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Loops in Breast Carcinomas: Possible Target for
Therapeutic Intervention

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13. ABSTRACT (Maximum 200 Words)
Hepatocyte growth factor (HGF) binding to its transmembrane receptor, Met, results in an increase in breast epithelial cell motility, morphogenesis and metastasis. ELISA methods have been used to assess the role of divalent metal cations on HGF/Met-IgG binding. Copper (II) and Zinc (II) significantly inhibited HGF/Met-IgG binding with IC50 values of 230 to 270 M each, while manganese (II) and magnesium (II) were less inhibitory with IC50 values between 5 to 15 mM and 30 to 70 mM, respectively. A concentration of 1 mM copper (II) incubated with HGF resulted in an HGF mobility shift in non-denatruing PAGE, indicating direct interaction of copper (II) with HGF, while 1 mM manganese (II) exhibited no effect. The activation of Met and cell scatter, functions both induced by exogenous HGF, were inhibited upon addition of HGF in the presence of 1 mM and 500 M copper (II), respectively. Chemical protonation, with DEPC, of HGF histidine residues impeded the ability of 500 M copper (II) to inhibit HGF/Met-IgG binding. A proposed ribbon diagram indicates that the HGF copper (II)- binding domain contains two histidine and these histidine are in close proximity to some key residues implicated in the Met binding domain. HGF-immunoreactive secretion products, 55 kDa and 32 kDa, of the human breast epithelial carcinoma cell line, MCF10A1T3B, were purified and inhibited HGF/Met-IgG binding. Sequencing of these products may also identify key residues implicated in the inhibition of HGF/ Met binding.

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Introduction:

Breast cancer is one of the leading causes of death in women, with 1 in every 9 North American women developing breast cancer in their lifetime. As cancer research has progressed, a number of proteins have been identified as being contributors to this disease. One key protein in breast cancer development is hepatocyte growth factor (HGF), a potent stimulator of cell survival, motogenesis 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 over-expression, leads to an HGF/Met autocrine loop that is implicated in tumourgenesis (6-9). High levels of HGF or Met are prognosticators of poor breast cancer patient survival (10-12).

HGF consists of a 69 kDa α-chain and a 34 kDa β-chain. The HGF α-chain is composed of an N-terminal hairpin loop (N), followed by four kringle domains (K1, K2, K3, K4), with each kringle domain containing three intra-molecular disulfide bonds. Following the four kringle domains and located at the C-terminus of the α-chain, is a disulfide bond linking the α– and β–chains of HGF. Met is a transmembrane protein expressed on the surface of epithelial cells. Met also consists of two disulfide-linked chains, a 50 kDa extracellular α-chain and a 140 kDa transmembrane β-chain. The intracellular domain on Met contains phosphorylation sites which interact with downstream SH2-domain proteins, such as Grb2, Gab1, ERK2, and PI-3K. The activation of Met, by its extracellular interaction with HGF, causes an upregulation of downstream proteins, resulting in cellular changes such as increased motility, proliferation and morphogenesis. My project is to specifically investigate the interaction between HGF and Met and to screen potential inhibitors of this binding.

We have previously demonstrated that HGF can be purified utilizing copper(II)-affinity chromatography (13). Since the K1 domain of HGF contains two histidine residues in the putative Met binding region, we hypothesized that copper(II) binding could potentially modulated HGF/Met binding. We have studied the copper(II) binding of HGF with the goal of modeling this interaction as a possible lead to inhibitory compounds of HGF/Met binding and downstream cellular functions.

Naturally occurring HGF isoforms, NK1 and NK2, are able to bind Met, but lack complete HGF activity (14-18). Also, a human breast carcinoma cell line (MCF10A1T3B) that over-expresses the *Ha-ras* oncogene, compared to the human breast control cell line (MCF10A1), has been shown to secrete HGF-immunoreactive degradation isoforms. Purifying and identifying these isoforms may lead to the development of small peptide mimics of NK1 and NK2, also yielding potential inhibitors of HGF/Met binding and downstream cellular functions.

Body:

Original Hypothesis and Objectives:

Our original hypothesis was that HGF interaction with, and subsequent activation of Met, is a major step in transition of epithelial cells to a malignant phenotype. Hence, disruption of this interaction may play an important role in inhibiting this functions.

Two overall objectives were proposed:

1) To identify, and assess the structural properties, of native HGF, HGF degradation products and isoforms secreted by breast carcinoma cell lines and tissues; then to further determine the effects of putative HGF degradation products and isoforms on Met activation and function in breast epithelial and carcinoma cells, and

2) To design high affinity peptides that either inhibit or promote the interaction between HGF and Met an analysis of these peptides on the modulation of HGF/Met function.

Objective 1:

MCF10A1T3B breast carcinoma cells secrete putative HGF degradation products (55 kDa and 32 kDa), identified by various polyclonal and monoclonal anti-HGF antibodies (Figure 1a). So far, only MCF10A1 and MCF10A1T3B cell lines have been shown to secrete these HGF degradation products (Figure 1b). Our lab has previously shown that full-length HGF can be purified using Cu(II)-affinity chromatography and identified with subsequent western blotting and HGF immunoreactivity. As mentioned in my report from 2002, this same technique was utilized in the partial purification of the putative HGF degradation products from MCF10A1T3B conditioned media (CM) (Figure 2a). Also in last year's report, we showed that a diethelaminoethylacetate (DEAE)-cellulose cation exchange column could be used as a subsequent purification step (Figure 2b). We have further refined this two-step purification method and are now able to isolate each of the individual HGF degradation products (Figure 3a). This refined purification method now makes it possible for us to assay and characterize the properties of each HGF degradation product.

A competitive ELISA assay was used to determine whether the individual HGF degradation products inhibit or facilitate the interaction of HGF with its receptor Met. HGF was immobilized on a 96-well ELISA plate. Added to the plate was a fixed concentration of Met-IgG alone or in the presence of the elution profile of each HGF degradation product. A mononclonal anti-human Met antibody (3D6) and a donkey anti-mouse HRP were used to assess Met-IgG binding by colour detection. The purified fractions exhibited an inhibition of Met-IgG binding to HGF (Figure 3b).

Recently we have begun to analyze the individual fragments by amino acid sequence. Obtaining this sequence may assist in locating key amino acids in the degradation products that bind to Met, thus preventing the binding of HGF. This information can then be used to design inhibitors of full-length active HGF.

Objective 2:

In my report from 2002 I showed that a number of various divalent metal cations can inhibit HGF binding to Met. I have since further analyzed this phenomenom. A competitive ELISA assay was used to determine how specifically divalent metal cations inhibit HGF/Met binding. Met-IgG was immobilized on a 96-well ELISA plate, with subsequent addition of a fixed concentration of HGF alone or in the presence a concentration titration of copper(II), manganese(II), zinc(II), or magnesium(II). A mononclonal anti-human HGF antibody (A3.1.2) and a donkey anti-mouse HRP were used to assess HGF binding by colour detection. Copper(II) and zinc(II) significantly inhibited HGF/Met-IgG binding with IC_{50} values of 230 to 270 μM each, while manganese(II) and magnesium(II) were less inhibitory with IC_{50} values between 5 to 15 mM and 30 to 70 mM, respectively (Figure 4). As previously mentioned in the report of 2002, HGF-inducible Met phosphorylation was inhibited by copper(II) (Figure 5), as was HGF-induced cell scatter (Figure 6). Also mentioned last year, HGF mobility, in a non-denaturing agarose gel was shown to shift upward upon addition of copper(II), indicating a change in structure or charge due to Cu(II) binding and has since been further explored. We have further refined this assay and showed that copper(II), but not manganese(II), induced an HGF gel-mobility shift in a non-denaturing acrylamide gel (Figure 7). Hence a change in structure and not charge is responsible for this upward gel-mobility shift.

Since HGF contains several histidine residues in its kringle domains that may play a role in binding copper(II), we decided to investigate whether copper(II) inhibition of the HGF/Met-IgG interaction is affected by this histidine residues. We modified histidine residues in HGF using diethyl pyrocarbonate (DEPC) which protonates histidine residues, decreasing their affinity for copper(II). An HGF stock was incubated in phosphate buffer (pH 6) either alone, or with 0.1% DEPC. Each treated fraction of HGF was diluted and incubated with immobilized Met-IgG alone, or in the presence of 500 μM copper(II) (Figure 8). The results showed that DEPC pre-treatment of HGF abrogates the inhibitory effect of copper(II) on HGF/Met binding. In contrast, DEPC pre-treatment of HGF without copper(II) results in similar HGF/Met-IgG binding to that of untreated HGF. These results indicate that hisidine residues are needed to bind copper(II) which in turn inhibits HGF interaction with Met, but these histidine residues are not directly involved in binding to Met.

Based, on these observations, we modeled a ribbon diagram for the first kringle domain of HGF (Figure 9). Copper could be easily accommodated by the kringle domain of HGF with reasonable interaction distances with the two histidine residues. The remainder of the coordination may be provided by water molecules, together allowing a binding site that is surface accessible. This model is consistent with what we currently know about HGF and kringle structures (19), particularly in light of the fact that histidine residues may be responsible for copper binding in HGF. Local conformation disturbance and introduction of positive charge upon copper binding would certainly have a large impact on its interaction with Met. This suggests that a surface patch containing a copper binding site is most likely responsible for Met binding. Our model supports the hypothesis that histidine residues are responsible for the specific binding of copper to HGF and inhibition of the HGF/Met interaction.

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Combining knowledge from both **Objectives 1 and 2** has given us further insight into both affinity domains of HGF and the inhibition of HGF/Met interaction. Instead of random screening of inhibitors, this information will allow us to selectively screen short synthetic or peptides that may bind to Met or HGF, according to the **original Objective 2.** We plan to use this information in the development of an efficient HGF/Met inhibitor, thus disrupting the autocrine loop present in breast carcinomas.

Key Research Accomplishments:

-Development of a two-step purification method that allows isolation of putative HGF degradation products from MCF10A1T3B breast carcinoma culture medium.

-These isolated fragments can competitively inhibit HGF/Met binding and their amino acid sequence may yield insight into development of HGF/Met binding inhibitors.

-Copper(II) and zinc(II) exhibit strong inhibition of HGF/Met binding, while manganese(II) and magnesium(II) exhibit weak inhibition.

-Copper(II) inhibits HGF-induced scatter and HGF-induced Met phosphorylation.

-Copper(II) directly interacts with HGF resulting in a gel-mobility shift, while manganese(II) does not.

-The chemical protonation of HGF histidine residues blocks the ability of copper(II) to inhibit HGF/Met binding.

-HGF contains a possible copper(II)-binding domain which is in close proximity and may even be within the Met-binding domain of HGF.

-Information on the copper(II)-binding domain of HGF and inhibitory HGF degradation products may lead to potential therapeutic inhibitory compounds or peptides that down-regulate HGF activity and subsequent cellular processes, specifically in carcinomas.

Reportable Outcomes:

-Supervised an undergraduate student on this project:
4th year thesis project (2003): **"Structural-functional relationship of aberrant HGF forms in breast carcinomas"**, Jonathon Foley

-Currently supervising another undergraduate student contributing to this project:
"Role of aberrant HGF on Met activity and downstream functions", Andrea Fung

-Poster presentation: **"Hepatocyte Growth Factor Interaction with its receptor, Met"**, Era of Hope, Orlando, Florida, USA, September 25-28, 2002

-Oral presentation: "HGF/Met Interaction: Can we break the loop?"
6th Annual Meeting for Basic & Research Trainees, Kingston, Ontario, Canada,
May 28, 2003

-Abstract accepted: "Inhibition by copper(II) binding of hepatocyte growth factor interaction with its receptor Met in breast carcinomas" accepted for AACR, Toronto, Ontario, Canada, April 5-9, 2003

-Abstract and poster presentation: "Inhibition by copper(II) binding of hepatocyte growth factor interaction with its receptor Met in breast carcinomas"
CBCRA Reasons for Hope, Ottawa, Ontario, Canada, October 25-28, 2003

-Paper submitted and accepted pending minor revisions: "Inhibition by copper(II) binding of hepatocyte growth factor interaction with its receptor Met"
J. Biol. Chem. 2003 (see appendix)

Conclusions:

We have identified HGF fragments secreted by human breast carcinoma cell lines. These products have been shown to be inhibitors of HGF/Met binding, and may play a regulatory role in the physiological function of HGF in epithelial cells. Additionally, identification of the amino acid sequences of the HGF fragments, and their derivation (eg degradation versus RNA splicing), will yield information that could lead to the development of specific peptide inhibitors. This information may also allow us to discriminately pre-screen protein peptides for putative peptide inhibitors.

We have identified a copper(II)-binding domain in HGF. Two histidine residues in K1 of HGF, separated by a single amino acid, can become oriented as to accommodate a copper(II) molecule. This copper(II)-binding domain is near a cluster of key amino acids implicated in Met binding, thus binding of copper(II) prevents HGF from binding to Met, probably due to a conformational change in HGF.

The copper(II)-binding domain of HGF and key Met-binding residues found in the degradation products yield further information on how HGF and Met interact. This knowledge will help to develop specific antagonists of this interaction and inhibit downstream cellular functions required for development of carcinomas.

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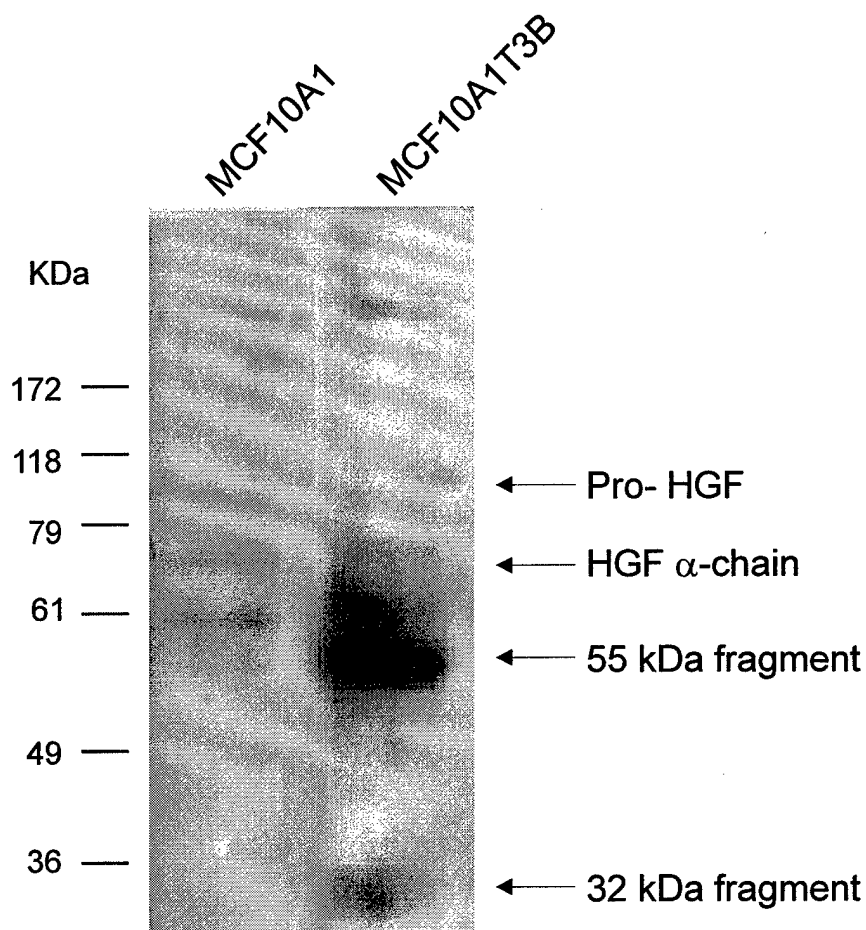
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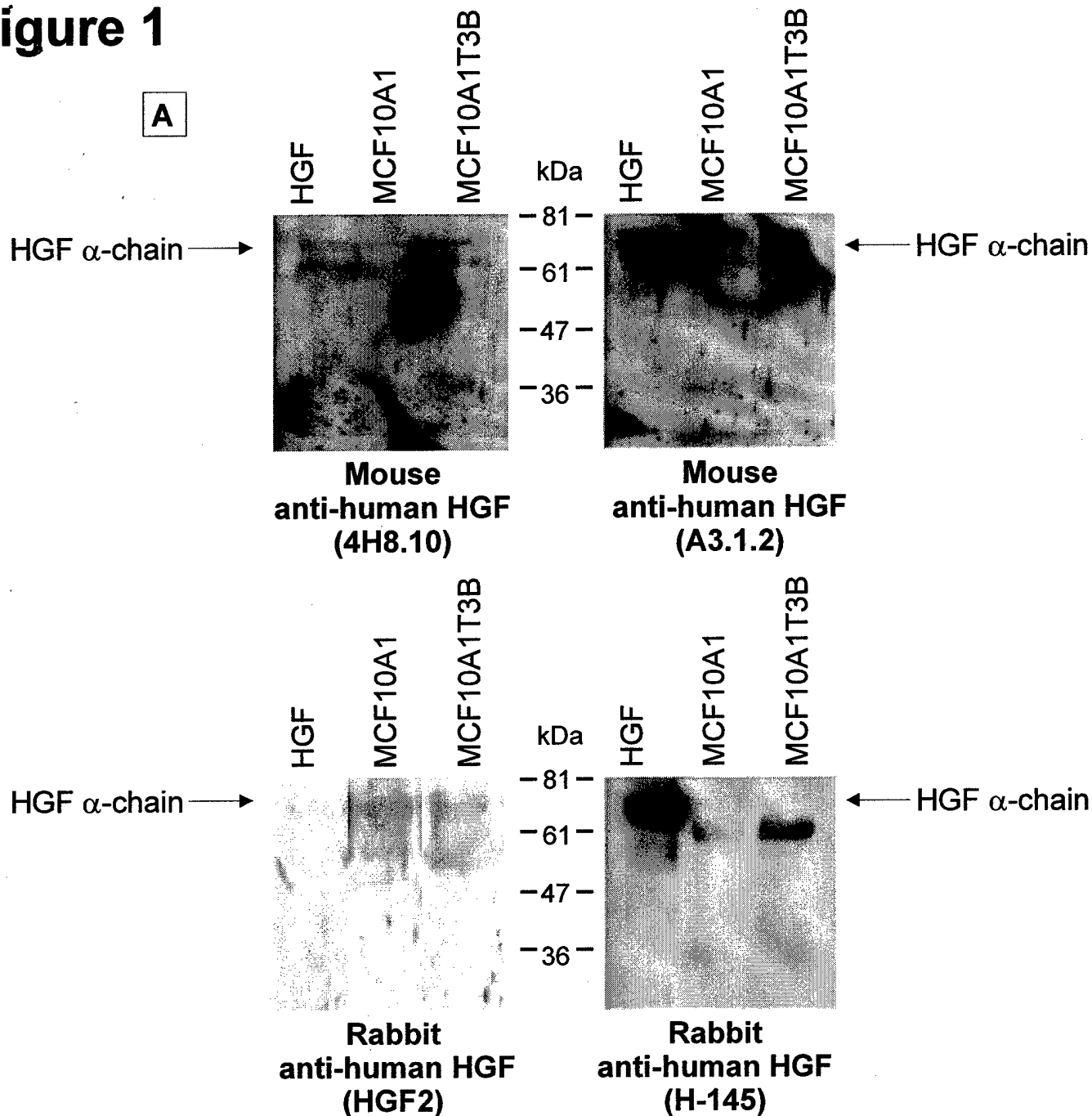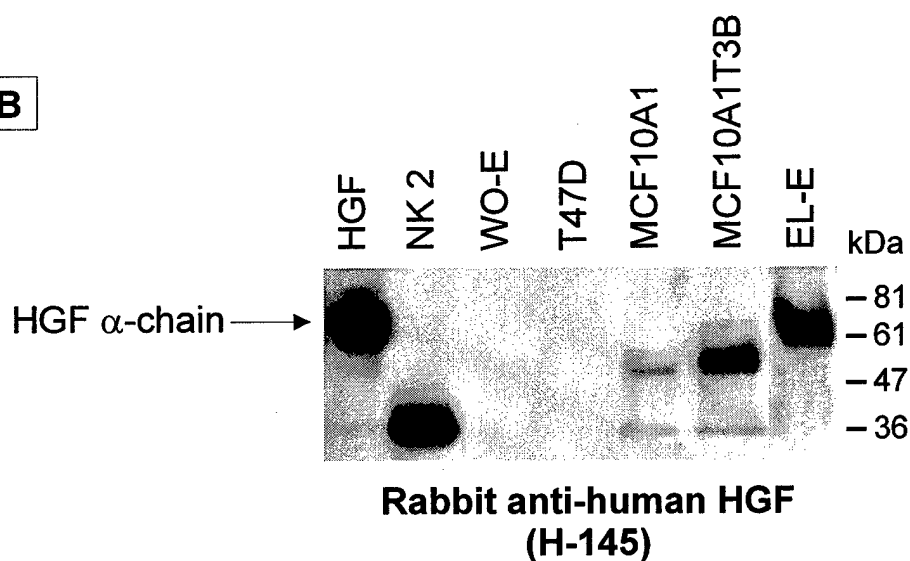
Figure 2

A: Step 1; Cu(II)-affinity

Rabbit
anti-human HGF
(HGF2)

B: Step2; DEAE
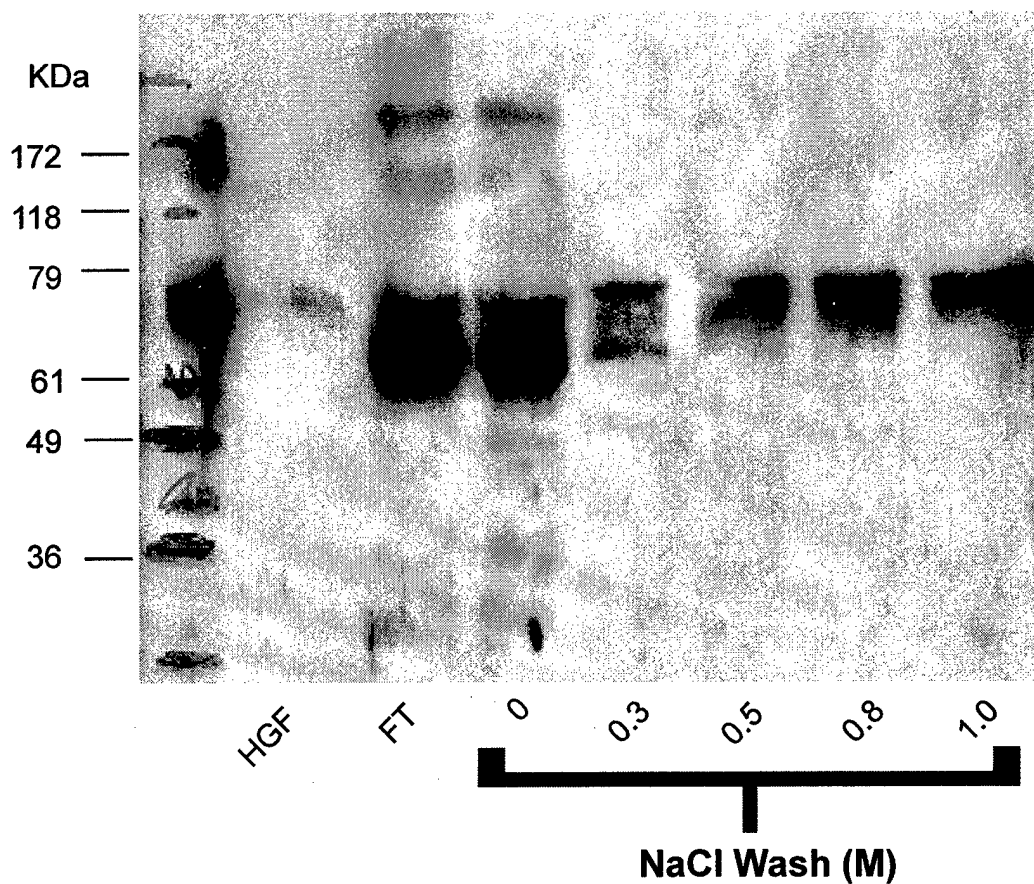cellulose-affinity Cu(II)

Rabbit
anti-human HGF
(HGF2)

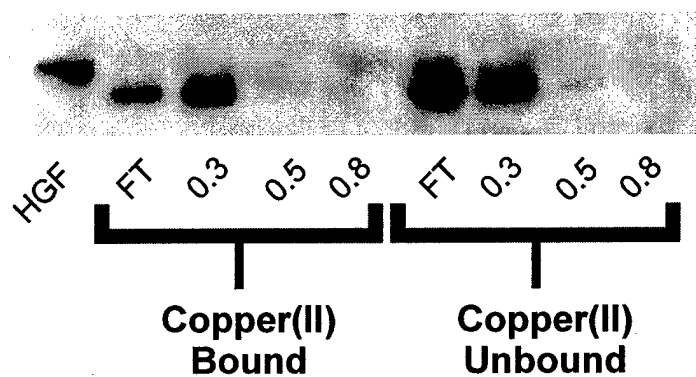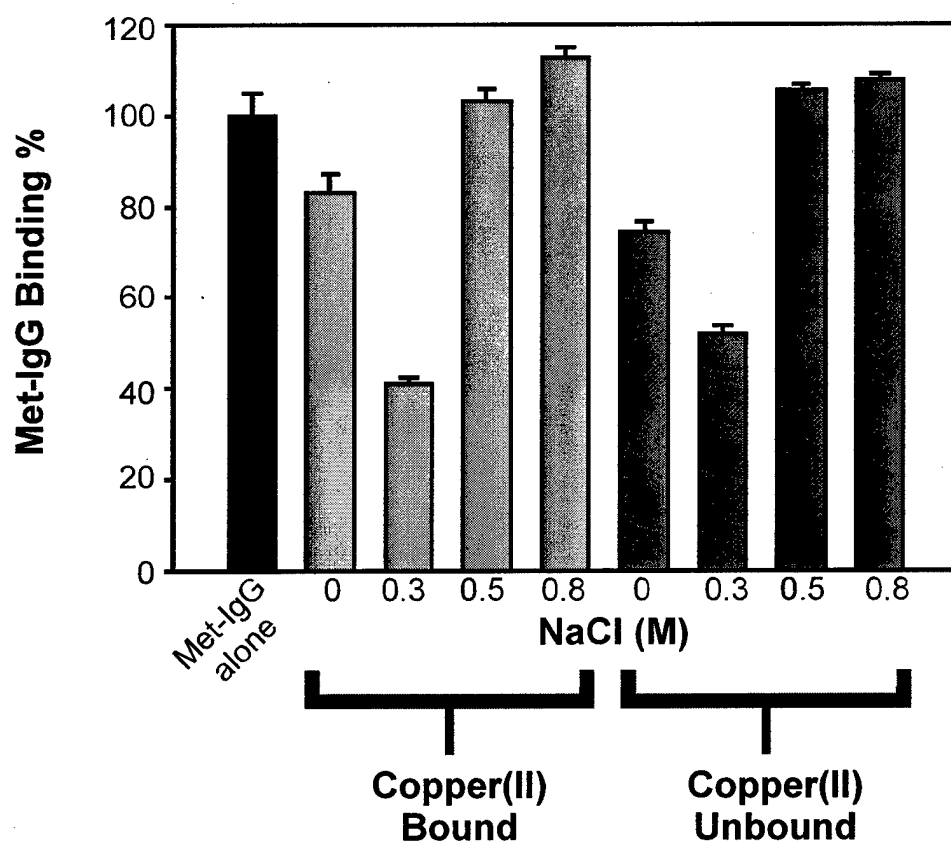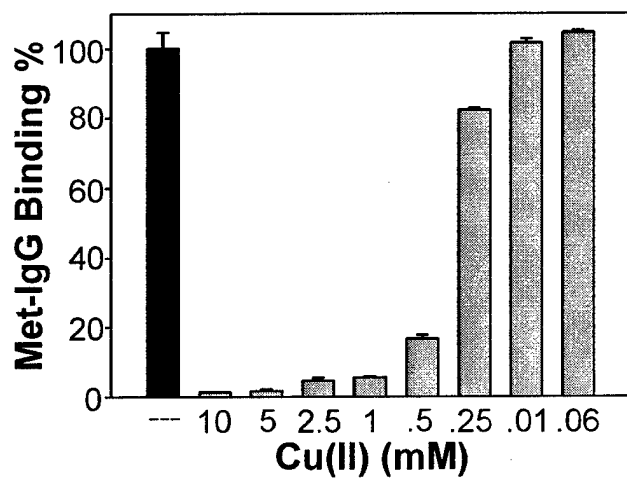# Figure 3

A

**Rabbit
anti-human HGF (H-145)**

HGF FT 0.3 0.5 0.8 FT 0.3 0.5 0.8

**Copper(II)
Bound**

**Copper(II)
Unbound**

B

**Copper(II)
Bound**

**Copper(II)
Unbound**

Figure 4

Figure 5

Figure 6

A: Untreated

B: HGF

C: Cu(II)

D: HGF +Cu(II)

Figure 7

Rabbit
anti-human HGF (H-145)

HGF ⟶

Untreated | EDTA | Cu(II) | Cu(II) + EDTA | Mn(II) | Mn(II) + EDTA
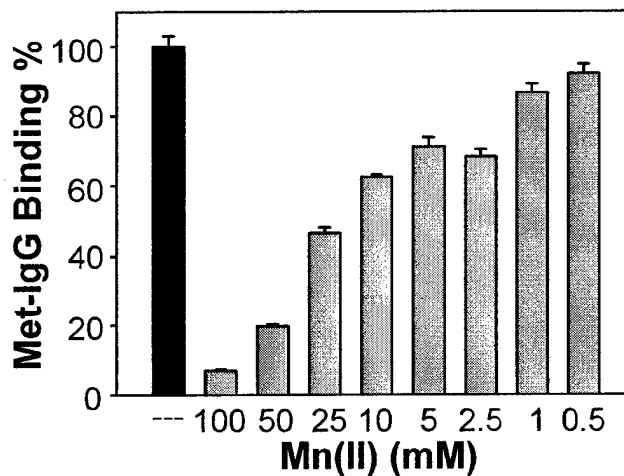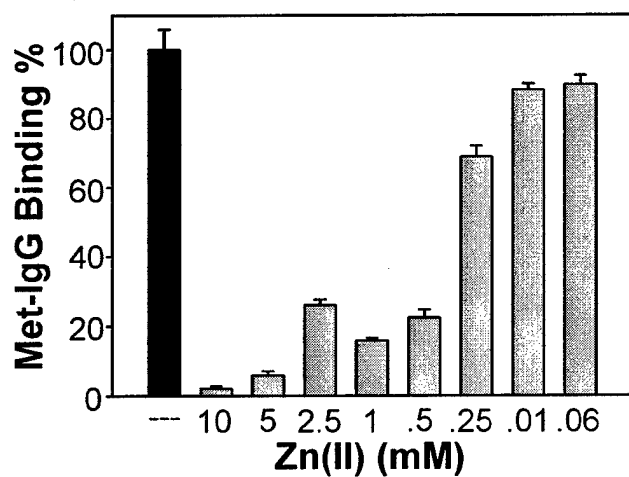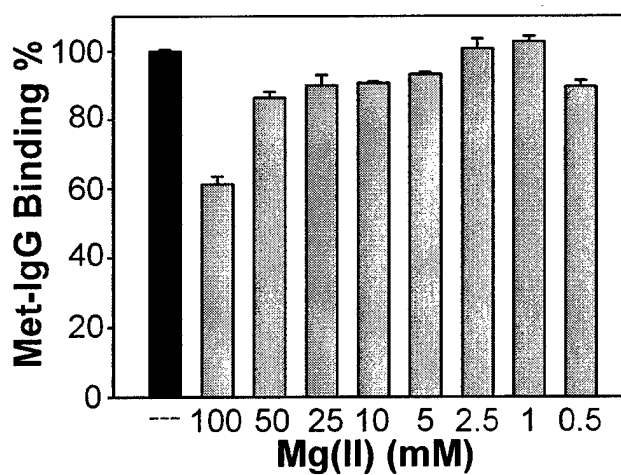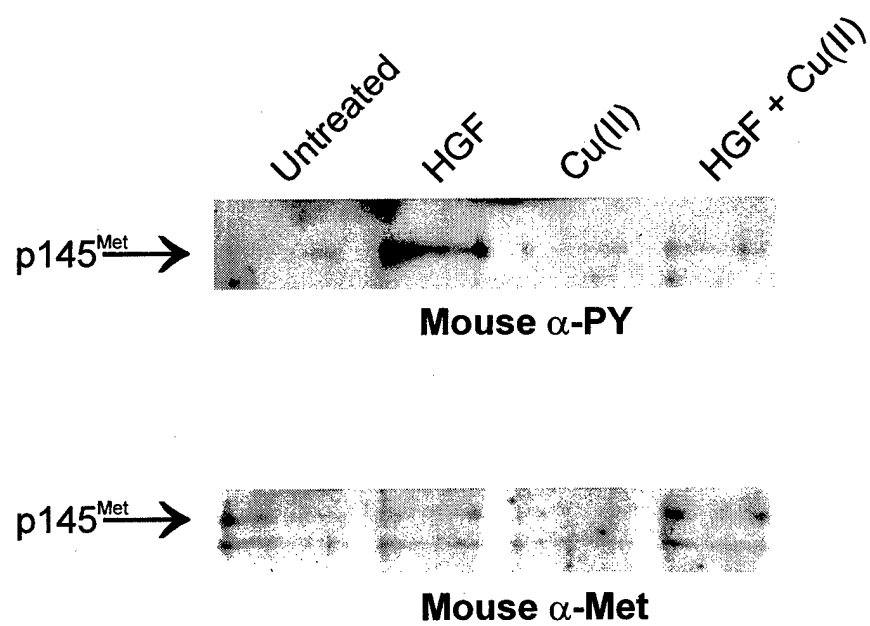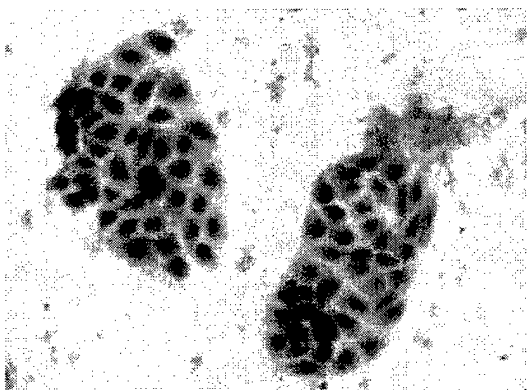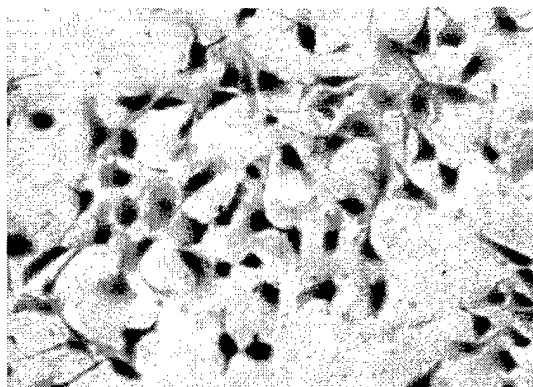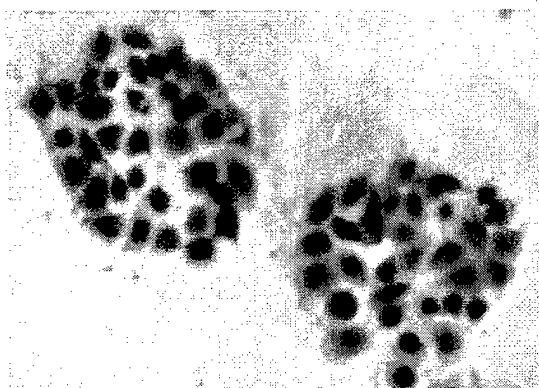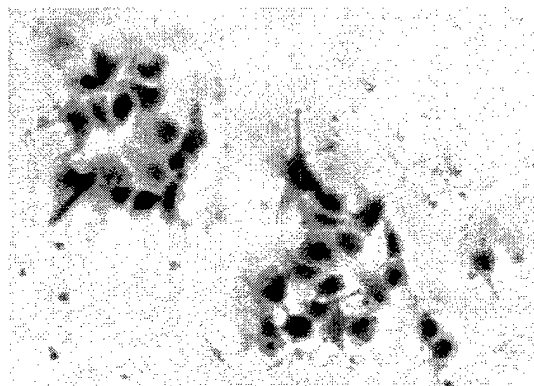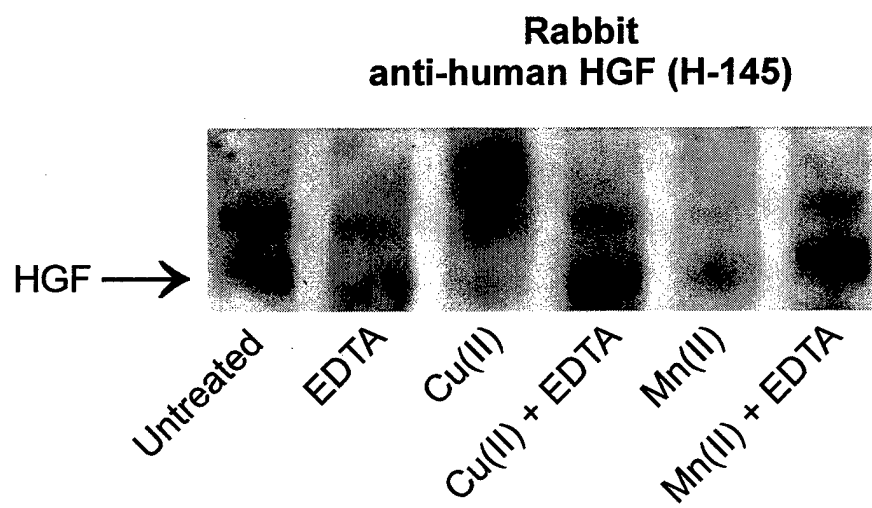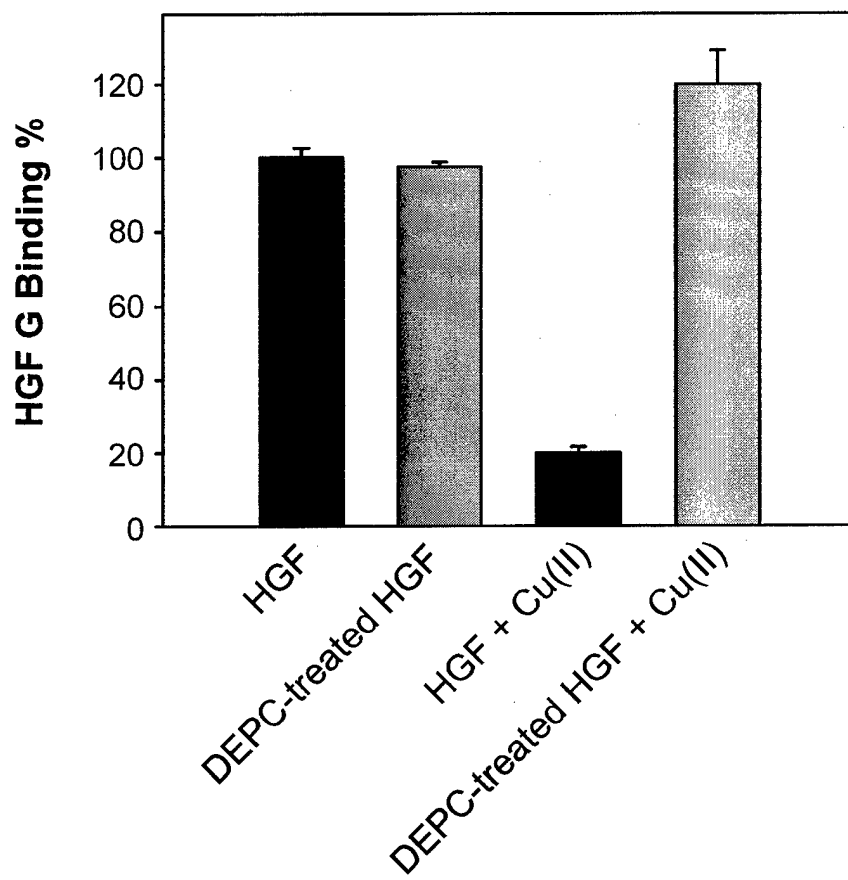
·Figure 8

Figure 9
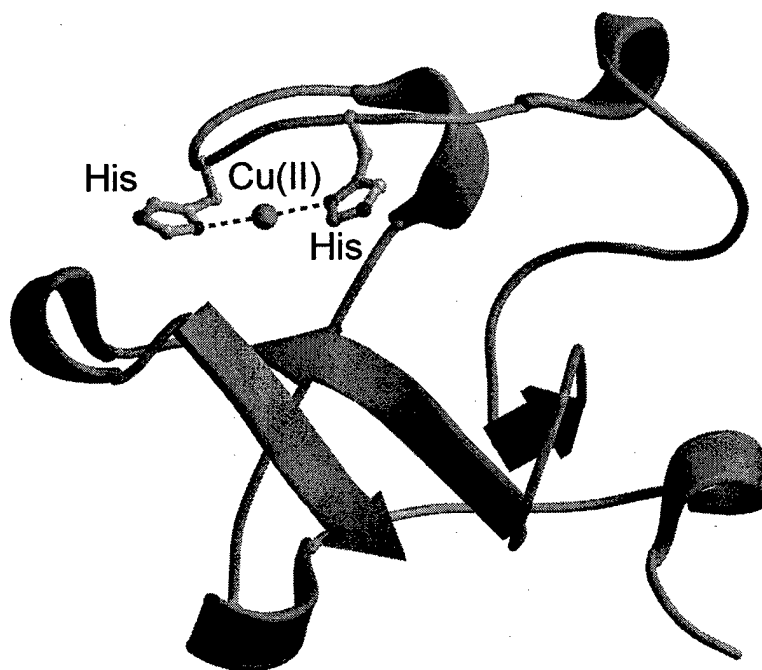
Inhibition by copper(II) binding of hepatocyte growth factor interaction with its receptor Met in breast carcinomas

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Running title: Copper(II) inhibits HGF binding to Met

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Summary

Over-expression 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 downregulation of tumourigenesis. 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 ELISA 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 to 270 μM, respectively, while manganese(II) and magnesium(II) were less inhibitory with 20- to 60 fold higher IC_{50} values. Incubation of 1 mM copper(II) with HGF resulted in an HGF mobility shift in non-denaturing PAGE, indicating direct interaction of copper(II) with HGF. 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 DEPC of HGF histidine residues impeded the ability of 500 μM copper(II) to inhibit the binding of HGF to immobilized Met-IgG. Based on modeling of the HGF copper(II)-binding domain, we propose that copper(II) interacts with HGF via two histidine residues in close proximity in the copper(II)-binding domain. 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.

Introduction

Hepatocyte growth factor (HGF) is a potent stimulator of cell survival, motogenesis 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 over-expression, leads to an HGF/Met autocrine loop that is implicated in tumourigenesis (6-9). A protein microarray ELISA approach was used to demonstrate elevated HGF levels in the sera of breast cancer patients (10), while 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 then undergoes processing by a number of factors such as tissue-type plasminogen activator (tPA) (14), urokinase-type plasminogen activator (uPA) (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 extra-cellular 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, while 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). Interestingly, 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, while 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 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.

We have previously demonstrated that HGF can be purified utilizing copper(II)-affinity chromatography (34). This observation suggests 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.

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Materials and Methods

Reagents:

ELISA, non-denaturing acrylamide gels and SDS-PAGE buffer materials were purchased from Fisher Scientific (Ottawa, ON), as were Harris Modified Hematoxylin and EDTA. Bovine serum albumin (BSA), O-phenylenediamine (OPD), $CuCl_2 \cdot 2H_2O$ and $ZnCl_2$ were supplied by Sigma-Aldrich (Oakville, ON). H_2SO_4, $MnCl_2 \cdot 4H_2O$ and $MgCl_2 \cdot 6H_2O$ were obtained from BDH (Mississauga, ON). Protease inhibitors were purchased from Roche Applied Science (Laval, QC), BDH and Fisher Scientific. Diethyl pyrocarbonate (DEPC) was purchased from ICN Biomedical Inc. (Aurora, OH). Protein A Sepharose Cl-4B (PAS) was purchased from Amersham Biosciences AB (Uppsala, Sweden). The Micro BCA protein determination kit was purchased from Pierce (Rockford, IL). Immobilon P membrane was supplied by Millipore (Bedford, MA). Western Lightning was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). Recombinant HGF, human IgG, Met-IgG fusion protein (35) and mouse anti-human HGF antibodies (A3.1.2 or 4H8.2) were generously donated by Dr. Ralph Schwall (Genentech Inc., San Francisco, CA). 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, ON). Donkey anti-mouse HRP antibody and donkey anti-rabbit HRP antibody were supplied by Amersham Pharmacia Biotech UK (Bucks, UK).

Cell Culture:

Tissue culture plates were supplied by Nunc (Burlington, ON). Madin Darby Canine

Kidney (MDCK) epithelial cells were maintained in DMEM, supplied by GibcoBRL

(Burlington, ON), supplemented with 10% FBS, with an atmosphere of 5% CO_2 at 37°C. A

spontaneous non-metastatic mouse mammary adenocarcinoma cell line, SP1, which expresses

both HGF and Met, was isolated at Queen's University and has been previously described (6).

SP1 cells were cultured in RPMI medium supplemented with 7% FBS at 37°C, with an

atmosphere of 5% CO_2.

Enzyme-linked Immunosorbant 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 (0.1M HCO_3 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 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 washed, as above. Either serial

dilutions or a fixed concentration (100 ng/ml) of HGF in HBS containing 1% BSA and 0.1%

Tween 20 were incubated (100 μl/well) in plates containing immobilized Met-IgG for 2 h at

room temperature with various screening conditions, as indicated in figure legends. When

required, HGF (12 μg/ml) was pretreated with 0.1% DEPC for 45 min prior to ELISA

incubation. Plates were washed, as above, and a mononclonal 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

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containing 1% BSA and 0.1% Tween 20 were incubated (100 µl/well) in plates containing immobilized HGF for 2 h at room temperature with various screening conditions, as indicated in 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 for 1 h at room temperature. Plates were again washed and either HGF or Met-IgG binding was assessed by colour detection with development buffer (0.4 M OPD in 50 mM Na_2HPO_4/25 mM citric acid containing 0.03% H_2O_2). The reaction was stopped with 2 M H_2SO_4 neutralizing acid (50 µl/well). Absorbance was determined at 490 nm and 630 nm.

Cell Scatter Assay:

Scattering of MDCK cells has been previously described (36). Cells were seeded at $6x10^3$ cells/well in a 24-well plate and cultured, in DMEM supplemented with 10% FBS, for 18 h. Medium was removed and cells were washed twice with PBS. Cells were supplemented with 500 µl serum-free DMEM alone, with 20 ng HGF, with 500 µM copper(II), or with 20 ng HGF and 500 µM copper(II). Cells were incubated for 24 h and subsequently stained with Harris Modified Hematoxylin. Representative images were captured with an inverted phase contrast Leica microscope equipped with imaging software.

Met Tyrosine Phosphorylation Assay:

SP1 cells were seeded at $5x10^4$ cells/well in 100 mm tissue culture dishes and incubated for 8 h. In order to reduce endogenous activation of Met, media was removed, cells washed once with PBS and subsequently incubated in serum-free RPMI overnight. Cells were then washed

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twice with PBS and supplemented with 5 ml 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 hr at 37°C, with 5%

CO_2 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 Na_3VO_4, 10 mM NaF, 2 µg/ml

aprotinin, 2 µg/ml leupeptin, and 1% Nonidet P40). Lyates were cleared of cellular debris by

centrifugation (14000 x 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) PAS was added to each supernatant and samples were incubated

with the rabbit anti-Met antibody (SP260; 1:50), overnight at 4°C. The PAS was then pelleted

(14000 x g) and washed twice with lysis buffer. A volume of 2X sample buffer, containing 5%

β-mercaptoethanol, was added to the pellets and boiled for 5 minutes. Samples were subjected

to 10% SDS-PAGE and were subsequently transferred to Immobilon P membrane. Western

blotting was performed with the mouse anti-mouse Met antibody (B2; 1:1000) or the mouse anti-

phosphotyrosine antibody (PY20; 1:200) followed by donkey anti-mouse HRP antibody.

Western Lightning was utilized for immunoreactivity detection.

HGF Gel-mobility Assay:

Aliquots (1 µg) of HGF were incubated in stabilizing buffer (20 mM Tris containing 500

mM NaCl) at 37°C for 2 h under various conditions. Conditions included HGF 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, samples were added, in a 1:1 ratio,

to a 50% sucrose sample buffer containing 0.1% bromophenol blue and subjected to non-

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denaturing 6% acrylamide gel electrophoresis. Gels were transferred to Immobilon P membrane.

Transferred gels were subjected to western blotting with the rabbit anti-human HGF antibody

(H-145; 1:500), and donkey anti-rabbit HRP antibody (1:1000), followed by Western Lightning

detection.

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), while Met-IgG exhibits very little binding affinity for immobilized human IgG.

Therefore, both ELISA methods can be used for detecting specific HGF/Met-IgG binding and

can be useful in the screening of potential inhibitors/mediators of this interaction.

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 (0.5-10 mM) (Figs. 2A and 2C). 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

approximately 250 μM. In contrast, a high concentration (100 mM) of manganese(II) inhibits

HGF/Met-IgG binding only to 10% that of control levels and binding is quickly recovered with decreasing concentrations of manganese(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, and 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).

Since 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 results of Fig. 2A, copper(II) at 500 µM almost completely inhibits binding (Fig 3). 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. 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 to immobilized HGF and free Met-IgG.

Copper(II) causes a gel-mobility shift of HGF and this effect is reversed by EDTA.

To further explore whether a direct interaction between HGF and copper(II) exists, we used a non-denaturing gel mobility assay. HGF (1 µg) was incubated under various conditions and subjected to non-denaturing PAGE and western blotting (Fig. 4). The presence of 1 mM copper(II) results in the upward mobility shift of HGF, which is reversed by 5 mM EDTA, while EDTA alone does not affect mobility of HGF. The presence of 1 mM manganese(II) alone or

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combined with 5 mM EDTA does not result in any detected HGF mobility shift. These results indicate that HGF specifically binds copper(II), which may alter the conformational properties of HGF.

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 immuno-precipitated for Met. Samples were subjected to SDS-PAGE and western blotting to assess the level of Met tyrosine phosphorylation (Fig. 5, top panel). Cells cultured in medium alone exhibit a base level of Met tyrosine phosphorylation and this level increases significantly with the addition of 100 ng HGF. HGF-inducible Met tyrosine phosphorylation is inhibited by 1 mM copper(II), while 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 tumourigenesis. 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 reduced cell-cell contacts and increased cell spreading (Fig. 6B). Compared to HGF-treated cells alone, the HGF-induced scattering phenotype is significantly inhibited by the addition of 500 μM copper(II), resulting in increased rounding of cells, increased cell-cell contacts and the presence of cell islets (Fig. 6D). The addition of 500 μM copper(II) alone has minimal effect on cell islets (Fig. 6C). Cell viability was maintained, as demonstrated by DAPI staining (data not shown). These results indicate that HGF activity is impeded at the cellular level by the presence of 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 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) (Figure 7). The results show that DEPC pre-treatment of HGF abrogates the inhibitory effect of copper(II) on HGF/Met binding. In contrast, DEPC pre-treatment 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*.

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Discussion

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 tumourigenesis, over-expression 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 signalling and function.

The domains of HGF involved in binding to Met have been identified using site-specific mutations. The β-chain of HGF is required for full activity, but is not required for binding to Met (23). 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 which form the hairpin loop, the first kringle and a significant portion of the second kringle (21,22). HGF binds to the extra-cellular domain of the transmembrane β-chain and the α-chain of Met (37,38). 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 (35), as a cell-free soluble form of Met. 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.

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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 antagonistic effects on full-length HGF to an agonistic function (39). 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 (40). Our laboratory has previously shown that copper(II) binds to HGF thereby allowing a single-step purification of HGF using copper(II)-affinity chromatography (34). Recently, HGF has been purified using immobilized copper ions on polyvinyldiene fluoride (PVDF)-based affinity membranes (41). 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 IC_{50} value in the range of 250 μM. HGF/Met-IgG binding is inhibited to a lesser extent by manganese(II) and magnesium(II), with IC_{50} values of 10 mM 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, while 500 μM copper(II) almost completely inhibits this interaction. 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 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 Figure 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 (unpublished results). 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 HGF and results in a gel-mobility shift, detected using non-denaturing PAGE. This finding is direct evidence that copper(II) causes 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 HGF or both. Manganese(II), which at 1 mM does not bind HGF in ELISA, also does not cause a mobility shift in HGF, suggesting that the shift induced by copper(II) is not the result of excess positive charge, but is a result of a specific interaction.

Since histidine residues are implicated in protein binding to copper(II) (42), 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). Therefore, histidine residues appear to mediate the copper(II) binding site of HGF. Based on this observation, we modeled the first kringle domain

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of HGF. As shown in Figure 8, copper could be easily accommodated by the kringle domain of HGF, having reasonable interaction distances with the two histidine residues, His158 and His160. The remainder of the coordination may be provided by water molecules, together allowing a binding site that is surface accessible. This model is consistent with what we currently know about HGF and kringle structures (15-17), particularly in light of the fact that histidine residues may be responsible for copper binding in HGF. Local conformation disturbance and introduction of positive charge upon copper binding would certainly have a large impact on its interaction with Met, which has been experimentally confirmed. This would in turn suggest that a surface patch containing a copper binding site is most likely responsible for Met binding. Indeed, mutagenesis results show that residues close to the copper(II) binding site, such as Glu159 and Ser161, are involved in Met binding (43). Our model supports the hypothesis that histidine residues are responsible for the specific binding of copper to HGF and inhibition of the HGF/Met interaction. We therefore suggest that the molecular basis for the inhibition by copper is that the kringle domain of HGF binds to copper and negatively regulates its interaction with the Met receptor.

HGF-induced phosphorylation of cellular Met is inhibited by 1 mM copper(II), showing that copper(II) inhibition of the HGF/Met interaction exhibits a consequence on Met activation. As mentioned above, the HGF β-chain is required for full HGF activity, but is not required for Met binding (23). Since HGF activity is required for Met activation (9) it appears that copper(II) specifically binds HGF in the N-terminus, causing a conformational change preventing HGF/Met binding and subsequent Met activation via the HGF β-chain. The inhibition by 500 μM copper(II) of the HGF/Met interaction also results in the inhibition of HGF-induced cell scatter, which is a downstream function of Met activation. Thus, copper(II) regulates HGF activity and

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subsequent Met activation, which can adversely affect cellular motility functions.

A variety of copper-binding proteins exist in humans, including Factor V, Factor VIII, superoxide dismutase and ceruloplasmin (44). Ceruloplasmin, the main human plasma copper carrier, maintains a copper concentration of ~10 μM (45,46), while human serum HGF levels have been shown to average ~350 pg/ml in healthy patients and can reach levels of 2 ng/ml in breast cancer patients (11). Therefore, the levels of HGF used in our assays range from 20- to 300-fold excess, while copper(II) levels were used at 50-fold excess that of physiological concentrations. However, the IC_{50} values of copper(II) and zinc(II) observed in our study are not far off physiological levels. Further experiments will be required to address physiological relevance of these findings.

In summary, HGF contains a copper(II) binding site located at the N-terminus of HGF, which modulates the HGF/Met interaction implicated in tumourigenesis. Our work shows that HGF specifically binds copper(II), probably via the first kringle domain, preventing the interaction with its receptor Met. This modulating copper(II) binding site may be useful in the design of potential HGF/Met inhibitors. Understanding the mechanisms of this interaction at physiological concentrations is an important next step in this study. Identifying key residues and binding affinities for the interaction of copper(II) and HGF can potentially assist in modeling putative inhibitors of the HGF/Met autocrine loop in cancers such as breast and lung.

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Acknowledgements

Figure Legends

Figure 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 human IgG (circles) or Met-IgG (triangles) was assessed by incubation with mouse anti-human HGF antibody, followed by donkey anti-mouse HRP and colour 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 colour detection. Points on the graphs represent the mean absorbance for triplicate wells +/- standard deviation. Experiments were performed a minimum of three times.

Figure 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 various 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) 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 colour detection. Results are expressed as percentages of mean

absorbances +/- standard deviation for HGF binding in the presence of compounds relative to the mean absorbance for HGF binding alone. Experiments were performed in triplicate.

Figure 3. 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 colour 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 colour detection. Each bar represents the mean absorbance for triplicate wells +/- standard deviation standardized to the percentage of HGF or Met-IgG binding alone. Experiments were performed in triplicate.

Figure 4. HGF gel-mobility is impeded by copper(II) and this effect is reversed by EDTA. HGF (1 µg) was incubated under various conditions at 37°C for 2 h. Conditions included HGF alone or in the presence of 5 mM EDTA, or 1 mM copper(II), or 1 mM copper(II) and 5 mM EDTA, or 1 mM manganese(II), or 1 mM manganese(II) and 5 mM EDTA. Samples were run on 6% non-denaturing acrylamide gel. The gel was transferred to a membrane, followed by western blot analysis. The blot was probed with rabbit anti-human HGF antibody, followed by donkey anti-rabbit HRP and chemiluminescence development. Experiments were performed in triplicate.

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Figure 5. HGF-induced tyrosine phosphorylation of Met is inhibited by copper(II). SP1 cells (1×10^5) were plated in 100 mm culture dishes. Prior to experimental conditions, culture medium was removed and cells were incubated for 16 h in serum-free medium. Cells were then incubated with medium alone, medium supplemented with 100 ng HGF, medium supplemented with 1 mM copper(II) or medium supplemented with 100 ng HGF and 1 mM copper(II). Cell lyses were immunoprecipitated with a rabbit anti-mouse Met antibody and samples were run on 10% SDS-PAGE. Gels were transferred to charged membranes, followed by western blot analysis. Blots were probed with either mouse anti-mouse HGF antibody or mouse anti-phosphotyrosine antibody, followed by donkey anti-mouse HRP and chemiluminescence development. Experiments were performed in triplicate.

Figure 6. HGF-induced cell scattering is inhibited by copper(II). MDCK cells (6×10^3) were plated in 24-well culture dishes and subsequently incubated with: A) medium alone, B) medium supplemented with 20 ng HGF, C) medium supplemented with 500 μM copper(II) or D) medium supplemented with 20 ng HGF and 500 μM copper(II). After 24 h, cells were fixed and stained with Harris Modified Hemotoxylin. Cell scattering was assessed visually and representative fields were photographed using a Leitz inverted microscope.

Figure 7. Pre-treatment 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

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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 colour detection. Each bar represents the mean absorbance for triplicate wells +/- standard deviation normalized to the percentage of untreated HGF binding alone. Experiments were performed at lease three times.

Figure 8. Ribbon diagram of the N-terminal kringle module in HGF. Side chains of the two histidine binding residues are shown, along with copper (sphere). The dotted lines indicate the interaction between histidines and copper. The N-terminal kringle of HGF structure was modeled based on the known crystal structure of apolipoprotein(a) kringle IV type 7 (47). Two histidine residues, His158 and His160, represent the most likely binding position for copper since there is no other place in the kringle that has multiple histidine residues positioned in close spatial proximity. A copper ion was inserted in between His158 and His160, the side chains of which were positioned to make appropriate interacting distance with the copper ion. The resulting model was subjected to extensive energy minimization using Sybyl software package (Tripos, St. Louis) and diagram was generated using Molscript (48).
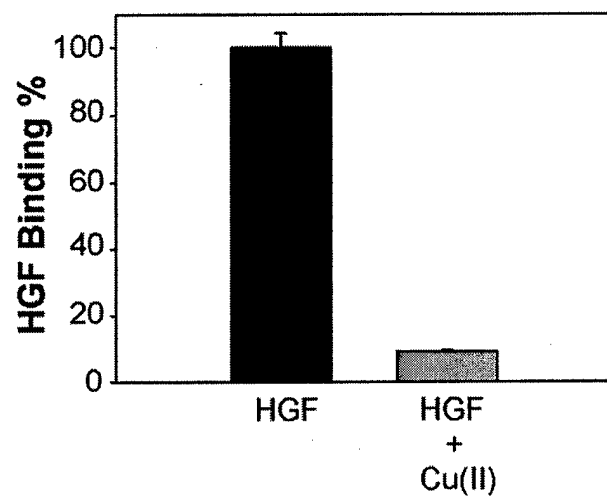
Figure 1

A

B

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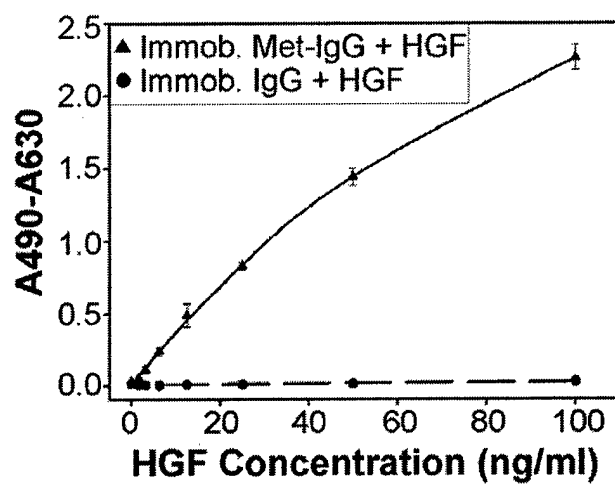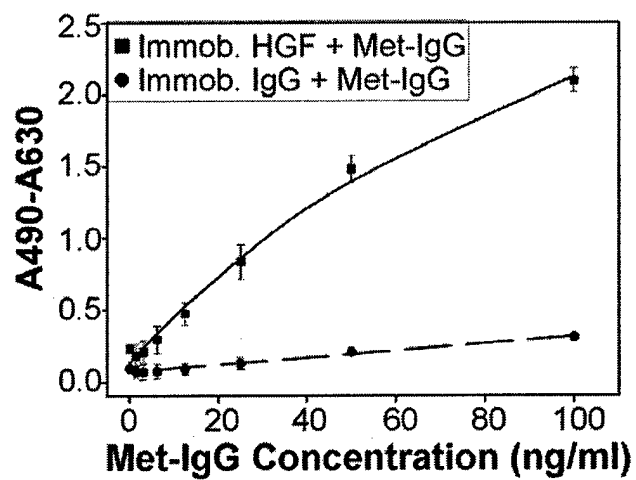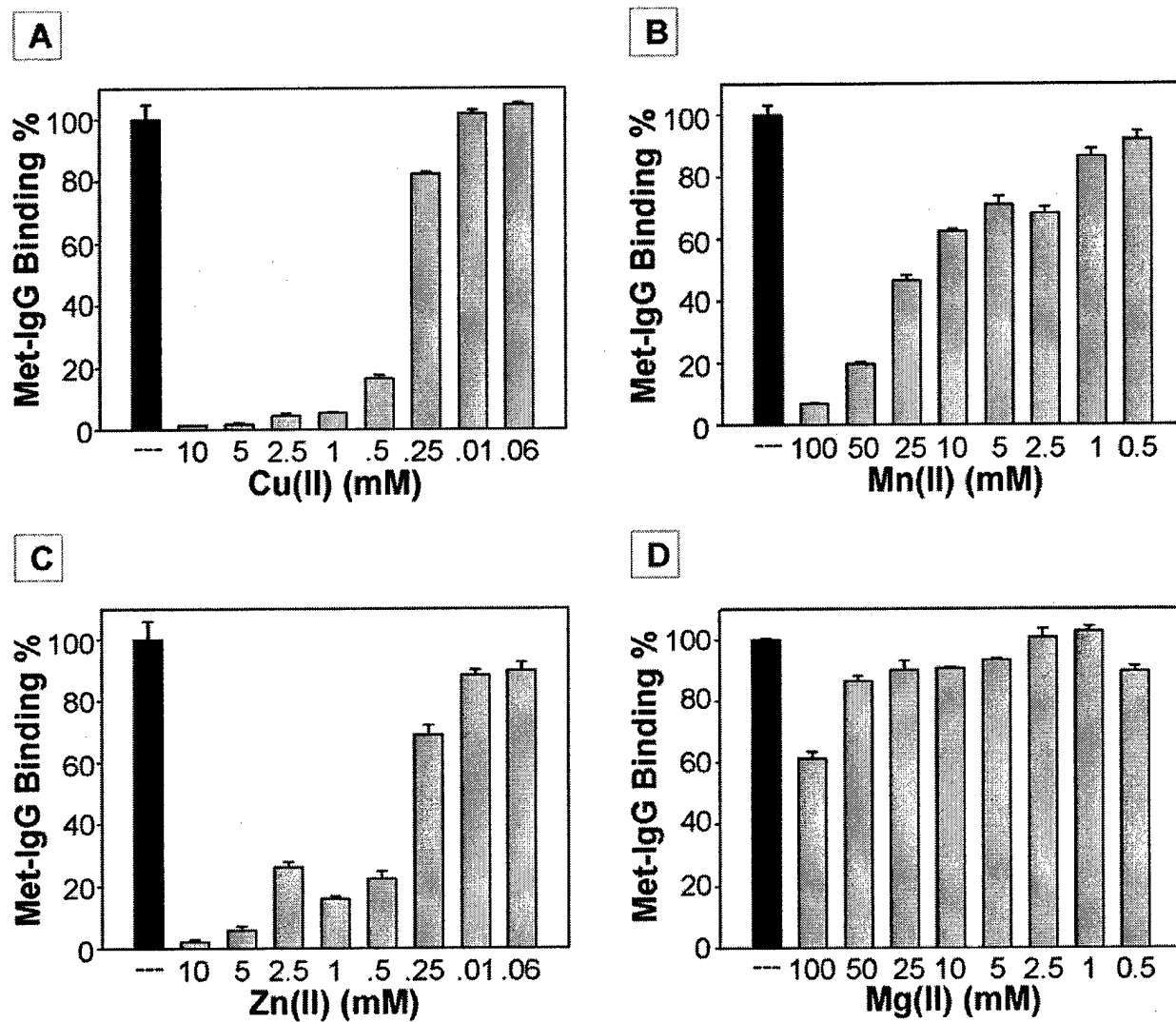
Figure 2

Figure 3

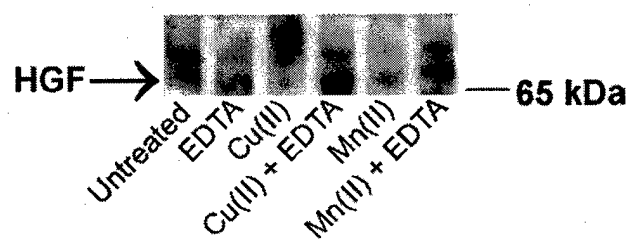## A: Immobilized Met-IgG

B: Immobilized HGF

Figure 4

HGF⟶   ——65 kDa

Untreated EDTA Cu(II) Cu(II) + EDTA Mn(II) Mn(II) + EDTA

Figure 5

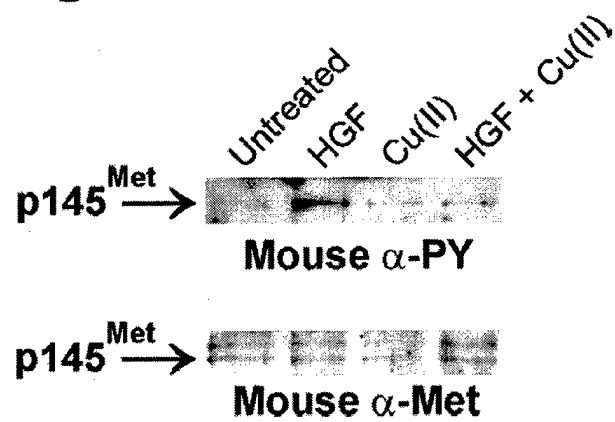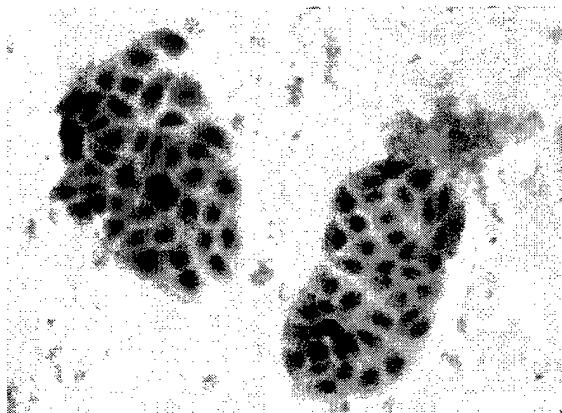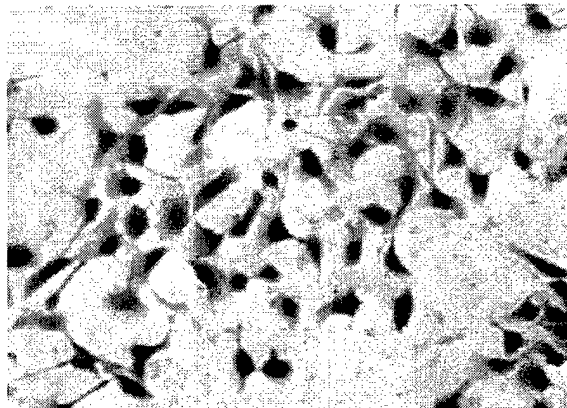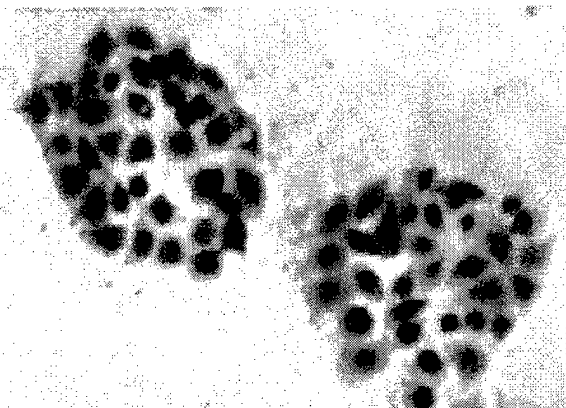# Figure 6

A: Untreated

B: HGF

C: Cu(II)

D: HGF +Cu(II)

Figure 7

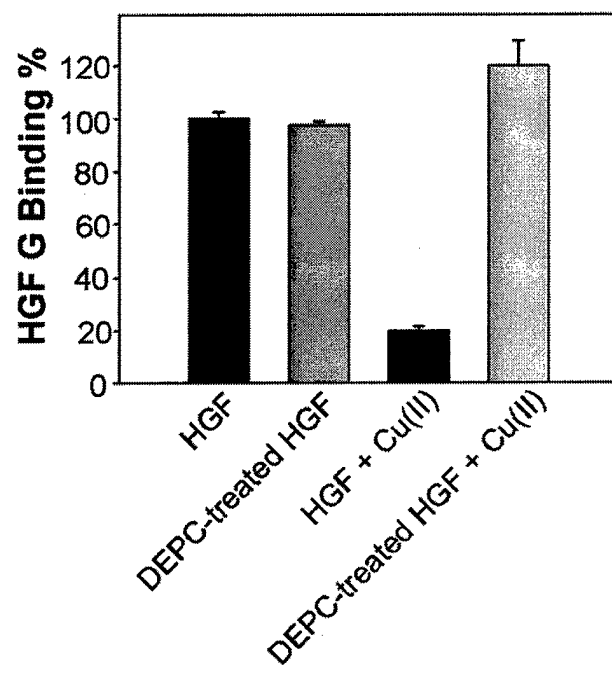# Figure 8