ERK activation (data not shown) or by cell migration across a porous Transwell membrane (Fig. 1). Even elevated concentrations of soluble exogenous heparin up to 50 ng/ml failed to have any effect (respective cells usually respond only to 1–10 ng/ml). However, upon the simultaneous addition of soluble exogenous heparin together with HGF/SF, these mutant cells then signal through ERK (Fig. 1A) and also acquire a migratory phenotype (Fig. 1B), although they do not incorporate [3H]thymidine (data not shown). Heparin, alone, in the absence of HGF/SF, had no effect (Fig. 1, A and B).

Both HS and DS are known to bind HGF/SF with similar high affinities (14, 15), even though there are significant structural differences between these two GAGs. Both HS and DS, as soluble exogenous ligands, promote HGF/SF-mediated motility of CHO pgsA-745 cells in a Transwell migration assay, and they appear to act with similar potencies over a 10-fold range of concentrations (Fig. 2).

HGF/SF Can be Covalently Cross-linked to Either HS or DS, and the Resulting Stable Conjugates Are Biologically Active—The crucial role of GAGs in the activation of Met by HGF/SF, as demonstrated in the CHO pgsA-745 cells, could result from a number of possible mechanisms. We have utilized a zero-length cross-linking technique which, by covalently linking HGF/SF to a bound GAG partner, can allow us to probe some of these putative mechanisms. Application of the zero-length cross-linking procedure gives rise to the efficient formation of covalent complexes between HGF/SF and all three GAG species that bind to it, i.e. HS (Fig. 3A), DS (Fig. 3B), and heparin (not shown). These complexes display a larger and more heterogeneous molecular size on SDS-PAGE than native HGF/SF, reflecting a stable conjugation of the protein with a heterogeneous GAG population. Treatment of HGF/SF-HS complexes with either low pH nitrous acid or a mixture of heparinase enzymes, to specifically degrade the HS component, leads to a reduction in molecular size of the complex toward that of the initial HGF/SF-HS conjugate; HGF/SF-DS conjugates can be reduced in size by digestion with chondroitinase ABC (Fig. 3B). Covalent HGF/SF-GAG conjugates are unable to bind heparin-agarose, unlike native HGF/SF (see Fig. 3C for HGF/SF-HS conjugates; HGF/SF-DS conjugates behave the same (not shown)). This proves that the GAG is correctly positioned and cross-linked into the putative GAG-binding site in HGF/SF, thereby blocking any additional GAG interactions. Importantly, this means that when the conjugates are introduced into cell cultures, there can be no subsequent interaction with any endogenous GAGs.
HGF/SF can also be effectively cross-linked to high affinity oligosaccharides such as HS- and DS-derived dp12–14s. However, there is only a very slight molecular weight shift on SDS-PAGE (not shown), and with smaller oligosaccharides it becomes imperceptible, because of the contrasting large size of HGF/SF (90 kDa). The formation of conjugates in these cases can only be confirmed by the proportionate loss of heparin affinity. As oligosaccharide size decreases the efficiency of cross-linking similarly decreases, and with dp8s only a small proportion of conjugate is formed, as assessed by binding to heparin-agarose (not shown). Importantly, the small size shifts upon SDS-PAGE after cross-linking with oligosaccharides of various sizes are only sufficient to indicate the formation of a 1:1 complex of HGF/SF and oligosaccharide, and there is no evidence of higher molecular weight oligomers. Even with intact GAGs the size of the great majority of the conjugate product would appear to be consistent with the presence of only one HGF/SF monomer per GAG chain (Fig. 3, A and B).

Cross-linked conjugates of HGF/SF with HS or DS are biologically active, in that they stimulate the activation of ERK (Fig. 4) and the consequent motility of CHO pgsA-745 cells in the Transwell assay (Fig. 4). They also stimulate the motility of MDCK cells in both the scatter and Transwell assays (data not shown). Again, HS and DS display comparable levels of activity when conjugated to HGF/SF, as they do when noncovalently mixed with HGF/SF (compare Figs. 1 and 4). However, in absolute terms, the HGF/SF-GAG conjugates, with either HS or DS, display about 40–50% of the activity of HGF/SF mixed with an identical concentration of free GAG in the CHO pgsA-745 cells (Fig. 4), and about 35–40% of the activity elicited by HGF/SF alone in the MDCK cells (data not shown). The potency of a conjugate with a HS dp12 is more apparent in excess of those used for assays of their biological activity.

**Molecular Size of HGF/SF-GAG Conjugates under Nonnaturating Conditions**—The elution positions of a HGF/SF-heparin conjugate were compared with HGF/SF and heparin alone on a size exclusion chromatography column calibrated with proteins of known $M_r$, and run under physiological conditions of pH and ionic strength (Fig. 5). The elution positions of free heparin and free HGF/SF corresponded to apparent $M_r$ values of 50,000 and 71,000, respectively. By comparison, the mass of the HGF/SF-heparin conjugate, which eluted as a monodisperse peak, was extrapolated to be ~151,000. This value is much closer to the additive mass of a HGF/SF-heparin monomer conjugate (121,000) than to a (HGF/SF-heparin)₂ dimer conjugate (242,000). Thus the HGF/SF-heparin conjugate does not appear to form dimers under nondenaturing and physiological conditions, even at concentrations of HGF/SF considerably in excess of those used for assays of their biological activity.
The Ectodomain of Met Has Only Very Weak Intrinsic Affinity for Heparin—It is clear from the above that HGF/SF can signal in vitro as part of a complex in which GAG-HGF/SF and HGF/SF-Met interactions are known to occur. Can an additional GAG-Met interaction take place? We have attempted to test experimentally whether native Met extracted from cell cultures has intrinsic affinity for heparin in vitro. A partially purified glycoprotein fraction from a detergent extract of CHO pgsA-745 cells was analyzed using an ectodomain-specific antiserum, and found to contain two Met species. From their respective sizes, and their differential recognition by the cytoplasmic domain-specific antibody DQ13, these two species appear to correspond to intact Met (Met190) and the cytoplasmically deleted form of Met (Met150) (Fig. 6A). This extracted Met population was then analyzed by affinity chromatography on heparin-agarose. As the Met has been extracted from the mutant CHO cells, it could not carry with it any bound endogenous PGs which could compete and block affinity for the immobilized heparin. Approximately half of the applied Met bound at physiological pH and ionic strength, and all of this bound fraction was subsequently eluted using only a 0.2 M NaCl step (Fig. 6B), indicating a very weak intrinsic affinity. No binding occurred to the control column (Fig. 6B). As both Met190 and Met150 appear to behave similarly, this very weak interaction would appear to be mediated specifically by the common ectodomain of Met.

A Minimum Length HGF/SF-binding Heparin Hexasaccharide Will Activate the Growth Factor—We know from our previous observations that a hexasaccharide appears to be the smallest even-numbered HS oligosaccharide required to occupy the major GAG-binding site on HGF/SF (12). We have therefore investigated the oligosaccharide size dependence of Met activation, to see if this suggests that any additional length of GAG may be necessary to sustain an additional obligate interaction with Met itself. Heparin oligosaccharides (dp2–12) were tested in combination with HGF/SF in functional assays with the CHO pgsA-745 cells. The smallest active size class which clearly potentiated ERK activation was found to be hexasaccharides (Fig. 7A). It was previously shown, by affinity chromatography, that HS dodecasaccharides bind to HGF/SF with markedly greater apparent affinity than the minimal hexasaccharides (12). However, Transwell motility studies with CHO pgsA-745 cells show that the activatory potential of heparin dodecasaccharides is only approximately twice that of the hexasaccharides, on a comparative molar basis (Fig. 7B). This is
This study conclusively demonstrates the GAG dependence of HGF/SF activity using the mutant CHO pgsA-745 cell system. This behavior mimics that reported for NK1 and NK2 (20, 21), indicating a unified GAG dependence for all the HGF/SF variants. Although a strict GAG dependence of HGF/SF activity was not shown previously in BaF-3 cells (20), these were chosen specifically on the basis of a lack of HS expression. This ignores the possible role of DS, and it has not been formally demonstrated that they are also devoid of DS.

Importantly, the use of the CHO pgsA-745 cell system has allowed a direct quantitative comparison to be made of the activities of HS versus DS. Both GAGs bind HGF/SF with similar high affinities (14, 15), and display similar behavior as either potentiators or inhibitors (depending upon the assay) of HGF/SF activity in MDCK cells (17). However, the CHO pgsA-745 cell system clearly demonstrates their functional equivalence as HGF/SF co-receptors in vitro. In tandem with the published abilities of DS to promote the activities of HARP/pleiotrophin (32) and, to a modest degree, FGF-2 (33), it is thus possible that DS may have a greater role than expected in the modulation of “HS-binding” growth factors.

For FGF-1 and -2 it has been proposed that HS stabilizes an activated, ligand-receptor complex, rather than it being an efficient intermediary for the capture and transfer of FGF to its receptor. A larger HS sequence was needed for activation of FGF-2 (34, 35) than was required solely to bind FGF-2 (36, 37). This suggested that HS may be required to dimerize FGF-2 (38, 39), thereby facilitating receptor dimerization and activation. An additional requirement for 6-O-sulfate groups to be present for activation (40–42), but not for FGF-2 binding, suggested that HS may alternatively need to interact with the receptor. A number of studies of complexes involving FGF-1 or -2 (43–46) have confirmed HS involvement, but have failed to deliver a consensus on the stoichiometry or molecular organization of the interacting components. We have attempted to probe the mechanism by which GAGs activate the HGF/SF-Met system, by comparison to the prototypic FGF system.

Zero-length cross-linking of HGF/SF to a GAG partner, allows two fundamental mechanistic questions to be addressed. First, can HGF/SF simultaneously interact with both GAG and Met, with retention of biological activity, or is a binary HGF/SF-GAG complex required to dissociate for an active HGF/SF-Met complex to subsequently form? Second, is GAG driven, HGF/SF dimerization a pre-requisite for activity? In zero-length cross-linking a direct amide linkage can be formed between the epsilon group of a lysine side chain and a uronate carboxyl group, if they are directly ion pair bonded (29). Thus cross-links only form within regions of direct protein-GAG contact. HS, DS, and heparin all cross-link to HGF/SF, with loss of heparin affinity, suggesting that all GAGs are occupying the same unique binding site within HGF/SF. There is no evidence, from denaturing SDS-PAGE, of apparent dimerization in which two HGF/SF monomers are cross-linked to the same GAG/oligosaccharide. There is also no evidence for a HGF/SF monomer noncovalently dimerized to a cross-linked HGF/SF-GAG conjugate. Such a dimer would be disrupted upon SDS-PAGE to give a free HGF/SF component. Also, it might be expected that the noncross-linked HGF/SF monomer would be able to interact with heparin-agarose. Similarly, the apparent monomeric size of the HGF/SF-GAG conjugates under non-denaturing conditions suggests they are not prone to dimerization, even at concentrations in excess of those which are biologically active.

The retained and potent biological activity of covalent HGF/SF-GAG conjugates indicates that HGF/SF can simultaneously interact with both GAG and Met without compromising biological activity, and that such a ternary complex may be the active complex in vivo. The potent activity of the monomeric HGF/SF-oligosaccharide conjugates suggests that HGF/SF dimerization is not a pre-requisite for Met activation. Previous studies using zero-length cross-linked HS-FGF-2 also found that monomeric conjugates were biologically active (47).

The simplest molecular model of a ternary complex would comprise a HGF/SF sandwiched by two independent binary interactions, i.e. HAG-HGF/SF and HGF/SF-Met. However, it is possible that a third binary interaction, i.e. GAG-Met, could consolidate the complex. Met contains a number of sequence clusters of basic residues distributed mainly throughout the ectodomain, but also within the cytoplasmic domain. Conformational clusters of basic residues cannot be assessed because of the present lack of knowledge of the tertiary structure of Met. Although there does appear to be an interaction between a monomeric native ectodomain of Met and heparin in vitro under physiological conditions, it is clearly of very low affinity. The affinity for HS is likely to be even lower still (as is often the case with heparin/HS-binding proteins). The absence of an intrinsic high affinity of the Met ectodomain for heparin/HS, which might suggest the likelihood of pre-formed complexes of receptor and co-receptor existing at the cell surface, does not preclude the possibility that a much weaker affinity interaction may come into play once Met and GAG have been brought into close proximity by the higher affinity GAG-HGF/SF and HGF/SF-Met interactions. It is also possible that appreciable GAG affinity may not be inherent, but could be expressed by Met as a consequence of a conformational change induced by the binding of HGF/SF. However, hexasaccharides are able to stimulate HGF/SF activity in CHO pgsA-745 cells. As these correspond to the minimum size of oligosaccharide which occupy the GAG-binding site on HGF/SF with measurable affinity (12), it would suggest that an additional interaction of GAG with Met is not strictly required for HGF/SF activity. The only caveat here being that HGF/SF and Met may conceivably bind to opposite faces of the same short oligosaccharide.

Intriguingly, the restoration of HGF/SF responsiveness by soluble GAG in CHO pgsA-745 cells (and also in NIH-3T3 fibroblasts depleted of their endogenous HS/D/DS by treatment with heparinases/chondroitinases; data not shown) is at variance with the failure to rescue by soluble GAG, and the specific requirement for a substratum-immobilized GAG, in chlorate-treated MDCK (17) or mammary myoepithelial cells (18). This dichotomy may reflect differences in cellular morphology and membrane distribution of Met. More polarized epithelial-like cells may express Met exclusively on the basolateral membrane, as shown with MDCK cells (48). Such receptors may be much more responsive to HGF/SF presented basally by a GAG-rich substratum. In contrast, cells with a more fibroblastic morphology (e.g. CHO cells) may have a less segregated distribution of Met which is more readily activated by HGF/SF and soluble GAG.

In conclusion, our observations are consistent with a model in which HGF/SF acts as a monomer, in complex with either HS or DS, to activate Met. This contrasts with the view from crystal structures of NK1 (49, 50) which suggests, at least for this fragment of HGF/SF, that a dimer is the functional unit. Activation of Met is likely to occur via an initial 1:1 HGF/SF-Met interaction, leading to the formation of signal-transducing Met dimers. Two separate monomeric HGF/SF-GAG complexes supporting evidence that a GAG oligosaccharide may only be required to occupy the binding site on HGF/SF for activation of Met to ensue, rather than to additionally and directly interact with Met as well.
may therefore be required to form a single, symmetric signaling complex with dimeric Met (as suggested for FGF-2 in Ref. 46). However, the requirement for Met dimerization does not prove that two HGF/SF molecules are needed; a single HGF/SF/Met complex may have the ability to asymmetrically engage a second nonligated Met to induce signal transduction (as suggested for FGF-2 in Refs. 43 and 44). The fact that hexasaccharides can activate suggests perhaps a role for GAG in bringing about a conformational change in HGF/SF, rather than additionally stabilizing a signaling complex, especially as there is no strong evidence that they can also bind directly to Met.

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REFERENCES

1. Bock, G. R., and Goode, J. A. (1997) Flaxseed-derived Growth Factors: Ciba Foundation Symposium 212, John Wiley, Chichester, UK
2. Delehedde, M., Sergeant, N., Lyon, M., Rudland, P. S., and Ferrig, D. G. (2001) Eur. J. Biochem. 269, 4425–4429
3. Schmidt, C., Bladt, F., Goeckede, S., Brinkmann, V., Zechiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. (1995) Nature 376, 699–702
4. Uehara, Y., Minowa, O., Mori, C., Shiota, K., Kuno, J., Noda, T., and Kitamura, Y. (1995) J. Biol. Chem. 270, 16871–16878
5. Zhou, H., Casas-Pinet, J. E., Coats, R., Kaufman, J. D., Stahl, S. J., Wingfield, P. T., Rubin, J. S., Bottaro, D. P., and Byrd, R. A. (1999) Biochemistry 38, 14793–14802
6. Walker, A., Turnbull, J. E., and Gallagher, J. T. (1994) J. Biol. Chem. 270, 23116–23121
7. Walker, A., Turnbull, J. E., and Gallagher, J. T. (1994) J. Biol. Chem. 269, 931–935
8. Walker, A., Turnbull, J. E., and Gallagher, J. T. (1994) J. Biol. Chem. 269, 931–935
9. Walker, A., Turnbull, J. E., and Gallagher, J. T. (1994) J. Biol. Chem. 269, 931–935
