Dissociation of Heparan Sulfate and Receptor Binding Domains of Hepatocyte Growth Factor Reveals That Heparan Sulfate-c-Met Interaction Facilitates Signaling*

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Hepatocyte growth factor (HGF) is a secreted, heparan sulfate (HS) glycosaminoglycan-binding protein that stimulates mitogenesis, motogenesis, and morphogenesis in a wide array of cellular targets, including hepatocytes and other epithelial cells, melanocytes, endothelial cells, and hematopoietic cells. NK1 is an alternative HGF isoform that consists of the N-terminal (N) and first kringe (K1) domains of full-length HGF and stimulates all major HGF biological activities. Within NK1, the N domain retains the HS binding properties of full-length HGF and mediates HS-stimulated ligand oligomerization but lacks significant mitogenic or motogenic activity. In contrast, K1 does not bind HS, but it stimulates receptor and mitogen-activated protein kinase activation, mitogenesis, and motogenesis, demonstrating that structurally distinct and dissociable domains of HGF are the primary mediators of HS binding and receptor activation. Despite the absence of HS-K1 binding, K1 mitogenic activity in HS-negative cells is strictly dependent on added soluble heparin, whereas K1-stimulated motility is not. We also found that, like the receptors for fibroblast growth factors, the HGF receptor c-Met binds tightly to HS. These data suggest that HS can facilitate HGF signaling through interaction with c-Met that is independent of HGF-HS interaction and that the recruitment of specific intracellular effectors that mediate distinct HGF responses such as mitogenesis and motility is regulated by HS-c-Met interaction at the cell surface.

Hepatocyte growth factor (HGF) is a secreted protein that stimulates proliferation, migration, and/or morphological differentiation of a wide variety of cellular targets, including hepatocytes and epithelial cells, melanocytes, endothelial cells, and hematopoietic cells (1). HGF shares several structural motifs and 38% amino acid sequence identity with plasminogen (2, 3). Each is synthesized as a single polypeptide chain that is cleaved at a conserved site to generate a biologically active, disulfide-linked heterodimer (4–6). The heavy chain of the dimer (~60 kDa in HGF) is derived from the N terminus of the precursor and contains multiple kringe domains (K) (four in HGF, five in plasminogen). Kringle domains (~80 amino acids) have a characteristic folding pattern determined by three internal disulfide bonds and additional conserved sequences (7). The HGF light chain (~34 kDa), like that of plasminogen, has the structure of a serine protease, but two nonconservative substitutions within the catalytic triad render HGF devoid of proteolytic activity (2).

The HGF gene encodes three distinct proteins: full-length HGF and two truncated HGF isoforms (NK1 and NK2) that consist of the N-terminal domain (N) linked in tandem with the first one (K1) or two (K1+K2) kringle domains, respectively (Fig. 1). NK1 appears to stimulate all of the biological activities of full-length HGF, whereas NK2 retains only mitogenic activity (8–10). All three isoforms bind to the HGF receptor c-Met, and NK2 appears to specifically activate a subset of the c-Met intracellular signaling pathways activated by HGF or NK1 binding (11).

HGF, NK1, and NK2, expressed in Escherichia coli, are misfolded and inactive, but controlled refolding of NK1 and NK2 resulted in complete reconstitution of their biological activities (9). Prokaryotic expression and refolding also facilitated the biological characterization of the HGF N domain. N retains the heparin binding properties of full-length HGF, and experiments performed with wild-type and HS-deficient CHO cells revealed that HS was essential for c-Met-NK1 interaction (12). Addition of heparin (a commercially available, commonly used substitute for HS glycosaminoglycans) to HS-deficient CHO cells not only restored ligand binding, but it also increased ligand-dependent c-Met activation and c-fos expression (12). These studies suggested that HS facilitated ligand oligomerization through interaction with the N domain and thereby enhanced receptor activation.

Using prokaryotically expressed and refolded protein, the three-dimensional solution structure of the HGF N domain was solved (13). The topology of the N-domain structure is unusual; the only similar structure found so far is the HS binding domain of vascular endothelial cell growth factor (14). Analysis of the surface charge distribution of N-domain, and extensive characterization of HS-N domain interaction using nuclear magnetic resonance spectroscopy have led to the identification of the HS binding site (13, 15). Recently, the crystal structure...
of NK1 was solved, revealing a dimer with only a few contacts between the N and K1 domains of each NK1 monomer; the dimer interface was composed of contacts between the linker peptides joining N and K1 (16, 17). This structural arrangement suggests that some degree of flexibility exists between N and K1, as well as some independence in their interactions with HS and c-Met.

Recombinant, refolded HGF K1 (residues 123–210) has also been expressed in bacteria, and circular dichroism studies revealed that its overall conformation is indistinguishable from the K1 domain of biologically active, intact NK1 (9). Unlike N domain, K1 was not retained by immobilized heparin and did not self-associate to form cross-linkable oligomers in solution in the absence or presence of heparin (12). Here, we report the biological properties of the isolated K1 domain: at concentrations of 30 nM or more, K1 stimulates receptor tyrosine kinase activation, MAP kinase activation, cell scatter, and DNA synthesis in HGF-responsive epithelial cells. Thus, structurally distinct domains of HGF appear to be the primary mediators of heparin binding and receptor activation.

We also examined signaling by K1 in 32D/c-Met cells, which lack cell surface HS. In that system, added heparin facilitates HGF signaling (11), through a mechanism that was presumed to involve its demonstrated ability to enhance HGF oligomerization (12, 18, 19). Despite its inability to bind heparin, we found that K1 mitogenic signaling in 32D/c-Met cells was completely heparin-dependent. These results suggested that HS may facilitate HGF signaling through a mechanism other than HGF-HS binding, such as a direct HS-c-Met interaction. Fibroblast growth factor (FGF) signaling has been shown to require not only the well characterized FGF-HS binding (20–22) but also an interaction between FGF receptor and HS (23). Crystalllographic analysis of FGF-FGF receptor interaction also supports a model in which HS binds to both ligand and receptor (24). Interestingly, c-Met shared several properties with other well studied heparin-binding proteins, and a comparison of the HS binding properties of a c-Met-Fc fusion protein and the HGF isoform NK1 confirmed the existence of a HS binding site(s) within the extracellular domain of the receptor. In contrast to K1-stimulated proliferation, K1-stimulated cell motility was completely HS-independent in the same HS-negative cell line. Together, these results suggest that HS facilitates HGF signaling through interactions with both HGF and c-Met and that the recruitment of intracellular effectors that mediate distinct cellular responses is regulated by HS-c-Met interaction at the cell surface.
Seaphorose-immobilized c-Met was washed five times in 300 volumes of cold lysis buffer before displacement with NaCl, soluble heparin or chondroitin sulfate. The amount of c-Met remaining immobilized throughout this treatment was visualized after SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-c-Met as described above.

ELISA of HS-c-Met Interaction—Purified NK1 (10 μg/ml, 50 μl), c-Met-Fc, or K1 (15 μg/ml; 50 μl) were added to a 96-well plate for 2 h (all incubations were at room temperature). Wells were decanted, blocked with carrier solution (4% bovine serum albumin in phosphate-buffered saline + Na3VO4) for 1 h, washed five times with TAPS (0.05% Tween 20 + 0.02% Na3VO4 in phosphate-buffered saline), and then incubated with biotin-HS (Neoparin) for 2 h. The wells were washed, incubated for 1 h with streptavidin-alkaline phosphatase (1 μg/ml in phosphate-buffered saline), washed again, and incubated with p-nitrophenyl phosphate (2 mg/ml in 1 mM MgCl2, 0.1 mM Na3VO4, pH 9.8) before measuring absorbance at 405 nm. For saturation binding experiments, serial dilutions of biotin-HS were added to wells in the presence or absence of 100-fold excess unlabeled HS (Neoparin); for competitive binding experiments, various concentrations of unlabeled HS (Neoparin, Calbiochem, or Seikagaku) were added with a single concentration of HS-biotin.

RESULTS

K1 Stimulates Biological Activity in HGF Target Cells—The ability of recombinant, prokaryotically expressed and refolded HGF K1 (residues 129–210) to stimulate DNA synthesis in Balb/MK keratinocytes was compared with that of full-length HGF, NK1, and N domain (Fig. 2). K1 mitogenic stimulation was dose-dependent and 100-fold less potent than that observed in response to NK1 (50% maximum at 3 μg/ml versus 30 ng/ml; Fig. 2A and data not shown). Interestingly, high doses of K1 were capable of stimulating the same maximum level of DNA synthesis as full-length HGF, whereas N-domain was relatively inactive under the same conditions (Fig. 2B). The effect of treatment of Balb/MK cells with both K1 and N domains simultaneously was similar to the effects of K1 alone, demonstrating that the potency of NK1 or HGF could not be reconstituted by its non-covalently bound components (Fig. 2B and data not shown).

K1 activated the HGF receptor tyrosine kinase, c-Met, in Balb/MK cells, although the kinetics of receptor activation appeared to be modestly delayed relative to activation stimulated by full-length HGF or by NK1 (Fig. 3A). These results are consistent with a direct interaction between K1 and the HGF receptor that is of lower affinity than receptor interaction with HGF or NK1. Under identical conditions, little or no receptor activation was stimulated by N domain (Fig. 3A). Similarly, K1 stimulated MAP kinase activation, whereas N domain did not (Fig. 3B). Consistent with their relative abilities to stimulate DNA synthesis and c-Met activation, K1 stimulated MAP kinase activation less potently than NK1 or HGF (Fig. 3B). N and K1 added together had no greater effect on MAP kinase activation than K1 alone (Fig. 3B).

The relative abilities of N and K1 to stimulate cell migration were compared using two different cellular targets and assay systems: MDCK cell scatter (31) and 32D/c-Met cell migration in a semipermeable barrier (11). As shown in Fig. 4, high doses of K1 stimulated MDCK cell scatter similar to that observed following NK1 treatment, whereas under the same conditions, N domain had no obvious effect relative to untreated controls. The potency of K1 in the MDCK cell scatter assay was ~30-fold lower than that of NK1, consistent with the suspected lower affinity of K1-c-Met interaction.

The murine interleukin 3-dependent hematopoietic cell line 32D expresses a very low level of c-Met and responds poorly to...
stimulation with human HGF. Ectopic expression of human c-Met in 32D cells (32D/c-Met) at levels similar to normal human epithelial cells reconstitutes potent human HGF signaling (11). Anchorage-independent 32D/c-Met cells traverse semipermeable membranes in response to HGF and NK2 (11), and this system was also used to compare cell migration responses to NK1, K1, and N (Fig. 5). In this system, K1 was only marginally less potent than NK1, whereas the effects of N domain were not significant (Fig. 5). The relative potency of K1 and NK1 parallels earlier observations made with this system, where NK2 and HGF showed similar potency in stimulating 32D/c-Met cell migration; in contrast, HGF was 10–30-fold more potent than NK2 in stimulating epithelial cell migration (11).

**K1 Mitogenic Activity Is HS-dependent in HS-deficient 32D/c-Met Cells**—Another feature of the 32D/c-Met cell system is that it lacks abundant cell surface HS. Thus, like other HS-deficient cell types, added soluble heparin can enhance signaling by HS-binding ligands (12, 19). The potency of HGF in stimulating DNA synthesis by 32D/c-Met cells was increased at least 3-fold in the presence of added soluble heparin (11) (Fig. 6B). Similar results were obtained using NK1 (data not shown). We examined K1-stimulated mitogenic activity in 32D/c-Met cells with the expectation that it would be HS-independent, in keeping with the lack of heparin binding by isolated K1. However, initial experiments suggested that K1 alone had little activity in this system, so K1 was tested over a wide range of added heparin concentrations (Fig. 6A). Whereas K1 activity was barely detectable in the absence of heparin, [3H]thymidine incorporation rose dramatically with increasing heparin concentrations (Fig. 6). Heparin alone was not mitogenic at any concentration tested (Fig. 6A). Heparin concentrations of 30 μg/ml or higher maximally promoted K1 signaling, and this heparin concentration was used to test the dose-responsiveness of K1 mitogenic activity in this system (Fig. 6B). In the presence of added heparin K1 was ~10-fold less potent than HGF without heparin and 30-fold less potent than HGF with heparin (Fig. 6B). At a concentration of 3 μg/ml, K1 reached ~75% of the maximum level of HGF-stimulated DNA synthesis (Fig. 6B).

Heparin is thought to facilitate signaling by heparin-binding ligands during early events such as receptor-ligand interaction and receptor activation. Therefore, we examined the effects of added heparin on the ability of K1 to stimulate c-Met autophosphorylation in intact 32D/c-Met cells. We found that the effect of added heparin was apparent within 5 min of K1 addition (Fig. 7). In the absence of added heparin, K1-stimulated receptor kinase activation was not detected, although it was observed after treatment with full-length HGF or NK1 (Fig. 7). In the presence of added heparin, c-Met activation by K1 was clearly observed, strongly suggesting that heparin acted by facilitating ligand-receptor interaction. N domain did not appear to stimulate receptor activation with or without added heparin, and heparin alone also had no effect (Fig. 7).
c-Met Binds Tightly to HS via Its Extracellular Domain—
The role of HS in signaling by heparin-binding growth factors has been extensively characterized for members of the FGF family, particularly FGF-1 and FGF-2. For FGFs, HS has been shown to directly facilitate ligand signaling in at least two ways: 1) ligand-HS interaction, which facilitates ligand dimerization (and in turn promotes receptor dimerization) and transactivation (33, 34), and 2) receptor-HS interaction, which facilitates ligand-receptor interaction and consequently receptor activation (23, 35). As K1 does not bind heparin, we examined whether heparin facilitated K1 signaling through direct and ligand-independent interaction with c-Met.

A preliminary analysis of c-Met-heparin interaction was performed using lysates prepared from Okajima cells, a gastric carcinoma cell line characterized by overexpression of the c-Met receptor protein (36). To test for c-Met-heparin interaction, we compared the binding of solubilized c-Met protein to heparin-Sepharose and Sepharose alone. Nonionic detergent extracts prepared from Okajima cells were incubated with either matrix, which was then washed extensively with lysis buffer. The amount of c-Met retained was determined after extraction with sample buffer, SDS-polyacrylamide gel electrophoresis, and immunoblotting with anti-c-Met antibodies. As an additional control, the same Okajima extracts were quantitatively immunoprecipitated with anti-c-Met antibody and protein-G Sepharose. We observed that c-Met was not retained by Sepharose alone, but heparin-Sepharose bound as much c-Met protein as the specific anti-c-Met antibody (data not shown). The bulk of heparin-Sepharose-immobilized c-Met was effectively eluted by salt concentrations equal to or greater than 0.5 M, and soluble HS displaced more than 90% of the immobilized c-Met, whereas chondroitin sulfate failed to displace immobilized receptor at any concentration tested (data not shown).

Although these results indicated that c-Met bound as tightly to immobilized heparin as many well characterized heparin-binding proteins, it remained unclear whether this interaction was mediated by the receptor extracellular domain or by an intracellular region that is normally inaccessible to cell surface HS. To address this issue, we analyzed HS-c-Met binding using a fusion protein consisting of the receptor extracellular domain and the Fc portion of IgG (25) and biotinylated HS in an ELISA format. Wells were coated with either c-Met-Fc fusion protein, the HGF isoform NK1 (which binds immobilized heparin-Sepharose in a manner indistinguishable from full-length HGF (12)), or the HGF K1 domain (which does not bind heparin-Sepharose (12)). As shown in Fig. 8A, saturable biotin-HS binding was observed in NK1 and c-Met-Fc-coated wells, but not in K1-coated wells, as anticipated. The specificity of the binding interactions between NK1 or c-Met-Fc and biotin-HS was analyzed in subsequent competitive binding experiments performed using a 50% saturating dose of biotin-HS in the presence of increasing concentrations of unlabeled HS (data not shown). Displacement of biotin-HS from either protein by the 5-kDa HS was 10-fold less efficient than displacement by high molecular mass HS (data not shown). Displacement of biotin-HS from either protein by the 5-kDa HS was 10-fold less efficient than displacement by high molecular mass HS (data not shown). This result was not unexpected, as the higher molecular weight HS provides more protein binding sites within a single polysaccharide chain than the lower molecular weight HS and thus greater possibility for multivalent binding having a higher apparent affinity. A comparison of NK1 and c-Met-Fc retention by immobilized heparin-TSK using high performance liquid chromatography gave results consistent with those obtained using the biotin-HS ELISA and also revealed that a control Fc protein was not retained by immobilized heparin (data not shown).
Thus, by all of the means used here to compare HS binding by ligand and receptor for the HGF/c-Met system, results were generally similar to those reported for other growth factor/receptor systems, such as FGFs 1, 2, and 7 (23, 35, 37, 38).

**DISCUSSION**

Like HGF, the truncated HGF isoform known as NK1 binds to c-Met with relatively high affinity (5, 28, 39) and activates this receptor kinase and downstream intracellular signaling pathways (9, 12, 28), thereby stimulating many well characterized HGF biological activities: cell proliferation, migration, and branching morphogenesis (8, 9, 28, 39). NK1 also binds to immobilized heparin with the same apparent affinity as HGF (12). These observations provided the rationale for further study of the separate NK1 components N and K1 with the goals of identifying the primary sites of HS and receptor binding on HGF and elucidating the role of HS in HGF signaling.

Studies using deletion mutants of HGF first implicated the N domain, and particularly the hairpin loop region, in the mediation of HGF-HS interaction (40, 41). Using bacterially expressed and refolded N and K1 domains, we later demonstrated that the isolated N domain bound to heparin-Sepharose as tightly as full-length HGF, whereas K1 was not retained by this matrix (12). That study further demonstrated that N domain formed high molecular mass oligomers in solution and that soluble heparin further enhanced N domain self-association (12). Although N domain did not appear to stimulate biological activity (9), HS binding, presumably mediated by N, was needed to reconstitute potent NK1-c-Met interaction and signaling in HS-deficient cells (12). The importance of HS in HGF signaling and the role of N domain in mediating HS-enhanced ligand oligomerization helped to shape the biological interpretation of the recently solved NK1 crystal structure (16). This model predicts that repeating units of HS polymer bind two NK1 molecules through interaction with their respective N domains, thereby facilitating ligand dimerization and, indirectly, the dimerization and transactivation of the associated receptor-kinase(s).

The participation of kringle domains in the protein-protein interactions of other proteins, such as plasminogen, suggested that the receptor binding site of HGF might be localized within these well defined structural units (7). Indirect evidence that K1 was essential for HGF-c-Met and NK1-c-Met interaction was provided by mutagenesis studies in which single amino acid substitutions in this domain substantially reduced HGF activity (42). Here, we provide direct evidence that K1 can mimic HGF activity, suggesting that the primary sites of HGF-c-Met interaction reside in K1. K1 stimulated two well characterized responses to HGF, DNA synthesis and cell motility, following the rapid activation of HGF receptor and MAP kinases, whereas under the same conditions N domain had little effect. The dosages at which K1 stimulated these activities are high, although this is not likely to be due to poor protein folding, because both circular dichroism and nuclear magnetic resonance studies show that isolated K1 has the same spectroscopic properties as the K1 domain of biologically active NK1 (9). Although it is possible that K1 was not as stable as NK1 in the biological assays used here, we believe that the difference in potency between NK1 and K1 reflects primarily the absence of a covalently linked domain capable of HS binding and HS-modulated self-association.

We observed that HGF and NK1 signaling persisted in HS-deficient 32D/c-Met cells in the absence of added heparin but showed significantly diminished potency in mitogenic assays. As expected, added heparin increased the potency of mitogenic signaling at least 3-fold (Fig. 6). These results were less extreme than the total heparin dependence we reported for NK1 in HS-deficient CHO cells, but this may be attributable to the higher apparent c-Met concentration in 32D/c-Met cells than in the CHO cells used in that study (11, 12). The idea that higher receptor expression may result in lower HS dependence is consistent with the hypothesis that HS enhances ligand signaling by facilitating ligand-receptor interaction and thereby receptor-receptor interaction; higher receptor expression could enhance signaling independently by facilitating receptor-receptor interaction. The impact of heparin on the motility of 32D/c-Met cells could not be determined because it interfered with the migration of these cells in the Boyden chamber assay system (data not shown). However, the relative potency of K1 compared with NK1 or HGF in this assay suggests that a lower overall level of c-Met pathway activation was sufficient to stimulate motility versus mitogenicity in these cells. In contrast to K1-stimulated motility, the strong heparin dependence of K1 mitogenic activity in 32D/c-Met cells implied that heparin (or HS) interactions at the cell surface are critical for K1-stimulated mitogenesis.

Our observations that K1 showed little stimulation of DNA synthesis in HS-deficient 32D/c-Met cells and the restoration of K1 mitogenic activity by added HS suggest that HS could facilitate HGF signaling by a mechanism other than HS-ligand interaction. The ability of added HS to promote rapid c-Met autophosphorylation by K1 in 32D/c-Met cells indicated that it acted at the level of receptor activation. Testing for hypothetical HS-receptor interaction revealed that, indeed, c-Met bound HS tightly, and this interaction involved the c-Met extracellular domain. Thus, the interaction between added HS and c-Met may have compensated for the lack of self-association of K1 and HS binding and facilitated K1-stimulated DNA synthesis in HS-deficient cells. We propose that an interaction between endogenous cell surface HS and c-Met enabled K1 signaling in Balb/MK keratinocytes. Cell surface HS may stabilize preformed c-Met oligomeric clusters, which exist in a variety of HGF target cell types, in both the presence and absence of ligand stimulation (43).

A critical role for receptor-HS interaction in growth factor signaling pathways has been demonstrated for FGF-1 and FGF-2 through FGF receptor 1 (23). More recently, HS binding by the keratinocyte growth factor (KGF) binding isoform of FGF receptor 2 was also observed, suggesting that this may be a common property of FGF receptors (37). In that study, the KGF receptor extracellular domain expressed as a soluble fusion protein with an immunoglobulin (Ig) heavy chain was retained by heparin-Sepharose almost as tightly as KGF itself (37). Ligand binding by that construct was not dependent on added heparin, whereas ligand binding and signaling by wild-type KGF receptor expressed on cell surfaces was significantly enhanced by HS (37). The explanation for this apparent discrepancy became clear after another, independent study of KGF receptor-HS interaction was published (38). Unlike the Ig-receptor chimera used by LaRochelle et al. (37), which contained the Ig hinge region and was secreted as a disulfide-linked receptor dimer, Hsu et al. (38) showed HS-dependent KGF binding by a soluble monomeric KGF receptor. The latter receptor construct formed a dimer in the presence of ligand and added heparin (38), suggesting that the normal HS dependence of KGF binding could be abrogated by the covalent linkage of two KGF receptors in the context of the Ig chimera. High

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[4] D. Bottaro and J. Rubin, unpublished observations.
affinity ligand binding to a soluble dimeric c-Met Ig fusion protein in the absence of heparin has been reported (25) and may also have been attributable to the existence of covalent receptor dimers.

Our results suggest that the role of HS in HGF signaling is more complex than previously predicted based on models of ligand-HS interaction alone. Whether simultaneous HS interactions with both HGF and c-Met are needed for potent, physiologic activities with both HGF and c-Met is unclear. The mature c-Met extracellular domain contains over 900 amino acids, and although it is tempting to speculate that some regions rich in basic residues may be involved in HS interaction, the structures of known sites of HS-protein interaction reveal that spatial proximity may be as important as sequence similarity to known HS binding motifs. The identification and specific substitution of HS binding residues in the c-Met extracellular domain, in conjunction with further analysis of the HS dependence of K1 signaling in 32D cells expressing these c-Met mutants, should help clarify the importance of HS in HGF signaling and offer insight into the role of HS in growth factor signaling in general.

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