Functional Characterization of Domains Contained in Hepatocyte Growth Factor-like Protein*

(Received for publication, March 31, 1997, and in revised form, September 15, 1997)

Susan E. Waltz‡, Susan A. McDowell‡, Rebecca S. Muraoka§, Ellen L. Air‡, Leah M. Flick‡, Ying-Qing Chen‡, Ming-Hai Wang‡, and Sandra J. Friezner Degen‡‡

From the ‡Division of Developmental Biology, Children’s Hospital Research Foundation, Cincinnati, Ohio 45229-3039, the §Graduate Program in Developmental Biology, University of Cincinnati, Cincinnati, Ohio 45229, and the ¶Department of Internal Medicine, Division of Pulmonary Medicine, Wayne State University School of Medicine, Detroit, Michigan 48201

To delineate the functional protein domains necessary for the biological activity of hepatocyte growth factor-like protein (HGFL), we created various site-directed and deletion mutated cDNAs coding for this protein. Wild-type and mutated versions of HGFL were produced after transfection of the corresponding cDNAs into tissue culture cells. The biological importance of the domains within HGFL was then examined by addition of recombinant wild-type or mutant forms of HGFL to assays aimed at elucidating regions involved in the stimulation of DNA synthesis, the induction of shape changes in macrophages, and the ability to stimulate cell scattering. Mutant proteins lacking the serine protease-like domain (light chain) were not biologically active in any of the assays tested and could not compete with wild-type HGFL in cell scattering experiments. These data, in addition to direct enzyme-linked immunosorbent assay analyses, suggest that the light chain may play an important role in the interaction of HGFL with its receptor, Ron. Elimination of the proposed protease cleavage site between the heavy and light chains (by mutation of Arg-483 to Glu) produced a protein with activity comparable to wild-type HGFL. Further studies with this mutated protein uncovered an additional proteolytic cleavage site that produces biologically active protein. Deletion of the various kringle domains or the amino-terminal hairpin loop had various effects in the multiple assays. These data suggest that the heavy chain may play a pivotal role in determining the functional aspects of HGFL.

Hepatocyte growth factor-like protein (HGFL)† was initially isolated by virtue of its sequence homology to domains in proteins involved in blood coagulation and fibrinolysis (1–3) and by virtue of its functional ability to induce macrophage activation (4, 5). HGFL has been shown to be synthesized as a single polypeptide chain that is proteolytically cleaved to an active disulfide-linked heterodimer. The large α or heavy chain encodes an amino-terminal hairpin loop structure, homologous to the preactivation peptide in plasminogen, and four kringle domains. Kringle domains are triple disulfide-bonded loop structures composed of approximately 80 amino acids that are thought to be important in protein:protein interactions. The small β or light chain contains a serine protease-like domain in which the three active site amino acids have been changed such that protease activity is unlikely. The cDNA for HGFL codes for a protein of approximately 80 kDa containing 711 amino acids and three potential N-linked carbohydrate addition sites (1). However, the exact size of the two chains of HGFL as determined by Western analysis has been placed at anywhere from 45 kDa to 62 kDa for the heavy chain and approximately 25–35 kDa for the light chain (6–8).

HGFL has been classified in the same growth factor family as hepatocyte growth factor (HGF), with both proteins having a strikingly similar domain structure composed of the NH₂-terminal hairpin loop, four kringle domains, and a serine protease-like domain. Because of this similarity, HGFL is thought to elicit a broad range of functions as has been determined for HGF. However, in contrast to the wide expression patterns of HGF, HGFL has been shown to be produced primarily by liver hepatocytes (9). To date, several functions for HGFL have been determined, including the ability to stimulate mouse resident peritoneal macrophage (4), to induce cellular proliferation (10–12), to induce cell motility (13), to bring about cellular apoptosis (12), and to stimulate bone resorption (14). These functions are thought to be brought about by the binding of HGFL to its membrane-bound receptor, Ron (10, 15). However, the various functions of HGFL appear to be dependent on cell type and/or Ron expression levels, with no single cell performing all functions.

As is the case for the ligands, the Ron gene product displays strong structural homology with the HGF receptor, Met (16). The Met receptor family encodes a variety of transmembrane tyrosine kinase receptors including the HGF receptor, Ron, and avian SEA (17). Ron is a heterodimeric transmembrane glycoprotein expressed relatively late in development in the central and peripheral nervous system, in cells of developing bones and in epithelia along the digestive tract, skin, and lungs (18, 19). Ron is synthesized as a single-chain precursor of 185 kDa. After synthesis, Ron is cleaved into a disulfide-linked heterodimer consisting of a 35-kDa α chain and 150-kDa β chain. The α chain and amino-terminal region of the β chain are present at the cell surface. The cytoplasmic portion of Ron
contains the kinase domain and phosphorylation sites required for eliciting activity through HGFL binding.

Based on the pleiotropic effects of HGFL, the aims of the experiments presented in this paper were to elucidate the essential regions or domains in HGFL that may be required to elicit preferential biological activities. To accomplish this goal, a series of HGFL cDNAs lacking various domains or containing point mutations were constructed. Representative recombinant proteins were then produced and assayed for their ability to elicit cellular proliferation, macrophage activation, and cell scattering functions. Our findings represent an initial dissection of HGFL protein function and suggest the necessity of the light chain containing the serine protease-like domain for HGFL activity. Further, our results demonstrate the requirement of the various kringle domains for modulating protein activity.

EXPERIMENTAL PROCEDURES

Generation of Mutants of the Wild-type Human HGFL cDNA—All mutants were generated using the pAlter1 mutagenesis kit (Promega, Madison, WI) with the appropriate mutagenic oligonucleotide listed in Table I and a wild-type human HGFL cDNA cloned into pAlter1. The wild-type HGFL cDNA is the cDNA presented in Han et al. (1), with the addition of nucleotides 508–578. The appropriate cDNA and a cysteine at this position in the gene. Because we were not present in the gene. This resulted in a tyrosine at amino acid 13 in the protein. HGFL, we performed site-directed mutagenesis using the Chameleon kit (Stratagene, La Jolla, CA) and oligonucleotide 48G. The appropriate sequence (lowercase).

A series of HGFL cDNAs lacking various domains or containing point mutations were constructed. Representative recombinant proteins were then produced and assayed for their ability to elicit cellular proliferation, macrophage activation, and cell scattering functions. Our findings represent an initial dissection of HGFL protein function and suggest the necessity of the light chain containing the serine protease-like domain for HGFL activity. Further, our results demonstrate the requirement of the various kringle domains for modulating protein activity.

### Table I

| Mutant | Nucleotides | HGFL sequence | Strand |
|--------|-------------|---------------|--------|
| ΔPAP  | 146–165, 235–254 | ATGGGCCAGATGCTGAAGCAGCAGGATGCTGACGCTCAAGTAGCA | Coding |
| ΔK1   | 308–327, 559–578 | AGAAGCAGCAGCGGCGGGGAGGCCGCGTGTGTCTG | Coding |
| ΔK2   | 539–558, 805–824 | GCTGGCGATCATAAATCCTGGGGTGGGTTGGGAGGGG | Coding |
| ΔK3   | 827–846, 1084–1103 | AAGAGCGAGGAGCAAGCTGAGCAAGAGCTGGGCG | Coding |
| ΔK4   | 1086–1107, 1364–1365 | ACCGAGGTGGGGGCAAGGACGGCAGTATGACGGCAGGCCAT | Coding |
| ΔKH2  | 308–327, 559–584 | AGAAGAGCAGCAGGCTGACGCGGGTCGGGAGGACAGGCCG | Coding |
| AL    | 1455–1464⁺ | GGTCCGAGAAGCACGcAGGGGGCAGGGGGGGCAT | Coding |
| K1K2  | 805–834⁺ | GGCGGGACGACGAcAGGGGGGAGGGGGGGCAT | Coding |
| Glu   | 1432–1464⁺ | CCAGGGTCAGAAGCTGACGCGGAGGACACGGGGTGGGGGGCAT | Coding |
| Xa    | 1423–1461⁺ | CTGGATCGAGCGGGCATcgagaAGGCGGGTGGGGGG | Coding |
| Ha    | 1423–1461⁺ | CTGGAGCAGCGGGCTGACGCGGGTGGGGGGCAT | Coding |
| 48G   | 24–53⁺ | CCAGGGAGCCCTACAGGATTGAGTAGAAGC | Non-coding |

* Inserted stop codon (lowercase).
* Nucleotide substitutions to replace activation site with Glu for Arg-483, thrombin (Leu-Val-Pro-Arg), or factor Xa (Ile-Glu-Gly)-recognition sequence (lowercase).
* Difference with Ref. 20.

**Mitogenic Assays**—CMT-93 cells were seeded in triplicate in 100 μl of complete medium at a concentration of 5 × 10³ cells/well in a 96-well tissue culture plate. Two days later, the medium was aspirated and 200 μl of serum-free DMEM was introduced into the abdominal cavity, immediately after the abdominal incision was made under sterile conditions. Ten milliliters of 5% fetal calf serum, 2 mM L-glutamine, and 50 μg/ml gentamicin. For production of recombinant HGFL and HGFL mutant proteins, the corresponding cDNAs were transfected into tissue culture cells as described previously (21). For transient protein expression, 293S cells were transfected and on the following day placed in serum-free medium for 5 additional days. For stable protein expression, CHO cells were transfected and placed in 400 μg/ml Geneticin (Life Technologies, Inc.). After selection, single colonies were isolated and cultured for 5 days in serum-free medium. The amount of recombinant HGFL protein from cell supernatants was measured by immunoblotting.

**Detection and Quantification of Wild-type and Mutant HGFL Proteins**—For analysis of secreted recombinant protein, cell culture supernatants were resolved by SDS-polyacrylamide gel electrophoresis on 10% gels under denaturing conditions. After electrophoresis, the proteins were transferred by electrophoresis to polyvinylidene fluoride membranes in 25 mM Tris-HCl, 192 mM glycine, and 20% methanol at 4°C for 4 h at 250 mA. The membrane was probed with a polyclonal anti-HGFL rabbit antibody. The polyclonal antibody was used for HGFL detection was raised in rabbits against a fusion protein of β-galactosidase and a 960-base pair cDNA fragment coding for 321 amino acids of human HGFL (including part of the second kringle to the carboxyl terminus of the protein, which included part of the serine protease-like domain). This antibody recognizes mouse, rat, and human HGFL (7). The membrane was then incubated with a biotinylated goat anti-rabbit antibody, followed by incubation with a PAP-complex and biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). A horseradish peroxidase luminescent visualization system (ECL, Amersham Life Science) was applied, and the membrane was exposed to x-ray film. A known amount of HGFL was used as a standard to determine the concentration of each recombinant protein by Western analysis. Recombinant protein was produced from all cDNA expression vectors with a range of concentrations from about 0.5 to 7 μg/ml in serum-free medium.

Alternatively, an enzyme-linked immunosorbent assay (ELISA) was developed for protein determination. Protein samples (native or reduced) were incubated in Nunc immunoplates overnight at 4°C. Plates were washed and incubated with primary antibody, washed again, and incubated with a donkey anti-rabbit secondary antibody conjugated with horseradish peroxidase. Peroxidase substrate was added, and the absorbance was measured at 490 nm after color development. Samples were assayed against a concentration curve of wild-type human HGFL protein as a standard.

In Vitro Cleavage of HGFL and Mutant Proteins—To analyze if activation of HGFL was important before or during the functional assays, HGFL functions were tested with recombinant protein, which was cleaved by porcine or human kallikrein (Enzyme Research Laboratories, South Bend, IN) at 50–100 nM. Kallikrein has been shown to cleave plasma HGFL into a functional heterodimer (22). No functional differences between kallikrein species were detected. Furthermore, the use of soybean trypsin inhibitor (STI) in macrophage stimulation assays has been shown to enhance function (11). All functional assays were also performed in combination with STI (Sigma). Identical results were obtained in all assays regardless of the use of kallikrein, STI, or a combination of both before or during biological testing.

**Mitrigenic Assays**—CMT-93 cells were seeded in triplicate in 100 μl of complete medium at a concentration of 5 × 10³ cells/well in a 96-well tissue culture plate. Two days later, the medium was aspirated and 200 μl of medium containing 0.5% serum was added. After 48 h, recombinant HGFL protein (a concentration of 200–400 ng/ml was found to be optimal) was added. Two days after recombinant protein addition, 10 μl of 5′-deoxy-5′- bromoadenosine (BrdU) was added for 16 h, followed by fixation. The amount of BrdU incorporation was determined using an ELISA-based BrdU labeling and detection kit from Boehringer Mannheim according to the manufacturer’s instructions.

**Macrophage Shape Change Assays**—For the isolation of mouse resident peritoneal macrophages, the mouse was anesthetized and a small abdominal incision was made under sterile conditions. Ten milliliters of serum-free DMEM was introduced into the abdominal cavity, immediately after the abdominal incision was made under sterile conditions. Ten milliliters of 5% fetal calf serum, 2 mM L-glutamine, and 50 μg/ml gentamicin.
experiments. However, it became apparent that processed preparations of peritoneal macrophages. Tissue culture dishes. Approximately 80% of the cells were lung macrophages. Centrifuged, resuspended in serum-free DMEM, and plated in six-well plates. The cells were then immediately withdrawn and transferred to a polypropylene tube. This step was repeated to a total perfusion volume of 10 ml. The cells were then centrifuged, resuspended in serum-free DMEM, and plated in six-well tissue culture dishes. Approximately 80% of the cells were lung macrophage. Recombinant protein or control medium was applied as for peritoneal macrophages.

Scattering Assays—A 293 cell line expressing the mouse Ron receptor was obtained by transfection with a full-length mouse Ron cDNA in pcDNA3 (Invitrogen). A clonal line (293/Ron) was isolated and used for further studies. For scattering assays, an approximately 70% confluent plate of 293/Ron cells was split 1:100 and placed in six-well tissue culture plates containing 5 ml of complete medium/well. The following day, the medium was aspirated and the cells were cultured with various concentrations of wild-type or deletion mutants of HGFL for 24–48 h. After culture, the cells were fixed in 3% paraformaldehyde and stained with hematoxylin.

Receptor Binding Assays—293/Ron cells were grown to approximately 70% confluence in 60-mm tissue culture plates in complete medium. The cells were washed three times with ice-cold PBS, and recombinant protein (or appropriate controls) was added for 1 h at 37 °C. Detachment from the tissue culture plates was accomplished in 2 ml of Versene in PBS. The cells were rinsed twice in ice-cold PBS, followed by resuspension in a solution of PBS containing 1% bovine serum albumin and a 1:1 mixture of antibodies directed against HGFL and an anti-rabbit-fluorescein conjugate. After a 30-min incubation period on ice, the cells were washed twice with ice-cold PBS followed by fixation in PBS containing 1% paraformaldehyde. The cells were then analyzed by measuring fluorescence intensity on a Becton Dickinson FACScan flow cytometer. Results are expressed as the mean channel fluorescence of 10,000 cells using logarithmic amplification.

Detection of Interaction of Purified Ron with Recombinant Variants of HGFL by Direct ELISA—The Ron protein was purified by an anti-Ron affinity column as described previously (42). To study the interaction of Ron with multiple HGFL recombinant proteins, the Ron receptor was purified, placed in a 96-well ELISA plate at 50 ng/well, and incubated overnight at 4 °C. Supernatants were collected from cells transfected with different HGFL variant cDNAs, and equal amounts of HGFL recombinant proteins were added to the wells and incubated at 37 °C for 90 min. To measure the amount of captured recombinant protein, an antibody against HGFL was added (42). For detection analyses, an anti-rabbit IgG antibody conjugated with horseradish peroxidase (Boehringer Mannheim) was applied to the wells. The reaction was developed with substrate, and the absorbance was measured in an ELISA plate reader. Each sample was tested in duplicate. The single-chain form of HGFL (WT HGFL up) was utilized initially for these experiments. However, it became apparent that processed preparations were needed for full activity. Thus, the wild-type (WT) HGFL in Fig. 9 contains significant amounts of proteolytically cleaved protein.

RESULTS

Generation and Expression of Recombinant HGFL Protein—In an effort to identify important structural domains involved in the function of HGFL, a panel of mutated cDNAs of HGFL were constructed. Fig. 1 shows a schematic representation of the structural domains contained within the wild-type human HGFL protein (wt). The schematics shown below wild-type HGFL consist of predicted translation products generated from transfection of HGFL mutant cDNAs. Eight deletion mutants and four site-directed mutants of HGFL were created by oligonucleotide-directed mutagenesis. The deletion mutants were created by deletion of the cDNA sequence corresponding to the HGFL domain of interest (ΔPAP, ΔK1, ΔK2, ΔK3, ΔK4, ΔK1K2, or sequence following kringle 2 as in K1K2). Deletion of the light chain of HGFL (ΔL) was accomplished by creating a stop codon (TAG) after amino acid 483, immediately preceding the activation site. To study the effect of proteolytic cleavage as it relates to HGFL function, a mutation that disrupts the putative cleavage site was also created with the substitution of Glu for Arg-483 at the activation site (Glu). Furthermore, two mutations were created in which an engineered processing site

FIG. 1. Schematic representation of HGFL deletion mutant proteins. The top diagram depicts the structural domains contained within the wild-type human HGFL protein (wt). The amino-terminal region (NH2) and carboxyl-terminal region (COOH) of the protein are indicated. The putative signal peptide (SP), amino-terminal hairpin loop containing a region similar to the preactivation peptide of plasminogen (PAP), the four kringle domains (labeled K1, K2, K3, and K4, respectively), and the serine protease-like region (unlabeled) are also shown. The *asterisks* correspond to the amino acids in the serine protease-like domain, which have been changed when compared with the active site residues found in biologically active serine proteases. The three CHO tags correspond to putative glycosylation sites, two of which are also found in mouse HGFL (for a more detailed structure and background of HGFL please refer to the review of Ref. 2). The arrow at top of the diagram marks the putative proteolytic cleavage site thought to be required for activation and the bar below is indicative of a potential disulfide bond between the two chains after cleavage. The schematics shown below wild-type HGFL consist of predicted translation products generated from transfection of HGFL mutant cDNAs. Deletion mutants are named for the deleted domains (for example, ΔK1 has the first kringle domain deleted); K1K2 refers to a mutant which contains the amino-terminal region of the protein including kringles 1 and 2. Glu, Xa, and Ila refer to site-directed mutants at the activation site, and 48G refers to a site-directed mutation in the signal peptide. Cleavage at the activation site of activation site mutants is indicated by the arrow; the X for activation of the Glu mutant indicates that activation at this site was removed.
for factor Xa (Xa) or thrombin (IIa) was created at the putative activation site of HGFL. The Xa processing site changed amino acids Ser-480, Lys-481, Gly-482 to Ile-480, Glu-481, and Gly-482, whereas the IIa site was created by changing these amino acids to Leu, Val, and Pro, respectively. Not shown in Fig. 1 is a mouse HGFL clone containing the full-length cDNA for expression of recombinant mouse HGFL (mwtHGFL; Ref. 3).

All of the cDNAs for the corresponding proteins in Fig. 1 were cloned into the eukaryotic expression vector pcDNA3 and transfected either stably into CHO cells or transiently into 293 cells. Serum-free culture medium from the transfected cells was used as a source of recombinant HGFL. Fig. 2 shows representative samples of culture medium from wild-type and mutant HGFLs separated under reducing conditions by SDS-polyacrylamide gel electrophoresis analysis and immunoblotted with a polyclonal antibody against HGFL. A majority of the protein products appeared to consist of the single-chain form of HGFL. In culture medium from untransfected cells, as well as both stable and transient transfections with wild-type and mutant forms of HGFL, variable nonspecific background was also seen. The band migrating at approximately 65 kDa may represent bovine serum albumin. Refer to Fig. 1 for a diagram of the mutated versions of HGFL.

Effects of Recombinant HGFL on the Induction of Shape Changes in Macrophage—HGFL has been shown to cause dramatic morphological effects on mouse peritoneal macrophage (4, 5). Among these effects are the ability to activate macrophage contractile proteins for the ingestion of complement coated erythrocytes and the ability to assume elongated shapes with increased phagocytic vesicles (4, 5). To identify domains within HGFL that are responsible for stimulating the morphogenetic phenotype of these cells, stimulation of both lung and peritoneal macrophage were examined. Recombinant supernatant from cells transfected with the wild-type and mutated forms of HGFL was applied to macrophage cultures. After 3 h, the number of stimulated macrophage were counted and photographed. Fig. 4 shows representative results for various recombinant mutants tested. Wild-type and various mutated versions of HGFL (such as Glu and Xa) showed an increased ability to stimulate peritoneal macrophage, as indicated by elongated shape changes. Deletion of the light chain, the amino-terminal hairpin loop (ΔPAP), or various kringle domains resulted in a lack of or reduced responsiveness of the macrophage. Further, no differences were seen with inclusion of kallikrein during incubation of the macrophage with wild-type HGFL conditioned medium as compared with wild-type HGFL medium alone. As is apparent, wild-type HGFL had no effect on macrophage isolated from the lung. Fig. 4B shows a summary of these results with the average number of stimulated macrophage from each recombinant preparation.

Mitogenic Effects of Various HGFL Mutant Proteins on CMT-93 Cells—The ability of wild-type and mutated versions of HGFL was examined for their capability to stimulate cell growth. Several reports have implicated Ron expression in the epithelia lining of the gastrointestinal tract (18, 19, 44). Although the function of Ron in this context is not known, Ron may be involved in the proliferative capacity of the intestinal epithelia, a cell layer that is constantly being turned over. We have identified three gastrointestinal cell lines that express the Ron receptor (44) and further examined one of these lines in detail for proliferative effects after HGFL exposure. Equal concentrations of wild-type and HGFL recombinants were applied to the rectal carcinoma cell line, CMT-93. Mitogenic activity was then determined based on the amount of nucleotide ana-
logue, BrdUrd, incorporated. The amount of background incorporation of BrdUrd as a result of medium from untransfected cultures was set to 1, and the -fold stimulation of each of the recombinant proteins was calculated. The average -fold stimulation of wild-type and recombinant forms of HGFL is shown in Fig. 5. Wild-type human HGFL (WT) along with the putative unprocessed forms of HGFL (Glu, Ila, and Xa) appeared to stimulate cell growth by 2-fold. The K1K2 mutant also stimulated growth in this range, whereas deletion of the light chain (ΔL) or deletion of kringle 2, kringle 4, or both kringles 1 and 2 abrogated the mitogenic effects.

Alternative Proteolytic Cleavage Site for Activation of HGFL—HGFL has been shown to circulate in plasma as an uncleaved precursor (4, 7, 11). Conversion of HGFL to its active heterodimeric form is thought to occur by proteolytic cleavage involving specific serine-proteases at an Arg-Val bond between amino acids 483 and 484. The precise mobility of the two chains has been estimated to be anywhere from approximately 45 to 53 kDa for the α chain and from 22 to 35 kDa for the β chain even within the same laboratory (2, 4, 8, 11, 23). Abolishment of the Arg site in HGFL, as in the Glu mutation, however, results in a protein with functional activities comparable to the wild-type protein as judged in mitogenic, macrophage, and cell scattering assays (Figs. 3–5). Alteration of this homologous cleavage site in HGF has been shown to result in a protein that binds well to its receptor but lacks the ability to stimulate mitogenic effects (24). To investigate the issue of aberrant size and alternative processing, we examined the functional activity of wild-type HGFL and the potentially unprocessed Glu mutation in more detail. Fig. 6A shows a Western analysis of recombinant wild-type HGFL cleaved for increasing amounts of time with kallikrein, a protease known to specifically cleave HGFL into its heterodimeric form (22). Two specific cleavage products from this digestion were obtained: a product of approximately 53 kDa (B, in Fig. 6A) and a product of approximately 45 kDa (C, in Fig. 6B). These results suggest that wild-type HGFL may have two distinct sites of proteolytic cleavage, producing α chain products of approximately 45 and 53 kDa. The β or light chain of HGFL is not detected in these assays as a result of the fact that the polyclonal antibody used for Western analyses is specific for the α chain.

It is interesting to speculate that cleavage at the alternative site may be responsible for the functional activity of the Glu mutation. To examine whether an alternative processing site exists in the Glu mutant, wild-type HGFL and the Glu recombinant protein were subjected to proteolytic digestion with kallikrein (Fig. 6B). In the presence of extended incubations with kallikrein, both proteins were cleaved to the 45-kDa product (C in Fig. 6B). These data suggest that the Glu mutant may have an alternative site of cleavage. Another plausible explanation is that proteolytic processing of HGFL is not required for function.

Previous studies have suggested that the addition of STI to assays of HGFL function may enhance activity by inhibiting nonspecific degradation. Fig. 6B shows the results of extended a copy of the mouse Ron cDNA was cultured in the presence of equal amounts of wild-type HGFL or mutated versions of this protein (as indicated). Conditioned medium from untransfected (UT) cells was used as a negative control. After 24 h, the cells were stained with hematoxylin and photographed. Refer to Fig. 1 for a diagram of the mutated versions of HGFL. B, the ability of recombinant HGFL and mutants to scatter 293/Ron cells was observed. The histogram shows the average ability of each protein to induce cell scatter compared with wild-type HGFL. The -fold scattering ability is shown in arbitrary units with untransfected cells set at 1. Multiple protein preparations were utilized and a number of scattering assays performed for each form of the protein. mWT refers to the wild-type mouse HGFL protein.
kallikrein digestion of wild-type HGFL and the Glu mutant in the presence of STI. Addition of this protease inhibitor appeared to cause enhanced digestion at the putative Arg\textsuperscript{483} site in the wild-type protein producing more of the 53-kDa product under prolonged digestion conditions (Fig. 6B, B and C). Digestions containing STI and kallikrein with the Glu mutant, however, appeared to partially inhibit cleavage at the putative alternative processing site (Fig. 6B, A and C). These data suggest that STI may be acting to cause selective cleavage by kallikrein at the Arg-483 site, whereas kallikrein alone appears to act at either site. Thus, these experiments further reinforce the idea that HGFL may have two activation sites.

To test whether the 45-kDa form of both the wild-type HGFL and Glu proteins could be functional, Glu and wild-type HGFL cell culture supernatants were subjected to an extended incubation with kallikrein. After incubation, the medium was used in cell scattering assays to access the selective activity of the 45-kDa digestion product. Western analysis of each supernatant determined that kallikrein digestion to this form was complete (Fig. 6B). Fig. 7 panels 1–7 demonstrates the ability of both 45-kDa products to induce cell scattering. Panel 1 contains 293/Ron cells with conditioned medium from untransfected cells. Panel 2 contains cells with conditioned medium containing wild-type HGFL without prior proteolytic cleavage or inhibitors, whereas panels 3 and 4 contain the same recombinant protein with prior cleavage by kallikrein or kallikrein and STI, respectively. Similar scattering studies were performed with the Glu mutant with cells in panel 5 containing the Glu mutant digested to the 45-kDa form with kallikrein. Panel 6 has the Glu mutant with prior digestion with STI and kallikrein, and panel 7 has the Glu mutant alone. As is apparent, no matter what combination of proteins were utilized, all were active in inducing scattering. These experiments raise a number of exciting suggestions about the processing and control of HGFL function. The significance of the two potential processing sites \textit{in vivo} is not known but may represent a means of regulating the biological activities of HGFL in a microenvironment.

\textbf{Binding of HGFL and Mutant Forms to 293/Ron Cells—}
Previous studies have shown that important receptor binding determinants in HGF are located within the amino-terminal domain (24–27). Further, deletion of the light chain of HGF appears to abolish biological activities but may retain receptor binding capabilities (24, 25, 28, 29). In contrast, our results suggest that, for HGFL, deletion of the light chain (ΔL) causes not only loss of biological activity but possibly loss of receptor binding. To further investigate receptor binding properties of HGFL and various mutants at the concentrations used in this study, flow cytometry and receptor binding competition experiments were performed. Competition experiments testing the ability of mutant forms of HGFL to compete for wild-type binding and function in cell scattering assays were performed. As determined previously, three mutants (namely ΔL, ΔK2, and ΔK3) were unable to produce a cell scattering phenotype.

**Fig. 4.** Effects of wild-type and mutated forms of HGFL to induce morphological shape changes in macrophages. A, macrophage were isolated from either the mouse pulmonary (indicated by Lung) or peritoneal cavities (unmarked) as described. Conditioned medium from untransfected (UT) cells or cells transfected with wild-type or mutated versions of HGFL was added as shown. Three hours later, the percent of activated macrophage were scored (based on the percentage of macrophage with elongations). WT + K refers to peritoneal macrophage with conditioned medium containing wild-type HGFL and kallikrein. The macrophage were then stained and photographed as described. Refer to Fig. 1 for a diagram of the various mutated versions of HGFL. B, for repeated experiments and multiple protein preparations, the average percent of morphologically altered macrophage was determined. For each recombinant protein (x axis), the percent of stimulated macrophage is shown (y axis). mWT refers to the wild-type mouse HGFL protein.
Lack of scattering function may be either a direct result of mutation of a region important for cell scattering or an indirect result of creating a mutant that loses the ability to bind to its receptor. Fig. 7 shows experiments investigating these possibilities. The scattering ability of conditioned medium from untransfected cells (panel 1) or wild-type HGFL (panel 2) is shown. For competition analyses, wild-type HGFL protein was tested in the presence of approximately equal amounts of ΔL (panel 8), ΔK2 (panel 10), or in the presence of an excess of conditioned medium from ΔL (panel 9), ΔK2 (panel 11), or ΔK3 (panel 12). In the presence of an excess of ΔK2 or ΔK3, wild-type HGFL had a reduced ability to stimulate cell scattering suggesting competition of ΔK2 and ΔK3 proteins for binding to the surface of the 293/Ron cells. In contrast, in the presence of ΔL, wild-type HGFL was not inhibited in its ability to induce cell scattering.

To investigate the ability of wild-type and mutant forms of HGFL to bind to its receptor under the conditions and concentrations utilized for the aforementioned biological assays, flow cytometry was employed. 293/Ron cells were incubated in the presence of conditioned medium from untransfected controls or in the presence of medium from cells that stably expressed wild-type or mutant versions of HGFL (Fig. 8). Binding was then assayed by flow cytometry after incubation with a primary antibody against HGFL followed by incubation with a fluorescent secondary antibody. Fig. 8 depicts two representative flow cytometry experiments with the wild-type and Glu mutant proteins producing a significant shift in fluorescence indicative of receptor binding (Fig. 8A). In contrast, the ΔL mutant appeared to have little effect on receptor binding (Fig. 8A). K1K2 and ΔK1K2 produced an increase in fluorescence intensity but to a lesser extent compared with levels seen with the wild-type HGFL protein (Fig. 8B). Data from multiple experiments are summarized and shown in Table II.

Because of the fact that our experimental system utilized crude protein preparations in limited quantities, $K_d$ values for the various mutants were not obtained. However, to directly access the quantitative ability of the recombinant HGFL proteins to associate with Ron, a quantitative ELISA assay was developed. For these analyses, the Ron receptor was affinity-purified and coated on the bottoms of a 96-well ELISA plate. Subsequently, equal amounts of recombinant wild-type and mutant HGFL proteins were added. After incubation, the amount of recombinant protein that remained bound to the Ron receptor was determined. Shown in Fig. 9 is the average amount of recombinant protein found bound to the Ron receptor. In these analyses, it became apparent that the single-chain unprocessed form of HGFL did not associate with the purified
receptor (WT HGFL up, Fig. 9). However, when processed HGFL was added in the direct ELISA experiments, binding to Ron was detected (Fig. 9, WT HGFL). These results are in contrast to those obtained from flow cytometry and in the biological assays in that prior processing of HGFL was not required for binding or activity. However, the flow cytometry experiments differ in that whole cells were utilized in these experiments and in the functional analyses, whereas only the Ron receptor was utilized in the direct ELISA experiments. These experimental data suggest that proteolytic processing is required before receptor binding and that the target cells in our functional assays are capable of this cleavage. Furthermore, the ELISA data correlate well with the flow cytometry data in that the kringle domains do not appear to be pivotal for receptor binding. Deletion of the light chain dramatically reduces the ability of HGFL to associate with Ron (Figs. 8 and 9).

Additional binding determinants that may aid in stabilizing receptor/ligand interactions may also be contained within the PAP domain (Figs. 8 and 9).

**DISCUSSION**

HGFL was identified as a potent mitogenic factor for liver hepatocytes (32–34). HGFL has also been shown to increase the motility and invasiveness of various epithelial and endothelial cells and to increase proliferation of multiple cell types (35–39). These various functional activities of HGFL have been extensively studied and mapped to specific structural domains and amino acid residues. An interesting characteristic of both HGF and HGFL is their striking structural similarity with distinct structural domains encoded by specific exons (2). With respect to the pleiotropic activities reported for HGF and the functional and structural similarities between HGF and HGFL, our investigations have focused on determining whether various functional activities for HGFL could be mapped to distinct regions in the protein. Utilizing site-directed mutagenesis and deletion analysis, cDNAs encoding a large set of mutant forms...
of HGFL were constructed. Representative wild-type and mutant proteins were expressed and utilized in functional assays consisting of cell growth, macrophage shape changes, and cell motility. Mutants of HGFL were engineered such that individual or multiple kringle domains were deleted (ΔK1, ΔK2, ΔK3, ΔK4, and ΔK1K2). Further deletions were constructed to test for the functional contribution of the light chain containing the serine protease-like domain (ΔL), the amino-terminal hairpin
loop (ΔPAP), or a construct containing the amino-terminal region of the protein through kringle 2 (K1K2). This latter mutant, K1K2, was created as a result of an analogous naturally occurring splice variant coding for sequences through the second kringle domain of human HGF, designated as HGF/NK2 (30, 31). However, a naturally occurring variant of HGFL analogous to that of HGF has not been found.

Deletion mutants of HGFL were also created with alterations designed to investigate proteolytic processing (IIa, Xa, and Glu) or which changed an amino acid residue in the signal peptide (48G). The 48G mutant was created as a result of a discrepancy between the reported cDNA sequence for HGFL (with a tyrosine at nucleotide position 48; Ref. 1) and the genomic sequence for HGFL (with a cysteine at nucleotide position 48; Ref. 1). Additionally, a full-length mouse HGFL construct was obtained to identify any species specific differences between the mouse and human proteins. Although we cannot exclude the possibility that any given mutation lacks biological activity because of disruption of proper protein folding, all of the mutants, excluding ΔL, had at least some nominal activity in one of the assays tested.

During biological testing, it became apparent that the activation site mutants, Glu, Xa, and IIa, were as functionally active as the wild-type HGFL protein (Figs. 3–5 and 7). These data suggest either that proteolytic processing is not required for binding to and activation of the HGFL receptor or that an amino acid sequence of HGFL for an alternative processing stems from the observation that the unprocessed single-chain form of HGFL does not associate with the “purified” Ron receptor (Fig. 9, WT HGFL up). In contrast, HGFL is capable of binding to the purified Ron gene product after proteolytic cleavage (Fig. 9, WT HGFL). Prior proteolytic processing was not required for WT HGFL to bind to the 293/Ron cells (Fig. 8), as judged by flow cytometry. These results suggest that proteolytic cleavage may occur on the cell membrane of target cells. These observations are reinforced by the fact that the unprocessed Glu mutant, which is functionally equivalent to WT HGFL, associates with the 293/Ron cells; however, no binding activity is detected using the purified receptor (Fig. 9). Thus, proteolytic processing appears to be a prerequisite for receptor binding.

A summary of the biological effects of the recombinant proteins is summarized in Table II. As is apparent, the 48G mutant and the mouse wild-type HGFL proteins had functional activities comparable to the human wild-type HGFL protein, suggesting that the discrepancy in DNA sequence and the differences between mouse and human HGFL were not important in our functional analyses. As indicated, mutant proteins devoid of the light chain were not effective in any of the biological assays tested. Flow cytometry and competition experiments suggest that this region appears to be important for stabilizing receptor interactions (Figs. 7 and 8), and this is confirmed by the direct ELISA results (Fig. 9). Our results are similar to those found with HGF mutants in that deletion of the light chain is also necessary for biological function (25, 26, 28,
In contrast, however, this mutant of HGF has been shown to retain its ability to bind to the receptor, albeit with a reduced affinity. Therefore, the light chain in HGF may play a role in mediating receptor phosphorylation (26, 29). Mutational analyses on HGF have established that the amino-terminal hairpin loop and the first and second kringle domains are important for binding to the c-Met receptor (26, 28, 29). For HGFL, however, this function appears to reside within the light chain.

Our receptor binding data are in agreement with the recent report of Wang and co-workers (43). In this report, mass preparations of recombinant HGFL were obtained. Utilizing kalikrein digestion and affinity purification, these investigators were able to obtain large amounts of unprocessed HGFL as well as the individual heavy and light chains of this protein. Receptor binding experiments were then undertaken to examine the ability of the α (heavy chain containing four kringles) and β (light chain containing the serine protease-like domain) chains of HGFL to bind to Ron. Radioiodination experiments suggested that the light chain was required for binding to the receptor but that this chain had no functional activity. Similarly, the heavy chain was not functional biologically as its ability to bind to the receptor was dramatically reduced. Further, these investigators determined that the unprocessed form of HGFL failed to associate with Ron. Taken together, the data support the fact that the light chain is the main determinant for receptor binding.

In addition to the light chain, absence of the amino-terminal hairpin loop (ΔPAP) or deletion of more than one kringle domain (ΔK1K2 and ΔK1K2) also had an effect on receptor binding (Figs. 8 and 9, Table II). Furthermore, the weak binding activity of ΔPAP and ΔK1K2 correlated with a decrease in biological activities (summarized in Table II). The K1K2 mutant, however, retained only a weak ability to associate with the HGFL receptor (Fig. 8 and 9, Table II). This mutant appeared to act in an agonistic fashion with respect to its ability to stimulate mitogenic and macrophage stimulating activities (Table II). These results are interesting in two regards. First, HGF/NK2, the naturally occurring HGF variant, has also been shown to serve as an agonist of HGF retaining the ability to bind to the c-Met receptor and to induce scattering of epithelial cells. This mutant however, lacks the mitogenic activity associated with wild-type HGF (26). Second, these studies are in agreement with a previous report, which investigated the ability of a similar two kringle variant of HGFL to induce tyrosine phosphorylation of a transfected Ron receptor in COS-1 cells (10). These studies demonstrated the ability of the similar K1K2 recombinant protein to induce tyrosine phosphorylation. In contrast to our detailed analysis, these investigators did not examine any functional activities associated with this mutant nor were any other HGFL mutations analyzed. Thus, the K1K2 HGFL mutant appears to act in a similar fashion to the naturally occurring variant of HGF in that it can bind to the receptor and activate selected activities, yet the primary binding capacity of HGFL appears to reside within the light chain.

With the exception of the light chain deletion, the various kringle domains appear to be critical for modulating the biological activities of HGFL. Kringles 2 and 3 appear to be essential for cell scattering functions (Table II). Deletions involving either of these two kringles result in a complete abolishment of this activity. Scattering was also reduced in response to deletion of kringle 4 and in the mutant that lacks the amino-terminal hairpin loop (ΔPAP). As mentioned previously, the contribution of ΔPAP may lie in enhancing interactions of the ligand with Ron, whereas deletion of kringle 4 may have a more functional role. In contrast to the cell scattering func-

---

**Fig. 9. Interaction of WT HGFL and HGFL mutant proteins with the purified Ron receptor.** The purified Ron receptor was utilized in ELISA assays to directly quantitate the affinity of wild-type and mutant forms of HGFL for Ron. Equal amounts of HGFL proteins were added to the wells of an ELISA plate coated with the purified Ron receptor. After a 1.5-h incubation, the amount of HGFL protein captured by the receptor was quantitated. For quantitation, an antibody against HGFL was added followed by incubation with a secondary antibody conjugated with horseradish peroxidase. The amount of binding was subsequently measured in an ELISA plate reader. The average amount of binding of each recombinant protein tested is shown in ng/well along with the standard deviation. WT HGFL up refers to an unprocessed form of HGFL, whereas WT HGFL refers to HGFL that has been activated before being assayed.
ations, kringle 1 or 3 and the inclusion of the amino-terminal hairpin loop appear to be pivotal in directing morphological shape changes in macrophage. Kringle domains 2 and 4 appear to be of lesser functional significance. Loss in activity as a result of deletion of kringle 2 or 3 may indirectly be hampered by the loss of a disulfide bond formed between the two domains (1). Furthermore, the stimulatory response of macrophage by HGFL was specific for peritoneal and not alveolar macrophage (Fig. 4A). Consistent with these results, the mouse Ron receptor has been shown to reside specifically on resident peritoneal macrophages and not on blood monocytes or other tissue macrophage (41).

Contrary to specific kringle requirements for cell scattering and macrophage stimulating functions, each kringle domain in HGFL appears to be important for propagating a mitogenic response. Excluding the mutants that examine proteolytic cleavage and also the K1K2 mutation, deletion of any kringle domain causes a reduction in or total loss of mitogenic activity (Table II). These results suggest that the mitogenic response elicited by HGFL may require a structurally intact protein.

Regardless of the complex structural requirements needed for biological function, another level of complexity associated with HGFL is its ability to act selectively on target cells. Recombinant HGFL was not able to scatter CMT-93 or HT-29 cells that express the endogenous Ron receptor, yet there was an induction of DNA synthesis in these cells in response to HGFL (data not shown and Ref. 44). Conversely, HGFL in-duced scattering phenotypes in 293/Ron cells and in NIH/3T3 cells that express the endogenous Ron receptor (44). These mutant studies of HGFL have pointed to an important role for biological function, another level of complexity associated with HGFL (1). Furthermore, the stimulatory response of macrophage by HGFL was specific for peritoneal and not alveolar macrophages (1). Moreover, the stimulatory response of macrophage by HGFL appears to be important for propagating a mitogenic and macrophage stimulating functions, each kringle domain in HGFL was specific for peritoneal and not alveolar macrophage (1). Furthermore, the stimulatory response of macrophage by HGFL was specific for peritoneal and not alveolar macrophage (1). Furthermore, the stimulatory response of macrophage by HGFL was specific for peritoneal and not alveolar macrophage (1). Furthermore, the stimulatory response of macrophage by HGFL was specific for peritoneal and not alveolar macrophage (1). Furthermore, the stimulatory response of macrophage by HGFL was specific for peritoneal and not alveolar macrophage (41).