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Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding

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Hepatocyte growth factor (HGF) is a potent mitogen for parenchymal liver, epithelial and endothelial cells. Structurally, it has similarities to kringle-containing serine proteases, although it does not possess proteolytic activity. A structure-activity relationship study of human HGF was performed by functional analysis of HGF substitution and deletion variants. Analysis of HGF variants was accomplished by defining their ability to induce DNA synthesis on hepatocytes in primary culture and to compete with wild-type HGF for binding to a soluble form of the HGF receptor. Three groups of variants were made: (i) substitutions at the cleavage site, (ii) substitutions within the protease-like domain and (iii) deletions of the β-chain and/or kringle domains. Our results show that: (i) single-chain HGF is a zymogen-like promitogen in that cleavage into a two-chain form is required for biological activity, however, the single chain form of HGF still retains substantial receptor binding capacity; (ii) certain mutations in the protease-like domain result in variants that are completely defective for mitogenic activity, yet exhibit apparent receptor binding affinities similar to wild-type HGF (K_d ~50-70 pM); and (iii) a variant containing the N-terminal 272 residues of mature HGF showed only a 4-fold increase in K_d when compared with wild-type HGF indicating that a primary receptor binding determinant is located within this sequence.

Key words: hepatocyte growth factor/hepatocyte growth factor receptor/mutagenesis/structure-function study/c-Met proto-oncogene

Introduction

Hepatocyte growth factor (HGF) was identified initially as a mitogen for hepatocytes (Michalopoulos *et al.*, 1984; Nakamura *et al.*, 1984; Russell *et al.*, 1984). The levels of HGF increase in the plasma of patients with hepatic failure (Gohda *et al.*, 1988) and in the plasma (Lindroos *et al.*, 1991) or serum (Asami *et al.*, 1991) of animals with experimentally induced liver damage. The kinetics of this response is rapid and precedes the first round of DNA synthesis during liver regeneration, suggesting that HGF may play a key role in initiating this process. More recently, HGF has been shown to be a mitogen for a variety of cell types including melanocytes, renal tubular cells, keratinocytes, certain endothelial cells and cells of epithelial origin (Igawa *et al.*, 1991; Kan *et al.*, 1991; Matsumoto *et al.*, 1991; Rubin *et al.*, 1991). Interestingly, HGF can also act as a 'scatter factor', an activity that promotes the dissociation of epithelial and vascular endothelial cells *in vitro* (Stoker *et al.*, 1987; Weidner *et al.*, 1990, Naldini *et al.*, 1991a). Moreover, HGF has recently been described as an epithelial morphogen (Montesano *et al.*, 1991). Therefore HGF has been postulated to be important in tumor invasion and in embryonic development.

Both rat and human HGF (huHGF) have been molecularly cloned and the sequence predicts a primary translation product of 728 amino acids (Miyazawa *et al.*, 1989; Nakamura *et al.*, 1989; Seki *et al.*, 1990; Tashiro *et al.*, 1990) with a characteristic hydrophobic leader sequence of 31 residues (Yoshiyama *et al.*, 1991). The mature form, corresponding to the major form purified from human serum, is a glycosylated disulfide-linked heterodimer derived by proteolytic cleavage of the human prohormone between R494 and V495. This cleavage process generates a molecule composed of a 69 kDa α-chain and a 34 kDa β-chain (Miyazawa *et al.*, 1989; Nakamura *et al.*, 1989). It is not known whether cleavage is required for either receptor binding or the mitogenic, motogenic or morphogenic activities of huHGF. Hepatocyte growth factor has no sequence homology with other known polypeptide growth factors. However, HGF does have ~40% overall sequence similarity with plasminogen (Nakamura *et al.*, 1989). Recently, an HGF-like cDNA has been isolated that has 44% amino acid homology with HGF, but the function of this polypeptide remains to be elucidated (Han *et al.*, 1991).

Secondary structure analysis of HGF predicts the presence of four kringle domains in the α-chain (Nakamura *et al.*, 1989). As yet, the function of these kringles has not been determined. The β-chain shows high homology to the catalytic domain of serine proteases. However, two of the three residues which form the catalytic triad of serine proteases are not conserved in HGF. Therefore HGF appears to have no proteolytic activity and the precise role of the β-chain remains unknown.

An antagonist of HGF, HGF/NK2, has recently been described which corresponds to an alternatively spliced form of the HGF transcript containing the coding sequences for the N-terminal two kringle domains of mature huHGF (Chan *et al.*, 1991; Miyazawa *et al*, 1991).

The HGF receptor has been identified as the product of the c-*met* proto-oncogene (Bottaro *et al.*, 1991; Naldini *et al*, 1991b), a 190 kDa heterodimeric (a disulfide-linked 50 kDa α-chain and a 145 kDa β-chain) membrane-spanning tyrosine kinase protein (Park *et al.*, 1987). The c-*met* protein is phosphorylated on tyrosine residues of the 145 kDa β-subunit upon HGF binding (Bottaro *et al*, 1991; Naldini *et al.*, 1991b).

We initiated a structure–activity relationship study of huHGF to identify functionally important domains and the residues that are responsible for the interaction of huHGF with its receptor.

In particular, we wished to address the role of cleavage of HGF for receptor binding and mitogenic activity, and to determine the function of the protease and kringle domains. We report here the expression of HGF variants in human fetal kidney 293 cells and the analysis of their ability to induce DNA synthesis of rat hepatocytes in primary culture and to bind in solution to the extracellular domain of the HGF receptor.

Results

Experimental strategy

A schematic representation of the α- and β-subunits of huHGF is shown in Figure 1A. Since the spatial relationship of one kringle to another is unknown, we could not represent

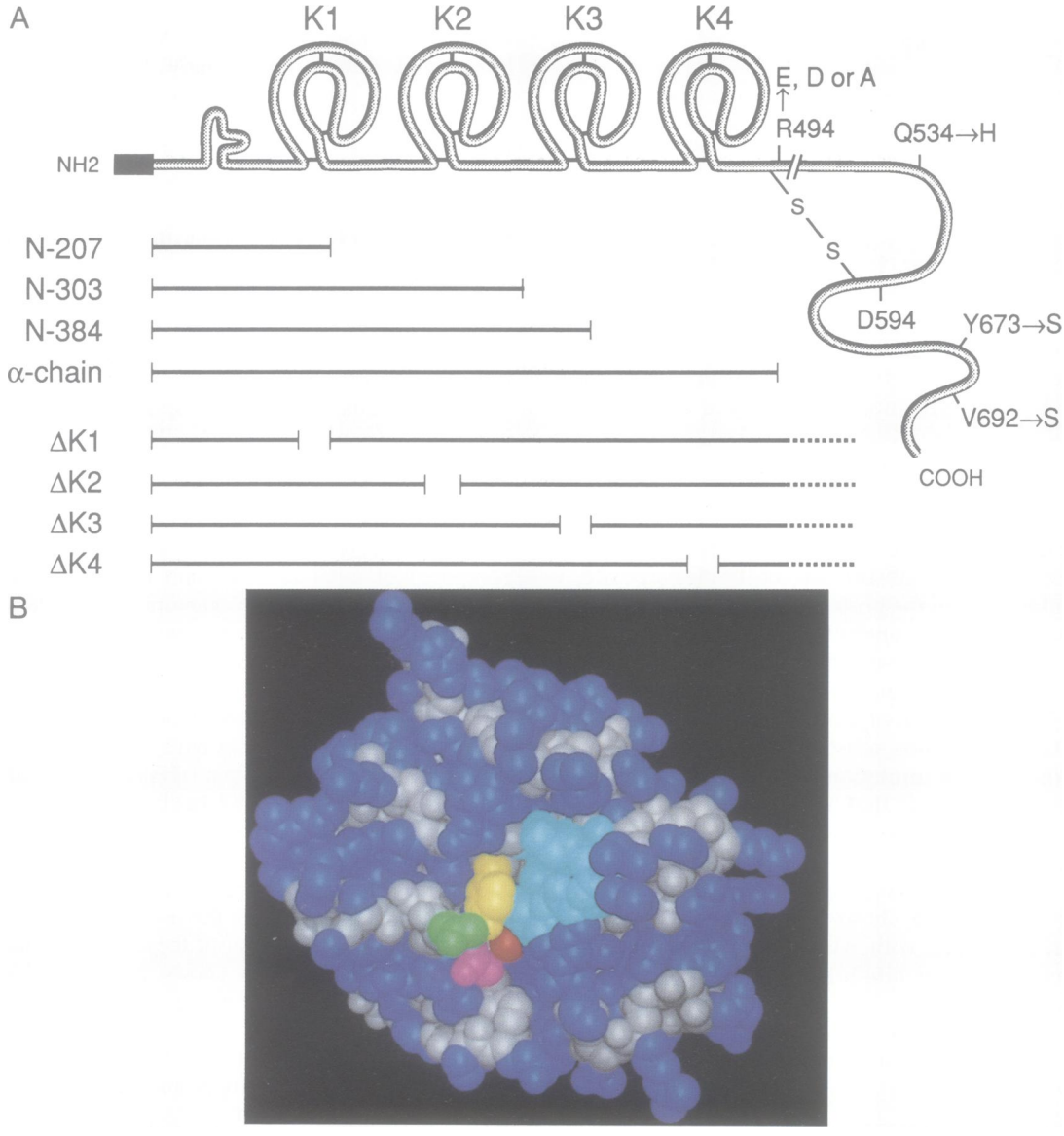

Fig. 1. **(A)** Schematic representation of the secondary structure prediction of huHGF. Shown in the α-chain are the signal sequence (boxed region) which encompasses amino acids 1–31 and four kringle domains, each with their respective three disulfide bonds. The cleavage site for generation of the heterodimeric α–β form of HGF immediately follows the P1 cleavage residue R494. This last residue has been specifically substituted with either E, D or A to generate HGF single-chain variants. The β-chain, which follows the cleavage site, contains homology to serine proteases. It is proposed that the α- and β-chains are held together by a unique disulfide bridge between C487(α) and C604(β) (Nakamura *et al.*, 1989). Three residues within the β-chain have been substituted individually or in combination to reconstitute the authentic residues of a serine protease. Schematic representations of the mature forms of the C-terminal truncation variants are depicted below: N-207, deleted after the first kringle; N-303, deleted after the second kringle; N-384, deleted after the third kringle and the α-chain. Also shown are the variants where deletions of each of the kringles (ΔK1, ΔK2, ΔK3 and ΔK4) were introduced. In each case, the deletions specifically remove the entire kringle from C1–C6. **(B)** Tertiary structure model of the serine protease-like domain (β-chain) of huHGF. The model is based on the crystal structures of the serine proteases trypsin (Kossiakoff and Spencer, 1981), chymotrypsin (Blevins and Tulinsky, 1985) and pancreatic elastase (Meyer *et al.*, 1988). The P1 binding pocket (cyan; Schechter and Berger, 1967), main-chain (grey) and side-chains (blue) are indicated. Highlighted are side-chains of highly conserved residues of serine proteases, e.g. those of the catalytic triad: Q534 (green) instead of H in serine proteases, D594 (purple) which is unmodified in HGF and Y673 (yellow) instead of S. Also highlighted is V692 (red); in serine proteases this position is usually an S which may be important for positioning the catalytic D in the active site.

the complete HGF molecule in a tertiary model. The α-chain contains a finger and four kringle domains, whereas the β-chain has homology with serine proteases (38% to the plasminogen serine protease domain). However, HGF does not appear to be an active protease because some of the residues proposed to be essential for proteolytic activity are changed. A model of the three dimensional structure of the protease-like domain is pictured in Figure 1B. This model is based on the crystal structures of trypsin (Kossiakoff and Spencer, 1981), chymotrypsin (Blevins and Tulinsky, 1985) and pancreatic elastase (Meyer *et al.*, 1988), and highlights the two amino acids in HGF, Q534 and Y673, which replace H57 and S195, respectively, in the catalytic triad of serine proteases. Also highlighted is V at HGF position 692 which replaces another highly conserved residue, S214; in serine proteases the S214 side-chain hydrogen-bonds to the catalytic D102 (D594 in HGF) and therefore helps to form the active site (Kossiakoff and Spencer, 1981; Blevins and Tulinsky, 1985; Meyer *et al.*, 1988).

A series of recombinant HGF (rhuHGF) variants was produced to determine the structural and functional importance of the cleavage of the prohormone to the α–β dimer, and of the kringle and protease-like domains. Mutations were introduced into the HGF cDNA in a CMV-based expression plasmid, and conditioned media from stable populations of human 293 cells expressing each variant were assayed by Western blotting in order to monitor the size and expression level of the HGF variants. The concentration of each HGF derivative was confirmed with two types of sandwich ELISA. The differences in expression levels found in ELISA correlated with those observed on Western blots. For most variants, the level of expression was in the range of 1–5 μg/ml. For variants with expression levels below 0.6 μg/ml, the conditioned medium was concentrated. The mitogenic activity on liver cells in primary culure and ability to bind to the HGF receptor were then determined. The extracellular domain of the HGF receptor was fused to the constant region (Fc) of a human IgG and binding was performed in solution (Mark,M., Lokker,N., Luis,E.A. and Godowski,P., in preparation).

Analysis of cleavage site mutants

The activation of the zymogen of serine proteases (e.g. plasminogen or tissue plasminogen activator) to active enzymes usually involves a specific cleavage of the single polypeptide chain (Tate *et al.*, 1987; Wun, 1988). The cleavage site commonly contains a basic residue at position P1 and two hydrophobic amino acid residues in positions P′1 and P′2, which follow the cleaved peptide bond. The proposed cleavage site of HGF (P1 R494, P′1 V495, P′2 V496) fits this consensus. We chose to try to block the cleavage of HGF by replacing the P1 R494 with either D, E or A. The major form of wild-type (wt) rhuHGF expressed in these cells is cleaved into two-chain material as judged by the presence of the α-chain with an apparent molecular mass of 69 kDa (Figure 2). Each of these mutations appeared to block processing of HGF because under reducing conditions these variants migrated as a single band at 94 kDa, the predicted size of single-chain HGF. These variants totally lacked the ability to induce the proliferation of hepatocytes in primary culture (Figure 3A). However, when these variants were analyzed for their ability to compete with wt rhuHGF for binding to the HGF receptor–IgG fusion protein, their inhibition curves were roughly similar to that of wt rhuHGF (Figure 3B). The K_d estimated from these curves showed that wt rhuHGF binds to the fusion protein with high affinity (50–70 pM) whereas all single chain variants showed an ~2- to 10-fold higher K_d (100–500 pM) than wt rhuHGF. Results from at least three independent assays are summarized in Table I as residual hepatocyte proliferative activity and receptor binding capacity compared with wt rhuHGF. A direct comparison of specific activity versus K_d ratios of all single chain variants showed they were inactive at the highest concentration tested (specific activity <3%) while receptor binding affinities were only decreased by a factor of two to ten. These results argue strongly that cleavage of HGF into the two-chain form is required for mitogenic activity, i.e. that single-chain HGF is a promitogen and that the uncleaved form of HGF binds to the HGF receptor, albeit with a reduced affinity.

Analysis of protease variants

We replaced HGF residues Q534 with H, Y673 with S, or V692 with S (Figure 1B) as either single, double or triple mutations. The analysis of their effects on mitogenic activity and receptor binding showed that the single mutation Q534H did not significantly alter either specific activity (5.2×10^4 Units/mg) or K_d (60 pM) when compared with wt rhuHGF (3.3×10^4 Units/mg and 70 pM, respectively) whereas Y673S and V692S exhibited specific activity reduced by ~5- and 10-fold, respectively. In fact, these two variants never reached the maximum plateau seen with wt rhuHGF (~50% of wt rhuHGF plateau). Interestingly, these variants showed a K_d similar to wt rhuHGF. All other double and triple variants also retained the ability to bind the HGF receptor but they clearly showed a reduced specific activity (Table I). The residual specific activity of the double variants Q534H,Y673S and Y673S,V692S and of the triple variant Q534H,Y673S,V692S were <3% of wt rhuHGF. However,

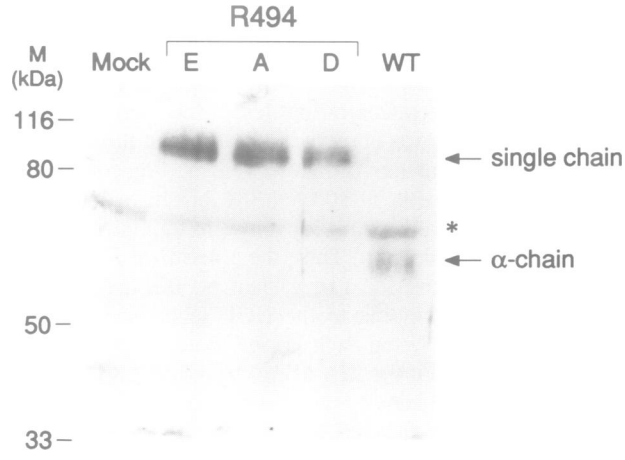

Fig. 2. Western blot of wt rhuHGF and single-chain variants. Conditioned media from mock transfected 293 cells or stable 293 cells expressing either wt rhuHGF (WT) or the variants R494E, R494A or R494D were fractionated under reducing conditions on an 8% SDS–polyacrylamide gel and blotted. The blot was incubated with polyclonal anti-HGF antisera which recognizes epitopes primarily in the α-chain. Molecular masses (kDa) of the marker are as indicated. Also indicated are the positions of the α-chain and uncleaved single-chain forms of HGF. Note that the polyclonal antibody cross-reacts with an unidentified band (*) present even in the control transfected 293 cells, which do not express detectable quantities of HGF.

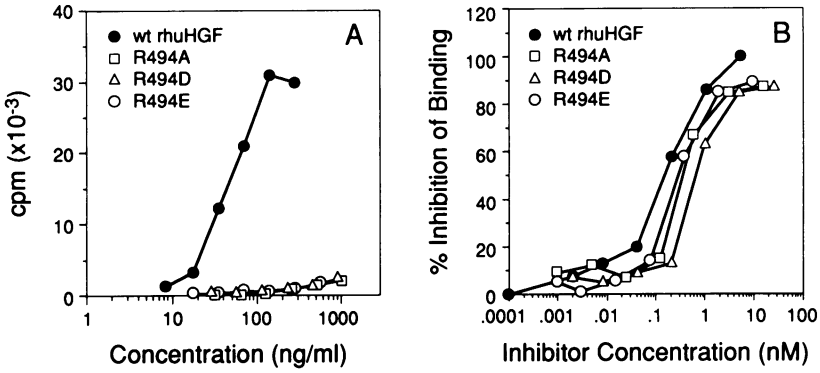

Fig. 3. Mitogenic activity (A) and competitive receptor binding (B) of wt rhuHGF and single-chain variants. **(A)** Biological activity was determined by the ability of wt rhuHGF and variants to induce DNA synthesis of rat hepatocytes in primary culture as described in Materials and methods. Shown are the mean c.p.m. from duplicates in a representative assay. Mock supernatant from control cells did not stimulate DNA synthesis in these cells (no c.p.m. increase above background levels). **(B)** To perform competitive binding, various dilutions of supernatants of human 293 cells containing wt rhuHGF or variants were incubated with 50 pM of the HGF receptor–IgG fusion protein as described in Materials and methods. Data represent the inhibition of binding as the percentage of any competing ligand from a representative experiment and were corrected by subtraction of background values from control 293 cells.

the K_d of these variants was not significantly different from wt rhuHGF (Table I). Thus variants with mutations in the β-chain of HGF lack mitogenic activity but are still able to bind to the HGF receptor.

Analysis of the C-terminal and kringle deletion variants

A number of C-terminal truncations of HGF were made by deleting either the β-chain or the β-chain in addition to a progressive number of kringles as depicted in Figure 1A. One variant (N-207), corresponding to the N-terminal domain with the first kringle, did not express the protein to levels detectable by either Western blotting or ELISA using a polyclonal antibody preparation and thus was not investigated further. Expression of the variants containing the first two kringles (N-303), three kringles (N-384) or the complete α-chain of HGF was as low as 250–600 ng/ml. A summary of the residual specific activity and K_d compared with wt rhuHGF of these variants is presented in Table I. At the concentration tested no activity above background levels was observed indicating that these variants lost their biological activity. However, binding competition showed that variants N-303, N-384 or the α-chain still retained substantial binding capacity (up to 23% compared with wt rhuHGF binding). Thus, the N-terminal 272 residues of HGF (the mature form of variant N-303) are sufficient for high affinity binding to the HGF receptor.

Results from deleting each kringle domain are shown in Table I. Deletion of the first kringle (variant ΔK1) of HGF affected biological activity most, showing at least a 100-fold reduction (specific activity <0.2% of wt rhuHGF). Similarly, binding of this variant was also affected as it failed to compete for binding with wt rhuHGF up to 2 μg/ml. Deletion of all other kringles (variants ΔK2, ΔK3 or ΔK4) also resulted in severely reduced mitogenic activity (Table I). However, the K_d values of these deletion variants remained close to that observed with wt rhuHGF.

Induction of tyrosine-phosphorylation of the HGF receptor

We determined if variants R494E or Y673S,V692S, which bind the HGF receptor *in vitro* but are defective for mitogenic activity, could stimulate tyrosine phosphorylation of the HGF receptor in A549 cells. Serum-starved cells were treated with purified wt rhuHGF or variants and immunoprecipitates of the HGF receptor were blotted and probed with phosphotyrosine antibodies. Stimulation with wt rhuHGF led to the phosphorylation of tyrosine on the 145 kDa β-subunit of the HGF receptor (Figure 4). Both variants exhibited a reduced ability to induce phosphorylation of the HGF receptor.

Table I. Residual biological activity and receptor binding capacity of single-chain, protease, C-terminal and kringle deletion rhuHGF variants compared with wt rhuHGF

Variants (var)	Specific activity of variant (var)/specific activity of wild-type (wt) ± SD	$K_d$wt/$K_d$var ±SD
Single-chain		
R494A	<0.03	0.32 ±0.18
R494D	<0.03	0.51 ±0.21
R494E	<0.02	0.31 ± 0.13
Protease		
Q534H	1.19 ± 0.44	1.48 ± 0.85
Y673S	0.27 ± 0.07[a]	1.35 ± 0.72
V692S	0.08 ± 0.04[a]	1.02 ± 0.13
Q534H,Y673S	<0.03	2.24 ± 1.11
Y673S,V692S	<0.02	1.76 ± 0.63
Q534H,Y673S,V692S	<0.02	1.91 ± 1.28
C-terminal truncation		
N-303	<0.05	0.23 ± 0.03
N-384	<0.05	0.25 ± 0.02
α-chain	<0.04	0.25 ± 0.03
Kringle deletion		
ΔK1	<0.002	<0.03
ΔK2	<0.05	0.41 ± 0.18
ΔK3	<0.03	0.56 ± 0.36
ΔK4	<0.07	0.86 ± 0.46

[a]Indicates that the plateau of the mitogenic activity curve of the variant was lower than for wt rhuHGF (~50%).
Ratios are mean ± SD from at least three assays. Protein contents from supernatants were determined in two different ELISAs and specific activity and K_d were calculated from mitogenic activity and inhibition curves as described in Materials and methods. When a < sign is used, this corresponds to the highest K_d or the lowest specific activity which could be defined.

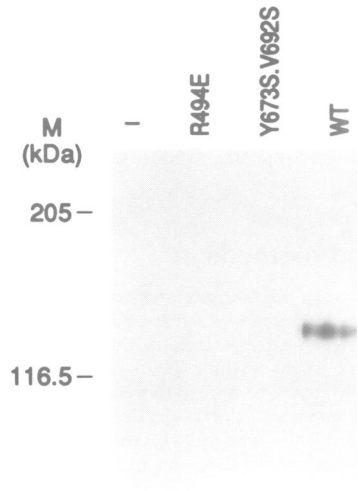

Fig. 4. Western blot of ligand-induced tyrosine phosphorylation on the 145 kDa β-subunit of the HGF receptor by wt rhuHGF, single-chain or protease variants. Lysates from A549 cells incubated for 5 min without (–) or with 200 ng/ml of purified wt rhuHGF (WT), single-chain (R494E) or double protease variants (Y673S,V692S) were prepared and immunoprecipitated with an anti-HGF receptor antibody. Western blots prepared from SDS–PAGE were probed with anti-phosphotyrosine antibodies. Molecular masses (kDa) are as indicated.

Discussion

In view of the pleiotropic activities of HGF, a molecule with a structure unlike any other known growth factor, it is important to understand the molecular interaction of this factor with its receptor. We initiated a structure–activity relationship of rhuHGF using the technique of site-directed mutagenesis and analyzing the HGF variants for their ability to induce DNA synthesis of hepatocytes in primary culture and to compete for binding to a soluble form of the HGF receptor.

Previous studies on HGF binding to hepatocytes have shown that huHGF could bind to its cell surface receptor with high affinity (K_d ~24–32 pM; Higushi and Nakamura, 1991). We preferred to examine HGF binding using a soluble form of the receptor because of the non-specific binding of HGF to cell surface heparin sulfate proteoglycans (Naldini *et al.*, 1991a). Our binding studies showed that wt rhuHGF bound to the soluble receptor fusion protein with a single class of high affinity binding sites (50–70 pM), similar to those found on hepatocytes by Higushi and Nakamura (1991). However, binding of HGF on cells may be slightly different since the soluble receptor is a dimer held together by the disulfide bridge of the hinge in the Fc portion of the IgG (M.Mark, N.Lokker, E.A.Luis and P.Godowski, in preparation).

Effects of mutations in the HGF cleavage site

We have shown that mutations that block cleavage of HGF into the two-chain form are completely defective for mitogenic activity, suggesting that cleavage is required for this activity (Table I). We cannot rule out the formal possibility that these mutations affect HGF activity independently of their effect on cleavage. However, we note that substitution of the basic hydrophilic R494 with either acidic hydrophilic residues E or D or the non-polar residue A result in variants with very similar phenotypes. It is noteworthy that variants R494E or R494D, which eliminate the trypsin-like cleavage site of HGF, generate a cleavage site for V-8 protease. Thus, two-chain forms of these variants can be generated *in vitro*. Preliminary analysis suggests that the V-8 cleaved forms of these variants are biologically active (E.A.Luis, unpublished). The major form of HGF isolated from placenta (Hernandez *et al.*, 1992) or expressed in transfected COS cells (Rubin *et al.*, 1991) is single-chain HGF. When tested in mitogenic assays, this single-chain form of HGF is found to be biologically active. Taken together with our data, this suggests that this single-chain HGF is activated to the two-chain form during the mitogenic assay.

A second observation is that the single-chain variants retain substantial capacity to bind to the HGF receptor, as suggested by our competition binding assays. This raises the interesting possibility that single-chain HGF may be bound to the cell surface HGF receptor *in vivo* in an inactive state and can subsequently be cleaved to the active double-chain form by the appropriate protease.

Effect of protease mutations

To elucidate the functional importance of the protease domain of HGF, several single, double and triple mutations were made in order to reconstitute a potential serine protease active site (Figure 1B). Variants with a single mutation in the active site residues exhibited wild-type (Q534H) or reduced (Y673S or V692S) mitogenic activity (Table I), similar to the results reported by Matsumoto *et al.* (1991). However, the variant in which both active site residues have been restored, was inactive in our mitogenic assay. This result differs from that published by Matsomoto *et al.* (1991). The reason for this discrepancy is unclear but may result from differences in the expression system or assay techniques used.

We have identified variants in the β-chain that are inactive in mitogenic assays yet still compete efficiently for binding to the HGF receptor. Thus, although the β-chain is not required for receptor binding, certain residues (e.g. Y673 and V692) are critical for the structure and/or activity of HGF. Substitution of the non-polar residue V692 with the polar residue S might have caused a structural transition if new hydrogen bonds to the active site residue D594, as found in serine proteases (Kossiakoff and Spencer, 1981; Blevins and Tulinsky, 1985; Meyer *et al.*, 1988), have been introduced. Substitution of Y673 with the smaller residue S might also introduce some local structural modifications. On the other hand, replacement of the polar residue Q534 by another polar residue H of similar size would not likely cause a drastic difference in the HGF conformation as this residue should be exposed (Figure 1B); indeed the activity of the Q534H variant was similar to rhuHGF (Table I). It will be interesting to determine if these variants restore proteolytic activity.

Effect of C-terminal and kringle deletions

In order to ascertain whether the α-chain is required for HGF binding or activity, C-terminal truncations were made that would result in variants containing either the α-chain alone, or variants truncated after the third (N-384) or second (N-303) kringles. All these variants lost mitogenic activity, which is consistent with our finding that the β-chain is important for biological activity. Moreover, these data show that kringles K3 and K4 are not required for receptor

binding. Two independent laboratories have described an alternatively spliced form of the huHGF cDNA transcript which we will refer to as HGF/NK2 (Chan *et al.*, 1991; Miyazawa *et al.*, 1991). It encodes 290 amino acids corresponding to the N-terminal finger and first two kringle domains of huHGF. This shortened form of HGF appears to be an antagonist of HGF therefore suggesting that the receptor binding domain of HGF is located within HGF/NK2. However, competitive receptor binding studies with HGF/NK2 were not presented and thus the affinity of this molecule for its receptor was not determined. Our data support the previous observations in the sense that variant N-303, which in amino acid sequence is very similar to HGF/NK2, retains the ability to compete efficiently for binding to the HGF receptor (K_d ~280 pM).

To characterize further the receptor binding domain of HGF, each of the kringles was also individually deleted (Table I). Similar to variants ΔK4 and ΔK3, ΔK2 did not exhibit a substantial reduction in receptor binding capacity whereas deletion of K1 increased the K_d >100-fold when compared with wt rhuHGF. The observations that N-303 is sufficient to bind to the receptor and that the second kringle is not required for binding the HGF receptor, in the context of the remainder of the molecule, suggest that the receptor binding domain is contained within the finger and first kringle of HGF. Unfortunately, we have not been able to detect expression of this variant using our polyclonal antisera suggesting that variant N-207 (deletion after the first kringle) was not expressed in 293 cells. Similar variants of plasminogen are structurally unstable which might be the explanation for the deficiency in expression of N-207 (F.Castellino, personal communication). It will be interesting in the future to describe which individual residues within the N-terminal 272 amino acids are indeed involved in receptor binding, because the knowledge of the active site of huHGF will be the first step in the rational design of novel therapeutics.

Ligand-induced phosphorylation of the HGF receptor

Stimulation of tyrosine phosphorylation on the HGF receptor β-subunit by HGF was previously reported (Bottaro *et al.*, 1991, Naldini *et al.*, 1991a). In the present paper, we have shown that variants R494E and Y673S,V692S can bind the soluble HGF receptor−IgG protein *in vitro* but are not efficient in stimulating tyrosine phosphorylation in A549 cells. One interpretation of this result is that these variants are capable of binding the HGF receptor on A549 cells, but are defective in a function required to induce efficient phosphorylation, e.g. receptor dimerization. It has been shown for other receptor proteins with an intrinsic tyrosine kinase, such as the epithelial and platelet-derived growth factor, that receptor−receptor interactions or dimerization is required for activation of kinase function (for review see Ullrich and Schlessinger, 1990). Alternatively, these variants may not be able to bind the cell-surface associated HGF receptor. Competition binding on cells and cross-linking experiments should be able to resolve this question directly.

Summary

The unique structure of HGF suggests that there may be multiple events that regulate the biological activity of this molecule. An early stage of regulation may be the cleavage step to generate the biologically active two-chain form. Interestingly, cleavage may not simply regulate receptor binding but rather control an event required for activating the HGF receptor. Our data also suggest that the β-chain, while not absolutely required for receptor binding, contributes to a receptor activation step. These variants may be useful in dissecting the signalling events at the HGF receptor.

Materials and methods

Bacterial strains, oligonucleotides and enzymes

Two *Escherichia coli* strains, DG98 and 294 TonA, were used to propagate and amplify mammalian expression plasmids.

Synthetic oligonucleotides used for *in vitro* mutagenesis and sequencing primers were prepared using the Applied Biosystems 380A DNA synthesizer as described (Matteucci *et al.*, 1981). The GeneAmp kit (Perkin−Elmer) was used for PCR and DNA sequencing was carried out using the sequenase kit (United States Biochemical Corporation).

Cloning of the HGF receptor (c-met proto-oncogene) cDNA

The c-*met* cDNA was cloned by standard methods (Maniatis *et al.*, 1982) and will be described elsewhere (M.R.Mark, N.A.Lokker, E.A.Luis and P.J.Godowski, in preparation). After sequencing of the complete c-*met* cDNA, an in frame deletion of 54 nucleotides in the extracellular domain of c-*met* was observed which corresponds to an alternatively spliced form identical to the sequence recently described (Rodrigues *et al.*, 1991).

Construction of plasmids and expression of huHGF variants

Plasmid DNA isolation, polyacrylamide and agarose gel electrophoresis were performed as reported (Maniatis *et al.*, 1982). Mammalian expression plasmid pRK 5.1 with a CMV promoter (Genentech, Inc.) was used for mutagenesis of huHGF allowing secretion of the HGF variants in the culture medium and directly assayed for biological activity and binding. The HGF cDNA used corresponded to the 728 amino acid form as published earlier (Seki *et al.*, 1990).

Mutagenesis was performed according to the method of Kunkel using the *dut*$^-$ *ung*$^-$ strain of *E.coli* (Kunkel *et al.*, 1987). Plasmids with the correct sequence were used to transfect human fetal kidney 293 cells by the calcium phosphate method. The cell monolayers were incubated for 4 h in the presence of the DNA precipitate, washed once with PBS and cultured in serum-free medium for 72 h. When stable populations were made, the HGF cDNA was subcloned in an episomal CMV driven expression plasmid pCisEBON (G.Cachianes, C.Ho, R.Weber, S.Williams, D.Goeddel and D.Lueng, in preparation) and populations were directly selected in neomycin selective medium.

Protein quantification of wild-type HGF and variants

A specific two-site huHGF sandwich ELISA using two monoclonal antibodies was used to quantify wt rhuHGF, single chain and protease substitution variants. Microtiter plates (Maxisorb, Nunc) were coated with 10 μg/ml of a monoclonal anti-rhuHGF antibody A 3.1.2 in 50 mM carbonate buffer, pH 9.6, overnight at 4°C. After blocking plates with 0.5% BSA (Sigma), 0.01% thimerosal in PBS (pH 7.4), subsequent washes and duplicate serial dilutions of HGF samples were prepared and in parallel a CHO-expressed rhuHGF (0.1−40 ng/ml) was used as a standard. 50 μl of these dilutions were simultaneously incubated with 50 μl of a 1:1500 diluted horseradish peroxidase conjugated monoclonal anti-rhuHGF antibody B 4.3 for 2 h at room temperature. The substrate was prepared by adding 0.04% *o*-phenylenediamine dihydrochloride (Sigma) and 0.012% (v/v) hydrogen peroxide (Sigma) to PBS and 100 μl were added to the washed plates for 15 min at room temperature. The reaction was stopped by adding 50 μl of 2.25 M H_2SO_4 to each well. The absorbance at 490 nm, with the absorbance at 405 nm subtracted as background, was determined on a microtiter plate reader (Vmax, Molecular Devices, Menlo Park, CA). The data were reduced using a four-parameter curve-fitting program developed at Genentech, Inc.

An HGF polyclonal sandwich ELISA was used to quantify all kringle deletion and C-terminal truncation variants. Briefly, microtiter plates (Nunc) were coated with 5 μg/ml guinea pig polyclonal (anti CHO-expressed rhuHGF) IgG antibody preparation (Genentech, Inc.) as described above. This antibody recognizes rhuHGF as well as HGF truncated forms when compared with visual inspection of Western blots, making it ideal for monitoring HGF variants. Plates were blocked and duplicate serial dilutions of 293 cell supernatants ($1 \times 10^3 - 6 \times 10^6$) were added and incubated overnight at 4°C. Purified CHO-expressed rhuHGF (0.78−100 ng/ml) was used as a standard and incubated in parallel. Plates were washed and

incubated with a 1:500 dilution of the same polyclonal antibody (~400 ng/ml) but in this case horseradish peroxidase conjugated for detection of the variants (see above).

Western blotting was performed to determine the size of the expressed HGF variants. For this, SDS-PAGE and Western blotting were performed using standard methods with the polyclonal IgG antibody preparation (500 ng/ml). A chemiluminescent detection method (Amersham) and a goat anti-guinea pig IgG-horseradish peroxidase conjugate (1:5000) were used for development of the blot as described by the manufacturer.

Soluble HGF receptor binding assay

Cell supernatants (concentrated on Amicon filters if the concentration was below 600 ng/ml) were tested for their ability to block in solution the binding of CHO-expressed [^{125}I]rhuHGF ($2-5 \times 10^3$ Ci/mmol, kindly provided by T.Zioncheck, Genentech, Inc.) to the extracellular domain of the HGF receptor fused to the Fc constant region of an human IgG, expressed and secreted from 293 cells (M.R.Mark, N.A.Lokker and P.J.Godowski, in preparation). The binding assay was performed in microtiter plates (Nunc) coated overnight at 4°C with 1 μg/ml of rabbit anti-human IgG Fc specific antibody (Jackson Immunoresearch) and plates were carefully washed with PBS containing 0.05% Tween 20 (Bio-Rad). After blocking with PBS containing 0.1% BSA, in this same buffer, 5 pM of [^{125}I]rhuHGF in 25 μl per well were added. To each well 50 μl of serial dilutions (1:25-1:6000) of cell supernatants, purified CHO-expressed rhuHGF (0.064-25 000 pM) or medium were added in duplicates. Subsequently, 25 μl of 50 pM of HGF receptor-IgG fusion protein were added and the plates were incubated with gentle shaking. After 4 h, when an equilibrium had been reached, plates were washed and wells were individually counted in a gamma-counter. The amount of non-specifically bound radiactivity was estimated by incubating [^{125}I]rhuHGF with a 500-fold excess of unlabelled rhuHGF. The dissociation constant (K_d) of each variant was calculated at the IC_{50} from fitted inhibition curves essentially as described (DeBlasi *et al.*, 1989) using the HGF concentration determined by ELISA.

Biological assay

The biological activity of wt HGF and variants was measured by their abilities to induce DNA synthesis of rat hepatocytes in primary culture.

Hepatocytes were isolated according to published perfusion techniques with minor modifications (Garrison and Haynes, 1975). Briefly, the livers of female Sprague Dawley rats (160-180 g) were perfused through the portal vein with 100 ml of Ca^{2+}-free HEPES-buffered saline containing 0.02% collagenase type IV (Sigma). After 20 min the liver was removed, placed in buffer, gently stirred to separate hepatocytes from connective tissue and blood vessels, and filtered through nylon mesh. Cells were then washed by centrifugation, resuspended at 1×10^5cells/ml in William's Medium E (Gibco) containing penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), trace elements (0.01%), transferrin (10 μg/ml) and aprotinin (1 μg/ml). Hepatocytes were incubated in 96-well microtiter plates (Falcon) in the presence of duplicate serial dilutions of either purified CHO-expressed rhuHGF (0.031-1 μg/ml), 293 supernatants (1:4-1:256) or medium. After 48 h incubation at 37°C, 0.5 mCi [^{3}H]TdR (15 Ci/mmol, Amersham) was added to each well and incubated for an additional 16 h. Cells were harvested on filter papers, which were washed, dried and counted in a Beckman counter after addition of scintillation liquid. For each HGF variant, the specific activity expressed in units/mg was calculated at half-maximal proliferation (defined as 1 unit/ml) using the HGF concentration obtained in ELISA.

Induction of tyrosine phosphorylations on A549 cells

Human lung carcinoma cells (A549) monolayers were cultured in RPMI 1640 medium containing 10% fetal bovine serum and maintained at 37°C in a humified atmosphere with 5% CO_2. Serum-starved cells were incubated with or without 200 ng/ml rhuHGF for 5 min at 37°C and extracted with lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100 and a cocktail of protease inhibitors. The lysates were immunoprecipitated with anti-Met COOH antibodies and blotted with anti-phosphotyrosine antibodies (see Western blotting above).

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References

Asami,O., Ihara,I., Shimidzu,N., Shimizu,S., Tomita Y., Ichihara,A. and Nakamura,T. (1991) *J. Biochem.*, **109**, 8-13.

Blevins,R.A. and Tulinsky,A. (1985) *J. Biol. Chem.*, **269**, 4264-4275.

Bottaro,D.P., Rubin,J.S., Faletto,D.L., Chan,A.M.-L., Kmiecik,T.E., Vande Woude,G.F. and Aaronson,S.A. (1991) *Science*, **251**, 802-804.

Chan,A.M.-L., Rubin,J.S., Bottaro,D.P., Hirschfield,D.W., Chedid,M. and Aaronson,S.A. (1991) *Science*, **254**, 1382-1384.

Deblasi,A., O'Reilly,K. and Motulssky,H.J. (1989) *Trends Pharmacol. Sci.*, **10**, 227-229.

Garrison,J.C. and Haynes,R.C. (1975) *J. Biol. Chem.*, **250**, 2269-2777.

Gohda,E., Tusubushi,H., Nakayama,H, Hirono,S., Sakiyama,O., Takahashi,K, Miyazaki,H. and Kitamura,N. (1988) *J. Clin. Invest.*, **81**, 414-419.

Han,S., Stuart,L.A. and Friezner Degen,S.J. (1991) *Biochemistry*, **30**, 9768-9780.

Hernandez,J., Zarnegar,R. and Michalopoulos,G. (1992) *J. Cell Physiol.*, in press.

Higushi,O. and Nakamura,T. (1991) *Biochem. Biophys. Res. Commun.*, **176**, 599-607.

Igawa,T., Kanda,S., Kanetake,H., Saitoh,Y, Ichihara,A., Tomita,Y. and Nakamura,T. (1991) *Biochem. Biophys. Res. Commun.*, **174**, 831-838.

Kan,M., Zhang,G., Zarnegar,R., Michalopoulos,A., Myoken,Y., McKeehan,W.L. and Stevens,J.I. (1991) *Biochem. Biophys. Res. Commun.*, **174**, 331-337.

Kossiakoff,A.A. and Spencer,S.A. (1981) *Biochemistry*, **20**, 6462-6474.

Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367-382.

Lindroos,P.M., Zarnegar,R. and Michalopoulos,G.K. (1991) *Hepatology*, **13**, 734-750.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Matsumoto,K., Tajima,H. and Nakamura,T. (1991) *Biochem. Biophys. Res. Commun.*, **176**, 45-51.

Matteucci,M.D. and Caruthers,M.H. (1981) *J. Am. Chem. Soc.*, **103**, 3185-3191.

Meyer,E., Cole,G., Radhakrishman,R. and Epp,O. (1988) *Acta Cryst. Sect. B*, **44**, 26-33.

Michalopoulos,G., Houch,K.A., Dalan,M.L. and Luetteke,N.C. (1984) *Cancer Res.*, **44**, 4414-4419.

Miyazawa,K., Tsabouchi,H., Naka,D., Takahashi,K., Okigaki,M., Gohda,E., Daikuhara,Y. and Kitamura,M. (1989) *Biochem. Biophys. Res. Commun.*, **163**, 967-973.

Miyazawa,K., Kitamura,A., Naka,D. and Kitamura,N. (1991) *Eur. J. Biochem.*, **197**, 15-22.

Montesano,R., Matsumoto,K., Nakamura,T. and Orci,L. (1991) *Cell*, **67**, 901-908.

Nakamura,T., Nawa K. and Ichira A. (1984) *Biochem. Biophys. Res. Commun.*, **122**, 1450-1459.

Nakamura,T., Nishizawa,T., Hagiya,M., Seki,T., Shimonishi,M., Sugimura,A., Tashiro,K. and Shimuzi,S. (1989) *Nature*, **342**, 440-443.

Naldini,L. *et al.* (1991a) *EMBO J.*, **10**, 2867-2878.

Naldini,L., Vigna,E., Narsimhan,R.P., Gaudino,G., Zarnegar,R., Michalopoulos,G.K. and Comoglio,P.M. (1991b) *Oncogene*, **6**, 501-504.

Park,M., Dean,M., Kaul,K., Braunn,M.J., Gonda,M.A. and Vande Woude G. (1987) *Proc. Natl. Acad. Sci. USA*, **84**,6379-6383.

Rodrigues,G.A., Naujokas,M.A. and Park,M. (1991) *Mol. Cell. Biol.*, **11**, 2962-2970.

Rubin,J.S. *et al.* (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 415-419.

Russell,W.E., McGowan,J.A. and Bucher,N.L.R. (1984) *J. Cell Physiol.*, **119**, 183-192.

Schechter,I. and Berger,A. (1967) *Biochem. Biophys. Res. Commun.*, **27**, 157-162.

Seki,T., Ihara,I., Sugimura,A., Shimonishi,M., Nishizawa,T., Asami,O., Hagiya,M., Nakamura,T. and Shimizu,S. (1990) *Biochem. Biophys. Res. Commun.*, **172**, 321-327.

Stoker,M.E., Gherardi,E., Perryman,M. and Gray,J. (1987) *Nature*, **327**, 239-242.

Tashiro,K.M., Hagiya,T., Nishizawa,T, Seki,T., Shimonishi,M.,

Shimizu,S. and Nakamura,T. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 3200–3204.
Tate,K.K., Higgins,D.L., Holmes,W.E.,Winkler,M.E., Heyneker,H.L. and Vehar,G.A. (1987) *Biochemistry*, **26**, 338–343.
Ullrich A. and Schlessinger,J. (1990) *Cell*, **61**, 203–212.
Weidner,K.M., Behrens,J., Vandekerckhove,J. and Birchmeier,W. (1990) *J. Cell Biol.*, **111**, 2097–2108.
Wun,T.C. (1988) *Crit. Rev. Biotechnol.*, **8**, 131–148.
Yoshiyama,Y. *et al.* (1991) *Biochem. Biophys. Res. Commun.*, **175**, 660–667.

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