Hairpin Loop and Second Kringle Domain Are Essential Sites for Heparin Binding and Biological Activity of Hepatocyte Growth Factor*

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Hepatocyte growth factor (HGF) has a strong affinity for heparin. About one fourth of HGF secreted from MRC-5 human embryonic lung fibroblasts was found to be associated with heparin and heparan sulfate proteoglycan on the cell surface and extracellular matrix. To identify heparin-binding sites within the HGF molecule, we constructed variously deleted mutant HGFs and examined their binding ability to an immobilized heparin column. Native HGF and mutant HGFs, including d-K1 (deletion of the first kringle domain), d-K2 (deletion of the second kringle domain), d-K3 (deletion of the third kringle domain), d-K4 (deletion of the fourth kringle domain), d-β (deletion of β-chain), and HKβ2 (consisting of the N-terminal hairpin loop and the first two kringle domains), tightly bound to a heparin column, but d-H (deletion of the N-terminal hairpin loop) and d-K2 (deletion of the second kringle domain) markedly decreased binding ability to the column. These observations suggest that the N-terminal hairpin loop and the second kringle domain are essential for the heparin-binding of HGF. The finding that HKβ2 competed the binding of 125I-HGF to immobilized heparin provided additional evidence that the N-terminal half of HGF α-chain is the principal heparin-binding site. The hairpin loop in HGF possesses a cluster of basic amino acid residues and a highly positive net charge, which, when compared with hairpin loop structures in the other proteins, plasminogen and HGF-like protein. The second kringle domain in HGF has the basic amino acid cluster in the central region. Thus, it is likely that the basic clusters in these domains cooperatively contribute to the binding of HGF to the anionic heparin or heparan sulfate molecule.

Hepatocyte growth factor (HGF)† was initially found as a potent mitogen for primary cultured hepatocytes in the serum of partially hepatectomized rats (1) and rat platelets (2). HGF, purified to homogeneity from rat platelets, is a disulfide-linked heterodimer composed of a 69-kDa α-chain and a 34-kDa β-chain (3, 4). Molecular cloning of cDNAs encoding human (5-7) and rat HGF (8) revealed that HGF has a considerable sequence homology (38%) with plasminogen and the two subunits are derived from a single chain precursor (reviewed in Refs. 9-12). The biologically inactive precursor is converted to an active two-chain form by extracellular serine protease through the single cleavage at the Arg-Val bond (13). The α-subunit of HGF has the characteristic domain structure consisting of a hairpin loop structure and four kringle domains. The β-subunit resembles serine proteases, but HGF has no protease activity because histidine and serine residues in the active site triad of serine proteases are replaced by glutamine and tyrosine residues, respectively (5).

Whereas HGF was first thought to be a mitogen with a narrow target cell specificity toward hepatocytes, ongoing studies revealed that HGF is a pleiotropic factor, stimulating growth, motility, and morphogenesis of various epithelial cells and inhibiting growth of several carcinoma cell lines (reviewed in Refs. 9-12). Two classes of HGF receptor have been detected on the surface of target cells. A class of high affinity HGF receptors has a Kd value of 20-30 pM (14-16) and was recently identified as a c-met proto-oncogene product with an intracellular tyrosine kinase domain (17-19). A class of lower affinity (Kd = 260-400 pM) binding sites with a large site number was also detected on the cell surface of both HGF-responsive and HGF-non-responsive cells (16, 20). These low affinity binding sites seem to be heparin or heparan sulfate proteoglycans present on the cell surface or in the extracellular matrix, since HGF bound to the low affinity sites was replaced by an excess of heparin (20). While expression of high affinity HGF receptor (c-met product) correlates well with the biological response of cells (16), less is known of the physiological role of low affinity heparin-like binding sites.

Basic fibroblast growth factor (bFGF), acidic fibroblast growth factor, and platelet-derived growth factor bind to heparin and heparan sulfate proteoglycan, which modulate stability, localization, and biological activity of these growth factors (21, 22). The interaction of bFGF with heparin is essential for its binding to the high affinity signaling receptor and for mitogenic activity (23, 24). As the physiological role of heparin binding of HGF has not been documented, we attempted to locate the site within the HGF molecule which is essential for heparin binding. We report here that both the N-terminal hairpin loop and the second kringle domain constitute the principal heparin-binding sites of HGF. The clusters of basic amino acid residues in these domains appear to contribute to the interaction of HGF with the anionic heparin and heparin-like molecules.

EXPERIMENTAL PROCEDURES

Cell Culture—MRC-5 (CCL 171) human embryonic lung fibroblasts were obtained from the Japanese Cancer Research Resources Bank.
Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum.

Measurement of Cell-associated HGF—MRC-5 cells were seeded on 24-well plates (Corning) at a density of 5 x 10^4 cells/cm^2 and then cultured for 24 h. After replacing the medium with 0.5 ml of fresh medium, these cells were cultured for 24 h and the amount of HGF secreted into the conditioned medium was determined by enzyme-linked immunosorbent assay (ELISA) (25). After washing once with 0.5 ml of phosphate-buffered saline (PBS), the cells were incubated for 10 min at 37 °C with 0.5 ml of PBS solution containing various concentrations of NaCl or heparin. The amount of HGF liberated from the cell surface was measured by ELISA. The number of cells was determined using a hemocytometer.

Construction and Expression of Mutant HGF cDNAs—Expression plasmids CDM(LeHGF) and CDM(dLeHGF) were used for expression of nat-HGF and del-HGF, as reported elsewhere (7). Mutant HGF cDNAs were constructed by oligonucleotide-directed in vitro mutagenesis system, using the TT-GEN in vitro mutagenesis kit (U. S. Biochemical Corp.) (26) From the expression plasmid CDM(LeHGF), the 2.3-kilobase pair BamHI-SalI fragment was transferred into M13mp19 and the single-stranded DNA from the resulting plasmid was used as a template. The constructed double-stranded mutant HGF cDNAs were inserted into the expression vector CDMmcs (26). COS-1 cells were transfected with 10 µg of expression plasmids, containing native or mutant HGF cDNA by the DEAE-dextran method, and cultured in COS medium 001 for 7 days at 37 °C. The amounts of mutant HGF in the culture medium were measured by ELISA, as reported elsewhere (25).

Heparin-Sepharose Chromatography—Aliquots of culture medium of 5 x 10^5 cells containing each mutant HGF were diluted to 1 ml with HTB buffer (10 mM Hepes, pH 7.2, 0.01% Tween 20, 0.01% (w/v) bovine serum albumin) and applied to a heparin-Sepharose CL-4B column (Pharmacia, 0.6 x 3.0 cm) equilibrated with the same buffer. The protein was eluted by a stepwise elution of 1 ml each of HTB buffer containing 0-2 M NaCl. One ml per fraction was collected. The amount of mutant HGF eluted in each fraction was measured by ELISA, as described (25).

Expression and Purification of HGF-like Protein (HLP)—Human HLP cDNA was cloned from human liver cDNA by polymerase chain reaction using primers designed based on nucleotide-sequence for human HLP (29). Expression plasmid pCDL-SRa inserted by human HLP cDNA was transfected into COS-7 cells. HLP was purified from the conditioned medium of COS-7 cells by three-step chromatography. Details for purification have been published elsewhere (45).

RESULTS

Association of HGF with Heparin or Heparan Sulfate Proteoglycan on the Cell Surface—HGF has a strong affinity for heparin, as was evidenced with affinity chromatography on a heparin-Sepharose column (3, 4, 30). We first examined whether the HGF secreted from producing cells is associated with heparin-like substances on the cell surface. After a 24-h culture of MRC-5 fibroblasts, 27-28 ng of HGF/ml/10^5 cells was detected in the culture media. The amount of cell-associated HGF, as measured by extraction with the solution containing a high concentration of NaCl, was 9 ng/ml/10^5 cells (Fig. 1, left). Thus,
about one fourth of the total secreted HGF was associated with the cell surface. Incubation with heparin (1–100 μg/ml) released most of the cell-associated HGF (Fig. 1, right), thereby indicating that most of the cell-associated HGF was bound to heparin-like molecules.

Location of Heparin-binding Site within the HGF Molecule—To search for the heparin-binding site within the HGF molecule, we measured the binding ability of variously deleted mutant HGFs to heparin-Sepharose. Fig. 2 illustrates the structures of native and variously deleted mutant HGFs that we constructed (26). nat-HGF designates the major form of native human HGF (7). del-HGF is a naturally occurring variant form with a deletion of 5 amino acid residues in the first kringle domain of nat-HGF (7). d-β (α), deleted with β-chain, consists of the entire α-chain of nat-HGF. d-H is deleted with the hairpin loop structure (amino acid residues 70 to 96) in the N-terminal region. d-K1, d-K2, d-K3, and d-K4, respectively, indicate mutant HGF with each kringle domain deleted. HK1K2 was constructed so as to have a structure similar to a naturally occurring HGF variant (HGF/NK2) (31, 32), consisting of a region from the N terminus to the end of the second kringle domain; HK1K2 has a C-terminal sequence of Cys-Ala in place of Cys-Glu-Thr in the naturally occurring variant HGF/NK2.

Native or each deleted mutant HGF was loaded onto a heparin-Sepharose column and chromatographed by stepwise elution with increasing concentrations of NaCl. Fig. 3 shows the elution profiles of native and deleted mutant HGFs, as monitored and quantified by ELISA using a specific antibody against HGF. Consistent with reported results (3, 4, 30), native HGF (nat-HGF) was adsorbed tightly to a heparin-Sepharose column, and eluted with a solution containing 0.9–1.0 M NaCl (fractions 13 and 14). Similar elution profiles were observed for a naturally occurring 5-amino acid deleted variant (del-HGF) and the artificially constructed mutant HGFs (d-β, d-K1, d-K2, d-K3, and HK1K2). On the other hand, d-H and d-K4 markedly decreased in heparin-binding ability and were eluted with a solution containing 0.2 M NaCl (fraction 5). Marked decrease in the affinity of d-H and d-K4 to a heparin-Sepharose column suggests that both the hairpin loop and the second kringle domain are required for heparin-binding of HGF molecule.

To further confirm involvement of the N-terminal hairpin loop and the second kringle domain in heparin binding of HGF, the competition assays of 125I-HGF binding to heparin were carried out. As shown in Fig. 4, nat-HGF competed 125I-HGF binding to the immobilized heparin, such that 50% inhibition was obtained at 2.8 nm. HK1K2, which is composed of the N-terminal hairpin loop and the first two kringle domains, also inhibited 125I-HGF binding to heparin with half-maximal inhibition at 22 nm. The strong competitive effect of HK1K2, which is comparable (about 8-fold molar excess) with the effect of nat-HGF, together with its high affinity binding to heparin-Sepharose, provided added evidence that the N-terminal half of HGF α-chain is the principal site for heparin-binding of HGF.

In contrast, a synthetic undecaepptide (P11; KAVFVD-KARKQ), corresponding to Lys-85 to Gln-95 within the hairpin loop of HGF (see Fig. 6), had no effect on the 125I-HGF binding to the immobilized heparin even at 10 μM (Fig. 4).

Structural Characteristics of Heparin-binding Site—The hairpin loop structure, an unique two-disulfide-linked struc-
ture consisting of 23-27 amino acid residues, has been found in sequences of three proteins: HGF, plasminogen (33), and HGF-like protein (29, 34) (Fig. 5A), but as yet in no other proteins. The domain structures and gene organization of these three proteins are similar (29, 33-35). A weak sequence homology was observed between hairpin loop structure and "apple" domains in coagulation factor XI and prekallikrein (36, 37) (Fig. 5A). Comparison of the amino acid sequences of hairpin loop structures in these three proteins revealed 10 conserved amino acid residues, including 4 cysteine residues (shaded in Fig. 6). There are, however, significant differences in other amino acid residues within the hairpin loop structure. HGF has 7 basic residues and 1 acidic amino acid residue within the hairpin loop, while plasminogen has 3 basic and 6 acidic residues and HGF-like protein has 2 basic residues and 1 acidic residue within the hairpin loop. Thus, the net charge of the hairpin loop, including the +0.5 positive charge of histidine residue, is calculated as +6 for HGF, -2.5 for plasminogen, and +2 for HGF-like protein (Fig. 6). In addition to the net charge, HGF has the clusters of basic amino acid residues (Arg-Cys-Thr-Arg, Asn-Lys, Lys-Ala-Arg-Lys), and plasminogen has a cluster of acidic residues (Glu-Glu-Asp-Glu) in the hairpin loop structure. When plasminogen and HGF-like protein was each applied to a heparin-Sepharose column under the same conditions as for Fig. 3, plasminogen eluted at 0.2 M NaCl (Fig. 7, top) and HGF-like protein eluted at 0.5 M NaCl (Fig. 7, bottom). Thus, the net charge and the existence of the cluster of basic or acidic amino acid residues in the hairpin loop substantially affect the heparin-binding ability of these proteins.

Fig. 5B shows alignment of amino acid sequences of four kringle domains of HGF. In the central region (amino acid residues 32-43) of the second kringle domain, there is a cluster of basic amino acid residues, and these may be involved in the interaction of HGF with heparin.

**DISCUSSION**

We obtained evidence that the N-terminal hairpin loop and second kringle domain within the HGF molecule are essential for heparin-binding of this growth factor. Since deletion of either the hairpin loop or the second kringle domain results in a marked decrease in heparin-binding ability, both domains seem to cooperatively constitute the principal heparin-binding site of HGF. The intramolecular interaction between these two domains probably serves to maintain the stable conformation for heparin binding. The findings that HK1K2 (containing both domains) tightly bound to heparin-Sepharose and competed 125I-HGF binding to heparin to the extent similar to nat-HGF further proved that the N-terminal half of HGF α-chain is the principal heparin-binding site of HGF. As often observed in sequences of heparin-binding sites of many polypeptide growth factors (38), a cluster of basic amino acid residues is present in the hairpin loop and second kringle domain of HGF. When we compared the three hairpin loop-containing proteins, the net charge and the presence of a cluster of basic amino acid residues within the hairpin loop correlated well with their potential to bind to heparin. HGF with a positively charged hairpin loop has a strong affinity for heparin, while plasminogen with a negatively charged hairpin loop has no heparin-binding ability. Thus, the hairpin loop seems to be at least one of the
important determinants for heparin-binding ability of this protein family. Kringle domain, a triple disulfide-linked structure consisting of about 80 amino acid residues, is present in several proteins involved in the fibrinolytic process, blood coagulation, and atherosclerosis. The kringle domains are thought to play a role in the protein-protein interactions essential for regulation of their biological functions, but their actual roles are not well understood. Recently the kringle domain of urokinase was identified to be the site essential for the heparin binding of this protein (27). The present finding is the second example of the kringle domain as the heparin-binding site.

As a trial to further locate the heparin-binding site in the HGF sequence, an undecapeptide, P11, which consists of a part of the hairpin loop of HGF, was synthesized and examined for competitive activity to 125I-HGF binding to heparin. This peptide, however, had no effect, even at a concentration of 5 x 10^{-7} M. Further studies using various peptides corresponding to the sequences of possible heparin-binding sites are required to define the location involved in heparin-binding within the HGF molecule.

There are two classes of HGF receptor with a high and low affinity on the target cells. While the high affinity receptor, which was recently identified as the c-met proto-oncogene product, corresponds to a signal-transducing receptor having an intracellular tyrosine kinase domain, the physiological role of low affinity binding sites has remained unknown. Since HGF binding to the low affinity receptor was replaced by heparin, the low affinity receptor was thought to be heparin or heparin-like proteoglycan present on the cell surface and extracellular matrix. Recent studies on the liver perfusion of 125I-labeled HGF in rats also demonstrated the presence of two kinds of binding sites on the liver cell surface: the heparin-washable lower affinity binding site and the heparin-resistant, acid-washable binding site with a much higher affinity (39). Sequstration of HGF activity in the extracellular matrix in the liver was also demonstrated by perfusion with solution containing 1 M NaCl (40). Taken together, these findings suggest that heparin or heparan sulfate proteoglycan on the cell surface and extracellular matrix serve as the reservoir to capture and retain HGF from the pericellular environment. This notion is supported by the fact that, whereas plasma clearance of 125I-HGF was rapid with a half-life of 4 min (39), the mitogenic effect of the intravenously injected HGF continued for over 12 h (41). Thus, it is postulated that the endogenously or exogenously supplied HGF is first captured on heparin-like substances on the cell surface in the liver and is eventually released and transferred to the closely situated c-met signaling receptor.

Interaction of bFGF with heparin was found to be essential for binding to a high affinity signal-transducing receptor and for biological activity (23, 24). It is not clear whether the interaction of HGF with heparin would modulate the binding to its high affinity c-met signaling receptor and hence its biological activity. Treatment of hepatocytes in primary culture with heparinase or heparitinase decreased the HGF-induced DNA synthesis of the cells, but this effect was not reverted by the addition of various concentrations of exogenous heparin, the result being different from the case of bFGF. Thus, the interaction of HGF with heparin-like substances on the cell surface seems to be important for HGF activity, but mechanisms of

\[^{3}\text{K. Mizuno and T. Nakamura, unpublished data.}\]
modulation differ from the case of bFGF.

Studies on the biological activity and c-met receptor binding of variously deleted mutant HGFs revealed that the N-terminal hairpin loop and the first two kringle domains in the α-subunit of HGF are required for c-met receptor recognition, and that the β-chain is indispensable for receptor activation and for biological activity (26, 42–44). Table I summarizes the results of biological activity and binding to c-met receptor and heparin of various mutant HGFs. The heparin-binding site in the HGF molecule identified in the present study is mostly shared by the domain essential for c-met binding and biological activity, a finding which suggests that the same region on the HGF molecule participates in binding to the c-met high affinity receptor and the heparin-like low affinity receptor. How HGF is captured by the heparin-like low affinity receptor and transferred to the c-met signal-transducing receptor is the subject of ongoing study.

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REFERENCES