Inhibition by Copper(II) Binding of Hepatocyte Growth Factor (HGF) Interaction with Its Receptor Met and Blockade of HGF/Met Function*

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Overexpression of hepatocyte growth factor (HGF) and its receptor Met often occurs in carcinoma cells, leading to establishment of an HGF/Met autocrine loop. Therefore, disruption of the HGF/Met autocrine loop may lead to down-regulation of tumorigenesis. To study the HGF/Met interaction, we have developed a cell-free system to detect HGF binding to a Met fusion protein, Met-IgG, using a modified enzyme-linked immunosorbent assay methodology. Since we previously showed that HGF can be purified by copper(II) affinity chromatography, we further explored the effect of copper(II) on the HGF/Met interaction. The divalent metal cations copper(II) and zinc(II) significantly inhibited HGF binding to immobilized Met-IgG with IC_{50} values of 230–270 μM, respectively, whereas manganese(II) and magnesium(II) were less inhibitory with 20–60-fold higher IC_{50} values. Incubation of 1 mM copper(II) with HGF resulted in nondenaturing and denaturing gel-mobility shifts, indicating that copper(II) binds directly to HGF. This interaction occurs at the N terminus of HGF, as incubation of 1 mM copper(II) with both HGF and the HGF derivative NK1 yielded similar results on SDS-PAGE. HGF-induced activation of Met and cell scattering were inhibited upon addition of HGF in the presence of 1 mM and 500 μM copper(II), respectively. Chemical protonation with diethyl pyrocarbonate of HGF histidine residues impeded the ability of 500 μM copper(II) to inhibit the binding of HGF to immobilized Met-IgG. Based on the NK1 domain structure, we propose that copper(II) may interact with HGF via the histidine residues in either N-terminal or kringle domains. The inhibition of HGF/Met interaction and subsequent downstream cellular functions may be through direct interference by copper(II), such as a change in charge or an induced local conformational change. This putative copper(II) binding domain may be the basis for developing potential inhibitors of HGF/Met binding and downstream functions and could lead to novel strategies for anti-cancer treatment.

Hepatocyte growth factor (HGF) is a potent stimulator of cell survival, mitogenesis, and invasion (1, 2). In normal development, HGF is secreted by stromal cells and stimulates the epithelial cell surface-expressed receptor Met in a tightly controlled paracrine manner (3–5). Aberrant expression of HGF in epithelial cells, coupled with Met overexpression, leads to an HGF/Met autocrine loop that is implicated in tumorigenesis (6–9). A protein microarray ELISA approach was used to demonstrate elevated HGF levels in the sera of breast cancer patients (10), whereas high levels of HGF or Met, specifically in human breast and lung carcinomas (11–13), are prognosticators of poor patient survival (11, 13). Therefore, targeting the HGF/Met interaction may be important in novel therapy designs for treatment of cancers in which an HGF/Met autocrine loop is implicated.

HGF is secreted as an immature pro-HGF, which undergoes processing by a number of factors such as tissue-type plasminogen activator (14), urokinase-type plasminogen activator (14), or HGF-converting enzyme (15). This post-translational processing yields mature HGF, consisting of a 69-kDa α-chain disulfide linked to a 34-kDa β-chain (16–19). The HGF α-chain consists of an N-terminal hairpin loop (N), followed by four sequential kringle domains, designated K1, K2, K3, and K4 (20). Located at the C terminus of the α-chain, following the kringle domains, is a cysteine residue involved in the disulfide linkage to the HGF β-chain (20). HGF interacts with the extracellular domain of Met, resulting in the activation of Met and subsequent downstream functions.

Deletion and mutational studies indicate that the HGF β-chain is not required for Met binding but is needed for complete activation of the receptor (21–23). Sequential C-terminal deletions of the HGF β-chain correlate with loss of HGF function, whereas no effectual single mutation has been identified, suggesting a role for the β-chain in conformational stabilization of the HGF/Met interaction (23). An HGF variant, NK4 (24), consisting of the α-chain N-terminal hairpin loop and the four kringle domains, antagonizes HGF-induced cell motility, proliferation, angiogenesis, and phosphorylation of Met (25–28). Most interesting, K1–4, an NK4 variant lacking the N-terminal hairpin loop, does not exhibit the full range of NK4 antagonistic behavior (27). Sequential deletion of the fourth and third kringles decreases HGF activity, whereas sequential deletion of the second and first kringles, along with the N-terminal hairpin loop, completely blocks HGF activity (21). Mutational analysis was used to map Met binding activity to the region of the HGF molecule spanning the hairpin loop, the first kringle, and the N terminus of the second kringle (22). The

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§ The abbreviations used are: HGF, hepatocyte growth factor; ELISA, enzyme-linked immunosorbent assay; DEPC, diethyl pyrocarbonate; TPA, 12-O-tetradecanoylphorbol-13-acetate; BSA, bovine serum albumin; HRP, horseradish peroxidase; MDCK, Madin-Darby canine kidney; DMM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; PBS, phosphate-buffered saline.
Copper(II) Inhibits HGF Binding to Met

HGF isoforms, NK1 and NK2, containing the hairpin loop and the first kringle or both the first and second kringle, respectively, exhibit both antagonistic and agonistic effects on HGF binding to Met and functional properties (29–33). The modulating effects of NK1 and NK2 are dependent on cell type and therefore may be influenced by the presence or absence of cellular stabilizing co-factors that affect HGF/Met binding. HGF binding was determined previously that HGF can be purified utilizing copper(II) affinity chromatography (34). Copper(II) binding to kringle-containing apolipoprotein(a) has also been documented recently (35). These observations suggest that HGF may contain copper(II)-binding sites, which could potentially modulate HGF activity. This paper investigates the binding of copper(II) to HGF, the effect of copper(II) on HGF binding to Met, the stabilization of this complex, and the effect on cellular functions. Our results indicate that copper(II) inhibits HGF/Met interaction and downstream cellular functions.

MATERIALS AND METHODS

Reagents—ELISA, non-denaturing gel, and SDS-PAGE buffer materials were purchased from Fisher, as were Harris modified hematoxylin and EDTA. Bovine serum albumin (BSA), O-phenylenediamine, 12-O-tetradecanoylphorbol-13-acetate (TPA), CuCl2·2H2O, and ZnCl2 were supplied by Sigma. H2SO4, MnCl2·4H2O, and MgCl2·6H2O were obtained from BDH (Mississauga, Ontario, Canada). Protease inhibitors were purchased from Roche Applied Science, BDH, and Fisher. Diethyl pyrocarbonate (DEPC) was purchased from ICN Biomedical Inc. (Aurora, OH). Protein A-Sepharose CI-4B was purchased from Amersham Biosciences. The Micro BCA protein determination kit was purchased from Pierce. Immobilon P membrane was supplied by Millipore (Bedford, MA). Western Lightning was purchased from PerkinElmer Life Sciences. Recombinant HGF, human IgG, Met-IgG fusion protein (36), and mouse anti-human HGF antibodies (A3.1.2 or 4H8.2) were generously donated by Dr. Ralph Schwall (Genentech Inc., San Francisco, CA). A recombinant HGF NK1 fragment was kindly provided by Dr. Ermanno Gherardi (University of Cambridge, UK). Rabbit anti-mouse Met antibody (SP260), mouse anti-mouse Met antibody (B2), and rabbit anti-human HGF (H-145) antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-phosphotyrosine antibody was obtained from Transduction Laboratories (Mississauga, Ontario, Canada). Donkey anti-mouse HRP antibody and donkey anti-rabbit HRP antibody were supplied by Amersham Biosciences.

Cell Culture—Tissue culture plates were supplied by Nunc (Burlington, Ontario, Canada). Madin-Darby canine kidney (MDCK) epithelial cells were maintained in DMEM (supplied by Invitrogen) supplemented with 10% FBS, with an atmosphere of 5% CO2 at 37 °C. A spontaneous nonmetastatic mouse mammary adenocarcinoma cell line, SP1, which expresses both HGF and Met, was isolated at Queen’s University and has been described previously (6). SP1 cells were cultured in RPMI medium supplemented with 7% FBS at 37 °C, with an atmosphere of 5% CO2.

Enzyme-linked Immunosorbent Assay (ELISA) for Measuring HGF–Met Binding—ELISA plates were obtained from Costar (Corning, NY). Either the Met-IgG fusion protein or HGF was diluted to a final concentration of 1 μg/ml in coating buffer (0.1 M HCO3, pH 9.6) and incubated (100 μl/well) in 96-well plates overnight at 4 °C. Plates were washed three times (150 μl/well) with PBS, pH 7.4, containing 0.1% Tween 20. Plates were then blocked (150 μl/well) with PBS containing 2% BSA for 2 h at 4 °C and again with blocking buffer, as above. Either serial dilutions or a fixed concentration (100 ng/ml) of HGF in HBS containing 1% BSA and 0.1% Tween 20 was incubated (100 μl/well) in plates containing immobilized Met-IgG for 2 h at room temperature with various screening conditions, as indicated in the figure legends. When required, HGF (12 μg/ml) was pretreated with 0.1% DEPC for 45 min prior to coating. Plates were washed, as above, and incubated (100 μl/well) in plates containing immobilized Met-IgG for 2 h at room temperature with various screening conditions, as indicated in the figure legends. Plates were washed, and a monoclonal anti-human HGF antibody (either A3.1.2 or 4H8.2) was added (100 μl/well; 1:2500) and incubated for 2 h at room temperature. When required, either serial dilutions or a fixed concentration (100 ng/ml) of Met-IgG in HBS containing 1% BSA and 0.1% Tween 20 was incubated (100 μl/well) in plates containing immobilized HGF for 2 h at room temperature with various screening conditions, as indicated in the figure legends. Plates were washed, and a monoclonal anti-human Met antibody (3D6) was added (100 μl/well; 1:2500) and incubated for 2 h at room temperature. All plates were washed, as above, and subsequently incubated (100 μl/well; 1:2500) with a donkey anti-mouse HRP antibody for 1 h at room temperature. Plates were again washed, and either HGF or Met-IgG binding was assessed by color detection with development buffer (0.4 M O-phenylenediamine in 50 mM Na2HPO4, 25 mM citric acid containing 0.03% H2O2, pH 5.0). The reaction was stopped with 2 μl H2SO4 neutralizing acid (50 μl/well). Absorbance was determined at 490 and 630 nm.

Gel-mobility Shift Assay—Aliquots (1 μg) of both HGF and NK1 were incubated separately in stabilizing buffer (200 mM Tris containing 500 mM NaCl, pH 7.4) at 37 °C for 2 h under various conditions. Conditions included either HGF or NK1 alone or with 5 mM EDTA, 1 mM copper(II), 1 mM copper(II) and 5 mM EDTA, 1 mM manganese(II), or 1 mM manganese(II) and 5 mM EDTA. Following incubation, either 50% sucrose sample buffer or 2x SDS sample buffer, containing 5% β-mercaptoethanol, was added to the sample. In a 1:1 ratio, and subjected to 7% nondenaturing gel electrophoresis or 10% SDS-PAGE, respectively. Proteins were then transferred to Immobilon P membranes. Transferred proteins were subjected to Western blotting with the rabbit anti-human antibody (H-145; 1:5000) and donkey anti-rabbit HRP antibody (1:1000), followed by chemiluminescence detection (Western Lightning).

Met-Tyrosine Phosphorylation Assay—SP1 cells were seeded at 1× 105 cells/well in 100-mm tissue culture dishes and incubated for 8 h. In order to reduce endogenous activation of Met, media were removed, and cells were washed once with PBS and subsequently incubated in serum-free RPMI overnight. Cells were then washed twice with PBS and supplemented with 5 ml of serum-free RPMI containing appropriate conditions. RPMI was either added alone, with 100 ng HGF, with 1 mM copper(II), or with 100 ng HGF and 1 mM copper(II). Cells were then incubated for an additional 1 h at 37 °C, with 5% CO2 atmosphere. The cells were washed twice with ice-cold PBS and lysed in 400 μl of lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 1 mM Na3VO4, 10 mM NaF, 1% phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 2 μg/ml leupeptin, and 1% Nonidet P-40, pH 7.4). Lysates were cleared of cellular debris by centrifugation (14,000 × g), and supernatant protein concentrations were determined by Micro BCA. The supernatants were adjusted, with lysis buffer, to equivalent protein concentrations. A 50-μl aliquot of a 50% (w/v) protein A-Sepharose was added to each supernatant, and samples were incubated with protein A-Sepharose at 4 °C overnight. Protein A-Sepharose was then pelleted (14,000 × g) and washed twice with lysis buffer. A volume of 2x SDS sample buffer, containing 5% β-mercaptoethanol, was added to the pellets and boiled for 5 min. Samples were subjected to 10% SDS-PAGE and were subsequently transferred to Immobilon P membrane. Western blotting was performed with mouse anti-Met antibody (B2; 1:1000) or mouse anti-phosphotyrosine antibody (PY20; 1:200), followed by donkey anti-mouse HRP antibody. Immunoreactivity was detected by chemiluminescence (Western Lightning).

RESULTS

HGF/Met-IgG Binding Can Be Assessed by ELISA—Two ELISA methods were initially utilized to assess HGF binding to its receptor Met. The first method required that titrations of HGF be incubated with a fixed concentration (1 mg/ml) of either immobilized human IgG or immobilized Met-IgG. HGF binds specifically to immobilized Met-IgG in a hyperbolic manner over a broad HGF concentration range (up to 100 ng/ml) (Fig. 1A). In comparison, HGF exhibits no binding affinity for immobilized human IgG. The second method required that titrations of Met-IgG be incubated with a fixed concentration (1 mg/ml) of either immobilized human IgG or immobilized HGF. Met-IgG binds specifically to immobilized HGF in a hyperbolic manner over a broad Met-IgG concentration range (up to 100 ng/ml) (Fig. 1B), whereas Met-IgG exhibits very little binding affinity for immobilized human IgG. Therefore, both ELISA methods can be used for detecting specific HGF/Met-IgG bind-
Copper(II) Inhibits HGF Binding to Met

Because it was determined that a copper(II) concentration as low as 500 μM could effectively inhibit HGF binding to immobilized Met-IgG, we explored whether 500 μM copper(II) could also inhibit Met-IgG binding to immobilized HGF. HGF (100 ng/ml) was incubated with immobilized Met-IgG (1 mg/ml) alone or in the presence of 500 μM copper(II). Similar to the results of Fig. 2A, copper(II) at 500 μM almost completely inhibits binding (Fig. 3A). Alternatively, Met-IgG (100 ng/ml) was incubated with immobilized HGF (1 mg/ml) alone or in the presence of 500 μM copper(II). In this ELISA method, 500 μM copper(II) decreases the binding level to only 60% that of the uninhibited binding level (Fig. 3B). These results indicate that copper(II) inhibits the HGF/Met-IgG interaction to a greater extent when HGF is free in solution and Met-IgG is immobilized, compared with immobilized HGF and free Met-IgG.

Copper(II) Causes Gel-mobility Shifts of Both HGF and NK1, and This Effect Is Reversed by EDTA—To explore further whether a direct interaction between HGF and copper(II) exists, we used a nondenaturing gel-mobility shift assay. HGF (1 μg) was incubated under various conditions, with or without copper(II), and subjected to nondenaturing gel electrophoresis and Western blotting (Fig. 4A). The presence of 1 mM copper(II) results in the upward mobility shift of HGF, which is reversed by 5 mM EDTA, whereas EDTA alone does not affect mobility of HGF. In contrast, the presence of 1 mM manganese(II) alone or combined with 5 mM EDTA does not result in any detectable mobility shift of HGF protein. To compare the effects of copper(II) on HGF and NK1, we utilized nonreducing SDS-PAGE. HGF and NK1 (1 μg each) were incubated separately under various conditions and subjected to SDS-PAGE and Western blotting (Fig. 4, B and C). The presence of 1 mM copper(II) results in the emergence of new protein bands mi-

Fig. 1. HGF binds to immobilized Met-IgG, and comparatively, Met-IgG binds to immobilized HGF. A, either human IgG or Met-IgG (1 mg/ml each) was immobilized on a 96-well ELISA plate overnight at 4 °C, followed by incubation with various concentrations of HGF at room temperature for 2 h. HGF binding to immobilized (Immob.) human IgG (circles) or Met-IgG (triangles) was assessed by incubation with mouse anti-human HGF antibody, followed by donkey anti-mouse HRP and color detection. B, either human IgG or HGF (1 mg/ml each) was immobilized on a 96-well ELISA plate overnight at 4 °C, followed by incubation with various concentrations of Met-IgG at room temperature for 2 h. Met-IgG binding to immobilized human IgG (circles) or HGF (squares) was assessed by incubation with mouse anti-human Met antibody, followed by donkey anti-mouse HRP and color detection. Points on the graphs represent the mean absorbance for triplicate wells ± S.D. Experiments were performed a minimum of three times.

Fig. 2. Titrations of copper(II) and zinc(II) inhibit HGF binding to Met-IgG. Met-IgG (1 mg/ml) was immobilized on 96-well ELISA plates overnight at 4 °C. HGF (100 ng/ml) was then incubated at room temperature under the following conditions. A, HGF alone or in the presence of a concentration titration of copper(II). B, HGF alone or in the presence of a concentration titration of manganese(II); C, HGF alone or in the presence of a concentration titration of zinc(II); or D, HGF alone or in the presence of a concentration titration of magnesium(II). HGF binding was assessed by incubation with mouse anti-human HGF antibody, followed by donkey anti-mouse HRP and color detection. Results are expressed as percentages of mean absorbances ± S.D. for HGF binding in the presence of compounds relative to the mean absorbance for HGF binding alone. Experiments were performed in triplicate.

Divalent Cations, Specifically Copper(II) and Zinc(II), Inhibit HGF/Met-IgG Interaction—Metal compounds are implicated in binding to HGF, specifically copper(II) which can be utilized in the purification of HGF (34). Therefore we screened potential metal ion modulators of the HGF interaction with Met-IgG. HGF (100 ng/ml) was incubated with immobilized Met-IgG (1 mg/ml) alone or in the presence of various metal ion titrations. Copper(II) and zinc(II) almost completely inhibit HGF binding to immobilized Met-IgG over a broad divalent cation concentration range (500 μM-10 mM) (Fig. 2, A and C). HGF/Met-IgG binding is recovered at a lower concentration (125 μM) of copper(II) or zinc(II), each exhibiting an IC_{50} value of ~250 μM. In contrast, a high concentration (100 mM) of magnesium(II) inhibits HGF/Met-IgG binding only to 10% that of control levels, and binding is quickly recovered with decreasing concentrations of magnesium(II) (Fig. 2B), with an IC_{50} value of 10 mM. A high concentration (100 mM) of magnesium(II) also reduces binding but only to 60% that of control levels. Binding is also quickly recovered upon decreasing concentrations of magnesium(II) (Fig. 2D), with an IC_{50} value of 50 mM. Thus, HGF interaction with Met-IgG appears to be mediated by divalent cations and is preferentially inhibited by copper(II) and zinc(II).
copper(II) inhibits HGF binding to immobilized Met-IgG to a greater extent than copper(II) inhibits Met-IgG binding to immobilized HGF. A, Met-IgG (1 mg/ml) was immobilized on a 96-well ELISA plate overnight at 4 °C. HGF (100 ng/ml) was incubated alone or in the presence of 500 μM copper(II) for 2 h. HGF binding was assessed by incubation with mouse anti-human HGF antibody, followed by donkey anti-mouse HRP and color detection. B, HGF (1 mg/ml) was immobilized on a 96-well ELISA plate overnight at 4 °C. Met-IgG (100 ng/ml) was incubated alone or in the presence of 500 μM copper(II) for 2 h. Met-IgG binding was assessed by incubation with mouse anti-human Met antibody, followed by donkey anti-mouse HRP and color detection.

These results indicate that both HGF and NK1 specifically bind copper(II), which may cause an alteration of their conformational properties.

HGF-induced Tyrosine Phosphorylation of Met Is Inhibited by Copper(II)—The spontaneous murine mammary carcinoma cell line SP1 expresses both HGF and Met, resulting in constitutively activated Met (6). Under serum-free medium conditions, SP1 cells exhibit a low level of Met-tyrosine phosphorylation, which can be increased upon addition of exogenous HGF. Therefore, this system was utilized to assess the role of copper(II) on HGF-inducible tyrosine phosphorylation of Met. SP1 cells were incubated under various conditions, then lysed, and immunoprecipitated for Met. Samples were subjected to SDS-PAGE and Western blotting to assess the level of Met-tyrosine phosphorylation (Fig. 5, top panel). Cytoskeletons from medium alone exhibit a base level of Met-tyrosine phosphorylation, and this level increases significantly with the addition of 100 ng of HGF. HGF-inducible Met-tyrosine phosphorylation is inhibited by 1 mM copper(II), whereas 1 mM copper(II), in the absence of HGF, shows no effect. Loading controls in each group were consistent throughout this experiment (Fig. 5, bottom panel). These results suggest that copper(II) inhibits HGF binding and activation of Met at the cellular level and can mediate HGF function.

HGF-induced Cell Scatter Is Inhibited by Copper(II)—Cell scattering is an important response regulated by HGF signaling and is an essential step in HGF-induced tumorigenesis. We therefore assessed whether copper(II) affects HGF-induced scattering of MDCK cells. In serum-free medium, MDCK cells grow in monolayers, with rounded cell islets (Fig. 6A). Addition of exogenous HGF (20 ng) results in marked cell scatter, with increased cell-cell contacts and increased cell spreading (Fig. 6C). Compared with HGF-treated cells alone, the HGF-induced scattering phenotype is significantly inhibited by the addition of 1 mM copper(II), resulting in increased rounding of cells, increased cell-cell contacts, and the presence of cell islets (Fig. 6D). The addition of 1 mM copper(II) alone has minimal effect on cell islets (Fig. 6B). Addition of TPA (100 nM) results in...
nonprotein-specific induction of cell scatter (Fig. 6), and this TPA-induced scattering phenotype is not inhibited by the addition of 500 μM copper(II) (Fig. 6F). Cell viability was maintained, as demonstrated by 4,6-diamidino-2-phenylindole staining (data not shown). These results indicate that HGF activity is impeded at the cellular level by a specific interaction with copper(II).

**Histidine Residues Are Involved in Copper(II) Inhibition of HGF/Met-IgG Binding**—HGF contains several histidine residues in its kringle domains that may play a role in binding copper(II), resulting in decreased affinity of HGF for Met. In order to investigate whether copper(II) inhibition of the HGF/Met-IgG interaction is affected by charge, we modified histidine residues in HGF using DEPC. An HGF stock (12 μg/ml) was incubated in 0.1 M phosphate buffer, pH 6, either alone, or with 0.1% DEPC. Each treated fraction of HGF was diluted (100 ng/ml) and then incubated with immobilized Met-IgG (1 mg/ml) alone or in the presence of 500 μM copper(II) (Fig. 7). The results show that DEPC pretreatment of HGF abrogates the inhibitory effect of copper(II) on HGF/Met binding. In contrast, DEPC pretreatment of HGF without copper(II) results in similar HGF/Met-IgG binding to that of untreated HGF, indicating that histidine residues are not directly involved in binding to Met per se.

**Fig. 6.** HGF-induced cell scattering is inhibited by copper(II). MDCK cells (6 × 10⁵) were plated in 24-well culture dishes and subsequently incubated with the following: A, medium alone; B, medium supplemented with 500 μM copper(II); C, medium supplemented with 20 ng of HGF; D, medium supplemented with 20 ng of HGF and 500 μM copper(II); E, medium supplemented with 100 nM TPA; or F, medium supplemented with 100 nM TPA and 500 μM copper(II). After 24 h, cells were fixed and stained with Harris modified hematoxylin solution. Cell scattering was assessed visually, and representative fields were photographed using a Leitz inverted microscope.

**Fig. 7.** Pretreatment of HGF with DEPC prevents copper(II) inhibition of HGF binding to immobilized Met-IgG. HGF (12 μg/ml) was incubated in 0.1 M phosphate buffer, pH 6, in the absence or presence of 0.1% DEPC for 45 min at room temperature. Met-IgG (1 mg/ml) was immobilized on a 96-well ELISA plate overnight at 4°C. Untreated HGF (100 ng/ml) was incubated alone, or in the presence of 500 μM copper(II), or DEPC-treated HGF (100 ng/ml) was incubated alone, or in the presence of 500 μM copper(II) for 2 h. HGF binding was assessed by incubation with mouse anti-human HGF antibody, followed by donkey anti-mouse HRP and color detection. Each bar represents the mean absorbance for triplicate wells ± S.D. normalized to the percentage of untreated HGF binding alone. Experiments were performed in triplicate.
Copper(II) Inhibits HGF Binding to Met

The interaction of HGF, secreted by stromal cells, with the epithelial expressed receptor tyrosine kinase Met regulates normal epithelial cell survival and morphogenesis (1, 2). In tumorigenesis, overexpression of Met and aberrant expression of HGF in epithelial cells leads to an HGF/Met autocrine loop, resulting in epithelial-mesenchymal transition and increased invasiveness (6–9). Inhibition or disruption of the HGF/Met interaction may cause a down-regulation of cellular invasion. We therefore studied HGF/Met interaction with the aim of identifying HGF-binding molecules, which may lead to the development of specific HGF antagonists. Our results show that HGF binding to Met can be inhibited with copper(II) or zinc(II) cations, and this inhibition can interfere with HGF/Met signaling and function.

The domains of HGF involved in binding to Met have been identified using deletion and site-specific mutations (21–23). The β-chain of HGF is required for full activity but is not required for binding to Met (20). In fact, HGF binding to Met has been targeted to the N terminus of the HGF α-chain, particularly the first N-terminal 272 residues that form the hairpin loop, the first kringle, and a significant portion of the transmembrane β-chain and the α-chain of Met (39, 40). In the present study we used a Met-IgG fusion protein, which consists of the extracellular HGF-binding domain of Met ligated to the constant region of human IgG (36) as a cell-free soluble form of Met. By using a modified ELISA technique, we established that soluble full-length HGF binds specifically to immobilized Met-IgG fusion protein. We also showed that soluble Met-IgG binds specifically to immobilized HGF. Together, these findings indicate that immobilizing either HGF or Met-IgG does not interfere with critical ligand/receptor interacting domains; therefore, either of the two ELISA methods can be utilized for screening potential antagonists.

HGF not only binds to Met but also contains binding sites for various other compounds. Heparin affinity chromatography has been used to demonstrate heparin binding properties of HGF and to purify both full-length HGF and the NK1 isoform (30). Heparin induces dimerization of NK1, reversing its antagonist effects on full-length HGF to an agonistic function (41). The heparin-binding site has been targeted to the HGF N terminus, particularly in a cluster of amino acids ranging from residues 60 to 78 of NK1 (42). Our laboratory has shown previously (34) that copper(II) binds to HGF thereby allowing a single-step purification of HGF using copper(II) affinity chromatography. Recently, HGF has been purified using immobilized copper ions on polyvinylidene fluoride-based affinity membranes (43). Therefore, in addition to the heparin binding N-terminal domain, HGF may also contain a binding domain for copper(II) and perhaps other divalent metal cations, which could mediate HGF/Met binding. Indeed our results show that the HGF/Met-IgG interaction is inhibited greatly by copper(II) and zinc(II), with each cation exhibiting an IC50 value in the range of 250 μM. HGF/Met-IgG binding is inhibited to a lesser extent by manganese(II) and magnesium(II), with IC50 values of 10 and 50 mM, respectively. The differences in inhibitory levels among these four divalent metal cations indicate specific binding affinities, with greater affinities attributed to copper(II) and zinc(II), and not simply to the charge of the cations.

A concentration of 250 μM copper(II) partially inhibits binding of soluble HGF to immobilized Met-IgG, whereas 500 μM copper(II) almost completely inhibits this interaction. Most interestingly, 500 μM copper(II) only partially inhibits soluble Met-IgG binding to immobilized HGF. This finding suggests that HGF in solution is more accessible to binding copper(II) than is immobilized HGF, possibly due to conformational constraints of immobilized HGF or lack of accessible copper(II) binding domains. In contrast, the Met binding domain of HGF is unaffected whether HGF is free or immobilized, as demonstrated in Fig. 1.

We believe that the copper(II) binding domain is located primarily in the N terminus of HGF. Our laboratory has purified fragments of HGF secreted from carcinoma cells, using copper(II) affinity columns. These fragments have been identified, by antibody mapping, as being N-terminal, supporting the suggestion that the copper(II) binding domain is located in the N terminus of HGF. Therefore, the Met-binding domain and the copper(II) binding domain of HGF are in close proximity but have very different modes of regulation.

Copper(II), at a concentration of 1 mM, binds to both HGF and NK1 and results in gel-mobility shifts. This finding is evidence that copper(II) binds directly to the HGF, specifically within the N terminus, causing a conformational change in HGF, which is prevented by the chelation of copper(II). This mobility shift is quite substantial and either represents a significant conformational change or a dimerization of both NK1 and HGF, or both. Manganese(II), which at 1 mM does not bind HGF in ELISA, also does not cause a mobility shift of either HGF or NK1, suggesting that the shift induced by copper(II) is not the result of excess positive charge but is a result of a specific interaction.

Because histidine residues are implicated in protein binding to copper(II) (44), we further investigated the role that histidine residues might have in copper(II) binding to HGF. We protonated histidine residues of HGF with DEPC, thereby neutralizing charge on the histidine residues. This chemical modification prevented 500 μM copper(II) from inhibiting binding of HGF to immobilized Met-IgG and actually increased binding up to levels of untreated and DEPC-treated HGF lacking...
Copper(II) inhibits HGF binding to Met

... copper(II) levels were used at 25°C, whereas human serum HGF levels have been shown to average 25 µM (19). HGF specifically binds copper(II), probably via the NK1 domain. Further experiments will be required to address the physiological relevance of these findings. Our work shows that residues close to the copper(II)-binding site, such as His-114, mediate binding to other single histidine residues such as His-114. The molecular basis for the inhibition by copper(II) is that the negative charge upon copper(II) binding would certainly have a large input of Dr. Gregory Ross is greatly appreciated. Isabel Renart helped establish preliminary ELISA methods.

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