The N-terminal Domain of Hepatocyte Growth Factor Inhibits the Angiogenic Behavior of Endothelial Cells Indepedently from Binding to the c-met Receptor*

Tatyana Merkulova-Rainon‡§, Patrick England‡, Shunli Ding‡, Corinne Demeren‡, and Gérard Tobelem‡

From the †Institut des Vaisseaux et du Sang, Centre de Recherche de l’Association Claude Bernard, Hôpital Lariboisière, 8 rue Guy Patin, 75475, Paris Cedex 10 and the §Plate-forme de Biophysique des Macromolécules et de leurs Interactions, Institut Pasteur, 28, rue du Docteur Roux, 75724 Paris Cedex 15, France

Hepatocyte growth factor (HGF) is a pleiotropic factor that plays an important role in complex biological processes such as embryogenesis, tissue regeneration, cancerogenesis, and angiogenesis. HGF promotes cell proliferation, survival, motility, and morphogenesis through binding to its receptor, a transmembrane tyrosine kinase encoded by the MET proto-oncogene (c-met). Structurally speaking, HGF is a polypeptide related to the enzymes of the blood coagulation cascade. Thus, it comprises kringle domains that in some other proteins have been shown to be responsible for the anti-angiogenic activity. To check whether the isolated kringles of HGF were able to inhibit angiogenesis, we produced them as recombinant proteins and compared their biological activity with that of the recombinant HGF N-terminal domain (N). We showed that (i) none of the isolated HGF kringle exhibits an anti-angiogenic activity; (ii) N is a new anti-angiogenic polypeptide; (iii) the inhibitory action of N is not specific toward HGF, because it antagonized the angiogenic activity of other growth factors, such as fibroblast growth factor-2 and vascular endothelial growth factor; and (iv) in contrast with full-length HGF, N does not bind to the c-met receptor in vitro, but fully retains its heparin-binding capacity. Our results suggest that N inhibits angiogenesis not by disrupting the HGF/c-met interaction but rather by interfering with the endothelial glycosaminoglycans, which are the secondary binding sites of HGF.

Received for publication, December 16, 2002, and in revised form, May 30, 2003
Published, JBC Papers in Press, July 7, 2003, DOI 10.1074/jbc.M212768200

* This work was supported in part by grants from the Ligue Nationale Contre le Cancer and from the Association pour la Recherche sur le Cancer (to G. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Tel.: 33-1-45-26-21-98; Fax: 33-1-42-82-94-73; E-mail: tatiana.merkulova-rainon@lrb.ap-hop-paris.fr.

¶ Recipient of a fellowship from the Association Franco-Chinoise pour la Recherche Scientifique & Technique.

The abbreviations used are: HGF, hepatocyte growth factor; N, N-terminal domain of HGF; K1, K2, K3, K4, the first, second, third, and fourth kringles of HGF, respectively; NK1, an HGF variant containing N domain and K1; NK2, an HGF variant containing N domain and the first two kringles; IPTG, isopropyl β-D-1-thiogalactopyranoside; NF heparin, non-fractionated heparin; LMW, low molecular weight; NHS, normal human serum; PBS, fetal bovine serum; PFG-2, fibroblast growth factor 2; VEGF, vascular endothelial growth factor; PDGF BB, platelet-derived growth factor BB; c-met/Fc, chimerical protein composed of mature hepatocytes and as a cytokine capable of inducing the dissociation (scattering) of epithelial cells. HGF has now been proven to be a pleiotropic factor that acts on a wide array of target cell types, including epithelial, endothelial, neuronal, and hematopoietic cells (3). HGF promotes cell proliferation, survival, motility, and morphogenesis through binding to its receptor, a transmembrane tyrosine kinase encoded by the MET proto-oncogene (c-met) (4, 5). The signaling cascade triggered by the binding of HGF to c-met contributes to complex biological processes, such as embryogenesis, tissue regeneration, and cancerogenesis (3, 6). Studies performed in the recent years have shown that HGF is also a potent angiogenic molecule (7–10).

HGF is a polypeptide structurally related to the enzymes of the blood coagulation cascade. In the cell it is synthesized as a biologically inactive single chain precursor that is then cleaved by specific serine proteases yielding a fully active disulfide-linked heterodimer composed of α- and β-chains (11, 12). The α-chain consists of an N-terminal domain (N) followed by four kringle modules and mediates the binding of HGF to the c-met receptor (13, 14). Additional structure-function studies indicated that the N-terminal domain and first kringle (K1) are primarily involved in this interaction (15). The α-chain is also involved in the high affinity binding of HGF to heparin. Based on deletion analysis, the hairpin loop in the N-terminal domain and the second kringle (K2) were shown to be implicated in heparin binding (16, 17). Recently x-ray crystal structures were determined for the complexes of heparin with NK1, a naturally occurring HGF variant containing N domain and K1, which suggested that some heparin binding activity is also located within K1 (18). Up to date, no discernible function has been demonstrated for the third (K3) and fourth (K4) kringles of HGF.

The kringle domain is an 80-amino acid triple-loop structure maintained by three intramolecular disulfide bonds highly conserved between different kringle containing proteins (19). Kringles are thought to play an important role in regulating the nature and strength of protein-protein interactions. Several reports have documented the anti-angiogenic activity of isolated kringles of different origin. The most known example is angiostatin, the three- to four-kringle containing fragment of human plasminogen (20, 21), and plasminogen kringle 5 (22). More recently the prothrombin kringle-2 domain has been...
shown to inhibit endothelial cell growth and angiogenesis in the choroidal membrane of chick embryos (23); a recombinant apolipoprotein (α) containing 18 kringle repeats has been implicated in the reduction of tumor microvessel density in transgenic mice (24). The molecular mechanism by which the kringles suppress blood vessel formation is still unknown. We hypothesized that one or more of the kringles of HGF might inhibit angiogenesis and that the mechanism of the anti-angiogenic activity of a kringle domain could be related to its ability to antagonize the HGF binding to c-met. To verify this hypothesis, we used Hybond C Extra membranes to express modules as recombinant proteins and analyzed their binding properties and biological activity in different assays related to angiogenesis.

EXPERIMENTAL PROCEDURES

Materials—The bacterial expression vectors pET15b and pET21b(+), the Escherichia coli expression strain BL21(DE3), and tetraacycline hydrochloride were purchased from Novagen (VWR International, Fontenay-sous-Bois, France). EZMix 2 × YT microbial medium, carbenicillin, chloramphenicol, IPTG, non-fractionated heparin (catalog number H 3390) and low molecular weight heparin (average molecular weight 3000, catalog number H 3400), a peroxidase conjugated antibody against goat IgG, protein A from Staphylococcus aureus, and 0.2% gelatin were all purchased from Sigma (St. Louis, MO). Pco DNA polymerase was purchased from Roche Diagnostics (Mannheim, Germany). TALON Superflow metal affinity chromatography was purchased from Clontech (Palo Alto, CA). The Ultrafree-4 and Ultrafree-15 Centrifugal Filter Devices with BioMax-5K filters and 0.22-μm Millex-GV filter units were obtained from Millipore (Bedford, MA). The BCA protein assay reagent, the IODOGEN Pre-Coated Iodination Tubes, and EZ-Link biotin-LC-hydrazide were all obtained from Pierce (Pierbio Sciences, Bezons, France). Nitrocellular weight heparin (average molecular weight 3000, catalog number H9262) was purchased from Novagen (VWR International, Fontenay-sous-Bois, France). NSO-expressed recombinant human HGF, recombinant polypeptides was generated by PCR using the pGEM/HGF-TM construct as a template (a generous gift of Dr. Pascale Briand at Institut Cochin de Ge (Cholet, France). The resulting precipitates were solubilized with 0.3N NaOH (0.5 ml/well), after an overnight incubation with stirring, and the protein solutions were concentrated using Ultrafree-15 Centrifugal Filter Devices and diafiltered against PBS containing 10% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.5 μg/ml amphotericin B, 7.5% NHS, and 7.5% FBS (regular medium). Cells—Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase (Roche Diagnostics) digestion (29). Cells were routinely grown in flasks covered with collagen, in M199 medium containing 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 7.5% NHS, and 7.5% FBS (regular medium). HUVECs from the second or third passage were used. The human colorectal carcinoma cell line DLD-1, the human osteogenic sarcoma cell line TE 85, and the human pancreatic adenocarcinoma cell line Capan-1 were obtained from the American Type Culture Collection and were cultured in RPMI supplemented with 10% FBS. Human aortic smooth muscle cells (hASMCs) and the corresponding growth medium (SmGM-2), and the human normal epidermal keratinocytes (NHEK) and the corresponding growth medium (KGM) were purchased from Clonetics (BioWhittaker, Walkersville, MD). Anti-angiogenic Activity of the N-terminal Domain of HGF

We hypothesized that one or more of the kringles of HGF might in HGF kringles (α-chain domains), or the plasmid encoding the HGF N domain were electroporated, respectively, into E. coli strain BL21(DE3), or into E. coli BL21(DE3) based strain ηRI (kindly provided by Dr. Janne L. Simonsen at Arhus University) carrying two additional plasmids: one encoding a TRNA pig specific for the rare isoleucine codon AUG and another for the LacZ repressor RP4. Bacteria were grown at 30 °C in EZMix 2 × YT medium containing 1 mg glucose and appropriate concentrations of antibiotics. Protein expression was induced with 1 mM IPTG at 25 °C. Bacterial cells were harvested by centrifugation and stored at −20 °C.

Bacterial pellets (5 g wet weight) were resuspended in 100 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, containing 0.2 mM imidazole, 10% glycerol, 1% Nonidet P-40, 2 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mg/ml lysozyme) and incubated for 20 min at room temperature with rotation. The suspensions were sonicated and centrifuged at 10,000 × g for 30 min. All HGF kringles were recovered in the supernatants and purified by cobalt-chelate affinity chromatography on a 2-ml TALON column under non-denaturing conditions. The pellet containing insoluble N was resuspended in 30 mM sodium phosphate buffer (20 mM sodium phosphate, pH 8, containing 10% glycerol, 2% Triton X-100 and subjected to three successive rounds of brief sonication, centrifugation (10,000 × g for 10 min), and resuspen- sation. The supernatants from all three steps were pooled and applied to a TALON column equilibrated with isolation buffer. To achieve the N domain renaturation, the column was washed with a 2 to 6 gradient of urea in IMAC buffer (20 mM Tris-HCl, pH 8, and 0.5 mM NaCl) containing 10 mM imidazole. Renaturated N was eluted by IMAC buffer containing 0.5 mM imidazole.

Further steps were all performed at 4 °C. Selected column fractions corresponding to the peaks of purified N, K1, K2, K3, or K4 were pooled and diluted with 10 volumes of refolding buffer (PBS containing 10% glycerol, 1 mM reduced glutathione, and 0.2 mM oxidized glutathione). Following an overnight incubation with stirring, the protein solutions were concentrated using Ultrafree-15 Centrifugal Filter Devices and dialyzed against PBS containing 10% glycerol for 2 days with at least three changes of buffer. The final protein preparations were sterilized using 0.22-μm Millex-GV filter units and stored at −80 °C until required. The protein concentration was determined using the BCA protein assay. The purified recombinant HGF-derived proteins were analyzed by SDS-PAGE and by circular dichroism as described previously (28).

Cells—Human umbilical vein endothelial cells (HUVECs) were iso- lated by collagenase (Roche Diagnostics) digestion (29). Cells were routinely grown in flasks covered with collagen, in M199 medium containing 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 7.5% NHS, and 7.5% FBS (regular medium). HUVECs from the second or third passage were used. The human colorectal carcinoma cell line DLD-1, the human osteogenic sarcoma cell line TE 85, and the human pancreatic adenocarcinoma cell line Capan-1 were obtained from the American Type Culture Collection and were cultured in RPMI supplemented with 10% FBS. Human aortic smooth muscle cells (hASMCs) and the corresponding growth medium (SmGM-2), and the human normal epidermal keratinocytes (NHEK) and the corresponding growth medium (KGM) were purchased from Clonetics (BioWhittaker, Walkersville, MD).
and the incorporated radioactivity was measured in a LS-6500 multipurpose scintillation counter (Beckman Coulter, Fullerton, CA).

Migration Assay—HUVEC migration was evaluated in a modified Boyden chamber assay. Transwell cell culture chamber inserts (BD Biosciences) with porous polycarbonate filters (8-μm pore size) were coated with 0.2% gelatin in PBS. HUVECs suspended in M199 medium supplemented with 2.5% FBS were added to the inserts at 4 × 10⁴ cells per well. The inserts were placed over chambers containing a chemotactic stimulus (10 ng/ml HGF, FGF-2, or VEGFα), and cells were allowed to migrate for 5 h at 37°C in a CO₂ incubator. For inhibition experiments one of the purified HGF α-chain-derived polypeptides (each at 1 μM) was added to the lower chamber. The filters were then rinsed with PBS, fixed with 1% (w/v) paraformaldehyde, and stained with 0.05% (w/v) crystal violet. The upper surface of the filters was scraped with a cotton swab to remove the non-migrant cells. The number of cells per high power field (×200) was recorded. Each experimental point was performed in triplicate, and 10 fields per filter were analyzed.

“In Gel” Three-dimensional Collagen Culture—Type I rat tail collagen-gel composed gels were formed according to the manufacturer’s recommendations. Briefly, an ice-cold collagen solution was neutralized by overlaying medium. Cultures were incubated for 48 h at 37°C in a humidified atmosphere containing 5% CO₂. Digital images were captured on confluent HUVECs in 24-well dishes, at 4°C. Cells were washed with PBS and preincubated for 30 min with 0.5 ml of binding medium. HUVECs suspended in M199 medium suplemented with 2.5% FBS were added to the lower chamber. The inserts were placed over chambers containing a chemotactic stimulus (10 ng/ml HGF, FGF-2, or VEGFα), and cells were allowed to migrate for 5 h at 37°C in a CO₂ incubator. For inhibition experiments one of the purified HGF α-chain-derived polypeptides (each at 1 μM) was added to the lower chamber. The filters were then rinsed with PBS, fixed with 1% (w/v) paraformaldehyde, and stained with 0.05% (w/v) crystal violet. The upper surface of the filters was scraped with a cotton swab to remove the non-migrant cells. The number of cells per high power field (×200) was recorded. Each experimental point was performed in triplicate, and 10 fields per filter were analyzed.

Radiolabeled Ligand Binding and Displacement Experiments—HGF (5 μg), VEGFα (10 μg), FGF-2 (10 μg), or recombinant HGF N-terminal domain (25 μg) were iodinated with 1 μCi of [125I]NaI using the Iodogen method and iodination Tubes according to the manufacturer’s instructions. Iodinated proteins were purified by chromatography on PD-10 columns equilibrated with 25 mM Tris-HCl, pH 7.4, containing 0.4 M NaCl, 5 mM EDTA, and 0.25% AlbuMAX and concentrated using the Ultrafree-4 Centrifugal Filter Device. The specific activities determined by trichloroacetic acid precipitation were 18 × 10⁶ cpm/pmol for [125I]HGF, 8 × 10⁶ cpm/pmol for [125I]VEGFα, 9 × 10⁶ cpm/pmol for [125I]FGF-2, and 5 × 10⁶ cpm/pmol for [125I]N.

Binding and displacement studies with the iodinated proteins were carried out on confluent HUVECs in 24-well dishes, at 4°C. Cells were washed with PBS and preincubated for 30 min with 0.5 ml of binding medium (M199 medium containing 0.1% AlbuMAX). To determine the binding parameters for N, increasing amounts of radiolabeled N were added to HUVECs, with or without a 100-fold molar excess of unlabeled N, and incubated for 6 h. For displacement experiments, constant amounts of radiolabeled HGF (1.5 nM), VEGFα (1.2 nM), or FGF-2 (1 nM) were added to HUVECs in the presence of increasing concentrations of unlabeled N for 4 h. At the end of incubation, cells were washed three times with binding medium and solubilized with 0.3 M NaOH. HUVEC-associated radioactivity was determined in a γ-counter, and the results were analyzed using the program Ligand included in the KELL package (Biosoft, Cambridge, UK). All experiments were done in triplicate. The Kᵢ values represent the means ± S.D. obtained from three to four experiments.

Anti-angiogenic Activity of the N-terminal Domain of HGF

Production and Purification of Recombinant HGF α-Chain Structural Modules—The HGF kringle domains were all expressed in E. coli in a soluble form. Recombinant N-terminal domain was mainly accumulated in insoluble inclusion bodies and was easily solubilized with 2 μM urea containing 2% Triton X-100. The proteins were purified to homogeneity using a simple one-step procedure on TALON metal affinity column (Fig. 1). They were subsequently subjected to oxidative folding using the glutathione-one-based oxidio-shuffling system. The final protein preparations were analyzed in SDS-PAGE under reducing and non-reducing conditions to ensure the presence of disulfide bonds (Fig. 1). We observed very little or no difference in the mobility of the non-reduced and reduced N, although this protein possesses two disulfide bonds. In contrast, the non-reduced K1 exhibited a substantial mobility shift compared with the reduced sample. Similar results had been previously reported for N and K1 (produced in E. coli (31)). K2, K3, and K4 also demonstrated an increased mobility under non-reducing conditions. These results indicate that our HGF kringle preparations have a compact conformation under non-reducing conditions and that the disulfide bonds were formed during the refolding procedure.

The circular dichroism analysis of N, K1, K2, K3, and K4 demonstrated that these proteins were folded. The far-UV CD spectra (180–260 nm) of N, K1, K2, K3, and K4 are all indicative of a high content in secondary structure. A broad band of positive ellipticity can be seen in the 230- to 260-nm region: it most probably originates from oxidized disulfides and aromatic residues involved in the characteristic hydrophobic core of the kringle-type folding. The near-UV CD spectra (240–340 nm) show bands in the 275- to 290-nm region, which are characteristic of an asymmetric orientation of aromatic residues (Trp and Tyr). These signals are typical for the folded tertiary structure of globular proteins (data not shown).
tion in a concentration-dependent manner. The influence of N on HUVEC proliferation could be detected at a concentration of 100 nM (about 10% of inhibition). Depending on the umbilical cord specimen, a maximum inhibition effect (up to 100%) was observed for a concentration of N of 1 \( \mu \)M.

Because our hypothesis was that kringles could modulate angiogenesis by competitive binding to the c-met receptor, we also studied the effect of recombinant HGF domains on the proliferation of HUVECs induced by HGF. The kringles of HGF had no effect on the proliferation of HUVECs in the presence of HGF (Fig. 2B). In contrast, N significantly inhibited the proliferation of HUVECs induced not only by HGF but also by two isoforms of VEGF: VEGF165 and VEGF121 (Fig. 2C). Thus, the anti-proliferative effect of N was not specific toward HGF.

We further tested the specificity of the anti-proliferative effect of N toward the endothelial cell type. In the preliminary experiments we showed that HGF was unable to stimulate the proliferation of hASM cells. In contrast, hASMC responded to PDGF BB (Fig. 2D), whereas PDGF BB had no effect on the proliferation of HUVECs (data not shown). The TE 85 and DLD-1 tumor cells did not respond to any of the growth factors tested in this study, and only DLD-1 cells could be slightly
The recombinant K1 of HGF, which had no effect on the HUVEC proliferation, migration, and morphogenesis, was not able to compete with HGF for binding to HUVECs (Fig. 5A). In contrast, N inhibited HGF, VEGF165, and FGF-2 binding to HUVECs in a concentration-dependent manner (Fig. 5). The results of two previous sets of experiments indicated that the HGF-derived kringles had no inhibitory activity, whereas the N-terminal domain appeared to be a potential anti-angiogenic molecule. To confirm the anti-angiogenic activity of N, we used a model known as “angiogenesis in vitro.” In this model, the HUVECs were suspended in a three-dimensional gel composed of type I collagen and underwent a number of events leading to the formation of a network of capillary-like tubes. The model reproduces several steps of the angiogenesis such as motility, morphogenesis, and the production of lytic enzymes, which makes the penetration of the extracellular matrix possible (33).

When grown in a three-dimensional collagen gel in the presence of FGF-2, VEGF165, or HGF, HUVECs rapidly underwent morphogenesis forming a network of cords and tubes. After 24 h, HUVECs showed a characteristic elongated phenotype and sprouting extensions. Some cells formed short structures made of several branched and associated elongated cells (data not shown). After 48 h, more cells were involved in structure formation, but some died (Fig. 4). After 72 h, virtually all the cells were dead. When 1 μM N was added to HUVECs grown in three-dimensional collagen, no formation of structures was observed, whatever the growth factor and the length of time tested (Fig. 4, shown for 48 h). In the presence of the HGF-derived kringles, the capillary-like structures formed normally (data not shown).

Recombinant HGF N-terminal Domain Prevents the Growth Factor Binding to HUVECs—Recombinant HGF N-terminal domain clearly antagonized the angiogenic response induced by at least three different cytokines. This inhibition could occur at several levels: (i) by inhibiting the interaction with binding sites on the cell surface; (ii) by inhibiting the receptor phosphorylation; and (iii) by inhibiting some signaling pathways downstream of the receptor activation. To clarify the molecular mechanism of N inhibitory activity, we undertook the displacement experiments with radiolabeled HGF, VEGF165, and FGF-2 (Fig. 5).

The recombinant K1 of HGF, which had no effect on the HUVEC proliferation, migration, and morphogenesis, was not able to compete with HGF for binding to HUVECs (Fig. 5A). In contrast, N inhibited HGF, VEGF165, and FGF-2 binding to HUVECs in a concentration-dependent manner (Fig. 5). In the three cases, the displacement kinetics was best described by a simple bimolecular reaction model. The $K_d$ values, calculated using a weighted non-linear curve fitting algorithm implemented in the KELL package, were of 17.1 ± 10.74 nm, 27.69 ±
The N-terminal domain of human HGF inhibits the binding of growth factors to HUVECs. Displacement analysis of 1.5 nM ¹²⁵I-labeled HGF (A), 1.2 nM ¹²⁵I-labeled VEGF₁₆₅ (B), and 1 nM ¹²⁵I-labeled FGF-2 (C) binding to HUVECs in the presence of unlabeled N or K1. Values on the y-axis are the mean values obtained from triplicate cultures. Results shown are representative of three or four experiments.

Fig. 5. The N-terminal domain of human HGF inhibits the binding of growth factors to HUVECs. Displacement analysis of 1.5 nM ¹²⁵I-labeled HGF (A), 1.2 nM ¹²⁵I-labeled VEGF₁₆₅ (B), and 1 nM ¹²⁵I-labeled FGF-2 (C) binding to HUVECs in the presence of unlabeled N or K1. Values on the y-axis are the mean values obtained from triplicate cultures. Results shown are representative of three or four experiments.

Fig. 6. The recombinant N-terminal domain interacts with a single class of binding sites on the HUVEC surface. ¹²⁵I-labeled N was added to HUVECs (0.1–40 nM) and incubated for 6 h at 4 °C. The inset shows a Scatchard plot obtained using Ligand analysis. Results shown are representative of three experiments.

Fig. 7. The N-terminal domain is the principal region involved in HGF-heparin interaction. SPR analysis of the interaction of purified recombinant N (0.7 µM), K1 (9.5 µM) (dashed line), K2 (10.1 µM) (bold line), K3 (6.1 µM), or K4 (12.2 µM) (thin dotted line) with heparin immobilized on the surface of streptavidin-coated SA sensor chips. Results shown are representative of two experiments.

27.52 nm, and 68.16 ± 17.5 nm for HGF, VEGF₁₆₅, and FGF-2 displacement, respectively. These values were in good agreement with a $K_d$ value of 67.9 ± 11.07 nm, obtained from the binding experiments between radiolabeled N and HUVECs. Scatchard analysis of the direct binding of N demonstrated the existence of a single class of binding sites (Fig. 6). Taken together these experiments indicated that N shares common binding sites with HGF, VEGF₁₆₅, and FGF-2 on HUVECs and that these sites are most probably of a low affinity nature.

SPR Analysis of the Interactions of HGF and Its Domains with Heparin and c-met—To gain insight into the molecular mechanism of the inhibitory activity of N, we compared the binding properties of this polypeptide with those of full-length HGF and of the four other isolated HGF α chain domains, using surface plasmon resonance (SPR) assays.

We first studied the interaction of purified HGF domains with immobilized non-fractionated heparin. Representative results are shown in Fig. 7: all five HGF α-chain domains were able to bind heparin, but their binding characteristics presented major differences. The analysis of the binding curves indeed showed that N bound heparin with a 1000-fold higher affinity than any of the four kringles, mainly because its association rate constant $k_a$ was much higher than that of the other HGF domains (Table I). N therefore clearly is the principal HGF domain involved in heparin binding.

| Analyte in solution | $k_a$ (µM) | $k_{off}$ (s⁻¹) | $K_a$ (µM) |
|---------------------|------------|-----------------|------------|
| N                   | 2.11 ± 0.40 | 1.03 ± 0.21     | 1.01 ± 0.09 |
| K1                  | 2.11 ± 0.40 | 1.03 ± 0.21     | 1.01 ± 0.09 |
| K2                  | 2.11 ± 0.40 | 1.03 ± 0.21     | 1.01 ± 0.09 |
| K3                  | 2.11 ± 0.40 | 1.03 ± 0.21     | 1.01 ± 0.09 |
| K4                  | 2.11 ± 0.40 | 1.03 ± 0.21     | 1.01 ± 0.09 |

We considered the interaction of N with immobilized heparin swapped, i.e., we analyzed the binding of soluble c-met or heparin molecules to HGF domains immobilized on the surface of CM5 sensor chips. Representative examples of the results obtained are shown in Fig. 8. Only full-length HGF was able to interact with c-met (Fig. 8A), whereas no detectable binding could be evidenced for the five HGF α-chain isolated subdomains (data shown only for N, Fig. 8B). The interaction of c-met with immobilized full-length HGF showed a complex kinetic behavior, which could be analyzed taking into account the dimeric (and therefore bivalent) nature of the c-met/Fc fusion. The kinetic parameters of the interaction were determined as follows: $k_{on} = 2.97 ± 0.57 \times 10^{4} M^{-1} s^{-1}$, $k_{off} = 2.11 ± 0.40 \times 10^{-3} RU^{-1} s^{-1}$, $k_{on} = 6.22 ± 1.01 \times 10^{-3} s^{-1}$, $k_{off} = 4.31 ± 0.56 \times 10^{-5} s^{-1}$. By analyzing the concentration...
to the c-met receptor in vitro. SPR analysis of the interactions of HGF(A and C) and its N-terminal domain (B and D) with c-met/Fc chimera (12, 97, and 770 nM) (A and B) and non-fractionated heparin (2, 17, and 139 nM) (C and D). Results shown are representative of two experiments.

![Image of SPR analysis](image-url)

dependence of the steady-state responses, a $K_d$ of 50 (±4) nM could be determined for the interaction between c-met and HGF; this value considers the binding process globally without making any assumption on the mechanism of the interaction.

We finally studied the interaction with immobilized HGF and N of two different commercial heparin preparations: NF heparin and LMW heparin (data shown only for NF heparin, Fig. 8, C and D). The kinetic parameters of the interactions are shown in Table II: they were not significantly dependent on the amount of immobilized ligand (data not shown). Interestingly, NF heparin bound ~25 times better than LMW heparin, both to HGF and N (Table II): NF heparin showed both higher association rates, which could be due to the fact that its global electrostatic charge is higher (and therefore more favorable) than that of LMW heparin, and lower dissociation rates, which could be explained by the fact that NF heparin is statistically more multivalent (and therefore more avid) than LMW heparin. It should also be noted that the affinity of heparin for N is slightly stronger than that for full-length HGF (Table II): this 4-fold difference, only due to changes in $k_{off}$, could be linked to slight conformational modifications in the heparin binding site(s) of N, whether this domain is isolated or in the context of full-length HGF.

**DISCUSSION**

HGF is a powerful endothelial cell mitogen, motogen, and morphogen (9). Abnormal HGF levels have been shown to contribute to the increased or impaired angiogenesis associated with several human cancers (34–37) and with some other pathological situations, such as diabetes (38), diabetic retinopathy (39), and arthritis and osteoarthritis (40). Therefore, HGF and its receptor represent very attractive targets for the development of new pro- or anti-angiogenic therapies. Indeed, several reports have demonstrated the effectiveness of HGF-based strategies in the promotion of therapeutic angiogenesis (38, 41, 42) or in the prevention of pathological angiogenesis (43).

Understanding the relationship between the structure and function of HGF could provide a way of creating potent HGF antagonists and agonists useful for the treatment of angiogenesis-associated disorders. At present, the significance of the modular structure of HGF and the mode of interaction of HGF with the c-met receptor are not completely understood. Protein engineering experiments and x-ray crystallography analysis have shown that the c-met receptor and heparin binding regions were located within the α chain, and more precisely, within NK1 (15, 16, 18). NK1 was further identified as a partial c-met receptor agonist in vivo (44). Recent studies have demonstrated that K1 alone is sufficient to bind to and activate the c-met receptor (45). Interestingly, the addition of the following kringles to NK1 changed the biological activity of the resultant polypeptide. Thus, NK2, another naturally occurring HGF variant composed of N and the two first kringles, antagonized the HGF-induced cell growth in vivo but facilitated cell motility (46). Finally, NK4, an entire HGF α chain, has been shown to compete successfully with the native HGF for the binding to the receptor and inhibit the diverse biological responses to HGF, including the angiogenic response (47–49). These observations suggested that an anti-angiogenic activity could be located within the HGF kringle domains. This inhibitory activity could depend on the ability to displace HGF from its receptor, and this mechanism could be common with anti-angiogenic kringles of other origin.

To understand the mode of interaction of HGF with its receptor and establish the regions with antagonistic activity, we produced all five HGF α-chain domains as recombinant proteins and tested their ability to inhibit the angiogenic response induced in HUVECs. Our results demonstrated that none of the isolated HGF kringles was capable of inhibiting the endothelial cell proliferation, migration, and morphogenesis in a three-dimensional collagen gel. In contrast, the N-terminal domain was identified as a new anti-angiogenic polypeptide.
The inhibitory activity of N was not specific toward HGF, because N successfully inhibited the HUVEC proliferation, migration, and morphogenesis induced by other growth factors, such as FGF-2 and VEGF<sub>165</sub>. Moreover, in contrast to the full-length HGF, the HGF N-terminal domain did not interact significantly with the c-met/Fc chimera <em>in vitro</em>. Therefore, the mechanism of anti-angiogenic activity of N was not dependent on the competitive binding to the HGF receptor.

SPR studies showed that none of the isolated domains of the α chain was capable to bind to the c-met receptor <em>in vitro</em>. This indicates that the c-met binding determinant in the native cytokine could involve several modules. Our results seem to be in disagreement with the findings that K1 is able to induce the c-met phosphorylation (45). Nevertheless, in the cited report, the authors showed that the c-met receptor activation in the presence of K1 was strongly dependent on the presence of heparin or heparan sulfate. The absence of K1 binding in our <em>in vitro</em> experiments using purified proteins could therefore be explained by the fact that heparan sulfate is required for the binding of K1 to the receptor <em>in vitro</em>

We have demonstrated in this work that K1 and three other HGF kringle were able to bind heparin <em>in vitro</em>, albeit with a relatively low affinity. These results are in agreement with the recently published x-ray crystal structure of NK1-heparin complexes, which have shown that heparin is in contact with several amino acid residues in N and in K1 (18). The K<sub>d</sub> calculated from our SPR experiments for the binding of different HGF kringle to heparin did not differ significantly between the kringle but were three orders of magnitude lower than that of N. From these data K2 does not seem to contribute in a particular way to the HGF interaction with heparin, in contrast with the conclusion drawn from earlier experiments with HGF deletion mutants (16).

The demonstration that N preserved the heparin binding activity of full-length HGF but did not bind the c-met receptor <em>in vitro</em> ruled out the possibility of contamination of the c-met/Fc preparations with cellular glycosaminoglycans (GAGs). Therefore, the HGF binding to c-met/Fc chimera observed in our SPR experiments is GAG-independent. Nevertheless, we obtained a K<sub>d</sub> value that was substantially lower than that obtained in the earlier <em>in vivo</em> studies (nanomolar against picomolar order). This is consistent with the data showing that HGF is less efficient at inducing the receptor activation in the heparan sulfate-deficient cells (45). Taken together these results indicate that the cell surface GAGs could increase the affinity of HGF toward c-met.

Despite the progress in the understanding of the role of heparin and other GAGs in the growth factor receptor signaling, the mechanisms through which they regulate this process remain incompletely understood. GAGs are believed to be physiologically required co-receptors for a number of growth factors, by providing secondary binding sites that complement the interaction with the specific receptor and strengthen the adhesive forces (50). GAGs also promote dimerization/oligomerization of growth factors. This allows the monomeric ligands such as FGF to induce receptor dimerization, which is widely accepted as a prerequisite for the receptor activation. According to this model, a monomeric receptor remains inactive until the oligomerized ligand binds and oligomerizes the receptor, allowing autophosphorylation of receptor-associated kinases (51). Nevertheless, an increasing number of data suggest that GAGs do not act only by oligomerizing either ligand or receptor and that their role could be to induce the conformational changes in ligand and/or receptor (52, 53).

Our results suggest that the anti-angiogenic activity of N is dependent on its high heparin binding capacity. The displacement experiments with HUVECs showed that N acts most probably at the level of growth factor binding to the secondary binding sites, presented by cell surface GAGs. Interestingly, our results demonstrated that N inhibited more efficiently the binding of HGF and VEGF<sub>165</sub> than that of FGF-2. This is in good agreement with the available data on the structure of the heparin binding sites of the three growth factors. Thus, the structure of the 55-residue heparin binding domain of VEGF<sub>165</sub> is remarkably similar to the structure of the hairpin-loop region within N domain of HGF, which forms part of the putative heparin binding site (17, 54). On the contrary, the folding pattern of the FGF-2 heparin binding site is quite different from that of the two other growth factors (55). Previous studies had also indicated that the heparin sequence that binds FGF-2 differs from the one that binds HGF. Thus, 2-O-sulfates in iduronic acid, N-sulfates in glucosamine, and the carboxylate group in iduronic acid units of heparin are involved principally in the interaction with FGF-2, whereas 6-O-sulfates in glucosamine units seem to be of less importance (55–57). In contrast, 6-O-sulfates are the major determinants of the heparin-HGF interaction, with 2-O-sulfates playing minor role (18, 58). Similarly, the VEGF<sub>165</sub>-heparin interaction involves principally the 6-O-sulfates and N-sulfates in glucosamine with a minor contribution of 2-O-sulfates in iduronic acid (59). Thus, our results are in agreement with the available data on the structural basis of the interaction of FGF-2, HGF, and VEGF<sub>165</sub> with sulfated polysaccharides.

N could also interfere with the pre-existing c-met/GAG interaction. Recent results suggest that the role of GAG in growth factor signaling is more complex than that predicted previously on the basis of ligand-GAG interaction alone. Thus, it has been shown that some growth factor receptors, including c-met, are themselves able to bind with high affinity to GAGs, which presumably could stabilize the pre-existing oligomeric receptor clusters (45, 60). Further elucidation of the molecular mechanism of N inhibitory action could contribute to the understanding of the physiological significance of the c-met/GAG interaction and to the design of new anti-angiogenic molecules targeting the GAGs involved in receptor tyrosine kinase signaling.

Acknowledgments—We are very grateful to Dr. Alain Chaffotte for the CD spectra of the HGF domains and to the maternity departments of the Lariboisière (Paris) and Jean Rostand (Ivy-sur-Seine) hospitals for providing umbilical cord specimens. We thank Dr. Pascale Briand for pGem/HGF-FM construct and Dr. Janne L. Simonsen for pRI expression strain.

References

1. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimomura, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989) Nature 342, 440–443
2. Stoker, M., Ghirardi, E., Perryman, M., and Gray, J. (1987) Nature 327, 239–242
3. Matsuzato, K., and Nakamura, T. (1997) CIBA Found. Symp. 212, 198–214
4. Park, M., Dean, M., Kaul, K., Braun, M. J., Gonda, M. A., and Vande, W. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6379–6383
5. Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M., Kmiecik, T. E., Vande, W. G., and Aaronson, S. A. (1991) Science 251, 892–894
6. Comoglio, P. M., and Trusolino, L. (2002) J. Clin. Invest. 109, 857–862
7. Bussolino, F., Di Renzo, M. F., Ziche, M., Bocchietti, E., Olivero, M., Naldini, J., Gaudino, G., Tamagnone, L., Coffier, A., and Comoglio, P. M. (1992) J. Cell Biol. 119, 629–641
8. Silvagno, F., Follenzi, A., Arese, M., Pratt, M., Giraudo, E., Gaudino, G., Camussi, G., Comoglio, P. M., and Bussolino, F. (1995) Arterioscler. Thromb. Vasc. Biol. 15, 1857–1860
9. Rosen, E. M., Lamzues, K., Laterra, J., Polverini, P. J., Rubin, J. S., and Goldberg, I. D. (1997) CIBA Found. Symp. 212, 215–229
10. Van Belle, E., Wittenbichler, B., Chen, D., Silver, M., Chang, L., Schwall, R., and Inner, J. M. (1998) Circulation 97, 381–390
11. Naldini, L., Tamagnone, L., Vigna, E., Sachs, M., Hartmann, G., Birchmeier, W., Dukuhara, Y., Tsucho, H., Blasi, F., and Comoglio, P. M. (1992) EMBO J. 11, 4825–4833
12. Miyazawa, K., Shimomura, T., Kitamura, A., Kondo, J., Morimoto, Y., and Kitamura, N. (1993) J. Biol. Chem. 268, 10924–10928
13. Hartmann, G., Naldini, L., Weidner, K. M., Sachs, M., Vigna, E., Comoglio, P. M., and Birchmeier, W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11574–11578

Anti-angiogenic Activity of the N-terminal Domain of HGF
