Isolation and Conformational Analysis of Fragment Peptide Corresponding to the Heparin-Binding Site of Hepatocyte Growth Factor

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ABSTRACT: Hepatocyte growth factor (HGF) is a potent mitogen for hepatocytes. The mitogenic activity of HGF is mediated by its binding to a high-affinity receptor, c-Met. Heparan sulfate is an initial binding site for HGF, based on its relative abundance on the cell surface. The binding of HGF to heparin or heparin-like molecules may induce oligomerization of HGF and facilitate c-Met-dependent mitogenesis [Zioncheck *et al.* (1995) *J. Biol. Chem. 270*, 16871−16878]. Thus, heparin binding is important for the biological activity of HGF. To identify the heparin-binding site of HGF, we isolated fragment peptides corresponding to the site by limited proteolysis and chemical degradation of recombinant human HGF (rhHGF). The heparin-binding ability of the peptides was expressed as their elution positions on heparin-affinity column chromatography with NaCl gradient elution. Because all of the heparin-binding peptides obtained in this study were isolated from the N-terminal hairpin-loop region (PyrGlu^{32}−Asn^{127}) of HGF, the region was identified as the heparin-binding site of HGF. One of the isolated peptides, Phe^{42}−Glu^{111}, containing the N-terminal hairpin-loop structure, was considered a suitable model peptide for the heparin-binding site of HGF. From the observation using circular dichroism spectroscopy, it was indicated that the secondary structure of the peptide changed from a random structure to a β-sheet-like structure upon heparin binding. In addition, oligomerization of HGF in the presence of heparin was observed by dynamic light scattering. Based on our evidence, it is considered that the conformational change in the heparin-binding site may induce the oligomerization of HGF.

Hepatocyte growth factor (HGF),[1] also referred to as scatter factor (SF), is a pleiotropic factor produced by mesenchymal cells. HGF has mitogenic, motogenic, and morphogenic activities on various epithelial and endothelial cell types in culture (Gohda *et al.*, 1988; Mizuno & Nakamura, 1993). HGF is a glycosylated polypeptide with an apparent molecular mass of approximately 85 kDa and is in a heterodimeric form held together by a disulfide bond. The heavy chain (PyrGlu^{32}−Arg^{494}, 54−65 kDa)[2] consists of a hairpin-loop region (PyrGlu^{32}−Asn^{127}) and four consecutive kringle domains (Cys^{128}−Cys^{206}, Cys^{211}−Cys^{288}, Cys^{305}−Cys^{383}, and Cys^{391}−Cys^{469}). The light chain (Val^{495}−Ser^{728}, 31−34 kDa) contains a serine protease-like domain.

The biological responses of deleted mutant HGFs have been studied to elucidate which domains of HGF are necessary to perform the biological activities (Matsumoto *et al.*, 1991; Okigaki *et al.*, 1992; Lokker *et al.*, 1992). These studies suggest that the N-terminal hairpin-loop region (PyrGlu^{32}−Asn^{127}) and the first two kringles (Cys^{128}−Cys^{288}) in the heavy chain are required for the biological activities, whereas the third and fourth kringles (Cys^{305}−Cys^{469}) are not. Deletion of the entire light chain reduces but does not abolish the biological activities (Okigaki *et al.*, 1992; Lokker *et al.*, 1992).

Similar to other known growth factors, HGF interacts with the specific receptors on the surface of target cells. Scatchard analysis of HGF revealed that the target cells possessed two classes of receptors with high affinity (K_d 4.6−17 pM) and low affinity (K_d 2−6.7 nM) (Naldini *et al.*,1991; Arakaki *et al.*, 1992; Komada *et al.*, 1992). The high-affinity receptor was identified as a c-*met* proto-oncogene product, c-Met. The receptor possesses a tyrosine kinase domain which is autophosphorylated by binding of HGF and transmits the intracellular signaling for the mitogenesis (Naldini *et al.*, 1991; Bottaro *et al.*, 1991; Komada *et al.*, 1992). The low-affinity receptor for HGF is considered to be a heparin-like molecule such as heparan sulfate (HS), a glycosaminoglycan structurally related to heparin, widely expressed in the form of proteoglycan species on the surface of most cells and secreted into the extracellular matrix. The heparin-like molecules play an important role as initial binding sites for the growth factors based on their relative abundance on the cell surfaces. That is, they do not transmit signals but play a role in the binding of growth factors to their high-affinity

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[1] Abbreviations: HGF, hepatocyte growth factor; rhHGF, recombinant human hepatocyte growth factor; HS, heparan sulfate; PyrGlu, pyroglutamic acid; V8 protease, Staphylococcal serine protease; PBS, phosphate-buffered saline; FGF, fibroblast growth factor; N-terminal, amino-terminal; C-terminal, carboxyl-terminal; S/E, substrate to enzyme ratio; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RPHPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; ESIMS, electrospray ionization mass spectrometry; MALDI-TOFMS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; IBA, *o*-iodosobenzoic acid; TrpL, 2,3-dihydroxytryptophan lactone; DTT, dithiothreitol; CD, circular dichroism.

[2] The amino acid residues of hepatocyte growth factor are numbered from the first methionine of the precursor protein. The N-terminus of the mature protein is PyrGlu-32.

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receptors (Rapraeger *et al.*, 1991; Olwin & Rapraeger, 1992; Yayon *et al.*, 1991; Ornitz *et al.*, 1992; Nugent & Edelman, 1992). With regard to HGF, HS *in vivo* is thought to be a modulator for the c-Met receptor binding and mitogenic activity of HGF. A recent study suggests that heparin and other highly sulfated oligosaccharides are capable of stabilizing HGF oligomers, which may facilitate c-Met receptor dimerization and activation (Zioncheck *et al.*, 1995).

Heparin is similar in composition and structure to HS (Gallagher & Walker, 1985). The affinity of an extracellular protein for heparin *in vitro* can signify a potential physiological interaction with HS. HGF has strong heparin affinity similar to many growth factors. Heparin competitively inhibits the binding of HGF to HS (Lyon *et al.*, 1994), as much as 85% of HGF bound on the cell surface is released by washing with heparin (Naka *et al.*, 1993), and HGF is commonly purified by heparin-affinity column chromatography; therefore, HGF has been considered to have specific sites for heparin binding in its structure, and the heparin-binding sites may be identical to the primary HS-binding site of HGF (Lyon *et al.*, 1994). Identification of the heparin-binding sites of HGF has been studied by analyzing the heparin affinity of deleted mutant HGFs (Okigaki *et al.*, 1992; Mizuno *et al.*, 1994). Mizuno *et al.* suggested that the N-terminal hairpin-loop region ($PyrGlu^{32}-Asn^{127}$) and the second kringle ($Cys^{211}-Cys^{288}$) were essential sites for the heparin binding of HGF. The N-terminal hairpin-loop region of HGF has many basic amino acid residues and is considered to locate on the surface of the molecule, thus facilitating the interaction of HGF with the heparin-like molecules on the cell surface. Therefore, the importance of the region on the heparin-binding properties of HGF is quite understandable. However, the contribution of the second kringle to heparin binding is not clear.

In the present study, we have directly investigated the heparin-binding sites of HGF by isolating the corresponding fragment peptides from HGF itself. Our results suggest that the hairpin-loop structure ($Cys^{70}-Cys^{96}$) in the N-terminal hairpin-loop region ($PyrGlu^{32}-Asn^{127}$) makes the major contribution to the heparin-binding properties of HGF. We also demonstrate that the secondary structure of a heparin-binding peptide, corresponding to the heparin-binding site of HGF, is changed from a random structure to a β-sheet-like structure during the formation of the complex with heparin in PBS. Moreover, we have observed by dynamic light scattering that HGF forms an oligomer in the presence of heparin. Therefore, we postulate that the conformational change in the heparin-binding site of HGF during binding to heparin or heparin-like molecules induces the formation of HGF oligomer, which may subsequently promote c-Met receptor activation.

MATERIALS AND METHODS

Materials. Reagents were obtained as follows: V8 protease and iodoacetamide from Wako Pure Chemical Industries; endoproteinase Asp-N from Boehringer Mannheim Biochemicals; *o*-iodosobenzoic acid and heparin (M_r 4000−6000) from Sigma Chemical Co.; electrophoresis calibration kit from Pharmacia LKB Biotechnology, Inc.; amino acid calibration mixture from Ajinomoto Co. The heterodimeric form of rhHGF was prepared as previously described (Naka *et al.*, 1992).

Preparation of Fragment Peptides from rhHGF by V8 Protease Digestion. The rhHGF solution (0.31 mg/mL) in 10 mM sodium phosphate, pH 7.5, containing 0.65 M NaCl, was concentrated and exchanged for 50 mM ammonium acetate, pH 4.0, to a final concentration of 1.3 mg/mL, using a Centricon-10 microconcentrator (Amicon). This solution was subjected to digestion with V8 protease [S/E = 25 (mol/mol)] for 20 h at 37 °C. The reaction mixture was boiled for 2 min and concentrated to 4.0 mL using a Speed-Vac concentrator (Savant). An aliquot of the digest was analyzed by SDS−PAGE, performed on a PhastGel Gradient 10−15 (Pharmacia) using a PhastSystem (Pharmacia).

Heparin-Affinity Column Chromatography of V8 Protease Digest of rhHGF. An aliquot of the digest was applied to a Tosoh TSK-gel heparin-5PW column (0.75 × 7.5 cm) equilibrated with 10 mM sodium phosphate, pH 7.5. The fragment peptides from rhHGF were eluted with a linear gradient from 0 to 2 M NaCl in 30 min at a flow rate of 0.7 mL/min, monitored at 215 nm.

Isolation of the Heparin-Binding Peptides by RPHPLC. Heparin-binding peptides from rhHGF, which were retained on the heparin-affinity column, were collected and analyzed by RPHPLC on a J. T. Baker Bakerbond C_8 Wide-Pore column (0.46 × 25 cm) equilibrated with 0.1% TFA in H_2O. The peptides were eluted with a linear gradient from 0% to 70% acetonitrile in 60 min at a flow rate of 1.0 mL/min, monitored at 215 nm. After examination of the elution positions of the fragment peptides, the total amount of the V8 protease digest was chromatographed on the reversed-phase column using the same conditions to isolate the heparin-binding peptides.

Amino Acid Analysis of the Isolated Peptides. An aliquot of each heparin-binding peptide isolated from the V8 protease digest of rhHGF was hydrolyzed for 24 h with 6 N HCl at 110 °C under vacuum using a Picotag Workstation (Waters). The hydrolysates were analyzed on a Model L-8500 amino acid analyzer (Hitachi).

Molecular Mass Measurement of the Isolated Peptides by ESIMS. An aliquot of each heparin-binding peptide was dissolved in the sampling solvent (5% acetic acid and 50% methanol in H_2O) and directly introduced into a Model TSQ-700 triple-quadrupole mass spectrometer (ThermoQuest) at a flow rate of 2 μL/min for ESIMS measurement.

Fragmentation of Peptide B with Endoproteinase Asp-N. One of the heparin-binding peptides, $Phe^{42}-Glu^{111}$ (peptide B), which was obtained by V8 protease digestion of the rhHGF, was further digested with endoproteinase Asp-N. The digestion was carried out under the conditions of S/E = 100 (weight/weight) for 17 h at 37 °C in 50 mM sodium phosphate, pH 7.5. The reaction mixture was boiled for 2 min and applied to the RPHPLC to isolate the peptide fragments. The elution positions of the peptide fragments were determined by heparin-affinity column chromatography using the conditions described above.

Preparation of Fragment Peptides from rhHGF with o-Iodosobenzoic Acid. For the cleavage at the C-termini of the tryptophanyl bonds of rhHGF, 10 nmol of rhHGF was reacted with 22 μmol of *o*-iodosobenzoic acid (IBA) in 2 mL of 80% aqueous acetic acid−4 M guanidine hydrochloride containing 10 μL of *p*-cresol for 20 h at room temperature (Fontana *et al.*, 1983). The reaction solution was desalted and exchanged for 50 mM ammonium acetate, pH 4.0, using a Centricon-3 microconcentrator (Amicon).

Table 1: Heparin-Binding Peptides Isolated from rhHGF

heparin-binding peptides	no. of basic amino acids	heparin-binding ability[a]
(A) PyrGlu^{32}−Glu^{41}	4	0.67
(B) Phe^{42}−Glu^{111}	17	1.0
RA[b]-Phe^{42}−Glu^{111}	17	0.85
(C) Phe^{42}−Ile^{53}	4	0.53
(D) Asp^{54}−Ala^{67}	4	0.40
(E) Asp^{68}−Glu^{111}	9	0.75
(F) PyrGlu^{32}−TrpL^{98} [c]	19	0.93

[a] Heparin-binding ability was expressed by the NaCl concentration (M) of the elution position in heparin-affinity column chromatography. [b] Reduced and alkylated. [c] The C-terminal tryptophan-98 was converted to 2,3-dihydroxytryptophan lactone.

A heparin-binding peptide was isolated from the fragment peptide mixture by heparin-affinity column chromatography and RPHPLC as described above. The peptide was characterized by MALDI-TOFMS using a Model TofSpec-E (Micromass).

Reduction and Alkylation of Peptide B. Peptide B (10 nmol) was reduced with DTT for 3 h at 60 °C in 0.5 M Tris-HCl, pH 8.6, followed by alkylation with iodoacetamide (1.5 times excess relative to DTT, mol/mol) for 30 min at 25 °C. The reaction solution was desalted and exchanged for 50 mM ammonium bicarbonate, pH 8.0, using a Centricon-3 microconcentrator. An aliquot of the solution was applied to amino acid analysis and ESIMS to examine the completion of the reaction. The elution position of the reduced and alkylated peptide B was determined by heparin-affinity column chromatography using the conditions described above.

Circular Dichroism Spectroscopy of Peptide B. CD spectra of peptide B were obtained using a Model J-600 spectropolarimeter (Jasco). Quartz cells of 0.1-cm path length were used. Peptide B (6.0 nmol) alone and peptide B (6.0 nmol) with heparin (6.0 nmol) were separately dissolved in 0.5 mL of PBS, and their CD spectra were recorded by scanning 10 times from 250 to 197 nm at 25 °C. The solvent background and CD contribution of heparin were subtracted out of the spectra.

Dynamic Light Scattering of HGF with or without Heparin. Dynamic light scattering of HGF was measured on a Model DynaPro-801TC dynamic light scattering instrument (Protein Solutions). rhHGF (12 nmol) alone and rhHGF (12 nmol) with heparin (60 nmol, 5 times molar excess relative to rhHGF) were dissolved in 1.0 mL of 10 mM sodium phosphate, pH 7.5, containing 1.0 and 0.45 M NaCl, respectively. After filtration using 0.22 μm membrane filters (Millipore), 150 μL of the sample solutions was introduced into the instrument controlled at 15 °C, and 16 time measurements were averaged.

RESULTS

Identification of the Heparin-Binding Sites of HGF. To identify the heparin-binding sites of HGF, rhHGF was subjected to protease digestion and chemical degradation with *o*-iodosobenzoic acid (IBA). Several fragment peptides from rhHGF obtained by the treatments showed specific heparin-binding ability (Table 1). The heparin-binding ability of the fragment peptides was expressed as their elution positions on heparin-affinity column chromatography with NaCl gradient elution. The elution of each heparin-binding peptide from the heparin-affinity column required a sufficient NaCl concentration to sever the electrostatic interaction between the peptide and the column.

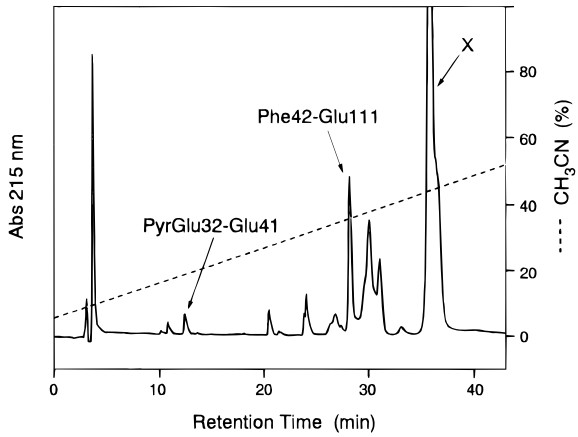

FIGURE 1: HPLC chromatogram of the V8 protease digest of rhHGF with a reversed-phase column. The column used was a Bakerbond C_8 Wide-Pore, 0.46 × 25 cm (J. T. Baker), equilibrated with 0.1% TFA in H_2O. The fragment peptides were eluted with a linear gradient of 0−70% acetonitrile in 60 min at a flow rate of 1.0 mL/min at room temperature, monitored at 215 nm. Peak X in the chromatogram is the large residual polypeptide after removal of PyrGlu^{32}−Glu^{41} (peptide A) and Phe^{42}−Glu^{111} (peptide B).

Two heparin-binding peptides, which were identified as PyrGlu^{32}−Glu^{41} (peptide A) and Phe^{42}−Glu^{111} (peptide B) by amino acid analysis and ESIMS, were obtained by the V8 protease digestion. The amino acid composition of these peptides corresponded with the theoretical values (data not shown). The observed mass of these peptides, 1320.7 and 7953.9 Da, corresponded with the theoretical values, 1320.5 and 7953.4 Da, respectively. The recovery through protease digestion and RPHPLC purification of each peptide was over 90%. The elution profile of the V8 protease digest containing these peptides on a reversed-phase column is shown in Figure 1, and the elution profile of the digest on a heparin-affinity column is shown in Figure 2a. As shown in Figure 2a, the peaks of peptide A and peptide B were observed at NaCl concentrations of 0.67 and 1.0 M, respectively, whereas the peak of HGF itself was not observed and no other peptide was bound to the heparin-affinity column. Peak X having no heparin-binding ability (Figure 2a) was identified as the large residual polypeptide after removal of peptide A and peptide B by SDS−PAGE and amino acid analysis (data not shown). Namely, other parts of HGF except for the N-terminal hairpin-loop region did not have heparin-binding ability independently. Therefore, it was indicated that the primary heparin-binding site of HGF was located in the N-terminal hairpin-loop region.

To study the heparin-binding sites of HGF in detail, peptide B was resubjected to further protease digestion by endoproteinase Asp-N. As a result of the treatment, three fragment peptides were obtained and identified as Phe^{42}−Ile^{53} (peptide C), Asp^{54}−Ala^{67} (peptide D), and Asp^{68}−Glu^{111} (peptide E) by amino acid analysis. These peptides were eluted from the heparin-affinity column at NaCl concentrations of 0.53, 0.40, and 0.75 M, respectively. Although peptide E containing the hairpin-loop structure (Cys^{70}−Cys^{96}) had the strongest heparin-binding ability among the three fragment peptides, its heparin-binding ability was reduced to 0.75 M by deletion of peptide C and peptide D from peptide B, which was eluted at 1.0 M (Figure 3). Therefore,

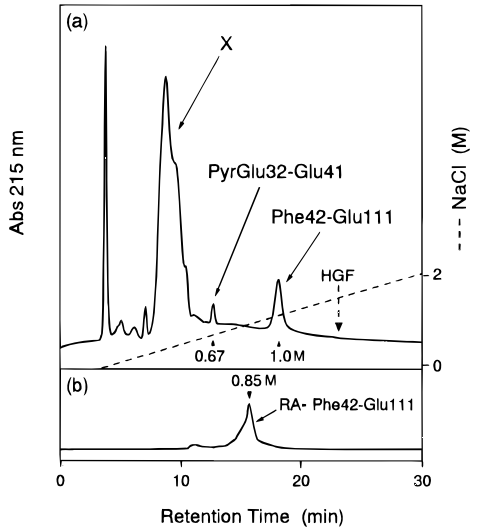

FIGURE 2: (a) HPLC chromatogram of the V8 protease digest of rhHGF with a heparin-affinity column. The column used was a TSK-gel heparin-5PW, 0.75 × 7.5 cm (Tosoh), equilibrated with 10 mM sodium phosphate, pH 7.5. The fragment peptides were eluted with a linear gradient of 0−2 M NaCl in 30 min at a flow rate of 0.7 mL/min at room temperature, monitored at 215 nm. Two heparin-binding peptides, PyrGlu^{32}−Glu^{41} (peptide A) and Phe^{42}−Glu^{111} (peptide B), were eluted at NaCl concentrations of 0.67 and 1.0 M, respectively. The elution position of HGF itself is marked in the chromatogram. Peak X is the large residual polypeptide after removal of peptide A and peptide B. (b) Reduced and alkylated peptide B (RA-Phe^{42}−Glu^{111}) was eluted at a NaCl concentration of 0.85 M, showing weaker heparin-binding ability than the original peptide B.

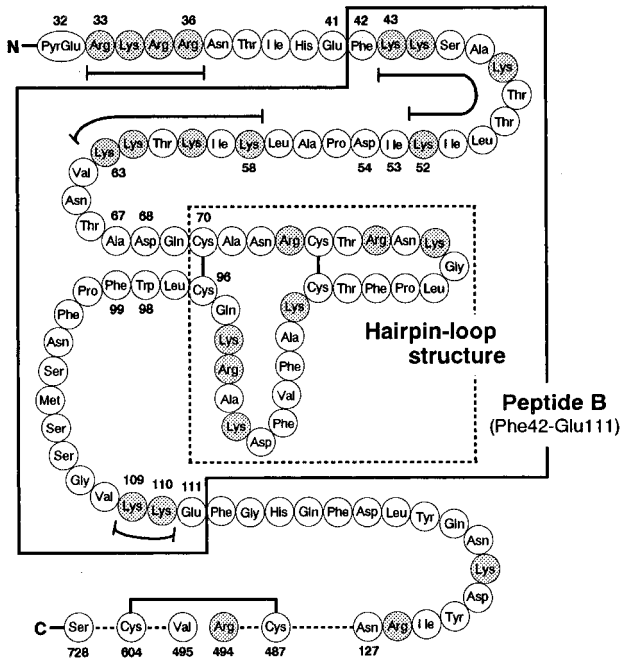

FIGURE 3: Schematic representations of the N-terminal hairpin-loop region of HGF. The basic amino acid residues are shaded, the basic amino acid rich parts are shown by lines along the sequence, the "hairpin-loop structure" is boxed with dotted lines, and "peptide B" is boxed with solid lines. The amino acid residues are numbered beginning with pyroglutamate-32, the N-terminus of mature HGF.

the hairpin-loop structure is essentially important for the heparin binding of HGF, but its neighboring part also has some contribution. Moreover, to ascertain the contribution of the N-terminal basic amino acid cluster (Arg^{33}−Arg^{36} in Figure 3) for heparin binding, a peptide containing both the positively-charged cluster and the hairpin-loop structure was prepared by chemical cleavage of rhHGF with IBA. As a result of the treatment, PyrGlu^{32}−TrpL^{98} (peptide F, the C-terminal tryptophan converted to 2,3-dihydroxytryptophan lactone) was isolated from the rhHGF. This peptide was identified by MALDI-TOFMS; the observed mass of the peptide (7860 Da) corresponded with the theoretical value (7864 Da). The peptide was eluted from the heparin-affinity column at a NaCl concentration of 0.93 M. Thus, the heparin-binding ability of peptide B and peptide F was almost equivalent for each of them and stronger than that of peptide E. Therefore, the N-terminal basic amino acid cluster (Arg^{33}−Arg^{36}) as well as the basic amino acid cluster (Lys^{109}−Lys^{110} in Figure 3) in Phe^{99}−Glu^{111}, which was found in peptide B but not in peptide F, is considered to play some role in the heparin binding of HGF.

Furthermore, to examine the significance of the two disulfide bonds (Figure 3) located in the hairpin-loop structure for heparin binding, peptide B was reduced and alkylated with a non-charge-changing reagent, iodoacetamide. In the heparin-affinity column chromatography of reduced and alkylated peptide B (RA-peptide B), the peak of the original peptide B was not detected (Figure 2b). In addition, on amino acid analysis of RA-peptide B, the peak of carboxymethylcysteine converted from acetamidated cysteine through hydrolysis was observed, while cystine peak was not detected. These results showed that the four cysteine residues constructing the two disulfide bonds in the original peptide B were completely reduced and acetamidated. The acetamidation of the cysteine residues of peptide B was also observed by ESIMS; the observed mass of RA-peptide B, 8179.0 Da (approximately 50%, estimated by the signal intensity) and 8235.0 Da (approximately 50%), corresponded with peptide B acetamidated at four and five positions, respectively. Thus, four or five acetamides were introduced in RA-peptide B (the fifth position acetamidated has not yet been identified). RA-peptide B was eluted from the heparin-affinity column at a NaCl concentration of 0.85 M (Figure 2b), showing weaker heparin-binding ability than the original peptide B, which was eluted at 1.0 M. Therefore, it was indicated that the hairpin-loop structure formed by the two disulfide bonds contributed to the heparin-binding properties of peptide B.

The heparin-binding peptides isolated in this study are shown to demonstrate a comparison of the strength of their heparin-binding ability in Table 1. All of them were derived from the N-terminal hairpin-loop region (PyrGlu^{32}−Asn^{127}) of HGF and contained basic amino acid residues contributing to their heparin-binding ability.

CD Spectra of Peptide B with or without Heparin. The CD spectra of peptide B with or without heparin are shown in Figure 4. The CD contribution of heparin was subtracted out of the spectra. While peptide B alone was in a random structure, that is, the specific large negative molar ellipticity ([θ]) between 197 and 205 nm was observed, the peptide with heparin presented a CD spectrum for a β-sheet-like structure, that is, the large negative molar ellipticity in the lower wavelength region was reduced, and the minimum of the molar ellipticity was shifted to a higher wavelength. Therefore, it was indicated that the secondary structure of peptide B changed upon heparin binding.

Dynamic Light Scattering of HGF with or without Heparin. The hydrodynamic radius (R_h) of HGF was 3.3 nm in

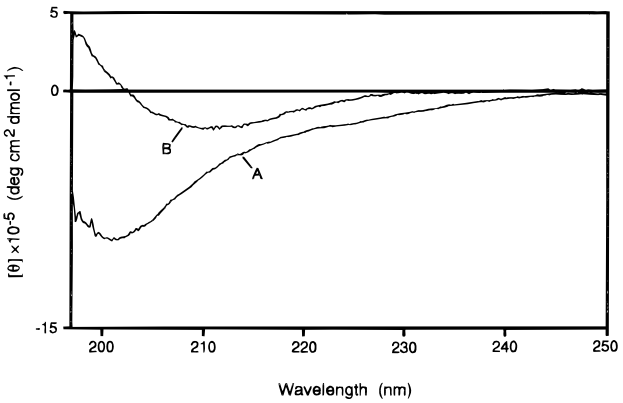

FIGURE 4: CD spectra of Phe[42]−Glu[111] (peptide B) with or without heparin. (A) 12 μM peptide B alone and (B) 12 μM peptide B with 12 μM heparin dissolved in PBS were measured by scanning 10 times from 250 to 197 nm at 25 °C. The solvent background and CD contribution of heparin were subtracted out of the spectra.

sodium phosphate buffer, while the R_h of HGF with heparin was 5.3 nm in the same buffer. Thus, the average size of HGF was enlarged by the addition of heparin. Therefore, it was indicated that HGF could self-associate in the presence of heparin in the buffer. The apparent molecular weight (M_w) was calculated from the R_h measured by dynamic light scattering using an empirically derived relationship between the R_h and M_w values for a number of well-characterized globular proteins in buffered aqueous solutions (Bloomfield, 1985). The apparent M_w values of HGF alone and HGF with heparin were calculated as 53 500 and 170 000 by the R_h vs M_w relationship, respectively.

DISCUSSION

We directly studied the heparin-binding sites of HGF by isolating the heparin-binding peptides from rhHGF with limited proteolyses and chemical degradation. It was shown that all of the isolated heparin-binding peptides were derived from the N-terminal hairpin-loop region (PyrGlu[32]−Asn[127]) of HGF, and the peptides contained basic amino acid residues which were required for their heparin-binding ability (Table 1 and Figure 3). The N-terminal hairpin-loop region of HGF has been considered as the heparin-binding site (Okigaki *et al.*, 1992; Lokker & Godowski, 1993; Mizuno *et al.*, 1994). Although Mizuno *et al.* suggested that the second kringle (Cys[211]−Cys[288]) of HGF was also essential for heparin binding (Mizuno *et al.*, 1994), no heparin-binding peptides were obtained from other parts of HGF except for the N-terminal hairpin-loop region in this study. Therefore, it is considered that the N-terminal hairpin-loop region is the primary heparin-binding site of HGF and the contribution of other parts of HGF to its heparin-binding ability is limited.

We further studied the heparin-binding ability of the N-terminal hairpin-loop region (PyrGlu[32]−Asn[127]) in detail. As shown in Figure 3, the potential heparin-binding sites in the region are the five basic amino acid rich parts: the hairpin-loop structure (Cys[70]−Cys[96], containing seven basic amino acid residues), the N-terminal cluster (Arg[33]−Arg[36]), and three other parts (Lys[43]−Lys[52], Lys[58]−Lys[63], and Lys[109]−Lys[110]). The heparin-binding ability is considered to require these basic amino acid clusters. The peptide showing the strongest heparin-binding ability among the isolated peptides in this study was Phe[42]−Glu[111] (peptide B), which contained the hairpin-loop structure and three other basic amino acid rich parts (Lys[43]−Lys[52], Lys[58]−Lys[63], and Lys[109]−Lys[110]).

From the Asp-N fragmentation and the reduction and alkylation of peptide B, it was observed that the hairpin-loop structure formed by the two disulfide bonds was the most important site for heparin binding of HGF. The observation of the heparin-binding ability of PyrGlu[32]−TrpL[98] (peptide F) and PyrGlu[32]−Glu[41] (peptide A) indicated that the contribution of the N-terminal cluster (Arg[33]−Arg[36]) to the heparin binding of HGF was also important. The contributions of three other basic amino acid rich parts are weak but not negligible. Consequently, it is considered that these basic amino acid rich parts additively contribute to the heparin binding of HGF.

In this study, peptide B is considered to be the most suitable model peptide for the heparin-binding site of HGF. The CD spectra of the peptide indicate that it forms a complex with heparin, in which the secondary structure of the peptide is a β-sheet-like structure (Figure 4). The complex of the peptide and heparin is considered to be formed by electrostatic interaction between orderly arranged basic amino acid residues in the peptide and the specific sulfated saccharides in the heparin molecule. It is likely that the conformational change in peptide B to the β-sheet-like structure is caused by an extension of the peptide chain accompanied by complex formation with heparin. Similar to our investigation, it was reported that a synthetic peptide for the heparin-binding site of antithrombin III (AT III) showed a conformational change from a random structure to a β-sheet-like structure during the formation of the peptide−heparin complex (Lellouch & Lansbury, 1992). The conformational change in peptide B upon heparin binding is similar to that of the AT III model peptide. The tendency for the negative molar ellipticity between 197 and 205 nm to be reduced upon heparin binding was also observed in the CD spectrum of HGF itself (data not shown). Thus, it is likely that a similar conformational change from a random structure to a β-sheet-like structure is induced in the heparin-binding site of HGF during the formation of the complex with heparin or heparin-like molecules.

From the dynamic light scattering analysis, the apparent M_w of HGF clearly increased with the addition of heparin (from 54 000 to 170 000). The apparent M_w value of 54 000 indicates that HGF without heparin exists in the monomeric form. Since the M_w value here is interpreted from the R_h value observed by dynamic light scattering, as mentioned under Results, it does not necessarily agree with the theoretical value. From the observation that the molar ratio of heparin (M_w 4000−6000)/HGF in the complex isolated by size exclusion chromatography is 2−3 (unpublished data), it is indicated that the increase in the apparent M_w of HGF is caused by oligomerization of HGF accompanied with heparin and not by formation of a complex composed of a monomer of HGF and a number of heparin molecules. Thus, the HGF−heparin complex may consist of a dimer or trimer of HGF and several heparin molecules. Similar to our evidence, Zioncheck *et al.* demonstrated that heparin or heparin-like molecules induced and stabilized HGF oligomerization based on size exclusion chromatography and analytical ultracentrifugation (Zioncheck *et al.*, 1995). They proposed a model in which the HGF oligomerization facilitated the c-Met-dependent mitogenesis. With regard to fibroblast growth factors (FGFs), it was reported that the

heparin-like molecules induced FGF dimerization and subsequent FGF−receptor dimerization and activation (Spivak-Kroizman *et al.*, 1994; Schlessinger *et al.*, 1995). These reports also support the model of HGF oligomerization upon heparin binding. Based on these reports and our evidence, it is considered that the conformational change in the N-terminal hairpin-loop region of HGF upon heparin binding induces the oligomerization of HGF.

In this study, we directly assigned the region involved in the heparin-binding properties of HGF and isolated the heparin-binding peptides from HGF. One of the isolated peptides corresponding to the hairpin-loop structure showed the strongest heparin-binding ability among the isolated peptides and a conformational change from a random structure to a β-sheet-like structure during the formation of a complex with heparin. Moreover, it was observed that HGF formed an oligomer in the presence of heparin. These results suggest that the conformational change in the heparin-binding site of HGF during binding to heparin or heparin-like molecules may induce the oligomerization of HGF.

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