Functional Characterization of Human Hepatocyte Growth Factor Mutants Obtained by Deletion of Structural Domains†

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ABSTRACT: Human hepatocyte growth factor (hHGF) consists of characteristic structural domains. In this study, we prepared mutant proteins lacking each of these domains and examined their biological activities for stimulation of hepatocyte DNA synthesis, inhibition of Meth A cell growth, and induction of MDCK cell dissociation. We also examined their interactions with the c-*met*/HGF receptor by competition analysis and by analysis of levels of tyrosine phosphorylation. The mutant proteins lacking the N-terminal, the first kringle, or the second kringle domain were not biologically effective and could not compete with hHGF bound to the c-*met*/HGF receptor. The results indicate that these domains are necessary for the biological activities of hHGF mediated by binding to the c-*met*/HGF receptor. The mutant proteins lacking the third or fourth kringle domain moderately retained biological activities and the receptor binding. The relative levels of the tyrosine phosphorylation of the c-*met*/HGF receptor by these mutant proteins correlated well with the relative potencies of the biological activities when compared with that of the wild-type hHGF. The mutant protein lacking the light chain was not effective in the biological activities and tyrosine phosphorylation of the c-*met*/HGF receptor, but competed with hHGF bound to the c-*met*/HGF receptor. These results suggest that the heavy chain plays an important role in the interaction of hHGF with the c-*met*/HGF receptor and that the light chain is further required for the tyrosine phosphorylation of the c-*met*/HGF receptor.

Human hepatocyte growth factor (hHGF)[1] is a heparin-binding glycoprotein originally purified from human plasma as a potent mitogen for hepatocytes in primary culture (Gohda et al., 1988). HGF is also mitogenic in melanocytes and endothelial and epithelial cells (Rubin et al., 1991) and inhibits the growth of some tumor cell lines (Higashio et al., 1990). Furthermore, HGF stimulates other biological responses such as dissociation of epithelial cells (Weidner et al., 1991) and induction of tubule formation by Madin-Darby canine kidney (MDCK) epithelial cells (Montesano et al., 1991).

Human HGF is synthesized as a 728 amino acid precursor (Miyazawa et al., 1989; Nakamura et al., 1989). The N-terminal 31 amino acid residues of the precursor probably function as a signal peptide (Yoshiyama et al., 1991). The precursor is cleaved at a specific proteolytic site to yield a heavy and a light chain of 463 and 234 amino acids, respectively, which are linked together by a disulfide bond. Human HGF consists of multiple putative domains that are homologous to those observed in plasminogen (Petersen et al., 1990). The N-terminal region of the heavy chain contains many basic amino acids and is homologous to the preactivation peptide of plasminogen. Four tandem repeats called kringles are present in the heavy chain region. The light chain is highly homologous to the catalytic domain of serine proteases, but the histidine and serine of the catalytic triad are replaced by glutamine and tyrosine, respectively. These characteristic domain structures are conserved in the rat HGF sequence (Okajima et al., 1990; Tashiro et al., 1990). Analysis of the genomic DNA encoding hHGF showed that these multiple domains are encoded as separate exons in the gene (Miyazawa et al., 1991a,b; Seki et al., 1991) localized to the chromosome segment 7q21.1 (Fukuyama et al., 1991).

HGF initiates its biological effects by interacting with specific receptors on the surface of its target cells. Recently, Bottaro et al. (1991) identified a HGF receptor as the c-*met* protooncogene product (the c-*met* protein) in the B5/589 human mammary epithelial cell line, whose growth is stimulated by HGF. The HGF receptor has also been identified as the c-*met* protein on the Meth A mouse sarcoma cell line, whose growth is inhibited by HGF (Komada et al., 1992), and on the A549 human lung carcinoma cell line, which is scattered by HGF (Naldini et al., 1991). The sequence of the c-*met* protein has features characteristic of the tyrosine kinase family of growth factor receptors (Park et al., 1987). HGF stimulated tyrosine phosphorylation of the c-*met* protein on all these target cells (Bottaro et al., 1991; Komada et al., 1992; Naldini et al., 1991). Thus, the biological effects of HGF on different target cells seem to be mediated by a common receptor, the c-*met* protein, and by different intracellular signal cascades, which may be initiated by tyrosine phosphorylation.

The interaction of hHGF with its receptor is probably mediated by the putative domain structures observed in hHGF. To elucidate the involvement of the domain structures on the biological effects of hHGF, we prepared a series of hHGF deletion mutant proteins and examined their biological activities and interactions with the c-*met* protein.

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[1] Abbreviations: hHGF, human hepatocyte growth factor; HGF, hepatocyte growth factor; MDCK, Madin-Darby canine kidney; BS^3, bis(sulfosuccinimidyl) suberate; ConA–Sepharose, concanavalin A–Sepharose; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Table I: Oligonucleotides Used for Mutagenesis[a]

primer#	mutants	Sequence
		32 111
		Pro Tyr Ala Glu Gly Gln Glu Phe Gly His Glu Phe
1	Δ N	CCC TAT GCA GAG GGA CAA GAA TTT GGC CAT GAA TTT GA
		127 207
		Asp Tyr Ile Arg Asn Ser Glu Val Glu Cys
2	Δ K1	GAC TAC ATT AGA AAC TCA GAA GTT GAA TGC
		210 289
		Gln Cys Ser Glu Val Glu Ala Asp Asn Thr Met Asn
3	Δ K2	CAG TGT TCA GAA GTT GAA GCT GAC AAT ACT ATG AAT
		304 384
		Pro Leu Glu Thr Thr Glu Asp Met Ser His Gly Gln
4	Δ K3	CCT TTG GAA ACA ACT GAA GAT ATG TCA CAT GGA CAA
		390 470
		Met Ser His Gly Gln Asp Glu Gly Asp Thr Thr Pro
5	Δ K4	ATG TCA CAT GGA CAA GAT GAA GGT GAT ACC ACA CCT
		494 495
		Lys Gln Leu Arg Val Val Asn Gly
6	Δ L	AAA CAA TTG CGA TAG GTT GTA AAT GGG

[a] The numbers correspond to the position of the amino acids in the published hHGF sequence (Miyazawa et al., 1989).

MATERIALS AND METHODS

Materials. Reagents were obtained as follows: Na^{125}I (IMS30), 5-[^{125}I]iodo-2′-deoxyuridine, and oligonucleotide-directed in vitro mutagenesis system (version 2) from Amersham Corp.; restriction endonucleases from Takara Shuzo Co. and Toyobo Co.; bis(sulfosuccinimidyl) suberate (BS^3) from Pierce Chemical Co.; heparin–Sepharose and ConA–Sepharose from Pharmacia LKB Biotechnology Inc.; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenylmethanesulfonyl fluoride, aprotinin, pepstatin A, and soybean trypsin inhibitor from Sigma; leupeptin from Peptide Institute Inc.; anti-phosphotyrosine monoclonal antibody PY20 from ICN ImmunoBiologicals; Trans-Blot poly(vinylidene difluoride) membrane from Bio-Rad; rabbit anti-(mouse Ig) and porcine anti-(rabbit Ig) monoclonal antibody–peroxidase conjugates from Dako; and TM Blue chromogenic substrate for peroxidase from Transgenic Sciences, Inc. Recombinant hHGF (Strain et al., 1991) and anti-hHGF monoclonal antibody (Miyazawa et al., 1991a) were provided from the Research Center, Mitsubishi Kasei Co. Antiserum against the c-*met* protein was prepared as previously described (Komada et al., 1992). Oligonucleotides were chemically synthesized using an Applied Biosystems 380B DNA synthesizer.

Construction of Expression Plasmids for hHGF Deletion Mutant cDNAs. The deletion mutant cDNAs were constructed according to the procedure of Nakamaye and Eckstein (1986) using oligonucleotide-directed in vitro mutagenesis system. From the plasmid pSRα-hHGF (Miyazawa et al., 1991a), the 870 bp *Bam*HI–*Sca*I fragment was transferred to M13mp18. This plasmid was used for deletion mutagenesis with oligonucleotide 1 (Table I) to delete the N-terminal region of the heavy chain (ΔN) and with oligonucleotide 2 (Table I) to delete the first kringle domain (ΔK1). After mutagenesis, the *Bam*HI–*Nco*I fragment was combined with the 1371 bp *Nco*I–*Kpn*I fragment and inserted into the expression plasmid pcDL-SRα296 as previously described (Miyazawa et al., 1991a). From the plasmid pSRα-hHGF, the 1245 bp *Bam*HI–*Bgl*II fragment was transferred to M13mp18. This plasmid was used for deletion mutagenesis with oligonucleotide 3 (Table I) to delete the second kringle domain (ΔK2). After mutagenesis, the *Bam*HI–*Bsp*1286I fragment was combined with the 1230 bp *Bsp*1286I–*Kpn*I and inserted into the expression plasmid. From the plasmid pSRα-hHGF, the 1428 bp *Eco*RV–*Kpn*I fragment was transferred to M13mp18. This plasmid was used for deletion mutagenesis with oligonucleotide 4 (Table I) to delete the third kringle domain (ΔK3), with oligonucleotide 5 (Table I) to delete the fourth kringle domain (ΔK4), and with oligonucleotide 6 (Table I) to delete the light chain (ΔL). After mutagenesis, the *Nco*I–*Kpn*I fragment was combined with the 831 bp *Bam*HI–*Nco*I fragment and inserted into the expression plasmid.

Expression and Partial Purification of the hHGF Deletion Mutant Proteins. The expression plasmids containing the cDNAs for the deletion mutants were transfected into Cos-7 cells by the calcium phosphate precipitation method as previously described (Miyazawa et al., 1991a). Four days after transfection, serum-free conditioned medium was harvested and centrifuged at 750*g* for 10 min at 4 °C. The supernatant (50 mL) was incubated with 25 μL of heparin–Sepharose for 24 h at 4 °C with gentle agitation. The heparin–Sepharose was collected by centrifugation at 750*g* for 10 min at 4 °C and washed twice with PBS (pH 7.4) containing 0.013% CHAPS. The protein bound to the heparin–Sepharose was eluted with 25 μL of 25 mM HEPES (pH 7.4) containing 1.5 M NaCl and 0.013% CHAPS. Cos-7 cells (5×10^7 cells) transfected with the deletion mutant cDNA for the N-terminal region of the heavy chain (ΔN) were suspended in 5 mL of PBS (pH 7.4) and sonicated three times for 10 s on ice. The sonicated cell suspension was centrifuged at 750*g* for 10 min at 4 °C. The supernatant was incubated with 25 μL of ConA–Sepharose for 24 h at 4 °C with gentle agitation. The ConA–Sepharose was collected by centrifugation at 750*g* for 10 min at 4 °C and washed twice with PBS (pH 7.4) containing 0.013% CHAPS. The protein bound to the ConA–Sepharose was eluted by 25 μL of 25 mM HEPES (pH 7.4) containing 0.3 M methyl α-D-mannopyranoside, 0.5 M NaCl, and 0.013% CHAPS.

Immunoblotting. hHGF deletion mutant proteins were resolved by SDS–PAGE in the presence of 2-mercaptoethanol on a 7.5% gel by the method of Laemmli (1970). After SDS–PAGE, proteins were transferred to a poly(vinylidene difluoride) membrane, according to the procedure of Towbin et al. (1979). The membrane was immunoblotted as previously described (Miyazawa et al., 1991a). The color was developed by soaking the filter in TM Blue.

Bioassays. DNA synthesis of adult rat hepatocytes in primary culture was determined as previously described (Miyazawa et al., 1991a). Cell growth inhibition activity was examined using Meth A cells as previously described (Komada et al., 1992). For cell scattering activity, MDCK cells (4×10^3) were cultured for 48 h with various concentrations of hHGF deletion mutants in 400 μL of DMEM containing 10% fetal calf serum in Corning 24-well flat-bottomed plates. After culture, the cells were washed twice with PBS (pH 7.4) and stained with Giemsa.

Cross-Linking of hHGF. ^{125}I-Recombinant hHGF was prepared as previously described (Komada et al., 1992). Meth A cells (5×10^5) were incubated with 50 pM ^{125}I-hHGF in the presence or absence of 200 or 400 pM unlabeled hHGF deletion mutants on ice for 2 h, then washed twice with PBS (pH 7.4) containing 1 mM $MgCl_2$, and treated with 100 μg/mL BS^3 in 0.5 mL of PBS containing 1 mM $MgCl_2$ on ice for 30 min. The reaction was terminated with 0.5 mL of 25 mM Tris·HCl (pH 7.4), 140 mM NaCl, and 1 mM EDTA. The cells were recovered by centrifugation at 2000*g* for 3 min at 4 °C and lysed on ice for 30 min in 15 μL of 50 mM Tris·HCl (pH 7.4) containing 140 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, 1 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin A, and 100 μg/mL soybean trypsin inhibitor. The cell lysate was centrifuged at 12000*g* for 30 min at 4 °C, and the supernatant was resolved by SDS–PAGE using a 6% gel by

the method of Laemmli (1970). The ^{125}I-hHGF binding protein complexes were detected by autoradiography.

Detection of Tyrosine Phosphorylation of the c-met Protein by hHGF Deletion Mutant Proteins. Meth A cells (4×10^7) were incubated with 200 pM hHGF deletion mutants in DMEM containing 25 mM HEPES (pH 7.4) and 0.5% bovine serum albumin at 37 °C for 15 min. Tyrosine phosphorylation of the c-*met* protein of the Meth A cells was detected by immunoblotting using anti-phosphotyrosine antibody as previously described (Komada et al., 1992). The amount of the c-*met* protein was estimated by immunoblotting using anti-*met* antiserum as previously described (Komada et al., 1992).

RESULTS

Construction and Expression of hHGF Deletion Mutant cDNAs. We previously isolated cDNAs for the full-size hHGF (Miyazawa et al., 1989) and a variant form of hHGF (Miyazawa et al., 1991a) and inserted them into the expression vector pcDL-SRα296 (Takebe et al., 1988). The expression plasmids generated the predicted translation products in the Cos cell transfection system. We therefore used this system to express hHGF deletion mutant cDNAs. Six deletion mutant cDNAs were constructed by oligonucleotide-directed mutagenesis. Five (ΔN, ΔK1, ΔK2, ΔK3, and ΔK4) of them were constructed by deleting the corresponding sequences of cDNA. The deletion mutant ΔL, which lacks the whole light chain sequence, was constructed by inserting a termination codon (TAG) into the proteolytic processing site between the heavy and the light chains. These cDNAs were inserted into the expression plasmid and transfected into Cos-7 cells. Figure 1 shows a schematic representation of the hHGF deletion mutant proteins. All of them, except for ΔN, were secreted into the Cos cell culture medium. They were partially purified from the culture medium by binding to heparin–Sepharose. Since the mutant ΔN was not secreted into the culture medium even though it retained the signal peptide sequence, it was obtained by sonication of the transfected cells. Because ΔN was not bound to heparin–Sepharose, it was partially purified by binding to ConA–Sepharose. The expression plasmids which contained cDNAs for the full-size (wild-type) hHGF and the variant form of hHGF were also transfected into Cos-7 cells. The proteins were partially purified by binding to heparin–Sepharose and were analyzed together with the deletion mutant proteins. The partially purified hHGF mutant proteins were analyzed by SDS–PAGE and immunoblotting (Figure 2). The monoclonal antibody used for the immunoblotting is directed against an epitope located between the N-terminal region and the first kringle, which is present on all the hHGF mutant proteins. The protein preparations of ΔN, ΔK1, ΔK2, and ΔL migrated as bands with the predicted molecular masses. However, ΔK3 and ΔK4 migrated as bands that were slower than those predicted. The reason for this remains unknown. Although a sensitive enzyme-linked immunosorbent assay (ELISA) system has been developed to quantify hHGF (Tsubouchi et al., 1991), it was not suitable to quantify some deletion mutant proteins. We thus quantified all of the mutant proteins by scanning densitometry of the immunoblots using recombinant hHGF as the standard.

Cell Growth Activities of hHGF Deletion Mutant Proteins. Cell growth activities were estimated by the amount of ^{125}I-deoxyuridine incorporated into the DNA of rat hepatocytes in primary culture (Figure 3). The wild-type hHGF showed concentration-dependent stimulation of DNA synthesis, and maximal response of DNA synthesis was observed at a concentration of about 50 pM. The concentration for the maximal response corresponded to that of natural hHGF purified from human plasma (Gohda et al., 1988). The deletion mutants ΔK3 and ΔK4 also showed concentration-dependent stimulation of DNA synthesis, and the concentrations for maximal response were also about 50 pM. But the potencies of the stimulation by ΔK3 and ΔK4 were about 25% and 10% of the wild-type hHGF, respectively. None of the other deletion mutants (ΔN, ΔK1, ΔK2, and ΔL) stimulated DNA synthesis, at least up to 200 pM. We

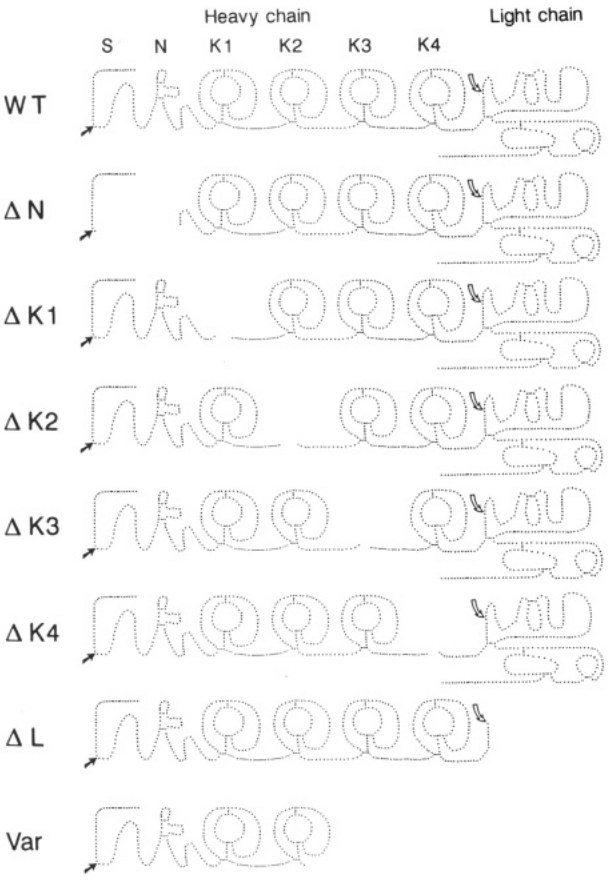

FIGURE 1: Schematic representation of hHGF deletion mutant proteins and a variant form of hHGF. S, signal peptide; N, the N-terminal region of the heavy chain; K1–K4, kringles 1–4; L, the light chain; WT, wild-type hHGF; ΔN, ΔK1–ΔK4, and ΔL, the mutant proteins lacking each domain; Var, the variant form of hHGF. Amino acid residues are represented by dots. The cleavage site between the putative signal peptide and the heavy chain is shown by an arrow. The cleavage site between the heavy chain and the light chain is shown by a white arrow.

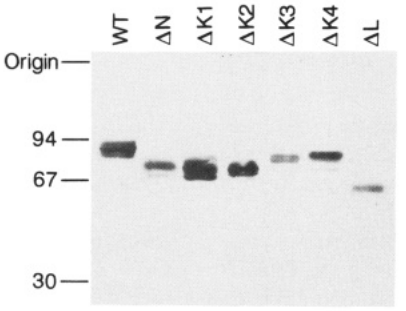

FIGURE 2: Immunoblotting analysis of the hHGF deletion mutant proteins. The partially purified deletion mutant proteins were separated on SDS–PAGE (7.5% gel) and transferred to a poly(vinylidene difluoride) membrane. The membrane was incubated with anti-hHGF monoclonal antibody and developed with rabbit anti-(mouse Ig) monoclonal antibody–peroxidase conjugate. The molecular mass standards were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), and carbonic anhydrase (30 kDa).

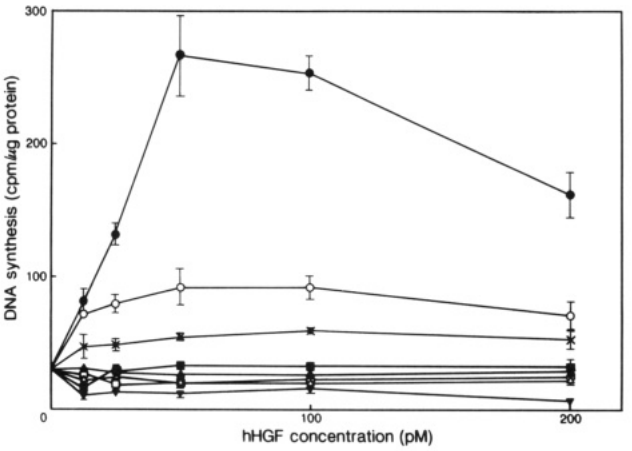

FIGURE 3: Effect of the hHGF deletion mutant proteins on DNA synthesis in cultured rat hepatocytes. Cells were incubated with various concentrations of the proteins, and DNA synthesis was determined by measuring the incorporation of ^{125}I-deoxyuridine. (●) WT; (▲) ΔN; (△) ΔK1; (■) ΔK2; (○) ΔK3; (×) ΔK4; (□) ΔL; (▼) Var. Each data point is the mean ± SD of triplicate measurements. When no error bar is shown, the error was less than the symbol size.

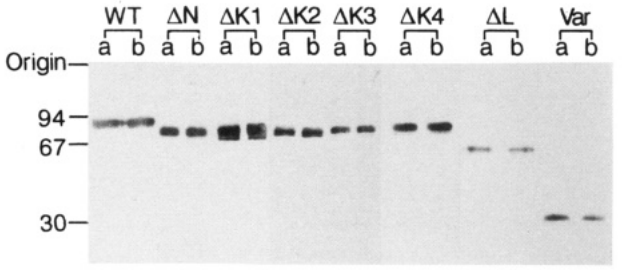

FIGURE 4: Analysis of the stability of the hHGF deletion mutant proteins in conditioned medium from cultured rat hepatocytes. The partially purified proteins were incubated in conditioned medium from cultured rat hepatocytes. After incubation, they were concentrated and separated by SDS–PAGE (10% gel). The separated proteins were analyzed by immunoblotting. Lane a, before incubation; lane b, after incubation.

previously showed that conditioned medium from the Cos cells transfected with the variant form of hHGF (Var) cDNA did not stimulate DNA synthesis (Miyazawa et al., 1991a). This result was confirmed using the variant form of hHGF partially purified by binding to heparin–Sepharose.

Decrease or loss of the activities for DNA synthesis of hepatocytes in primary culture may be due to proteolytic degradation of the mutant proteins during the assay. To examine the stabilities of the mutant proteins under the assay conditions, they were incubated for 24 h at 37 °C in the conditioned medium of rat hepatocytes in primary culture which had been cultured for 24 h. After the incubation, they were concentrated by binding to heparin–Sepharose or ConA–Sepharose and subjected to immunoblotting analysis. As shown in Figure 4, all deletion mutant proteins as well as the variant form of hHGF were as stable as the wild-type hHGF.

Cell Growth Inhibition Activities of hHGF Deletion Mutant Proteins. Cell growth inhibition activities were examined by means of a colorimetric assay for viable cell number using Meth A cells (Figure 5). The viable cell number increased about 150-fold in 5 days culture in the absence of hHGF. Wild-type hHGF showed concentration-dependent inhibition of the cell growth, and maximal inhibition (about 80% inhibition) was observed at about 100 pM. The deletion mutants ΔK3 and ΔK4 also showed concentration-dependent inhibition of the cell growth, and the concentrations for maximal inhibition were about 100 and 50 pM, respectively. The potencies of the inhibition by ΔK3 and ΔK4 were about

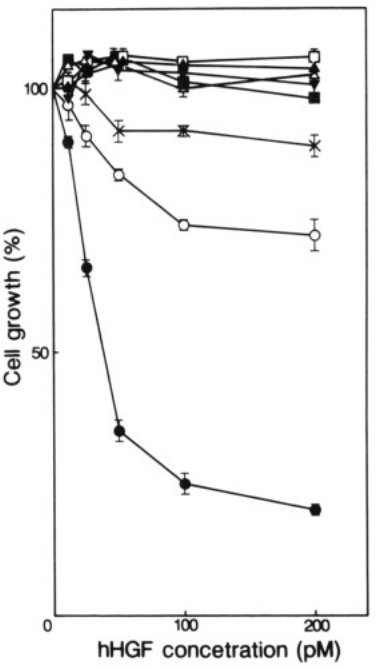

FIGURE 5: Effect of the hHGF deletion mutant proteins on the growth of Meth A cells. Cells were incubated with various concentrations of the hHGF deletion mutant proteins, and cell growth was examined by a colorimetric assay using MTT. Cell growth was calculated as (A_{570} of sample treated with the mutant protein)/(A_{570} of sample without the mutant protein) × 100. (●) WT; (▲) ΔN; (△) ΔK1; (■) ΔK2; (○) ΔK3; (×) ΔK4; (□) ΔL; (▼) Var. Each data point is the mean ± SD of triplicate measurements. When no error bar is shown, the error was less than the symbol size.

30% and 10% of that of wild-type hHGF, respectively. The potencies were similar to those observed in cell growth activities. All other deletion mutants (ΔN, ΔK1, ΔK2, and ΔL) as well as the variant form of hHGF (Var) had no effect upon the cell growth at least up to 200 pM.

Scattering Activities of hHGF Deletion Mutant Proteins. Scattering activities were examined using the MDCK epithelial cell line which is a highly sensitive target for the activity. In tissue culture, MDCK cells form tight epithelial monolayers with junctional complexes (McRoberts et al., 1981). When added to cultured MDCK cells, purified hHGF causes the breakdown of intercellular junctions and a transition from an epithelial to a fibroblastic morphology (Gherardi et al., 1989; Weidner et al., 1990). Figure 6 shows the morphological changes of MDCK cells treated with the deletion mutant proteins. In the absence of hHGF, MDCK cells showed epithelial morphology (Figure 6A). MDCK cells were scattered by wild-type hHGF (Figure 6B,G,H). A completely scattered morphology was observed at a concentration of about 200 pM (Figure 6B). The deletion mutants ΔK3 and ΔK4 also scattered cells (Figure 6E,F). But a completely scattered morphology was not observed with ΔK3 and ΔK4 even in the presence of 400 pM proteins. The morphology observed after incubation with 200 pM ΔK3 and 400 pM ΔK4 roughly corresponded to that with 100 and 50 pM wild-type hHGF, respectively. None of the other deletion mutant proteins (ΔN, ΔK1, ΔK2, and ΔL) nor the variant form of hHGF (Var) affected the morphology of MDCK cells (Figure 6C,D, and not shown).

Effects of hHGF Deletion Mutant Proteins on ^{125}I-hHGF Binding to the c-met Protein. To examine the interaction of mutant proteins with membrane proteins of target cells, the effects of mutant proteins on ^{125}I-hHGF binding to the c-*met* protein of Meth A cells were analyzed. When ^{125}I-hHGF

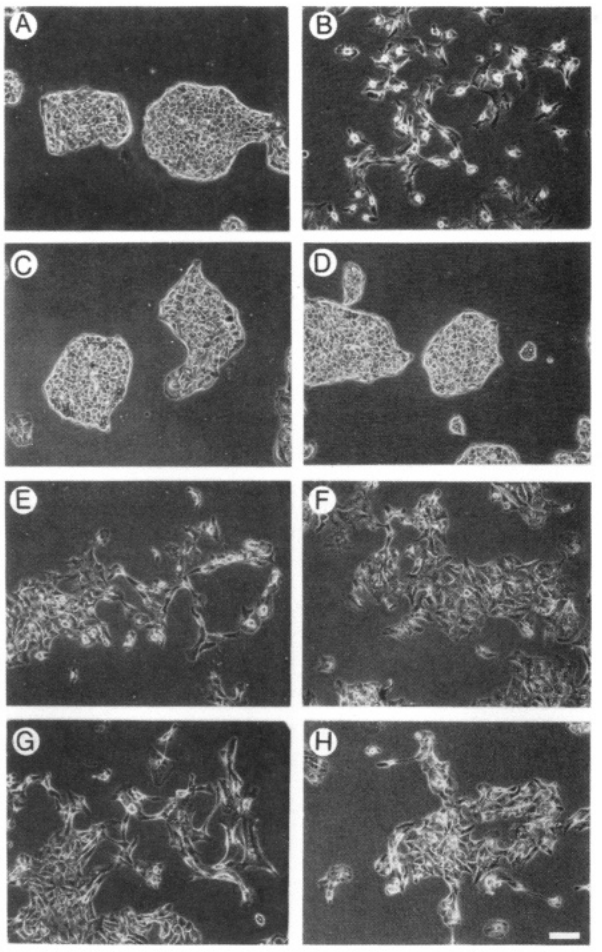

FIGURE 6: Effect of the hHGF deletion mutant proteins on morphology of MDCK cells. Cells were incubated with or without various concentrations of the mutant proteins and stained with Giemsa. A, without the protein; B, 200 pM wild-type hHGF; C, 400 pM ΔK1; D, 400 pM ΔK2; E, 200 pM ΔK3; F, 400 pM ΔK4; G, 100 pM wild-type hHGF; H, 50 pM wild-type hHGF. Bar, 100 μm.

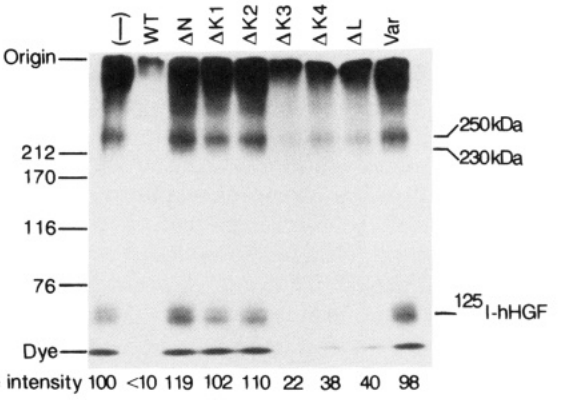

FIGURE 7: Competition with ^{125}I-hHGF bound to the c-*met* protein by the hHGF deletion mutant proteins. Meth A cells were incubated with 50 pM ^{125}I-hHGF in the presence or absence (–) of 400 pM unlabeled protein and chemically cross-linked. The cross-linked products were analyzed by SDS–PAGE under nonreducing conditions. ^{125}I-hHGF binding complexes (230 and 250 kDa) were detected by autoradiography. Relative intensities of the bands are shown below the autoradiogram. The molecular mass standards were myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), and transferrin (76 kDa).

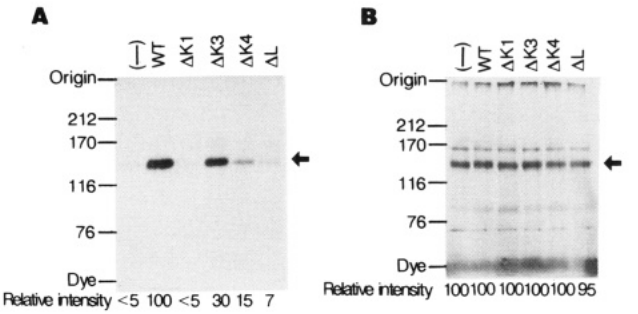

FIGURE 8: Tyrosine phosphorylation of the c-*met* protein on Meth A cells by the hHGF deletion mutant proteins. Cells were treated or untreated (–) with 200 pM proteins and immunoprecipitated with an anti-*met* antiserum. The immunoprecipitates were separated by SDS–PAGE (6% gel) under reducing conditions and immunoblotted with anti-phosphotyrosine antibody (A) or anti-*met* antiserum (B). The position of the β-subunit (140 kDa) of the c-*met* protein is indicated by an arrow. Relative intensities of bands of the tyrosine-phosphorylated β-subunit of the c-*met* protein are shown below the immunoblots. The band of 170 kDa in (B) probably corresponds to the precursor (unprocessed) form of the c-*met* protein, and the other bands are unidentified.

was cross-linked to binding proteins on Meth A cells, two complexes with molecular masses of 250 and 230 kDa were detected. We previously demonstrated that the 250-kDa complex is formed by binding of hHGF to the c-*met* protein and the 230-kDa complex is formed by binding of hHGF to the truncated form of the c-*met* protein (Komada et al., 1992). The results of competition with ^{125}I-hHGF bound to the c-*met* protein by unlabeled proteins are shown in Figure 7. ^{125}I-hHGF was almost completely competed in the presence of 400 pM unlabeled wild-type hHGF. The mutant proteins ΔK3 and ΔK4 at 400 pM also competed with ^{125}I-hHGF, but their effects were weaker than that of the wild-type hHGF. The effect of ΔK3 and ΔK4 roughly corresponded to that of 200 pM wild-type hHGF and to that of 200 pM ΔK3, respectively (data not shown). Competition with ^{125}I-hHGF by mutant protein ΔL was also observed even though ΔL had no activity in three biological assays. The effect of ΔL corresponded to that of ΔK4. The other deletion mutant proteins (ΔN, ΔK1, and ΔK2) and the variant form of hHGF (Var) had no measurable effects on the competition with ^{125}I-hHGF.

Stimulation of the Tyrosine Phosphorylation of the c-met Protein by hHGF Deletion Mutant Proteins. HGF stimulates the tyrosine phosphorylation of the c-*met* protein on its target cells. Tyrosine phosphorylation seems to be involved in the signal transduction of the biological activities of HGF. We therefore examined the effects of the mutant proteins on the tyrosine phosphorylation of the c-*met* protein of Meth A cells. As shown in Figure 8A, the tyrosine phosphorylation of the c-*met* protein was markedly stimulated by the wild-type hHGF. The deletion mutants ΔK3 and ΔK4 also stimulated tyrosine phosphorylation, but at potencies of about 30% and 15% that of the wild-type hHGF, respectively. Tyrosine phosphorylation was slightly stimulated by the deletion mutant ΔL, but the potency was much lower than that of ΔK4. No stimulation was observed on the Meth A cells treated by ΔK1. The amounts of the c-*met* protein were unchanged among the cells treated with the deletion mutant proteins (Figure 8B).

DISCUSSION

Human HGF consists of characteristic structural domains which are encoded as separate exons in the gene. In this study, we prepared mutant proteins lacking each of these domain structures and examined their biological properties. The mutant proteins lacking the N-terminal region of the heavy chain (ΔN), the first kringle domain (ΔK1), or the second kringle domain (ΔK2) were not effective in stimulation of hepatocyte DNA synthesis, inhibition of Meth A cell growth,

and MDCK cell dissociation. In addition, these mutant proteins could not compete with the full-size hHGF bound to the c-*met* protein on Meth A cells, thereby suggesting that they have no binding affinity for the target cells. From these observations, we conclude that a whole or a part of each of these domains is necessary for the biological activities of hHGF which are mediated by binding to a specific receptor. However, the sequence of the N-terminal and the first two kringle domains is not sufficient for the biological activities, because the variant form of hHGF (Var), which consists of only these domains, did not exhibit these activities. The deletion mutant protein ΔN did not bind to heparin–Sepharose, whereas all other deletion mutant proteins as well as the wild-type hHGF did. There are two classes of HGF binding sites with high affinity and low affinity on the surfaces of target cells (Naldini et al., 1991; Komada et al., 1992). Naldini et al. (1991) found that the low-affinity binding was eluted by excess heparin, and they suggested that the binding sites most likely corresponded to matrix- or cell-associated heparan sulfate proteoglycans. Thus, the mutant protein ΔN probably has no affinity to the low-affinity binding site. Although involvement of the low-affinity binding in the high-affinity binding is not known, loss of the biological activities of ΔN may be due to the inability of ΔN to bind to heparin.

The mutant proteins lacking the third (ΔK3) or fourth kringle domain (ΔK4) retained moderate activities in all three biological assays using different target cells (4- to 10-fold less active than the wild-type hHGF). Competition analysis followed by cross-linking indicated that these two proteins bound to the c-*met* protein on Meth A cells, although with a lower affinity than the wild-type hHGF. Moreover, tyrosine phosphorylation of the c-*met* protein was stimulated by both of these mutant proteins. The relative levels of the tyrosine phosphorylation correlated well with the relative potencies of the biological activities when compared with that of wild-type hHGF. The mutant protein which consisted of only the heavy chain (ΔL) was not effective in the biological activities. The level of the tyrosine phosphorylation of the c-*met* protein was much lower than that with ΔK4, although it was slightly higher than the basal level. These results, together with those obtained for ΔK3 and ΔK4, suggest that the efficiency of the tyrosine phosphorylation of the c-*met* protein results in the biological activities of hHGF. Competition analysis followed by cross-linking indicated that affinity of ΔL to the c-*met* protein was the same as that of ΔK4. Thus, a whole or a part of the light chain sequence is required for the tyrosine phosphorylation of the c-*met* protein.

Kringle structures are present in other proteins involved in blood coagulation and fibrinolysis. They have been implicated as domains involved in the interaction with other proteins. The first kringle in plasminogen and the second kringle in tissue plasminogen activator function as binding sites for fibrin (Lerch et al., 1980; van Zonneveld et al., 1986; Ichinose et al., 1986). The hHGF mutant proteins lacking the first or the second kringle have lost the binding activity to the c-*met* protein, and the protein consisting of only the heavy chain retained the binding activity. These results suggest that the kringle domains in hHGF play an important role in the interaction of hHGF with its receptor. Recently, Chan et al. (1991) reported that the variant form of hHGF (Var) can bind to the c-*met* protein. However, our results showed that the heavy chain exhibited higher affinity for the c-*met* protein than the variant form of hHGF. In addition, the mutant proteins ΔK3 and ΔK4 did not have full affinity to the c-*met* protein found with the wild-type hHGF. Thus, the third and the fourth kringles also contribute to receptor binding.

Even at high concentrations, the mutant proteins ΔK3 and ΔK4 gave a suboptimal maximal response in all three bioassays. This property is characteristic of a partial agonist. Generally, mutant forms of some of other growth factors with altered biological function exhibit reduced receptor affinity but induce the full biological response found with the wild-type molecule (Tager et al., 1980; Cascieri et al., 1988; Clements et al., 1991). In contrast, Zurawski et al. (1990) revealed partial agonism of some interleukin-2 proteins with amino acid substitutions, and they suggested existence of a third receptor component other than the α and β chains of the receptor. Thus, a second receptor component for HGF may exist other than the c-*met* protein. However, only the c-*met* protein and its truncated form have been identified as cell surface proteins bound to HGF (Komada et al., 1992; Prat et al., 1991). Further characterization of the receptor molecules will be required to understand the partial agonism of ΔK3 and ΔK4. These characterizations will also elucidate the molecular mechanisms for receptor recognition of hHGF.

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