Generation and Characterization of a Competitive Antagonist of Human Hepatocyte Growth Factor, HGF/NK1*

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Our previous studies have suggested that a derivative of hepatocyte growth factor (HGF), HGF/NK2, containing the coding sequences for the N-terminal hairpin and first two kringle domains, is sufficient to mediate high affinity binding to the HGF receptor. Here, we wished to test directly whether HGF/NK1 (N-terminal hairpin and first kringle domains) could bind the receptor and/or mediate receptor signaling. HGF/NK1 was expressed in Escherichia coli and purified to homogeneity using heparin-affinity and fast protein liquid chromatography. Biological characterization of HGF/NK1 showed that it can compete for binding to the HGF receptor on human lung carcinoma A549 cells and to a soluble form of the HGF receptor. HGF/NK1 is inefficient at promoting autophosphorylation of the HGF receptor, although some activity was detected at very high concentrations. HGF/NK1 fails to exhibit mitogenic properties even at very high concentrations. However, HGF/NK1 can act as a potent competitive antagonist in this assay. Our data demonstrate directly that a receptor binding determinant of HGF is located within the N-terminal 32-212 residues of HGF. HGF/NK1 will serve as a powerful tool for (i) generating neutralizing antibodies, (ii) in determining x-ray crystallographic and nuclear magnetic resonance structures, and (iii) for in vivo studies as an HGF antagonist.

Hepatocyte growth factor (HGF) exhibits mitogenic and/or motogenic activities on a variety of cells (for review see Matsumoto and Nakamura, 1991) and has recently been shown to possess potent angiogenic activity in vivo (Bussolino et al., 1992). Structurally, HGF has similarities to kringle-containing serine proteases that are involved in coagulation and fibrinolysis (Sotttrup-Jensen et al., 1978; Patthy, 1985; Tulinsky, 1991). The mature form of HGF is a heterodimer of a 69-kDa α-chain and a 34-kDa β-chain (Miyazawa et al., 1989; Nakamura et al., 1989). The primary amino acid sequence of HGF predicts a hairpin-containing and four kringle domains in the α-chain and a serine protease-like domain in the β-chain. However, HGF apparently does not possess proteolytic activity, presumably because residues within the catalytic triad are not conserved (Nakamura et al., 1989).

The HGF receptor is the product of the c-Met proto-oncogene, a membrane-spanning tyrosine kinase receptor (Bottaro et al., 1991; Naldini et al., 1991). The 190-kDa precursor of the HGF receptor is proteolytically processed within the extracellular domain to a heterodimer consisting of a 50-kDa α-subunit and a 145-kDa β-subunit (Park et al., 1987). The HGF receptor is autophosphorylated on tyrosine residues of the 145-kDa β-subunit upon HGF binding (Naldini et al., 1991, Lokker et al., 1992). We have expressed a chimeric protein containing the extracellular domain of the HGF receptor fused to the constant region of a human IgG heavy chain. This soluble HGF receptor binds HGF with an affinity similar to that of authentic, membrane-associated receptor, and is thus sufficient to mediate HGF binding (Lokker et al., 1992; Mark et al., 1992).

Kringles are known to be independent structural and functional domains (Castellino et al., 1981; Tregler and Patthy, 1983) that have been suggested to participate in protein-protein interactions (for review, see Tulinsky (1991)). For example, kringle 2 of tissue plasminogen activator (Van Zonneveld et al., 1986; Verhejen et al., 1986; Bennett et al., 1991; Byeon et al., 1991) and kringles 1 and 4 of plasminogen (Thorsen, 1975; Thorsen et al., 1981; Motta et al., 1987; Petros et al., 1988) have been shown to interact with fibrin. In other proteins, including HGF, the detailed functions of kringles are poorly understood. Moreover, HGF is a mitogen, motogen and morphogen (Matsumoto and Nakamura, 1991) and thus differs in that respect from all the kringle-containing proteases involved in the blood cascade.

To assess the role of the hairpin-containing and kringle domains in the HGF α-chain, a functional analysis of HGF variants was performed (Lokker et al., 1992). We showed that a variant containing the N-terminal hairpin and first two kringles of HGF could compete for binding to the HGF receptor with only a 4-fold increase in Kd when compared to HGF. This variant is similar to a naturally occurring variant, HGF/NK2, that arises from an alternatively spliced transcript (Chan et al., 1991). Analysis of additional deletion variants suggested that both the N-terminal hairpin-containing region and kringle 1 are essential for mediating receptor binding of HGF (Lokker et al., 1992).

We wished to determine whether HGF/NK1 itself might be sufficient for HGF receptor binding. Since we were unable to express the HGF/NK1 variant of HGF in eukaryotic cells, we decided to express this fragment in procaroytic cells. We report here the expression and purification of HGF/NK1 from Escherichia coli. In addition to the biochemical characterization of HGF/NK1, we analyzed HGF/NK1 for binding to a soluble and membrane-associated HGF receptor on A549.

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1 The abbreviations used are: HGF, hepatocyte growth factor; FPLC, fast protein liquid chromatography; HGF/NK1, N-terminal hairpin and kringle 1 of hepatocyte growth factor; rhoHGF recombinant human hepatocyte growth factor; PAGE, polyacrylamide gel electrophoresis; sII, heat-stable enterotoxin II; PCR, polymerase chain reaction; IEF, isoelectric focusing.

2 N. Lokker, unpublished observations.
cells, for induction of HGF receptor autophosphorylation, for mitogenic properties, and for the ability to antagonize HGF-induced mitogenesis.

EXPERIMENTAL PROCEDURES

Materials—Heparin-Sepharose was purchased from Bio-Rad. Mono S cation-exchange columns and the FPLC equipment were from Pharmacia LKB Biotechnology Inc. SpectraPor/10 dialysis tubing (molecular weight cut off 10,000) was from Spectrum. All restriction enzymes were obtained from New England Biolabs and used according to the manufacturer's instructions. Anti-Flag monoclonal antibody M2 was from International Biotechnologies, Inc. Recombinant human HGF (rhuHGF) was provided by Steve Chamow (Genentech). Purified kringle 4 of plasminogen was a gift of Frank Castellino.

Purification of NK1—The expression plasmid pf-NK1 outlined in Fig. 1 was used to transform the E. coli protease-deficient strain 27C7 (tonA phoA ΔE15 ΔargF-lacI69 ptr3 degP41 KanR ompT ρ0) (Lukker et al., 1992). The recombinant plasmid contained a heat-stable enterotoxin (stII) signal sequence, a modified Flag epitope sequence of 10 amino acid residues (CGG S D D D D K), and a StuI endonuclease restriction site (see Fig. 1). The Flag epitope (Hopp et al., 1987) is a 30-residue sequence, which is used as a strain marker, for transformation of E. coli. The Flag epitope fusion protein was purified on an affinity column (2.5 cm). Bound proteins were sequenced in the blot cartridge. Peaks were integrated with Justice Innovation software using Nelson analytical mass spectrometer. Multiple charged ions of horse myoglobin (m/z 17146) were used for instrument calibration.

Analysis of Proteins by SDS-PAGE—Fractionation of the HGF/NK1 samples was performed by SDS-PAGE with glycine-containing 8-16% Tris-glycine gradient gels (Novex) in a Novex minigel apparatus. Western blotting, proteins were transferred onto nitrocellulose with a Pharmacia Novablot apparatus. The blot was blocked in 3% dry milk/Tris-buffered saline overnight at room temperature. The M2 monoclonal antibody (1 pg/ml, International Biotechnologies, Inc.) was used for detection of the Flag-HGF/NK1 fusion protein (2 h, room temperature). After three washes in Tris-buffered saline, the blot was incubated with a horseradish peroxidase-conjugated antibody to mouse IgG (1:5000; Amersham Corp.) for 20 min at room temperature and washed four times. The Western blot was developed with a chemiluminescent detection system as described by the manufacturer (Amersham Corp.).

Amino Acid Analysis—Peptides were hydrolyzed for 24 h with 6 N constant boiling HCl at 110°C under vacuum using a Millipore Pictostat worktation. The hydrolysates were dried on a Savant Speed-Vac concentrator and analyzed on a Beckman model 6300 amino acid analyzer equipped with a ninhydrin detector using on-line phenylthiohydantoin analyzers. Electroblotted proteins were sequenced in the blot cartridge. Amino acid analyses were performed on models 470A and 477A Applied Biosystems sequencers equipped with on-line phenylthiohydantoin analyzers. Electroblotted proteins were sequenced in the blot cartridge. Peaks were integrated with Justice Innovation software using Nelson analytical 760 interfaces. Sequence interpretation was performed on a VAX 8600 (Digital, 1987).

RESULTS

Characterization of E. coli-expressed HGF/NK1—To follow the expression and purification of HGF/NK1 in E. coli, we constructed a gene containing the coding sequences for immunoreactive "Flag" epitope fused upstream of residues 32-210 of human HGF (Nakamura et al., 1989) as diagrammed in Fig. 1. Since HGF/NK1 contains 10 cysteine residues, we used a stII leader sequence to direct secretion of the protein into the periplasmic space where the redox potential for disulfide formation is more favorable. This also allowed us to purify the soluble form from the osmotic shock fraction.
Fig. 1. Outline of expression plasmid pf-NK1. For bacterial expression of HGF/NK1, an expression plasmid containing an alkaline phosphatase promoter (phoA) adjacent to the coding sequence for the stI1 leader peptide to direct secretion of the expressed protein into the periplasmic space was used. The coding sequence for the Flag epitope was included to follow expression and purification steps. This Flag sequence was followed by the coding sequence for mature HGF/NK1 (hatched segment) as indicated. The corresponding DNA and amino acid sequence of the stI1 leader, Flag epitope (italics) and HGF/NK1 portions (N- and C-terminal, boldface) are shown.

![Diagram](image)

A. 

**Uninduced**

| kDa | 106 | 80 | 49 | 32 | 19 | 8 |

**Induced**

| kDa | 106 | 80 | 49 | 32 | 19 |

![Coomassie staining](image)

HGF/NK1

B.

**Uninduced**

| kDa | 106 | 80 | 49 | 32 | 19 |

**Induced**

| kDa | 106 | 80 | 49 | 32 | 19 |

![Coomassie staining](image)

HGF/NK1

Coomassie staining suggested that efficient induction of HGF/NK1 was achieved by growing the transformed strain in low phosphate medium (Fig. 2A). The induced protein was also immunoreactive in Western blot analysis using an anti-Flag monoclonal antibody (data not shown). The level of HGF/NK1 expression is estimated between 100 and 500 mg/liter. Using the protocol outlined under "Experimental Procedures," approximately 500 μg of HGF/NK1 were purified from the soluble osmotic shock fraction of 100 g of cell paste. The HGF/NK1 preparation from the final FPLC Mono S cation-exchange chromatography step has an apparent molecular mass of 22 kDa as determined by SDS-PAGE (Fig. 2B). Moreover, the purified HGF/NK1 migrates as a monomer on PAGE under nonreducing conditions and was also immunoreactive with a monoclonal antibody to the Flag sequence confirming its identity as HGF/NK1 (data not shown).

Biochemical Characterization of Purified HGF/NK1—The isoelectric point (pI) of HGF/NK1 ranges between 8.2 and 8.6 as judged from the IEF gel (data not shown). The isolated protein has the predicted amino acid composition and N-terminal amino acid sequence of correctly processed HGF/NK1 (Fig. 1). The molecular mass was determined to be 21,872 Da by electrospray ionization mass spectrometry. This number is identical to the calculated molecular mass of the 32-210 fragment of human HGF (with the formation of five disulfide bonds) linked to the 10-amino acid Flag epitope.

Receptor Binding of HGF/NK1—We assayed the HGF/NK1 preparation for competitive binding to a soluble form of the HGF receptor as well as for binding to the cell surface-associated HGF receptors on A549 cells. Inhibition curves from representative experiments are shown in Fig. 3, A and B, and indicate that HGF/NK1 is able to compete for the binding of radiolabeled wild-type HGF to the HGF receptor, albeit with reduced affinity (8-11-fold when compared to wild-type HGF). As expected, purified kringle 4 from human plasminogen did not compete for binding to either the soluble or cell-associated HGF receptor. Dissociation constants (Kd) estimated from these curves in at least three independent assays indicate that in solution HGF/NK1 binds with a Kd of 1.10 ± 0.04 nM versus 0.10 ± 0.02 nM for wild-type HGF. Similarly, on A549 cells, HGF/NK1 binds with a Kd of 1.60 ± 0.08 nM compared to 0.21 ± 0.04 nM for wild-type HGF. These data demonstrate that the NK1 region of HGF is sufficient to mediate binding to the HGF receptor. The reason for the reduced affinity of HGF/NK1 compared to intact HGF is not clear, but may indicate that other regions of HGF contribute directly or indirectly to receptor binding. We note

5 B. Henzel, personal communication.
that HGF/NK2, expressed in mammalian cells, also exhibits a somewhat reduced affinity for binding the HGF receptor (Lokker et al., 1992).

**Ligand-induced Autophosphorylation of the HGF Receptor**—The HGF receptor undergoes autophosphorylation of the 145-kDa β-subunit upon binding of ligand (Naldini et al., 1990; Lokker et al., 1992). In A549 cells, the maximal response was observed at a concentration of 1–4 nM (Fig. 4). At these concentrations of HGF/NK1, autophosphorylation of the HGF receptor was not detectable. However, at higher concentrations (20 and 100 nM; Fig. 4) some autophosphorylation could be detected.

**Biological Properties of HGF/NK1**—Whereas HGF stimulates [3H]thymidine incorporation in primary cultured hepatocytes with an half-maximal effect (IB_{50}) at 0.64 nM, HGF/NK1 under identical conditions causes no enhancement of DNA synthesis at concentrations as high as 110 nM (Fig. 5A). We subsequently tested HGF/NK1 for antagonistic activity using hepatocytes in primary culture (Fig. 5B). HGF/NK1 completely antagonizes HGF-induced mitogenic activity with an IB_{50} of 6 nM, corresponding to a 10-fold molar excess of HGF/NK1 over HGF to neutralize 50% DNA synthesis in hepatocytes. As a control, we showed that purified kringle 4 of human plasminogen failed to antagonize HGF-induced mitogenesis. Moreover, the effect of HGF/NK1 was specific since it failed to neutralize epidermal growth factor-promoted mitogenesis (data not shown). Thus, we conclude that HGF/NK1 is a potent and specific antagonist of HGF activity.

**DISCUSSION**

Growth factor antagonists can function by a variety of mechanisms, including direct inhibition of the binding of the ligand to its cognate receptor. Competitive antagonists of a variety of growth factors including human growth hormone (Fuh et al., 1992), bovine growth hormone (Okada et al., 1992), and mouse interleukin-2 (Zurawski and Zurawski, 1992) have been generated. Some growth factor antagonists have shown function to in vivo experiments; e.g. an interleukin-1 receptor antagonist (Conti et al., 1992). Antagonists of HGF might have therapeutic potential in the treatment of certain forms of malignancies. For example, the HGF receptor, c-Met, has been shown to be overexpressed in a substantial fraction of colorectal carcinomas (Di Renzo et al., 1991; Liu et al., 1992). HGF has been shown to promote invasiveness of a number of transformed cells in vitro, perhaps promoting a more malignant invasive phenotype (Weidner et al., 1990; Giordano et al., 1993). Finally, blocking the angiogenic activity of HGF (Bussolino et al., 1992) might be useful in the treatment of certain types of solid tumors. Here, we describe the high level expression, purification, and characterization of a competitive antagonist of HGF.

To assess the structure and function of HGF, we previously attempted to express HGF/NK1 in mammalian expression systems (Lokker et al., 1992). We were unable to detect HGF/NK1 expression by either 35S labeling or Western blot analysis using polyclonal or monoclonal antibodies. Expression in E. coli of complex proteins with disulfide bonds has been reported (e.g. Carter et al., 1992). Several kringle domains have been successfully expressed in E. coli including kringle 1 of plasminogen (Mehnhart et al., 1991) and kringle 4-type 2 of apolipoprotein (Zhigao et al., 1992). Kringle 2 but not kringle...
HGF/NK1 for heparin is similar to that previously reported deletion of 5 amino acid residues (F162-L-P-S-S166) in the heparin (Shima et al., 1989). Previous data have shown that have affinity for heparin (Nakamura et al., 1987). Analysis by SDS-PAGE of the corresponding to the amount of HGF required for incorporation.

Fig. 5. Effect of HGF/NK1 alone (A) or together with rhuHGF (B) on DNA synthesis of hepatocytes in primary culture. Hepatocytes were exposed to increasing concentrations of rhuHGF or HGF/NK1 alone (A) or increasing concentrations of HGF/NK1 together with a fixed concentration of HGF (0.64 nM corresponding to the amount of HGF required for 50% [3H]thymidine incorporation (B)). Shown are representative curves from three independent experiments.

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HGF/NK1 was purified to homogeneity by a three-step procedure combining heparin affinity and FPLC Mono S cation-exchange chromatography. HGF has been shown to have affinity for heparin (Nakamura et al., 1987; Zarnegar and Michalopoulos, 1989). Previous data have shown that deletion of 5 amino acid residues (F162-L-P-S-S166) in the first kringle of human HGF reduces the affinity of HGF for heparin (Shima et al., 1991). It appears that the affinity of HGF/NK1 for heparin is similar to that previously reported for HGF (Zarnegar and Michalopoulos, 1989) since both HGF and HGF/NK1 eluted at identical salt concentrations, suggesting that a heparin binding site is located within the N-terminal 179 residues of mature HGF.

Purified HGF/NK1 was able to compete with 125I-rhuHGF for binding to the HGF receptor. This represents the first direct evidence for a receptor-binding determinant located within the N-terminal HGF/NK1 domain of HGF. HGF/NK1 binds the HGF receptor with an affinity similar to that of HGF/NK2 (Lokker et al., 1992). Only about a 5-fold reduction in Kd was observed for HGF/NK2 and a 10-fold reduction for HGF/NK1 when compared to wild-type HGF. The lowered affinity suggests that other regions of the HGF molecule contribute to the interaction of HGF and its receptor. For example, other regions of HGF might be required to maintain structural integrity and, thus, high affinity binding of the NK1 portion of HGF. Alternatively, other regions of HGF might directly interact with the HGF receptor.

HGF/NK2 is a naturally occurring HGF variant identified in human fibroblasts which derives from an alternative transcript of HGF (Chan et al., 1991). It has been described as a competitive antagonist of HGF which lacks mitogenic activity but specifically inhibits HGF-induced mitogenesis in B6/889 cells. We have shown here that HGF/NK1 alone can act as a potent antagonist of wild-type HGF. As little as a 10-fold molar excess of HGF/NK1 neutralizes 50% of wild-type HGF activity. These antagonistic properties are very similar to those reported for HGF/NK2 (Chan et al., 1991).

A general mechanism for activation of growth factor recep-
tors appears to involve ligand-induced oligomerization (for review see Ulrich and Schlessinger (1989) and Schlessinger and Ulrich (1992)). One mechanism involves receptor dimerization resulting from the binding of a dimeric ligand, as proposed for the platelet derived growth factor receptor (Hart et al., 1988; Heldin et al., 1988, 1989). A second mechanism involves allosteric activation of pre-associated receptors induced upon binding of a monomeric ligand, as exemplified by epidermal growth factor (Greenfield et al., 1989; Lax et al., 1991) (for review see Canals (1992)). Finally, receptor activation could result from the binding of a monomeric ligand that contains two receptor binding sites, as shown for growth hormone (Cunningham et al., 1991; de Vos et al., 1992). In this context, it is likely that the antagonistic activity of HGF/NK1 results from the failure to induce efficient HGF receptor oligomerization subsequent to ligand binding. Our results support previous studies indicating that regions outside of HGF/NK1 are required for efficient activation of the HGF receptor. For example, we have identified HGF variants with amino acid substitutions in the β-subunit that bind to the HGF receptor with affinities similar to wild-type HGF but are defective in receptor activation (Lokker et al., 1987).

It is interesting to note that although HGF/NK1 was defec-
tive at stimulating receptor autophosphorylation at low concentrations, a small amount of autophosphorylation was observed at high concentrations. Hartmann et al. (1992) have reported that the HGF/NK2 variant at similar elevated concentrations could also stimulate tyrosine autophosphorylation. The molecular mechanism by which HGF activates its receptors has not been elucidated.

The detailed description of ligand-receptor complexes are useful in designing possible small molecule antagonists or agonists which might have therapeutic potential. The availa-
bility of HGF/NK1 will facilitate a precise analysis of the interaction of HGF and its receptor. First, detailed mutational analysis together with high resolution x-ray crystallographic and nuclear magnetic resonance studies of the HGF/NK1 fragment will shed light on the HGF ligand-receptor interaction. In addition, HGF/NK1 will help in generating neutralizing polyclonal and monoclonal antibodies. Finally, HGF/NK1 may be useful for in vivo studies as an HGF antagonist.

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