Analysis of deleted variant of hepatocyte growth factor by alanine scanning mutagenesis: identification of residues essential for its biological function and generation of mutants with enhanced mitogenic activity on rat hepatocytes

Masahiko Kinosaki, Kyoji Yamaguchi*, Akihiko Murakami, Tomonori Morinaga, Masatsugu Ueda, Kanji Higashio

Research Institute of Life Science, Snow Brand Milk Products Co. Ltd., 519 Ishibashi-machi, Shimotsuga-gun, Tochigi 329-0512, Japan

Received 30 May 1998; revised version received 23 July 1998

Abstract To understand the structure-function relationship of hepatocyte growth factor (HGF) in more detail, we analyzed one of the other forms of HGF, deleted variant of HGF (dHGF), by alanine scanning mutagenesis. We show here that there are at least four sites important for dHGF to stimulate DNA synthesis in cultured adult rat hepatocytes, and that the residues of HGF essential for exerting its biological activity are not identical to those of dHGF. In addition, two mutants showed a decrease (approximately three-fold) in EC_{50} compared with wild-type dHGF in an assay of mitogenic activity on rat hepatocytes.

Key words: Hepatocyte growth factor; Deleted variant of hepatocyte growth factor; Alanine scanning mutagenesis

1. Introduction

Hepatocyte growth factor (HGF) is a heparin binding basic protein initially identified as a potent mitogen for hepatocytes [1–3]. The molecular cloning of HGF cDNA has predicted its primary and tertiary structures [4,5]. Subsequently, both scatter factor and fibroblast-derived tumor cytotoxic factor were found to be identical to HGF [6–10].

Recent studies have revealed that HGF is biologically multifunctional. It is a mitogen not only for hepatocytes but also for various epithelial cells [11–14] and endothelial cells [9,13–15], whereas it suppresses the growth of tumor cells [7,9,13,14]. HGF executes these activities through its receptor, the c-*met* proto-oncogene product [13,14,16–18].

HGF is a disulfide-linked heterodimer composed of an α chain with a molecular mass of 52–56 kDa and a β chain of 30–34 kDa [19,20]. HGF consists of six major domains: the N-terminal domain (including the hairpin loop) and four kringle domains in the α chain, and a serine protease-like domain in the β chain [4,5].

A naturally occurring HGF variant (deleted variant of hepatocyte growth factor, dHGF) that arises from an alternatively spliced transcript and lacks five amino acids (Phe-Leu-Pro-Ser-Ser) in the first kringle domain was also found in many cells or cell lines [10,21]. It has been reported that dHGF is distinguishable from HGF in biological activity and tertiary structure [10,22,23].

Numerous HGF mutants have been prepared in investigations of the HGF structure-function relationship [22,24–28]. Through these studies, it has been established that the N-terminal domain and the first and second kringle domains are required for its biological functions [22,24–26]. The importance of the first two domains has been demonstrated by the finding that a variant consisting of the N-terminal domain and the first kringle domain binds to the receptor and has a low level of scatter activity [27]. More recently, a series of point mutations were systematically introduced into the first two domains of HGF to identify the residues essential for the mitogenic activity on rat hepatocytes [28]. Of more than 50 HGF mutants constructed in many laboratories, no mutant has been found to have specific activity significantly higher (two-fold or more) than that of HGF.

Since no structure-function study has been carried out with dHGF, we conducted a mutational analysis of dHGF. To analyze the role of the residues in the two N-terminal domains, alanine scanning mutagenesis was employed [29]. We describe here the identification of the residues of dHGF required for stimulating DNA synthesis in hepatocytes. We also show that these residues are not identical to those of HGF. In addition, two mutants showed a decrease in EC_{50} (dose required for half-maximal cell proliferative response) in an assay of mitogenic activity on rat hepatocytes. The two dHGF mutants are, therefore, the most potent mitogen for hepatocytes among the HGF and dHGF variants constructed to date.

2. Materials and methods

2.1. Cells

Adult rat hepatocytes were prepared by the method of Seglen [30]. NFS-60 cells (murine myeloblastic cells) were kindly provided by Dr. T. Suda (Kumamoto University, Japan). OK cells (American opossum kidney epithelial cells) and Chinese hamster ovary (CHO) cells were purchased from American Type Culture Collection (ATCC).

2.2. Construction of expression vectors for dHGF and its mutants

The plasmid to express human dHGF, designated pSRαTCF, was constructed as follows. An SR α promoter-based vector termed pcDL-SR α 296 [31] (a gift from Dr. Y. Takebe) was digested with restriction enzymes *Pst*I and *Kpn*I, and the ends were blunted using DNA blunting kit (Takara Shuzo). dHGF cDNA fragment was excised from pUCTCF, a plasmid with the entire coding sequence for

*Corresponding author. Fax: (81) (285) 53-1314.
E-mail: fvbd7042@mb.infoweb.or.jp

Abbreviations: dHGF, deleted variant of hepatocyte growth factor; HGF, hepatocyte growth factor; ELISA, enzyme-linked immunosorbent assay; IMDM, Iscove's modified Dulbecco's medium; FPLC, fast protein liquid chromatography; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; EC_{50}, dose required for 50% maximal cell proliferative response

PII: S0014-5793(98)00971-5

human dHGF inserted between the *Bam*HI and *Sph*I sites of pUC 18 (Takara Shuzo), by digesting with *Bam*HI and *Sph*I. The fragment was then treated with the DNA blunting kit, and inserted into the blunt-ended pcDL-SR α296 vector. Site-directed mutagenesis was carried out by a recombinant polymerase chain reaction (PCR) technique as described by Higuchi [32]. pUCTCF was used as a template for the PCRs. A *Bst*PI/*Eco*RV fragment of pSRαTCF, which encodes the N-terminal region and the first kringle domain, was replaced with the *Bst*PI/*Eco*RV fragment derived from each PCR product to generate the various vectors to express dHGF mutants. CHO cells were transfected with a mixture of 200 μg of each expression plasmid and 10 μg of pSV2bsr, an expression vector for blasticidin S resistant gene (Funakoshi), by electroporation, and cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS at 37°C. After 3 days of incubation, the cells suspended in IMDM containing 10% FBS and 5 μg/ml of blasticidin S (Funakoshi) were seeded at an initial density of 10^4 cells/200 μl/well in 96-well plates. Two weeks after the inoculation, the amount of mutant dHGF in the cultured medium in each well was measured by an ELISA as previously described [33], and the clones which expressed mutant proteins were selected.

2.3. Nomenclature of mutants

The mutations or mutants are defined by the following, in the order shown: (a) residue(s) to be replaced, (b) the position of the replaced amino acid or the first residues of the mutated sequences in the amino acid sequence, and (c) the residue or the sequence generated by the mutagenesis (e.g. R42A or KIKTKK27AIATAA). The position of each mutation is described by indicating the residue number of the first residue of the cluster in the amino acid sequence and the original sequence (e.g. 42R or 27KIKTKK). Amino acid residues are numbered starting at the amino-terminus of the mature protein. The deduced amino acid sequences of HGF and dHGF together with the positions of the mutations in dHGF mutants are shown in Fig. 1.

2.4. Heparin Sepharose chromatography

The affinity of wild-type and dHGF mutants for heparin was determined by FPLC on a HiTrap heparin column (Pharmacia). Conditioned medium containing each dHGF mutant was applied to the column (1 ml) equilibrated with 20 mM Tris-HCl (pH 7.5)-0.01% Tween 80 (Sigma). The protein was eluted with a 45 min linear gradient of 0–1.5 M NaCl in the buffer at a flow rate of 0.5 ml/min, and fractions (0.5 ml) were collected. The concentration of the dHGF mutant in each fraction was determined by an ELISA employing a rabbit anti-dHGF polyclonal antibody.

2.5. Purification

CHO cells producing dHGF mutants were cultured in IMDM with 5% calf serum at 37°C for 1 week. Each mutant in the conditioned medium was purified as previously described [23] with the following modification. The conditioned medium was applied to an S-Sepharose column (25×80 mm, Pharmacia) at a flow rate of 4 ml/min. The column was washed with an equilibration buffer (10 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl and 0.01% Tween 20) and then eluted with the buffer containing 2 M NaCl. The fractions containing the mutant were pooled and dialyzed against 10 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl and 0.01% Tween 80. The mutants were further purified to homogeneity by the combination of Mono S (Pharmacia) and heparin-5PW FPLC (Tosoh) as previously described [23]. The protein concentration was determined by the method of Lowry using bovine serum albumin as a standard protein.

2.6. Biological assays

Adult rat hepatocytes suspended in William's E medium containing 10% FBS and 10 mM dexamethasone were seeded at an initial cell density of 10^4 cells/100 μl/well in 96-well plates. The cells were incubated for 24 h at 37°C. dHGF and dHGF mutants were partially purified with heparin Sepharose, resuspended in phosphate-buffered saline (PBS) containing 0.01% Tween 80 and concentrated with Centricut (Kurabo). Each dHGF mutant was serially diluted with the medium and added to each well. The plates were incubated for 22 h at 37°C. Subsequently, 1 μCi/10 μl of [*methyl*-^{3}H]thymidine (85 Ci/mmol, Amersham) was added to each well and the plates were further incubated for 2 h. The cells were washed with PBS and then trypsinized. The radioactivity in the wells was measured using a Direct Beta Counter, Matrix 96 (Packard). OK cells were maintained in DMEM containing 10% FBS. The cells suspended in the medium were seeded at a cell density of 10^4 cells/100 μl/well and were incubated at 37°C for 24 h. The medium was replaced with 100 μl of fresh serum-free DMEM and the cells were further incubated for 48 h. Subsequently, the medium was replaced with 50 μl of fresh serum-free DMEM, and then serially diluted dHGF or each mutant in the serum-free medium containing 0.1% bovine serum albumin was added to each well of the plates (50 μl/well). After the incubation at 37°C for 22 h, 1 μCi/10 μl of [*methyl*-^{3}H]thymidine (5 Ci/nmol, Amersham) was added to each well and the plates were further incubated for 2 h. The cells were washed with PBS and trypsinized. The radioactivity incorporated into the cells was determined using Matrix 96. NFS-60 cells were maintained in RPMI 1640 (Life Technologies) containing 10% FBS and 10% conditioned medium of WEHI-3 cells. The cells suspended in RPMI 1640 with 10% FBS were seeded at a cell density of 10^4 cells/50 μl/well. Serially diluted dHGF or each mutant was added to each well (50 μl/well), and the plates were further incubated. Twenty-four hours later, 10 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well and the plates were further incubated for 4 h. Subsequently, 100 μl of 10% SDS in 0.01 M ammonium chloride was added to each well. The plate was then incubated overnight at 37°C to solubilize the cells. The optical density at 590 nm of each well of the plates was measured.

3. Results

3.1. Identification of residues essential for the biological function of dHGF by alanine scanning mutagenesis

Mutants were produced in which multiple or single charged residue(s) in the N-terminal domain and the first kringle domain was/were replaced with alanine, and their mitogenic activity was determined. The specific activity of the mutants to stimulate DNA synthesis in rat hepatocytes at a concentration of 1.25 ng/ml is shown in Fig. 2A. The results are expressed as activity relative to that of wild-type dHGF. Of the N-terminal domain mutants, DKARK59AAAAA and ENKD89ANAA were inactive. Next, mutants in which each of the charged residues in 59DKARK and 89ENKD was replaced with alanine (with an exception of RK62AA, where both 62R and 63K were replaced with alanine residues) were generated. As shown in the left two columns in Fig. 2B, all six mutants maintained the biological activity, suggesting that individual residues are not functionally crucial. In contrast, none of the

```
          N-terminal basic region
HGF    1:QRKRRNTIHEFKKSAKTTLIKIDPALKIKTKKVNTADQCANRCTRNKG 48
dHGF   1:QRKRRNTIHEFKKSAKTTLIKIDPALKIKTKKVNTADQCANRCTRNKG 48
          AAAA   A      A    AIA   AIATAA     A    A  A A
          2      9      16   21    27         37   42 4547

HGF   49:LPFTCKAFVFDKARKQCLWFPFNSMSSGVKKEFGHEFDLYENKDYIRN 96
dHGF  49:LPFTCKAFVFDKARKQCLWFPFNSMSSGVKKEFGHEFDLYENKDYIRN 96
                A    AAAAA               AAA  AAFA  ANAA  A
                54   59                  78   83    89    95


          First kringle domain
HGF   97:CIIGKGRSYKGTVSITKSGIKCQPWSSMIPHEHSFLPSSYRGKDLQENY 145
dHGF  97:CIIGKGRSYKGTVSITKSGIKCQPWSSMIPHEHS-----YRGKDLQENY 140
             AGA          A   A          AAA        AGAA  A
             101          113 117        127        132   138

HGF  146:CRNPRGEEGGPWCFTSNPEVRYEVCDIPQCSEVE               179
dHGF 141:CRNPRGEEGGPWCFTSNPEVRYEVCDIPQCSEVE               174
          A  AGAA          AVAYA  A     AVA
          142145           159    166   172
```

Fig. 1. Alignments of the amino acid sequences of human HGF and dHGF (excluding the signal peptide). The amino acids are numbered starting at the amino-termini of the mature proteins. Positions of alanine substitutions in dHGF mutants and their sequences after mutagenesis are shown in the bottom row.

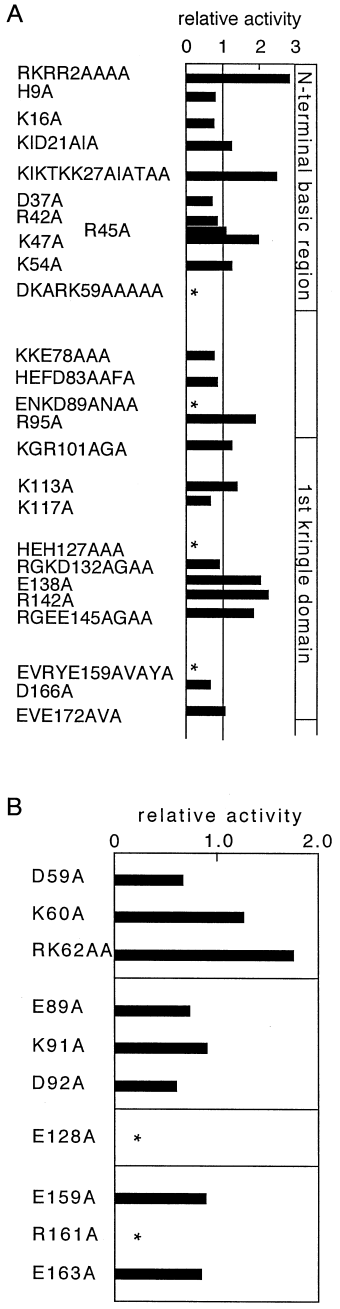

Fig. 2. Biological activity of alanine substitution mutants relative to dHGF. A: Biological activity of the alanine substitution dHGF mutants relative to dHGF. Asterisks indicate lack of detectable activity. B: Relative biological activity of dHGF mutants with single or double (62RK) substitution(s) in the four mutation-sensitive regions identified by the experiment shown in A. Asterisks indicate lack of detectable activity.

mutations in the hairpin loop structure, D37A, R42A, R45A, K47A and K54A, markedly affected the biological activity (Fig. 2A). The mutation at two sites in the first kringle domain, HEH127AAA and EVRYE159AVAYA, abolished the biological activity. To determine the residues essential for the activity, a single amino acid substitution was introduced into these two 'mutation-sensitive' sites. As shown in the right two columns in Fig. 2B, two mutants, E128A and R161A, were inactive, indicating that the residues 128E and 161R are required for dHGF to exert its biological activity.

As shown in Fig. 2A, several mutants were found to be more potent than dHGF in stimulating DNA synthesis in rat hepatocytes. These mutants were purified to homogeneity (purity was over 95%) and their biological activity was evaluated. In this second screening, two mutants, RKRR2AAAA and KIKTKK27AIATAA, reproducibly showed increases in specific activity (see below) and were characterized further. Mutants RKRR2AAAA and KIKTKK27AIATAA were renamed #2 and #27, respectively.

3.2. *Stimulation of DNA synthesis in adult rat hepatocytes by purified mutants #2 and #27*

Fig. 3 shows the ability of the two mutants to stimulate DNA synthesis in cultured adult rat hepatocytes. In a dose range of 1–16 ng/ml, the two mutants were significantly higher in specific activity than dHGF. The EC_{50} values for #2 and #27 were approximately three-fold lower than that for dHGF. The maximum stimulation levels for #2 and #27 were the same as that for dHGF.

3.3. *Biological activities of mutants #2 and #27 on NFS-60 and OK cells*

As shown in Fig. 4A, the two mutants were less potent than dHGF in the stimulation of NFS-60 (a mouse bone marrow cell line) cell proliferation in a dose range of 0.5 to 32 ng/ml. Mutants #2 and #27 showed increases in EC_{50} by approximately three- and eight-fold, respectively, compared with wild-type dHGF. Similarly, the ability of the mutants to stimulate DNA synthesis in NFS-60 was lower than that of dHGF in terms of EC_{50} (data not shown). As shown in Fig. 4B, the two mutants were more potent than dHGF in the stimulation

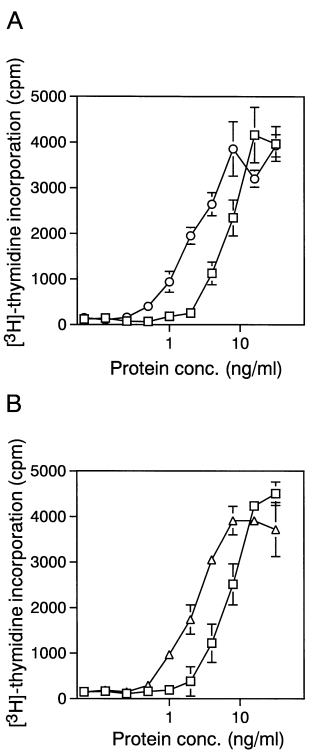

Fig. 3. Effect of dHGF mutants, #2 or #27, on the stimulation of DNA synthesis in rat hepatocytes. Cells were incubated with various concentrations of dHGF, mutants #2 (A) or #27 (B). A: dHGF (□) and mutant #2 (○). B: dHGF (□) and mutant #27 (△). Each value represents the mean ± S.D. of triplicate experiments.

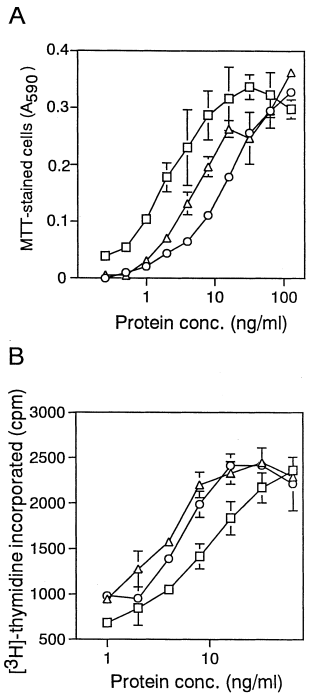

Fig. 4. Mitogenic activity of dHGF mutants, #2 and #27, on (A) NFS-60 (murine myeloblastic cells) or (B) OK (American opossum kidney cells). Cells were incubated with various concentrations of dHGF (□), mutant #2 (○) or mutant #27 (△). Each value represents the mean ± S.D. of the triplicate experiments.

of DNA synthesis in OK cells. In the tested range of 1–64 ng/ml, mutants #2 and #27 showed decreases in EC_{50} by approximately 1.5- and three-fold, respectively, compared with wild-type dHGF. However, the maximum response levels of the two mutants on both NFS-60 and OK cells were comparable to those of dHGF.

3.4. Amino-terminal sequences of mutants #2 and #27

Four positively charged residues are replaced with alanine residues in mutants #2 and #27. These amino acid substitutions may cause a conformational change that can eventually expose a possible protease-sensitive site. Mutant #2 has a predicted amino acid sequence of QAAAA in its N-terminus, which is a possible substrate for the signal peptidase. To examine whether any protease digestion had occurred in the cell or in the conditioned medium, the N-terminal amino acid sequences of the two mutants were determined. The amino-termini of the α chains of both mutants were blocked, as is the case with dHGF. The amino acid residues from the second to the fifth positions of #2 were AAAA, and those of #27 were RKRR. The amino-terminal sequences of the β chains of both mutants were VVNGI. These results indicate that the substitutions of amino acid residues did not induce the susceptibility to protease.

3.5. Affinity of mutants #2 and #27 to heparin

Conditioned medium of cells expressing #2 or #27 was loaded on a HiTrap heparin column and the bound protein was eluted with NaCl-containing buffer. As shown in Fig. 5, the mutants #2 and #27 were eluted from the column at NaCl concentrations of 0.78 M and 0.82 M, respectively, whereas dHGF was eluted at that of 1.14 M.

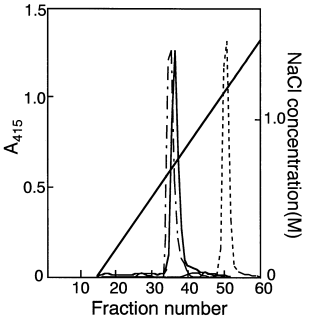

Fig. 5. HiTrap heparin column chromatography of dHGF and dHGF mutants #2 and #27. Conditioned medium of a cell line expressing dHGF (dotted line), dHGF mutants #2 (dot-dashed line) or #27 (solid line) was loaded on a HiTrap heparin column as described in Section 2.

4. Discussion

It has been reported that dHGF is distinguishable from HGF in several ways. First, dHGF is more potent than HGF in the stimulation of DNA synthesis in cultured adult rat hepatocytes [10,22] and in epithelial cells [23]. Conversely, HGF is more potent in the stimulation of DNA synthesis in mesenchymal cells [23]. Second, dHGF is over 70-fold less soluble than HGF in PBS [23]. Finally, several monoclonal antibodies raised against dHGF do not recognize HGF or 2-mercaptoethanol-reduced dHGF [23], demonstrating that the two forms of HGF are different in tertiary structure. In the present study, to obtain further insight into the structure-function relationships of HGF and dHGF, we analyzed dHGF by alanine scanning mutagenesis.

Our analysis of mutants with alanine substitution(s) in the N-terminal domain of dHGF has identified two sites important for its biological activity. One of the sites, 59DKARK, is in the hairpin loop structure. This suggests that the hairpin loop structure of dHGF is a structurally or functionally important region, as is the case with HGF [22,34]. However, the finding that the five other point mutations in the hairpin loop structure, D37A, R42A, R45A, K47A and K54A, did not affect the biological activity (Fig. 2A) indicates that the integrity of the structure is not crucial for the mitogenic activity. The other mutant, ENKD89ANAA, which has no detectable mitogenic activity on rat hepatocytes, has mutations in the region between the hairpin loop structure and the first kringle domain. Unexpectedly, none of the single point mutations in 59DKARK and 89ENKD abolished the biological activity (Fig. 2B), suggesting that no specific residue in the two stretches is directly involved in the interaction with the receptor. Lokker et al. demonstrated that the production levels of HGF (not dHGF) mutants with mutations at the positions corresponding to 59DKARK and 89ENKD (termed D90A,K91A,R93A,K94A and E121A,N122A,K123A,D124A, respectively in [28]) are low in human kidney 293 cells [28]. Taken together, these results suggest that the two regions may be important for the folding of both HGF and dHGF into proper structures.

A comparison of the results obtained from the mutational study of dHGF (present study) and that of HGF [28] clarifies the similarities of and the differences between the two forms of HGF. An HGF mutant, K52A,D54A, had lower mitogenic activity than did wild-type HGF on hepatocytes [28], while the

corresponding dHGF mutant (KID21AIA) had a specific activity comparable to that of wild-type dHGF (Fig. 2A). This suggests that the role of the KID sequence in the biological activity is different between HGF and dHGF. HGF mutants, H114A,E115A,D117A and D171A, could not stimulate DNA synthesis in rat hepatocytes, due to their inability to bind to the receptor [28], whereas the corresponding dHGF mutants (HEND83AANA and RGKD132AGAA, respectively) maintained the biological activity (Fig. 2A). These results strongly suggest that the residues involved in the binding to the receptor are different between dHGF and HGF. The mutational analysis in the first kringle domain also identified two sites, HEH127 and EVRYE159, as sequences essential for the biological function of dHGF. Our analysis of the mutants with a single point mutation in these regions revealed that 128E and 161R are crucial for the biological function of dHGF. Similarly, the alanine substitution of 128E and 166R of HGF, residues that correspond to 128E and 161R of dHGF, respectively, resulted in a loss of receptor binding capability [28]. Therefore, these may be the key residues for both HGF and dHGF to exert the mitogenic activity on rat hepatocytes.

Two mutants (termed #2 and #27) had increased mitogenic activity on rat hepatocytes and OK cells. Replacement of four basic amino acid residues in the N-terminal region with alanine residues resulted in a marked decrease in affinity for heparin (Fig. 5). All of the HGF mutants with no affinity for heparin lose the biological activity [24,34], suggesting that binding to heparin is crucial for the biological function of HGF. Naka et al. showed that cell surface heparin-like molecules are important in the formation of the high-affinity binding sites for HGF [35]. The functional importance of binding to heparin has been reported for other cytokines [36]. However, our analysis of mutants #2 and #27 demonstrated that an about 30% reduction in the heparin binding capability does not diminish the biological activity of dHGF. Further analysis of these two mutants will shed light on the relationship between the heparin binding capability and the biological activity of dHGF.

In some cytokines, mutations in the N-terminal region result in an increase in their biological activity. These include human granulocyte colony-stimulating factor (G-CSF) [37–39], mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) [40] and human tumor necrosis factor-α (TNF-α) [41]. These examples demonstrated that an alteration of the N-terminal region can increase the structural stability or affinity for their signaling receptors. Similar to these cytokines, the mitogenic activity of dHGF on rat hepatocytes was increased when four amino acid residues in the N-terminal region were replaced with alanine residues. Whether or not the mutations influence the protein stability and/or affinity for the receptor is currently under study. It is also of great importance to examine the possibility that the reduction in heparin binding capability is related to the increase in mitogenic activity of the two mutants on rat hepatocytes and OK cells (but not NFS-60).

A previous study has shown that dHGF is not as potent as HGF in stimulating DNA synthesis in mesenchymal cells including NFS-60. However, dHGF is more potent than HGF in stimulating DNA synthesis in epithelial cells including OK cells [23]. Such features of dHGF seemed to be maintained or rather enhanced in mutants #2 and #27. The two mutants may therefore have some advantage in the treatment of diseases that involve epithelial cells in organs such as liver and kidney. Indeed, preliminary in vivo experiments showed that these two mutants are more potent than dHGF in the stimulation of liver function as judged from protein synthesis (M. Kinosaki et al., in preparation).

Acknowledgements: We thank Dr. M. Goto for amino acid sequencing, Fumiko Kobayashi, Fumie Kobayashi, T. Satake and N. Kobayashi for their excellent assistance and Drs. N. Shima and E. Tsuda for their helpful discussions.

References

[1] Nakamura, T., Nawa, K. and Ichihara, A. (1984) Biochem. Biophys. Res. Commun. 122, 1450–1459.
[2] Russel, W.E., McGowan, J.A. and Bucher, N.L.R. (1984) J. Cell Physiol. 119, 183–192.
[3] Thaler, J. and Michalopoulos, G.K. (1985) Cancer Res. 45, 2545–2549.
[4] Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K. and Shimizu, S. (1989) Nature 342, 440–443.
[5] Miyazawa, K., Tsubouchi, H., Naka, D., Takahashi, K., Okigaki, M., Arakaki, N., Nakayama, H., Hirano, S., Sakiyama, O., Takahashi, K., Gohda, E., Daikuhara, Y. and Kitamura, N. (1989) Biochem. Biophys. Res. Commun. 163, 967–973.
[6] Stoker, M.E., Gherardi, E., Perryman, M. and Gray, J. (1987) Nature 327, 239–242.
[7] Higashio, K., Shima, N., Goto, M., Itagaki, Y., Nagao, M., Yasuda, H. and Morinaga, T. (1990) Biochem. Biophys. Res. Commun. 170, 397–404.
[8] Weidner, K.M., Arakaki, N., Hartmann, G., Vandkerckhove, J., Weingart, S., Rieder, H., Fonatsch, C., Tsubouchi, H., Hishida, T., Daikuhara, Y. and Birchmeier, W. (1991) Proc. Natl. Acad. Sci. USA 88, 7001–7005.
[9] Shima, N., Itagaki, Y., Nagao, M., Yasuda, H., Morinaga, T. and Higashio, K. (1991) Cell Biol. Int. Rep. 15, 397–408.
[10] Shima, N., Nagao, M., Ogaki, F., Tsuda, E., Murakami, A. and Higashio, K. (1991) Biochem. Biophys. Res. Commun. 180, 1151–1158.
[11] Igawa, T., Kanda, S., Kanetake, H., Saitoh, Y., Ichihara, A., Tomita, Y. and Nakamura, T. (1991) Biochem. Biophys. Res. Commun. 174, 831–838.
[12] Gherardi, E. and Stoker, M. (1990) Nature 346, 228.
[13] Zarnegar, R. and Michalopoulos, G.K. (1995) J. Cell Biol. 129, 1177–1180.
[14] Matsumoto, K. and Nakamura, T. (1997) Biochem. Biophys. Res. Commun. 239, 639–644.
[15] Morimoto, A., Okamura, K., Hamanaka, R., Sato, Y., Shima, N., Higashio, K. and Kuwano, M. (1991) Biochem. Biophys. Res. Commun. 179, 1042–1049.
[16] Park, M., Dean, M., Kaul, K., Braun, M.J., Gonda, M.A. and Vande Woude, G. (1987) Proc. Natl. Acad. Sci. USA 84, 6379–6383.
[17] Naldini, L., Vigna, E., Narsimhar, R.P., Gaudino, G., Zarnegar, R., Michalopoulos, G.K. and Comogilo, P.M. (1991) Oncogene 6, 501–504.
[18] 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.
[19] Nakamura, T., Nawa, K., Ichihara, A., Kaise, N. and Nishino, T. (1987) FEBS Lett. 224, 311–318.
[20] Gohda, H., Tsubouchi, H., Nakayama, H., Hirano, S., Sakiyama, O., Takahashi, K., Miyazaki, H., Hashimoto, S. and Daikuhara, Y. (1988) J. Clin. Invest. 81, 414–419.
[21] Seki, T., Ihara, J., Sugiyama, A., Shimonishi, M., Nishizawa, T., Asami, O., Hagiya, M., Nakamura, T. and Shimizu, S. (1990) Biochem. Biophys. Res.Commun. 172, 321–327.
[22] Matsumoto, K., Takehara, T., Inoue, H., Hagiya, M., Shimizu, S. and Nakamura, T. (1991) Biochem. Biophys. Res. Commun. 181, 691–699.
[23] Shima, N., Tsuda, E., Goto, M., Yano, K., Hayasaka, H., Ueda,

M. and Higashio, K. (1994) Biochem. Biophys. Res. Commun. 200, 808–815.
[24] Okigaki, M., Komada, M., Uehara, Y., Miyazawa, K. and Kitamura, N. (1992) Biochemistry 31, 9555–9561.
[25] Lokker, N.A., Mark, M.R., Luis, E.A., Bennett, G.L., Robbins, K.A., Baker, J.B. and Godowski, P.J. (1992) EMBO J. 11, 2503–2510.
[26] Hartman, G., Naldini, L., Weidner, K.M., Sachs, M., Vigna, E., Comoglio, P.M. and Birchmeier, W. (1992) Proc. Natl. Acad. Sci. USA 89, 11574–11578.
[27] Lokker, N.A. and Godowski, P.J. (1993) J. Biol. Chem. 268, 17145–17150.
[28] Lokker, N.A., Presta, L.G. and Godowski, P.J. (1994) Protein Eng. 7, 895–903.
[29] Bennet, W.F., Paoni, N.F., Keyt, B.A., Botstein, D., Jones, A.J.S., Presta, L., Wurm, F.M. and Zoller, M.J. (1991) J. Biol. Chem. 266, 5191–5201.
[30] Seglen, P.O. (1976) Methods in Cell Biology, Vol. 13, pp. 29–83, Academic Press, New York.
[31] Takebe, Y., Seiki, M., Fujisawa, J.I., Hoy, P., Yokota, K., Arai, K.I., Yoshida, M. and Arai, N. (1988) Mol. Cell. Biol. 8, 466–472.
[32] Higuchi, R. (1990) PCR protocols, pp. 177–183, Academic Press, New York.
[33] Shima, N., Higashio, K., Ogaki, H. and Okabe, K. (1991) Gastroenterol. Japon. 26, 477–482.
[34] Mizuno, K., Inoue, H., Hagiya, M., Shimizu, S., Nose, T., Shimohigashi, Y. and Nakamura, T. (1994) J. Biol. Chem. 269, 1131–1136.
[35] Naka, D., Ishii, T., Shimomura, T., Hishida, T. and Hara, H. (1993) Exp. Cell Res. 209, 317–324.
[36] Yayon, A., Klagsbrun, M., Esko, J., Leder, P. and Ornitz, D.M. (1991) Cell 64, 841–848.
[37] Nagata, S., Tsuchiya, M., Asano, S., Yamamoto, O., Hirata, Y., Kubota, N., Oheda, M., Nomura, H. and Yamazaki, T. (1986) EMBO J. 5, 575.
[38] Kuga, T., Komatsu, Y., Yamasaki, M., Sekine, S., Miyaji, H., Nishi, T., Sato, M., Yokoo, Y., Asano, M., Okabe, M., Morimoto, M. and Ito, S. (1989) Biochem. Biophys. Res. Commun. 159, 103–111.
[39] Okabe, M., Asano, M., Kuga, T., Komatsu, Y., Yamasaki, M., Yokoo, Y., Itoh, S., Morimoto, M. and Oka, T. (1990) Blood 75, 1788–1793.
[40] Shanafelt, A.B. and Kastelein, R.A. (1989) Proc. Natl. Acad. Sci. USA 86, 4872–4876.
[41] Masegi, T., Kato, A., Kitai, K., Fukuoka, M., Ogawa, H., Ichikawa, Y., Nakamura, S., Watanabe, N. and Niitsu, T. (1995) Jpn. J. Cancer Res. 86, 72–80.