HEPARAN SULFATE GLYCOSAMINOGLYCAN REQUIREMENT FOR Met BINDING AND SIGNALING*  

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Hiromi Sakata, Stephen J. Stahl†, William G. Taylor, Jared M. Rosenberg, Kazushige Sakaguchi‡, Paul T. Wingfield‡, and Jeffrey S. Rubin‡

From the Laboratory of Cellular and Molecular Biology, NCI, the ‡Protein Expression Laboratory, NIAMSD, and the $Glycobiology Program, NIDR, National Institutes of Health, Bethesda, Maryland 20892

Hepatocyte growth factor/scatter factor (HGF/SF) is a heparin-binding polypeptide that stimulates cell proliferation, motility, and morphogenesis by activation of its receptor, the c-Met tyrosine kinase. HGF/SF consists of a series of structural units, including an amino-terminal segment with a hairpin loop, four kringle domains, and a serine protease-like region. In this study, we demonstrate that the amino-terminal (N) domain retains the heparin-binding properties of full-length HGF/SF. In contrast to a previous hypothesis, selected basic amino acid residues in the hairpin loop are not critical for heparin binding, although alanine substitution at a subset of these sites markedly reduced the biological activity of the HGF/SF isoform, HGF/NK1. Covalent cross-linking experiments performed with wild-type and heparan sulfate glycosaminoglycan (HSGAG)-deficient Chinese hamster ovary (CHO) cells revealed that Met-HGF/NK1 binding was strongly dependent on HSGAG. Addition of heparin to HSGAG-deficient CHO cells not only restored ligand binding, but also increased ligand-dependent Met tyrosine phosphorylation and c-fos expression. Moreover, our results showed that heparin stimulated ligand oligomerization through an interaction with the N domain. These findings establish the importance of the N domain for heparin-ligand and ligand-ligand interactions, and demonstrate a crucial role for HSGAG in receptor binding and signal transduction.

Hepatocyte growth factor/scatter factor (HGF/SF) is a heparin-binding polypeptide that stimulates cell proliferation, motility, and morphogenesis by activation of its receptor, the c-Met tyrosine kinase. HGF/SF consists of a series of structural units, including an amino-terminal segment with a hairpin loop, four kringle domains, and a serine protease-like region. In this study, we demonstrate that the amino-terminal (N) domain retains the heparin-binding properties of full-length HGF/SF. In contrast to a previous hypothesis, selected basic amino acid residues in the hairpin loop are not critical for heparin binding, although alanine substitution at a subset of these sites markedly reduced the biological activity of the HGF/SF isoform, HGF/NK1. Covalent cross-linking experiments performed with wild-type and heparan sulfate glycosaminoglycan (HSGAG)-deficient Chinese hamster ovary (CHO) cells revealed that Met-HGF/NK1 binding was strongly dependent on HSGAG. Addition of heparin to HSGAG-deficient CHO cells not only restored ligand binding, but also increased ligand-dependent Met tyrosine phosphorylation and c-fos expression. Moreover, our results showed that heparin stimulated ligand oligomerization through an interaction with the N domain. These findings establish the importance of the N domain for heparin-ligand and ligand-ligand interactions, and demonstrate a crucial role for HSGAG in receptor binding and signal transduction.

During the past dozen years, several mitogens have been identified that possess the distinctive property of binding to heparin or closely related heparan sulfate glycosaminoglycan (HSGAG)† (1, 2). Heparin-based affinity chromatography has been a convenient tool for purification of these factors, and several studies have shown that this binding phenomenon has functional relevance as well (2–10). HSGAG-dependent stabilization (3) and/or localization of the growth factor on the cell surface may promote interaction with less abundant, higher affinity tyrosine kinase receptors involved in signal transduction (2, 4, 5). Coupling of heparin or HSGAG either to the growth factor or its tyrosine kinase receptor may induce conformational changes in these molecules that augment signaling (3, 6). Several laboratories have reported that heparin binding can provide a framework for ligand oligomerization, which may enhance signaling by stimulating dimerization of the tyrosine kinase receptor (5, 8–10).

Hepatocyte growth factor/scatter factor (HGF/SF) is a heparin-binding polypeptide that can function as a mitogen, motogen, or morphogen on a broad spectrum of cellular targets (11–13). HGF/SF is related to plasminogen and macrophage-stimulating protein, sharing with these molecules approximately 40–45% amino acid sequence identity and several structural motifs (14, 15). HGF/SF is synthesized as an inactive monomer which undergoes internal proteolysis to yield a bio logically active, disulfide-linked heterodimer (16–18). The heavy chain of the HGF/SF dimer (~60 kDa) is derived from the amino terminus of the precursor and contains an amino-terminal segment followed by four kringle domains. A kringle consists of ~80 amino acids and has a characteristic folding pattern defined by three internal disulfide bonds and additional conserved residues (19). Two alternative transcripts have been identified that encode truncated variants of HGF/SF, terminating after either the first or second kringle domain (20–23). These smaller isoforms, designated HGF/NK1 and HGF/NK2, respectively, behave as antagonists or partial agonists, depending on the molecule and assay conditions (20, 22–25). All of these HGF/SF isoforms bind with high affinity to the tyrosine kinase transmembrane HGF/SF receptor, the Met proto-oncogene product (20, 22, 23, 26, 27).

As with other heparin-binding growth factors, the higher capacity, lower affinity cell surface binding sites for HGF/SF were attributed to heparan sulfate proteoglycan (27–30). Based on deletion analysis, kringle 2 (K2) and the hairpin loop in the amino-terminal (N) domain were implicated in heparin binding (31, 32), although recent work raised doubt about the importance of K2 in this regard (23). The potential significance of HGF/SF-heparin interactions has been inferred from a variety of observations. HGF/SF-Met covalent cross-linking was diminished following treatment of primary hepatocytes with heparinase, indicating that HSGAG might be required for binding (33). Administration of heparin to cells altered the biological response to HGF/SF, although the impact on DNA synthesis was either positive or negative depending on the assay system (34–37). Recent evidence indicated that heparin...
stimulated dimerization of HGF/SF and HGF/NK1 in a cell-free setting, raising the possibility that a similar effect on the cell surface could facilitate Met dimerization and activation (25, 37) as suggested for FGF-1 and FGF-2 (5, 9, 10).

In the present study, we have investigated the hypothesis that heparin binding of HGF/SF isoforms is dependent on K2 and specific basic amino acid residues in the hairpin loop of the N domain. We also provide new evidence that HSGAG is required for the binding of HGF/SF variants to Met and subsequent signal transduction. Finally, we demonstrate that heparin stimulates the oligomerization of HGF/SF isoforms in both cell-free and cell culture models, and that the N domain has an important role in this process.

EXPERIMENTAL PROCEDURES

Cell Culture—BALB/MK mouse epidermal keratinocytes were maintained as described previously (38, 39). Madin-Darby canine kidney cells (MDCK), kindly provided by Dr. R. Purlong (ICRF, Cambridge University Medical School, Cambridge, United Kingdom) were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Biofluids). Chinese hamster ovary (CHO) cell lines, both wild-type (WT) and mutant pgaA-745 (hereafter designated CHO-745), which is defective in glycosaminoglycan chain initiation (40), were grown in Ham's F-12 medium (Biofluids) supplemented with 5% fetal calf serum (Biofluids) and 0.1 ml was applied to heparin-Sepharose CL-6B resin (Pharmacia Biotech Inc., catalog no. sc-162) for 2 h at 4 °C. After immunoprecipitation, SDS-PAGE and transfer to Immobilon filters, blotting was performed with anti-mouse Met polyclonal antiserum (1 μg/ml; Santa Cruz Biotechnology, Inc.) for 2 h at 4 °C, lysed in anti-phosphotyrosine RIPA buffer (20 mM Tris-HCl, pH 7.5, with 1% Nonidet P-40, plus protease inhibitors, pH 7.5), and immunoprecipitated with anti-phosphotyrosine monoclonal antibody 4G10 bound to agarose beads (50 μg/ml of beads/lystate; Upstate Biotechnology, Inc.). Immunoprecipitated proteins were resolved by 7.5% SDS-PAGE and immunoblotted for mouse Met as described above.

Heparin-Sepharose Chromatography—HGF/SF and its derivatives were treated with HGF/NK1 (100 ng/ml) and/or heparin (0.3 μg/ml) for 5 min at 37 °C, lysed in anti-phosphotyrosine RIPA buffer (20 mM Tris-HCl, pH 7.5) containing 1 μg/ml of Met monoclonal antibody 4G10 bound to agarose beads (50 μg/ml of beads/lystate; Upstate Biotechnology, Inc.), and immunoprecipitated with antiphosphotyrosine monoclonal antibody 4G10 bound to agarose beads (50 μg/ml of beads/lystate; Upstate Biotechnology, Inc.). Immunoprecipitated proteins were resolved by 7.5% SDS-PAGE and immunoblotted for mouse Met as described above.

c-fos Gene Expression in CHO-WT and CHO-745 Cells—Cell cultures were serum-starved overnight, exposed to 100 ng/ml HGF/NK1 and/or heparin at either 0.3 or 3 μg/ml for 5 min at 37 °C, lysed in anti-phosphotyrosine RIPA buffer (20 mM Tris-HCl, pH 7.5) containing 1 μg/ml of Met monoclonal antibody 4G10 bound to agarose beads (50 μg/ml of beads/lystate; Upstate Biotechnology, Inc.), and immunoprecipitated with antiphosphotyrosine monoclonal antibody 4G10 bound to agarose beads (50 μg/ml of beads/lystate; Upstate Biotechnology, Inc.). Immunoprecipitated proteins were resolved by 7.5% SDS-PAGE and immunoblotted for mouse Met as described above.

RESULTS

N Domain Retains Heparin-Binding Properties of Full-length HGF/SF—To analyze the structural features of HGF/SF responsible for its interaction with heparin, we determined the NaCl concentration required to elute various HGF/SF isoforms and derivatives from heparin-Sepharose resin. Immunoblotting of fractions from each step in the salt gradient revealed that the N domain eluted at 0.9 M NaCl (Fig. 1). To further localize the heparin-binding site of HGF/SF, we produced the N and K1 domains separately in a bacterial expression system, and purified them to homogeneity. Blotting was performed with anti-mouse Met polyclonal antiserum (0.5 μg/ml; Sigma Chemical Co.) for 2 h at 4 °C. After immunoprecipitation, SDS-PAGE and transfer to Immobilon filters, blotting was performed with anti-mouse Met polyclonal antiserum (0.5 μg/ml; Sigma Chemical Co.) for 2 h at 4 °C.

Biological Assays—DNA synthesis by BALB/MK cells was measured by [(H)]thymidine incorporation as described (45), except that IGF-1 (200 ng/ml; Pepro Tech Inc., catalog no. 100-11) was included in the assay medium. The scatter assay was performed with a subclone of MDCK cells, according to published methods (46). After 20 h, the cells were fixed for 20 min with 2.5% formaldehyde, and for an additional 20 min with methanol/formaldehyde/phosphate-buffered saline (7/2/1) prior to staining with Giemsa (pH 6.8).

HGF/NK1 Radioliodination and Covalent Affinity Cross-linking—Recombinant human HGF/NK1 was radiolabeled with [125I]NaI (Amersham) by the chloramine-T method as described (44). Cross-linking was performed with 10 μg of protein and 1 mCi of [125I]NaI yielded tracer with a specific activity of ~65 μCi/μg.

Cross-linking was performed as described (44), with the following modifications. Confluent serum-starved monolayers were incubated for 45 min at room temperature in HEPES binding buffer (44) containing 0.9 M NaCl, 25 μM EDTA, 10 μM NaF, 10 μM sodium pyrophosphate, 1 mM sodium vanadate, 1% Nonidet P-40, plus protease inhibitors (pH 7.5). After immunoprecipitation, SDS-PAGE and cross-linking complexes were detected by autoradiography of dried gels exposed to Kodak X-Omat AR film at -70 °C. Met Autophosphorylation—Confluent monolayers (10-cm dishes) of CHO-WT and CHO-745 cells were serum-starved overnight, exposed to 100 ng/ml HGF/NK1 and/or heparin at either 0.3 or 3 μg/ml for 5 min at 37 °C, lysed in anti-phosphotyrosine RIPA buffer (20 mM Tris-HCl, pH 7.5) containing 1 μg/ml of Met monoclonal antibody 4G10 bound to agarose beads (50 μg/ml of beads/lystate; Upstate Biotechnology, Inc.), and immunoprecipitated with antiphosphotyrosine monoclonal antibody 4G10 bound to agarose beads (50 μg/ml of beads/lystate; Upstate Biotechnology, Inc.). Immunoprecipitated proteins were resolved by 7.5% SDS-PAGE and immunoblotted for mouse Met as described above.

Cell-free Cross-linking/Oligomerization Analysis—Experiments were performed according to a protocol similar to one described in Ref. 25. 1 μg HGF/NK1, N, or K1 domain was incubated in 20 μl of PBS for 20 min at room temperature, with or without cross-linking reagent bis(sulfosuccinimidyl) suberate (1 mM; Pierce) in the presence or absence of heparin (1 μg/ml). Next, 5 μl of 1 x Tris-HCl, pH 6.8, was added, followed by 6 μl of 5 x SDS-sample buffer containing 10% β-mercaptoethanol. After boiling for 3 min, samples were resolved by SDS-PAGE in a 4–20% mini-gel (Novex). Protein was detected by staining with coomassie blue (Novex).

RESULTS

N Domain Retains Heparin-Binding Properties of Full-length HGF/SF—To analyze the structural features of HGF/SF responsible for its interaction with heparin, we determined the NaCl concentration required to elute various HGF/SF isoforms and derivatives from heparin-Sepharose resin. Immunoblotting of fractions from each step in the salt gradient revealed that the N domain eluted at ~0.2 M NaCl, while the N domain eluted at

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the same concentration as HGF/SF (Fig. 1). These data suggest that the determinants of HGF/SF heparin binding are confined to the N domain.

Modification of Basic Residues in the Hairpin Loop Has Only a Slight Effect on Heparin Binding, but a Significant Impact on Biological Activity—In their investigation of the sites required for heparin binding, Mizuno et al. (32) observed that deletion of the hairpin loop region (amino acid residues 70–96) in the N domain resulted in loss of heparin binding. After comparing the heparin-binding properties and hairpin loop sequences of HGF/SF and the structurally related molecules, plasminogen (14) and macrophage-stimulating protein (15), they noted a correlation between the net charge of the hairpin region and avidity for heparin. Specifically, the net charge of this region in HGF/SF was +6 and the protein eluted from heparin-Sepharose with ~1.0 M NaCl, while the corresponding values of these parameters for plasminogen were ~2.5 and ~0.1 M NaCl, and for macrophage-stimulating protein were +2 and ~0.4 M NaCl. This led to the suggestion that clusters of basic amino acid residues in the HGF/SF hairpin loop mediated the interaction with heparin. We tested this hypothesis directly by substituting alanine for these residues in a series of site-directed mutants. As illustrated in Fig. 2, HGF/NK1 derivatives were generated that had 2, 3, or 5 alanine substitutions, as follows: R76A and K78A (2A/NK1), K91A, R93A, and K94A (3A/NK1), or a combined set (5A/NK1). When these reagents were chromatographed on heparin-Sepharose, they all bound tightly to the resin and eluted either with 0.6–0.8 M (2A/NK1 and 5A/NK1) or 0.8–1.0 M (3A/NK1) NaCl (Fig. 1). Although replacement of R76 and K78 resulted in slightly earlier elution of protein, our findings indicated that the five basic residues did not have a critical role in heparin binding of HGF/SF isoforms.

The effect of alanine substitution on HGF/SF biological activity was evaluated by testing the 2A, 3A, and 5A/NK1 derivatives in a[^3H]thymidine incorporation bioassay using BALB/MK cells (Fig. 3A). 5A/NK1 was approximately 30-fold less potent than wild-type HGF/NK1 in this assay. This decrease in activity could not be attributed to the small reduction in heparin affinity associated with this molecule, because 2A/NK1, which exhibited a comparable decline in affinity to heparin-Sepharose, was as active as wild type HGF/NK1. Moreover, the derivative that most closely retained the heparin-binding characteristics of HGF/SF, 3A/NK1, was markedly less potent than HGF/NK1 in the mitogenic bioassay. A similar pattern of relative potency was observed with these reagents in the standard scatter assay using MDCK cells (Fig. 3B). At a concentration where wild type HGF/NK1 and 2A/NK1 elicited a marked scatter response, 3A/NK1 and 5A/NK1 stimulated only modest cellular retraction and fewer signs of the spiky morphology characteristic of the scatter phenomenon (46). Preliminary experiments suggest that the diminished activity of 3A/NK1 and 5A/NK1 may result from reduced stability of these derivatives during overnight incubation at 37°C. Nonetheless, regardless of the loss in biological activity, alanine substitution of the HGF/SF-specific basic residues in the hairpin loop had little impact on heparin binding.

Proteoglycan Is Important for Binding of HGF/NK1 to Met—In addition to characterizing the structural elements responsible for heparin binding of HGF/SF isoforms, we also explored the relevance of the heparin interaction for ligand binding to Met. To address this issue, we performed a series of experiments with wild type and glycosaminoglycan-deficient CHO cells (CHO-745). The latter are lacking in enzymatic activity required for synthesis of heparan sulfate (40), and have been used extensively to analyze the role of HSGAG in FGF-

![Fig. 1. Heparin-Sepharose chromatography of HGF/SF isoforms and derivatives. Various HGF/SF-related polypeptides were applied in parallel to columns containing 0.3 ml of heparin-Sepharose resin equilibrated in PBS. After the resins were washed twice with 1.5 ml of PBS, protein was eluted with a stepwise gradient of increasing NaCl molar concentrations. Aliquots of starting material (lane 1), eluted fractions (lanes 2–10), and heparin-Sepharose beads following chromatography (lane 11) were processed for SDS-PAGE and immunoblotting as described under “Experimental Procedures.” Molecular size markers are indicated at the right.](http://www.jbc.org/)

![Fig. 2. Amino acid sequences of hairpin loop region from wild type human HGF/NK1 and site-directed mutants. Positively charged residues are indicated by + in the wild type (WT) sequence, and sites of alanine substitutions are designated by shaded circles labeled A in the mutant sequences (2A, 3A, and 5A). Residues in the wild type sequence are numbered according to Rubin et al. (36).](http://www.jbc.org/)
protein
deficient CHO strains expressed similar levels of hamster Met
cells (Fig. 4). Moreover, the wild type and glycosaminoglycan-
deficient CHO cells. Lysates from BALB/MK, CHO-WT, and CHO-745 cells were immunoprecipitated and immuno-
blotted with an antiserum raised against the carboxyl-terminal se-
quence of murine Met. The arrow indicates a 145-kDa protein band
corresponding to the Met β-subunit in murine and hamster cells.

Covalent cross-linking experiments performed with 125I-
HGF/NK1 and BALB/MK or CHO cells revealed a high mole-
cular mass band with an estimated mass of 165 kDa in lysates of
BALB/MK and wild type CHO cells (Fig. 5A). However, no
band was observed in lysates from the glycosaminoglycan-de-
ficient CHO-745 cells. The ligand specificity of this binding
interaction was verified by competition with an excess of unla-
belled HGF/NK1 but not IGF-I (Fig. 5A). Immunoprecipitation
of the cross-linked complex with mouse Met peptide antiserum
in the absence but not presence of peptide confirmed that Met
was in the complex (Fig. 5B). To demonstrate that the lack of
HGF/NK1-Met cross-linking in CHO-745 cells was due to a
deficiency in HSGAG, the experiment also was performed in
the presence of varying concentrations of added soluble hepa-
rin. Consistent with earlier observations (33), when heparin
was added to cells such as BALB/MK and wild type CHO,
which have an ample supply of HSGAG on their surfaces,
cross-linking of ligand to Met was diminished (Fig. 5A). In
contrast, heparin at 0.3 μg/ml (and to a lesser extent at 3
μg/ml) facilitated cross-linking of 125I-HGF/NK1 to Met in the
glycosaminoglycan-deficient CHO-745 cells. These results es-

established that the shortage of endogenous HSGAG in CHO-745
cells was specifically responsible for the paucity of their HGF/
NK1-Met binding.

Signal Transduction Is Enhanced by Addition of Heparin to
Glycosaminoglycan-deficient Cells—To explore the functional
significance of HGF/NK1 binding to Met observed in response
to exogenous heparin, we measured tyrosine phosphorylation
of Met following treatment of CHO cells with HGF/NK1 in the
presence or absence of heparin. As shown in Fig. 6, incubation
of wild type CHO cells with HGF/NK1 for 5 min resulted in a
prominent Met protein band in the anti-phosphotyrosine immu-
no precipitate. When heparin also was present in the HGF/
NK1-containing medium, there was little or no additional stim-
ulation. In contrast, while HGF/NK1 and heparin each by itself
caus ed only a slight increase in the amount of Met immuno-
precipitated from CHO-745 cells by phosphotyrosine antibody,
together they stimulated a large increase in the amount of
immunoprecipitated Met protein. This result strongly sug-
gested that the addition of heparin to glycosaminoglycan-defi-
cient CHO cells not only enhanced HGF/NK1 binding to Met,
but also triggered tyrosine kinase activity.

Further evidence of signaling was obtained by analysis of
c-fos expression. c-fos induction is a sensitive measure of rapid

cellular responses to mitogenic stimulation (51), which served
as a convenient indicator of HGF/NK1 signaling. When wild-
type CHO cells were incubated with HGF/NK1 at 100 ng/ml, an
increase in c-fos transcript was observed after 15 min and was
more pronounced at 60 min (Fig. 7). Heparin alone did not
stimulate c-fos expression, and had only a modest positive
effect when tested in combination with HGF/NK1 on CHO-WT
cells. In contrast, HGF/NK1 alone stimulated only a slight
increase in c-fos expression by CHO-745 cells after either 15 or
60 min. However, when heparin (3 μg/ml) was included in the
incubation medium, c-fos induction in CHO-745 was much
stronger after 60 min, comparable to that observed with the
wild-type cells. Heparin itself had no effect on c-fos expression
in this assay. These findings indicated that the heparin-de-
pend ent interaction of HGF/NK1 with Met in CHO-745 cells
was capable of activating the signal transduction process.
Heparin Binding and Oligomerization of HGF/SF Isoforms

FIG. 5. Covalent affinity cross-linking of 125I-HGF/NK1 to Met in BALB/MK, CHO-WT, and CHO-745 cells. A, cross-linking of 125I-HGF/NK1 in the absence or presence of 25 nM unlabeled HGF/NK1, heparin (0.3 or 3.0 μg/ml), or 25 nM IGF-I. Following incubation with tracer and the indicated reagents, cells were treated with diisocyanatodiphenyl suberate and subsequently lysed. Proteins were resolved by 6% SDS-PAGE and gels dried for autoradiography. B, Met specificity of 125I-HGF/NK1 cross-linking. After cross-linking 125I-HGF/NK1 to three cell lines as described in A, 10% of each lysate was removed and the remainder divided in half for immunoprecipitation with Met antisera in the absence (−Met) or presence (+Comp) of competing peptide (10 μg/ml). For CHO-745, cross-linking was performed in the presence of heparin at 0.3 μg/ml. In both A and B, the arrow indicates a 165-kDa band corresponding to 125I-HGF/NK1-Met complex with an apparent stoichiometry of 1:1. Positions of molecular mass markers are at right.

N Domain Mediates Heparin-induced Ligand Oligomerization—Because of earlier reports that heparin enhanced the activity of FGFs (8–10) and HGF/SF (37) by promoting ligand oligomerization, we investigated the possibility that the positive impact of heparin or cell surface heparan sulfate proteoglycan on Met-ligand binding and signaling might involve oligomerization of HGF/SF isoforms. We first examined the effect of heparin on HGF/NK1 oligomerization in a cell-free setting. In the absence of heparin, there was little evidence of cross-linked HGF/NK1 dimer as detected by Coomassie Blue staining after SDS-PAGE. However, when heparin was present during the incubation with cross-linking agent, subsequent analysis revealed that a substantial portion of HGF/NK1 protein migrated as a dimer (Fig. 8, left panel). Weak bands corresponding to higher order oligomers occasionally were also seen. When similar experiments were performed with separately prepared recombinant N and K1 domains, the ability to oligomerize correlated unequivocally with the N domain. As shown in the middle panel of Fig. 8, a small amount of putative N domain dimer was evident in the absence of heparin, and in the presence of heparin a set of bands were observed which represent a series of oligomers. Frequently the series included bands corresponding in size to hexamers and even octamers. In contrast, there was no evidence of oligomerization of the K1 domain corresponding to 125I-HGF/NK1-Met complex with an apparent stoichiometry of 1:1. Positions of molecular mass markers are at right.

FIG. 6. Tyrosine phosphorylation of Met in response to HGF/NK1 and/or heparin in CHO-WT and CHO-745 cells. Serum-starved cells were treated with 100 ng/ml HGF/NK1, 3 μg/ml heparin, both, or neither reagent for 5 min at 37 °C. Whole cell lysates were immunoprecipitated with phosphotyrosine antibody bound to agarose beads, resolved by 6% SDS-PAGE, blotted, and probed with Met antisera. The band migrating at 145 kDa corresponds to the β-subunit of Met; the positions of molecular mass markers are at right.

either in the absence or presence of heparin (right panel, Fig. 8).

Ligand oligomerization also was observed when incubations were performed with cells in culture. Cross-linked 125I-HGF/NK1 dimer was readily detected when tracer was added to BALB/MK or CHO-WT cells, but was far less conspicuous with the glycosaminoglycan-deficient CHO-745 cells (Fig. 9). The addition of 25 nM unlabeled HGF/NK1 increased the amount of dimer in lysates of all three cell types, and even trimer and tetramer were seen with BALB/MK and CHO-WT cells. Exogenous heparin only promoted oligomer formation in CHO-745 lysates, while it inhibited oligomerization in cultures of cells having normal levels of proteoglycan. These findings suggested that a moderate amount of heparin or endogenous heparan sulfate proteoglycan could facilitate oligomerization on the cell surface, but that excessive heparin would block this process.

DISCUSSION

Several reports suggest that the interaction of heparin-binding growth factors with heparan sulfate proteoglycan has a major impact on their biological effects (2–5, 7–10, 25, 34–37). In practical terms, the clinical utility of such growth factors may be strongly influenced by their binding to the proteoglycan on blood vessel walls and in extracellular matrix, which could hamper delivery of factor to cellular targets. The present study was undertaken to identify the heparin-binding site(s) of HGF/SF and investigate the role of heparin in growth factor binding and signal transduction. Mapping of the primary hep-
In designing mutants with altered heparin affinity, such derivatives would be useful in testing the importance of the proteoglycan interaction for growth factor activity and might have greater efficacy in therapeutic applications.

Previous analyses of HGF/SF deletion mutants had implicated the N domain (31) and more specifically the hairpin loop (32) in heparin interactions. However, in deletion studies loss of function could result not only from removal of the targeted domain but also from improper folding of the residual protein that could reduce its stability or alter tertiary structure. For instance, the K2 deletion mutant also reportedly bound poorly to heparin (32), suggesting a critical role for K2 in this interaction that was inconsistent with our results. By showing that the N domain retained the binding properties of full-length HGF/SF, we directly demonstrate that the heparin-binding site of HGF/SF resides in this domain.

Because the heparin-binding motifs of many proteins involve basic amino acid residues clustered in a linear sequence (52, 53), the proposed role of two such sites in the hairpin region of the N domain was tested. The failure of alamine substitution at these positions to have much impact on the interaction with heparin-Sepharose suggested that other residues must be responsible. Two clusters of basic residues near the amino terminus of the N domain, RKRR (residues 33–36) and KKSAK (residues 43–47), probably can be excluded from consideration because corresponding sequences were absent from a truncated form of rat HGF/SF, which bound tightly to heparin-Sepharose (14, 54). Two other segments, KIKTKK (residues 58–63) and KK (residues 109–110), are worthy of investigation, as well as other nearby residues that may contribute to heparin binding (52, 53, 55).

While our evidence that HSGAG is important for Met-ligand binding complements other recent work, it would appear to be in conflict with earlier observations made in a cell-free setting. When Mark et al. (56) detected high affinity binding of HGF/SF isoforms to a fusion protein containing the extracellular domain of Met and the heavy chain Fc portion of immunoglobulin G, no heparin or HSGAG had been added to the assay. These ostensibly contradictory observations could be reconciled in several ways. First, HSGAG may have inadvertently co-purified with the Met-IgG fusion protein, and consequently been present in the cell-free assay. Such co-purification is plausible given the evidence that the extracellular domain of another heparin-binding growth factor, tyrosine kinase receptor can itself bind heparin (6). Second, it is possible that nonspecific adsorption of HGF/SF isoforms in the solid phase binding assay used by Mark et al. (56) resulted in high local concentrations of ligand, obviating one of the potential mechanisms of HSGAG action in promoting high affinity receptor binding (5). Third, heparin-dependent protection of ligand from proteolytic degradation may not have been important in a cell-free binding assay, where proteases presumably are scarce. Finally, if the HSGAG requirement for Met-ligand binding involves receptor dimerization as has been suggested both for FGFs (5, 8–10) and HGF/SF isoforms (25, 37), this probably would have been unnecessary in the cell-free assay because immunoglobulin fusion proteins typically already exist as dimers.

The present findings indicated that heparin or endogenous HSGAG was required both for binding and activation of receptor signaling. The potent stimulation of tyrosine phosphorylation and c-fos expression in CHO-745 cells elicited by the combination of HGF/NK1 and exogenous heparin established the biological relevance of heparin-dependent binding reconstituted in these cells. Occasionally weak responses to HGF/NK1 alone were observed in CHO-745 cells, presumably due to a low level of receptor binding that was below the sensitivity of detection by cross-linking analysis. Such binding might arise either from small amounts of endogenous HSGAG on CHO-745 cells or occur independent of HSGAG. Similarly, heparin itself at times appeared to elicit a minor tyrosine phosphorylation signal. While not a consistent finding, this may reflect a capacity of the glycosaminoglycan to promote tyrosine phosphorylation, as has been reported in cells expressing the tyrosine kinase receptor FGFR-4 (57).

Our results emphasized the importance of heparin not only for Met binding but also ligand oligomerization. As recently reported for HGF/NK1 (25), cross-linking experiments performed in a cell-free setting suggested that HGF/NK1 and especially the N domain itself have an intrinsic tendency to oligomerize. This predisposition is markedly enhanced by the addition of heparin. For the N domain, a series of oligomers ranging up to octamers formed in the presence of heparin. When 125I-labeled HGF/NK1 (Fig. 9) or N domain3 was incubated with cells and cross-linking agent, oligomers again were observed if endogenous heparan sulfate proteoglycan or added heparin was present. Because the latter experiments were

FIG. 8. Oligomerization of HGF/NK1, N, and K1 in a cell-free setting. Cross-linking reactions were performed as described under “Experimental Procedures.” Protein was incubated alone or with cross-linking agent bis(sulfosuccinimidyl)suberate in the absence or presence of heparin. Subsequently, protein was resolved by SDS-PAGE and detected by staining with colloidal Coomassie Blue. In addition to positions of molecular mass markers are at the right.

FIG. 9. Oligomerization of 125I-HGF/NK1 in cell culture. Covariant cross-linking of HGF/NK1 to itself was analyzed by incubating tracer and cross-linking agent with BALB/MK, CHO-WT, or CHO-745 cells in the presence or absence of various combinations of unlabeled HGF/NK1 (500 ng/ml) and heparin (0.3 or 5 μg/ml). After the reaction was terminated, cell lysates were resolved by SDS-PAGE and labeled proteins detected by autoradiography of dried gel. Position of HGF/NK1 dimer (a), trimer (b), and tetramer (c) is indicated by label at left; location of molecular mass markers is shown at right.
performed with whole cells and ng/ml concentrations of tracer, the potential physiological relevance of ligand oligomerization was reinforced. Of note, the effect of exogenous heparin on ligand oligomerization varied with cell type: while heparin increased dimer formation on glycosaminoglycan-deficient CHO cells, it reduced oligomerization on BALB/MK and wild type CHO cells. In the latter instances, HGF/SF ligands probably were dispersed among the abundant heparin molecules such that ligand-ligand interactions were less likely to occur. Thus, the impact of heparin on oligomerization is dependent on the content of endogenous HSGAG. If ligand oligomerization is important for signal transduction, the effect of added heparin on the biological activity of HGF/SF ligands will vary with the quantity and perhaps composition of proteoglycan on the cell surface.

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Heparin Binding and Oligomerization of Hepatocyte Growth Factor/Scatter Factor Isoforms: HEPARAN SULFATE GLYCOSAMINOGLYCAN REQUIREMENT FOR Met BINDING AND SIGNALING

Hiromi Sakata, Stephen J. Stahl, William G. Taylor, Jared M. Rosenberg, Kazushige Sakaguchi, Paul T. Wingfield and Jeffrey S. Rubin

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