Preparation and Characterization of Functional Domains of Porcine Plasma Fibronectin

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Summary

The relations between surface hydrophobicities and binding properties of the functional domains of porcine plasma fibronectin were investigated. Porcine and human plasma fibronectins were adsorbed on a hydrophobic column with butyl or phenyl ligands in the presence of 0.5 M ammonium sulfate, and recovered in a single peak by decreasing the concentration of ammonium sulfate to 0 M, indicating that both fibronectins have very high surface hydrophobicities. Limited proteolysis of porcine plasma fibronectin with thermolysin yielded five fragments, of 140–150 kDa, 43 kDa, 25 kDa, 17 kDa and 14 kDa. Analysis of the digests by high performance hydrophobic interaction chromatography and affinity chromatography on heparin- or gelatin-Sepharose, together with previous results, indicated that the 140–150 kDa fragment has cell-attachment and heparin-binding domains, the 43 kDa fragment a collagen-binding domain, the 25 kDa fragment a heparin-binding domain, the 17 kDa fragment a fibrin-binding domain and the 14 kDa fragment a heparin-binding domain. The three heparin-binding fragments were found to have a wide range of surface hydrophobicities, of which the 140–150 kDa fragment had the lowest, the 25 kDa fragment higher, and the 14 kDa fragment the highest among all the fragments. The 43 kDa and 17 kDa fragments had surface hydrophobicities as high as that of fibronectin. Carbohydrate analysis and lectin binding studies using horseradish peroxidase conjugated lectins showed that porcine plasma fibronectin has 10 biantennary *N*-linked carbohydrate chains on the 43 kDa and 140–150 kDa fragments. It is noteworthy that the 43 kDa collagen-binding fragment contributes to the high surface hydrophobicity of intact fibronectin in spite of the high content of carbohydrates (60% of the total carbohydrates).

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Abbreviations: HRP, horseradish peroxidase; LCA, *Lens culinaris* agglutinin; Pea, *Pisum sativum* agglutinin; PHA-L, *Phaseolus vulgaris* agglutinin-L; alloA, *Allomyrina dichotoma* agglutinin; ConA, concanavalin A; WGA, wheat germ agglutinin; HPLC, high-performance liquid chromatography; HP-HIC, high-performance hydrophobic interaction chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacryl amide gel electrophoresis.

Introduction

Plasma fibronectin is a high molecular weight glycoprotein which is structurally, chemically and antigenically closely related to a cellular fibronectin. It consists of two subunits of molecular weights close to 230 kDa, held together by two disulfide bridges located near their carboxyl termini [1]. Fibronectin is involved in a large variety of cell functions such as cell-cell and cell-substrate adhesion, cell spreading, cellular motility, differentiation, opsonization and wound healing [2]. These pleiotropic functions of fibronectin are primarily based on its ability to bind to a number of biomolecules including collagen (gelatin), glycosaminoglycans, fibrin, DNA and cell surface receptors. Intensive studies in various laboratories have revealed that fibronectin has a complex molecular structure composed of multiple specific binding sites [3]. Some of these binding sites have been found in specific fragments (structural domains) generated by limited proteolysis, and their localization within the fibronectin polypeptide has been determined [4]. Furthermore, the complete amino acid sequence of the fibronectin has been determined from cloned cDNA [5, 6] and protein levels [1, 7–10], and a high degree of sequence homology has been found in fibronectins from various species [11]. It has also been demonstrated that fibronectin has the essential cell attachment site consisting of Arg-Gly-Asp(-Ser) (RGD(S)) [12] which is common to another cell adhesive glycoprotein, vitronectin [13]. Therefore, it has been assumed that the oligopeptide specifically binds to complementary structures on the cell surface receptors that belong to the integrin superfamily of proteins [4, 14]. It has also been assumed that fibronectin binds electrostatically to heparin, simply because the 24 kDa and 16 kDa heparin-binding domains of hamster fibronectin were shown to have high pI values, 8.40–8.60 and 8.50–9.00, respectively [15]. However, the precise properties of the specific binding between these domains and biomolecules have not been completely elucidated, even for the bindings of fibronectin with collagen (gelatin) and heparin, which have been efficiently utilized for the affinity chromatographic purification of fibronectin. Generally, the specific bindings consist either of hydrogen bonds, van der Waals, electrostatic and hydrophobic interactions. The present paper describes the hydrophobic properties of fibronectin and its functional domains which may contribute to the specific bindings to biomolecules.

Materials and Methods

Materials

Materials obtained commercially included thermolysin (type X) from

Sigma Chemical Co., gelatin (bovine skin) and heparin (porcine intestinal mucosa) from Wako Pure Chemical Ind. Ltd. (Osaka, Japan), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and sodium cyanoborohydride ($NaCNBH_3$) from Nakarai Chemicals Ltd. (Kyoto, Japan), Sepharose 4B from Pharmacia Fine Chemicals, TSKgel Phenyl-5PW and Butyl Toyopearlpak from Tosoh Co. Ltd. (Tokyo, Japan), 10–20% polyacrylamide continuous gradient gel from Daiichi Pure Chemicals (Tokyo, Japan), horseradish peroxidase (HRP) from Toyobo Co. Ltd. (Osaka, Japan), *Lens culinaris* agglutinin (LCA), *Pisum sativum* agglutinin (Pea), and *Phaseolus vulgaris* agglutinin-L (PHA-L) from Honen Oil Ltd. (Tokyo, Japan), and *Allomyrina dichotoma* agglutinin (alloA) from Cosmo Bio Ltd. (Tokyo, Japan). Concanavalin A (ConA) and wheat germ agglutinin (WGA) were prepared as described previously [16]. Heparin-Sepharose 4B (20 mg of heparin/ml of packed gel) was prepared by coupling the carboxyl groups of heparin with amino-Sepharose with the aid of EDC as described previously [17]. Gelatin-Sepharose 4B (36 mg of gelatin/ml of packed gel) was prepared by reductive amination [18] between the amino groups of gelatin and formyl-Sepharose, which was obtained by periodate oxidation of epoxy-activated Sepharose [19] as described previously. Porcine plasma was obtained by centrifugation at 9000 rpm for 20 min of fresh blood anticoagulated with EDTA (1 mg/ml) and human plasma was kindly donated by a Japanese blood bank. These were kept at −20°C until use. Fibronectin was isolated from the plasma by gel chromatography on Sepharose 4B and subsequent affinity chromatography on gelatin-Sepharose essentially according to the method of Isemura et al. [20]. On SDS-polyacrylamide gel electrophoresis under the reduced conditions, the fibronectin preparation gave a single band corresponding to a 230 kDa protein like human plasma fibronectin and no glycine-rich gelatin-binding protein [21].

High-performance Hydrophobic Interaction Chromatography (HP-HIC)

High-performance liquid chromatography (HPLC) was performed using a Tosoh HPLC system model HLC-803 equipped with a column of TSKgel Phenyl-5PW (150×21.5 mm) or Butyl-Toyopearlpak (200×22 mm), a gradient generator model GE-4 and a variable wavelength UV detector model UV-8. The fibronectin sample and its proteolytic digests were dissolved in 0.5 M ammonium sulfate in 10 mM Tris-HCl buffer (pH 7.5) and clarified by centrifugation before application to the column. Elution was performed with a 40 min or 60 min linear gradient of ammonium sulfate from 0.5 M to 0 M in 10 mM Tris-HCl buffer (pH 7.5) at a flow rate of 4 ml/min at room temperature. The effluent was monitored at 280 nm.

Enzymatic Digestion

Thermolysin digestion of plasma fibronectin was performed essentially according to Sekiguchi and Hakomori [22]. Fibronectin dissolved in 25 mM Tris-HCl buffer, (pH 7.6) containing 0.5 mM EDTA, 50 mM NaCl and 2.5 mM $CaCl_2$, was digested using an approximate weight ratio of enzyme to substrate of 1 : 200, at 22°C, 25°C or 37°C. The digestion was terminated by adding 0.2 M EDTA to give a final concentration of 5 mM EDTA. For the preparative scale, the digestion was performed at 22°C for 4 h.

Affinity Chromatography

The digests of fibronectin were applied to a column (bed volume, 2 ml) of gelatin- or heparin-Sepharose equilibrated with 10 mM Tris-HCl buffer, (pH 7.5) and extensively washed with the same buffer. The fragments adsorbed on a gelatin-Sepharose column were eluted with 4 M urea in the same buffer, and those adsorbed on a heparin-Sepharose column were eluted sequentially with 0.145 M NaCl, 0.2 M NaCl and 2.0 M NaCl in the same buffer. Fractions of 2 ml were collected and assayed for protein by absorbance at 280 nm.

Amino Acid and Carbohydrate Analysis

Amino acid and amino sugar analyses were performed with a Hitachi 835 amino acid analyzer as described previously [23]. Sialic acid content was estimated by the resorcinol reaction [24]. Sialic acid composition was analyzed after hydrolysis with 0.1 M H_2SO_4 at 80°C for 30 min, by ion-exclusion HPLC [25] with a column of Shodex Ionpak KC-811 (300×8 mm) in which the effluent was monitored at 205 nm [26]. Neutral sugar composition was analyzed by HPLC with a column of Finepak GEL SA-121 as described previously [27].

SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis of fibronectin and its proteolytic digests was performed in the presence of 1% SDS according to the method of Laemmli [28] using 5%, 7.5%, 9.5%, 12.5% or gradient (10–20%) acrylamide gels. The samples were treated with 5% 2-mercaptoethanol in a boiling bath for 10 min. Molecular weights were estimated using Sigma molecular marker. The protein bands were stained with Coomassie brilliant blue R-250 and traced densitometrically at 620 nm using a Hiranuma model HAD-501 densitometer.

Interaction of Fibronectin and Its Domains with HRP-lectins

ConA, WGA, LCA, PHA-L, alloA and Pea lectin were coupled with HRP in the presence of corresponding inhibitory sugars (0.15 M) according

to the method previously reported [29]. HRP-conjugated lectins (HRP-lectins) were purified by gel chromatography on a Toyopearl HW-55 column (74×3 cm) using 10 mM acetate buffer (pH 7.0) containing 0.15 M NaCl as a column buffer. The preservation of the lectin activity of all the HRP-lectins was confirmed by the reactivity toward porcine thyroglobulin. HRP-lectins were diluted to 1–20 μg/ml with buffer A (10 mM Tris-HCl buffer (pH 7.5) containing 0.05% Tween 20 and 0.15 M NaCl) to get a suitable color development. Proteins on a gel were transferred to nitrocellulose sheets in 25 mM Tris, 192 mM glycine, 20% methanol (v/v), at 250 mA for 1 h and 300 mA for 2 h. The nitrocellulose sheet with blotted

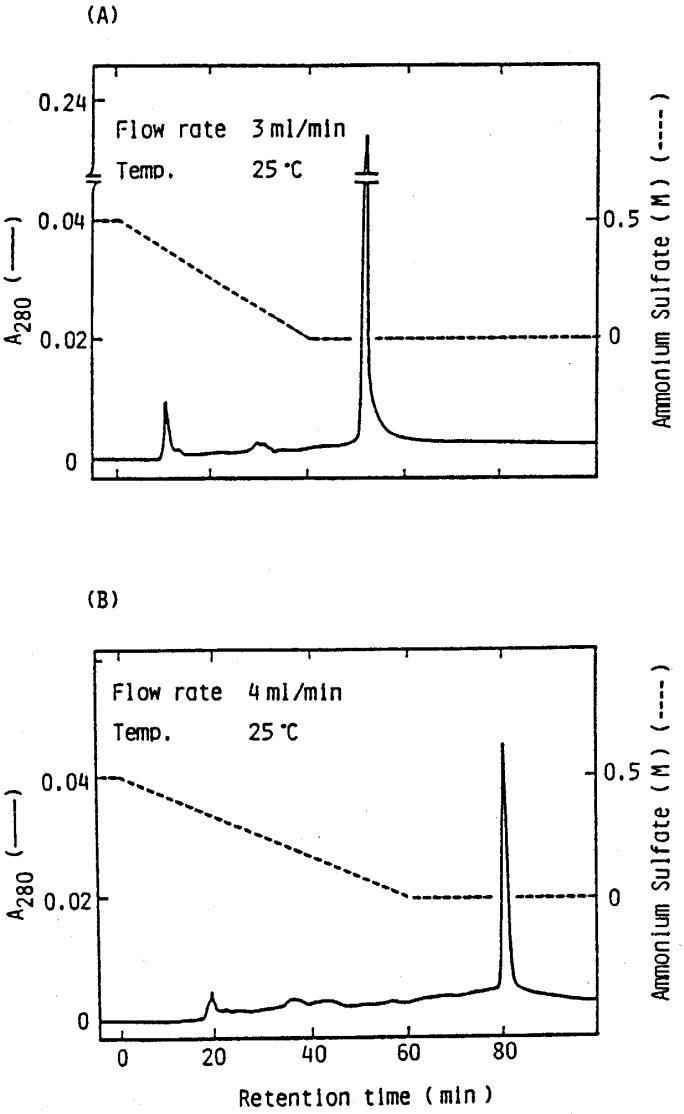

Fig. 1. HP-HIC of porcine plasma fibronectin. Porcine plasma fibronectin (0.5–1 mg) dissolved in 0.5 M ammonium sulfate in 10 mM Tris-HCl buffer (pH 7.5) was loaded on columns of TSKgel Phenyl-5PW (A) and Buthyl-Toyopearlpak (B). Chromatographic conditions are described in the text.

proteins was washed twice in 30 ml of buffer A and then cut by lane and soaked in each HRP-lectin solution for 1 h at room temperature. The treated nitrocellulose sheet was washed four times in buffer A, and then reacted with 4-chloro-1-naphthol or 3, 3′-diaminobenzidine-HCl/H_2O_2 solution. After staining, the nitrocellulose sheet was washed several times with water and dried.

Results

Hydrophobicity of Intact Plasma Fibronectin

On HP-HIC, porcine and human plasma fibronectins were completely adsorbed on the hydrophobic adsorbent with phenyl or butyl ligand, in 10 mM Tris-HCl buffer (pH 7.5) containing 0.5 M ammonium sulfate, and were recovered in a single peak by elution with 10 mM Tris-HCl buffer (pH 7.5) as shown in Fig. 1. The results indicate that both plasma fibronectins have very high surface hydrophobicities.

Proteolytic Fragmentation of Porcine Plasma Fibronectin with Thermolysin

The time course of digestion of porcine plasma fibronectin with thermolysin is shown in Fig. 2. At an early stage of digestion at 22°C, three major fragments, the doublet 150–140 kDa, 60 kDa and 25 kDa fragments,

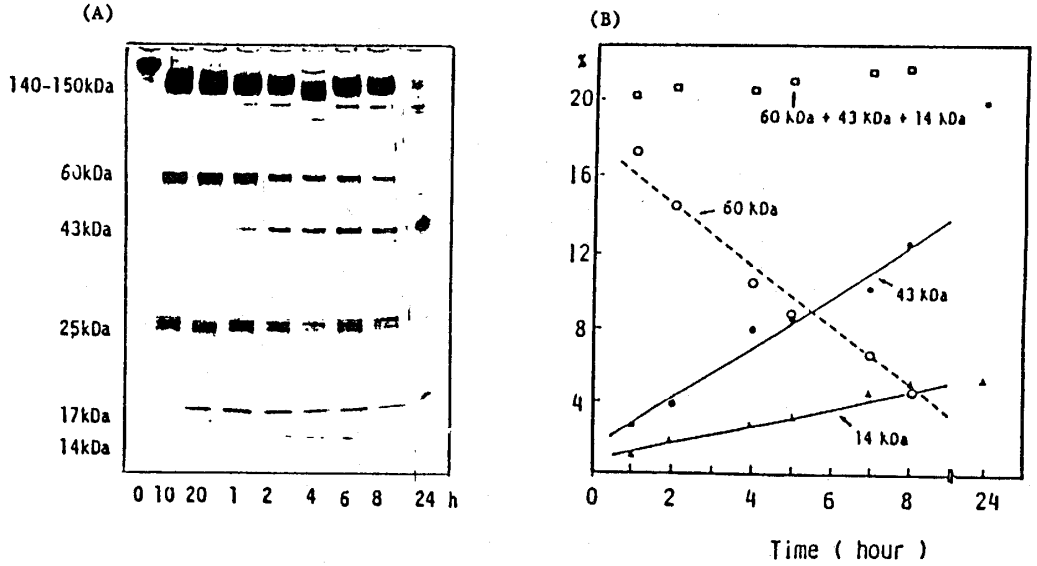

Fig. 2. Time course of thermolysin digestion of porcine plasma fibronectin at 22°C. The thermolysin digests of porcine plasma fibronectin (10 μg) were subjected to SDS-PAGE on a 10-20% polyacrylamide gel in the presence of 2% mercaptoethanol. The protein bands were stained with Coomassie brilliant blue R-250 (A). The mobilities of molecular weight markers are indicated at left. The relative amount of each protein band was calculated from the densitometry of the gel (B).

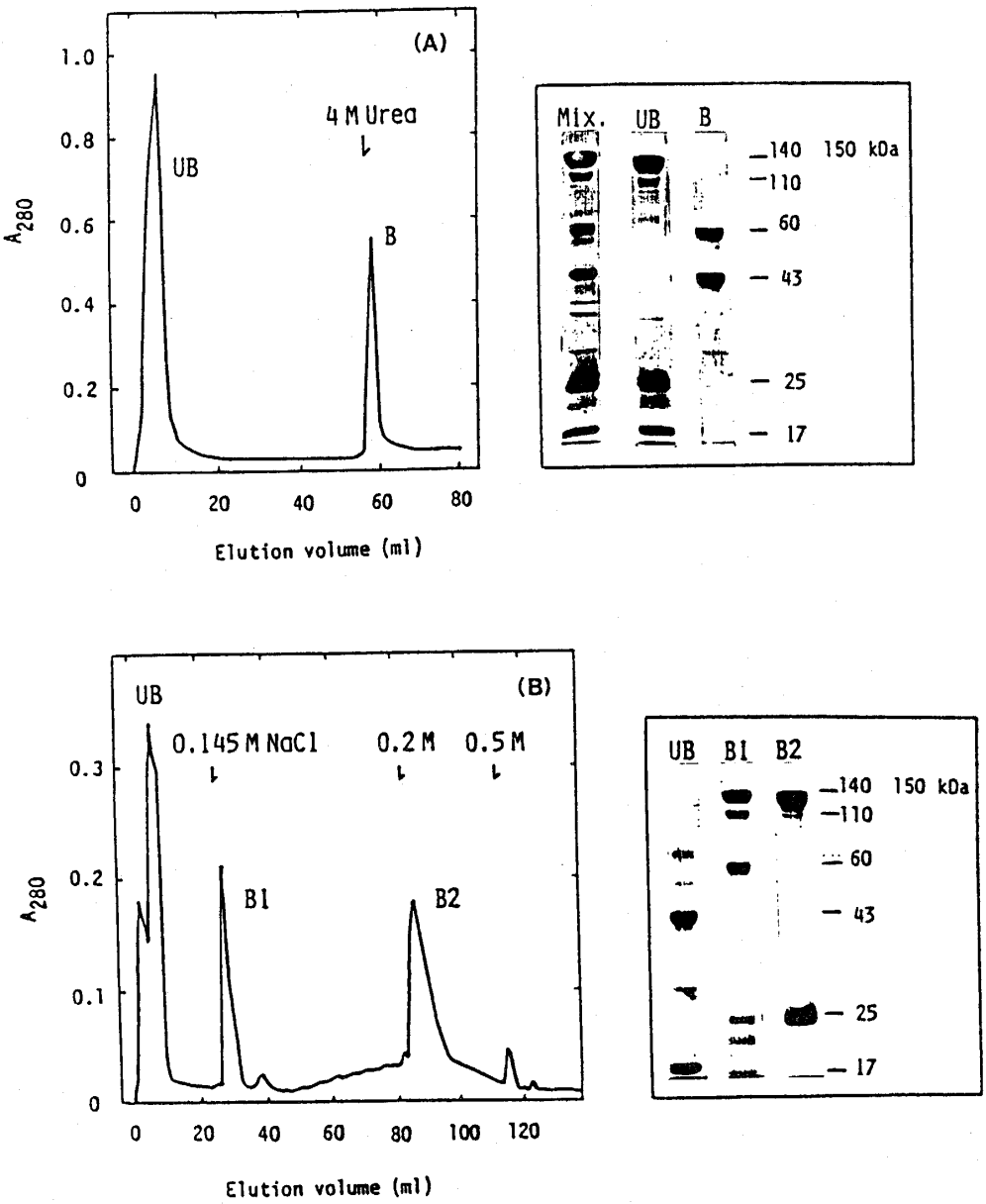

Fig. 3. Affinity chromatography of porcine plasma fibronectin digestion mixture on gelatin-Sepharose 4B (A) and heparin-Sepharose 4B (B). The thermolysin digests of porcine plasma fibronectin were applied to a column (bed volume, 2 ml) of gelatin- or heparin-Sepharose equilibrated with 10 mM Tris-HCl buffer (pH 7.5). The elution conditions are described in the text. The patterns of SDS-PAGE of the fraction on a 9.5% acrylamide gel in the presence of 2% mercaptoethanol are shown at right side on each chromatogram. The mobilities of molecular weight markers are indicated in the right lane. Mix., the digestion mixture; UB, unbound fraction; B, the bound fraction on a gelatin-Sepharose column; B1, the fraction bound and eluted by 0.145 M NaCl on a heparin-Sepharose column; B2, the fraction bound and eluted by 0.2 M NaCl on a heparin-Sepharose column.

and one minor fragment, 17 kDa fragment, were observed (Fig. 2A). With further incubation, the 60 kDa fragment gradually disappeared and 43 kDa and 14 kDa fragments newly appeared as shown in Fig. 2B. After digestion for 24 h, the 60 kDa fragment disappeared completely. The results indicate that the 60 kDa fragment is composed of 43 kDa and 14 kDa fragments. The 140–150 kDa fragments were also found to be degraded to 110 kDa and 40 kDa fragments. On digestion with thermolysin at 25°C or 37°C, minor and low molecular weight fragments were produced in addition to the above-mentioned fragments. The fragments obtained by digestion at 22°C for 4 h were investigated further.

Binding Properties of Fragments

To identify collagen-binding and heparin-binding fragments, the thermolysin digests of porcine fibronectin were subjected to affinity chromatography on a gelatin-Sepharose column and a heparin-Sepharose column, respectively. The 43 kDa and the 60 kDa fragments were adsorbed on the gelatin column, but not the 140–150 kDa, 110 kDa, 25 kDa and 17 kDa fragments (Fig. 3A). The results indicate that the 60 kDa and 43 kDa fragments share the same gelatin (collagen) binding domain. On the other hand, 140–150 kDa, 110 kDa, 25 kDa and 14 kDa fragments were adsorbed on the heparin column, but not 43 kDa and 17 kDa fragments (Fig. 3B). The results indicate that there are three kinds of heparin-binding domains in porcine plasma fibronectin. The 25 kDa fragment has one of the heparin-binding domains. The 140–150 kDa and 110 kDa fragments share another heparin-binding domain and also the same cell attachment domain. And the 60 kDa and 14 kDa fragments share the other heparin-binding domain.

Hydrophobicity of Porcine Plasma Fibronectin Fragments

The thermolysin digests of porcine plasma fibronectin were separated into four fractions (I–IV) upon HP-HIC on TSKgel Phenyl 5PW with decreasing ammonium sulfate concentration (Fig. 4A). The order of elution of the fractions directly reflects their surface hydrophobicities, that is, fraction I has the lowest surface hydrophobicity and fraction IV the highest. Each fraction was analyzed by SDS-PAGE as shown in Fig. 4B. Fraction I gave no significant bands, suggesting that it contains very small fragments which cannot be detected in this system. Fraction II contained the 140–150 kDa cell-attachment/heparin-binding fragments. Fraction III contained the 25 kDa heparin-binding fragment. Fraction IV contained the 43 kDa gelatin-binding fragment and the 17 kDa fibrin-binding fragment. Among the fragments obtained on the HP-HIC, the surface hydrophobicity was lowest in the 140–150 kDa cell-attachment/heparin-binding fragments, higher in the 25 kDa heparin-binding fragment, and

highest in the 43 kDa collagen binding and the 17 kDa fibrin binding fragments, being as high as in intact fibronectin.

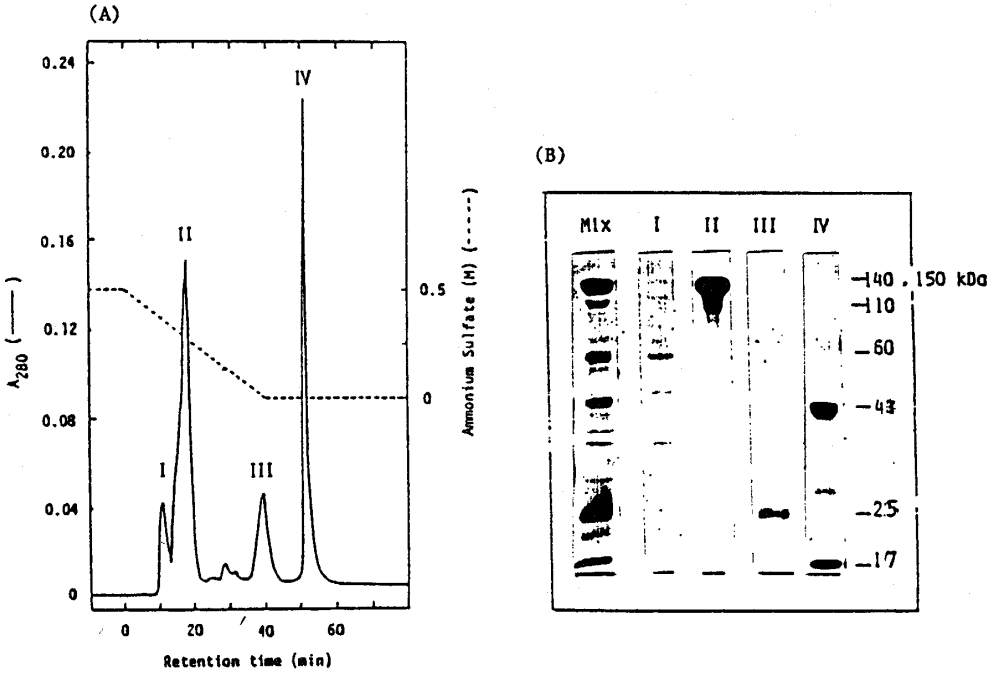

Fig. 4. HP-HIC of porcine plasma fibronectin digestion mixture on TSKgel Phenyl-5PW (A) and SDS-PAGE of peak I–IV (B). The thermolysin digest of porcine plasma fibronectin dissolved in 0.5 M ammonium sulfate in 10 mM Tris-HCl buffer (pH 7.5) was loaded on the column of TSKgel Phenyl-5PW. Elution was performed as described in the text. The digestion mixture and peak I–IV were subjected to SDS-PAGE on 9.5% acrylamide gel in the presence of 2% mercaptoethanol.

Isolation of 43 kDa Collagen Binding Domain from Fraction IV

To isolate the 43 kDa collagen-binding fragment, fraction IV from HP-HIC on TSKgel-Phenyl 5PW was applied to a gelatin-Sepharose column. The 17 kDa fragment was found in the passed-through fractions, and the 43 kDa fragment bound to the column and was recovered by elution with 4 M urea.

Amino Acid and Carbohydrate Composition of Porcine Plasma Fibronectin and Its 43 kDa Collagen-binding Domain

Amino acid compositions of porcine plasma fibronectin and its purified collagen-binding 43 kDa domain were similar to those reported for porcine plasma fibronectin by Isemura et al. [20] and those obtained from human [30] bovine [31] and hamster [32] fibronectins. The results of the porcine 43 kDa fragment were also similar to those of other 43 kDa fragments [11].

Porcine plasma fibronectin and its 43 kDa collagen-binding domain had very similar carbohydrate compositions, as shown in Table I. They had D-glucosamine, D-mannose, D-galactose, L-fucose and *N*-acetylneuraminic acid

Table I. Carbohydrate composition of porcine plasma fibronectin and its 43 kDa collagen-binding domain

Monosaccharide	230 kDa Subunit	43 kDa Domain
D-Glucosamine	19.5 (3.6)	13.1 (4.3)
D-Mannose	16.2 (3.0)	9.1 (3.0)
L-Fucose	4.4 (0.8)	2.9 (1.0)
D-Galactose	12.9 (2.4)	8.1 (2.7)
D-Glucose	0.7 (0.1)	0.4 (0.1)
N-Acetylneuraminic acid	8.5 (1.7)	— —

Values are mol per mol subunit or domain. Molar ratios calculated by taking the value of mannose as 3.0 are shown in parentheses.

in an approximate molar ratio of 4 : 3 : 2 : 1 : 2 and no detectable amounts of D-galactosamine and *N*-glycolylneuraminic acid. These results were consistent with those reported by Isemura et al. [20] except for the D-glucose content, which seems to be a contaminant. The 43 kDa fragment contained about 60% of the carbohydrates of the fibronectin.

Interacion of Porcine Plasma Fibronectin and Its Fragments with HRP-lectins

Porcine plasma fibronectin and its thermolysin fragments were transferred, after SDS-PAGE, to nitrocellulose sheets and allowed to react with HRP-lectins. Three kinds of fragments corresponding to the 140–150 kDa cell-attachment/heparin binding fragments, the 43 kDa and 60 kDa collagen-binding fragments, as well as the intact fibronectin, showed high affinity to HRP-ConA, HRP-WGA, HRP-Pea, HRP-LCA and HRP-alloA, and low but significant affinity to HRP-UEA-I, and no affinity to HRP-PHA-L. These results combined with the carbohydrate composition, support the conclusion that porcine plasma fibronectin molecule has 10 complex type, biantennary fucosylated *N*-glycosidic carbohydrate chains. The findings that the fibronectin bound to LCA and UEA-I indicate that the fucosyl residues linked to the innermost *N*-acety-D-glucosamine residue bound to asparagine [33, 34]. In addition, the fibronectin strongly reacted with allo-A. This result suggests that the *N*-acetylneuraminyl residues were linked to the galactose residues by $\alpha(2\rightarrow6)$ linkages [35] at the non-reducing terminal ends.

Discussion

In this study, we have demonstrated by using HP-HIC that porcine and human plasma fibronectins have very high surface hydrophobicities. This is consistent with the previous report that human plasma fibronectin

was adsorbed on a octyl-Sepharose clumn [36]. In this study, we have further elucidated the hydrophobic properties of the functional domains of the fibronectin. The biological multifunctionality of fibronectin is attributable to the specific binding properties of its structural domains with a variety of biomolecules, and its hydrophobic properties would be expected to contribute to these specific binding properties. To elucidate the relation between the binding properties and surface hydrophobicity of each domain, the proteolytic fragments of porcine plasma fibronectin were investigated by HP-HIC.

First, the porcine fibronectin was subjected to limited proteolysis with thermolysin. Analysis of the digestion products by SDS-PAGE showed that the enzyme cleaves porcine fibronectin mainly into five fragments, of 140–150 kDa, 43 kDa, 25 kDa, 17 kDa and 14 kDa fragments. The 60 kDa fragment observed at an early stage of the digestion was cleaved into 43 kDa and 14 kDa fragments by further digestion. Thus the cleavage of porcine plasma fibronectin by thermolysin is essentially the same as those of human and hamster plasma fibronectins [9, 15] but seems to be somewhat depressed, with the linkage between the 43 kDa collagen-binding domain and the 14 kDa heparin-binding domain being particularly resistant to the enzyme. This might be due to the presence of fucosylated carbohydrate chains on the 43 kDa collagen-binding domain which are not found on the other fibronectins.

Second, the digestion products were subjected to affinity chromatography on gelatin- and heparin-Sepharose. Based on the results and those of previous studies, the binding properties of each fragment were identified as follows: the 140–150 kDa fragment includes the cell-attachment domain and one of the heparin-binding domains (Hep II), the 43 kDa fragment has the collagen-binding domain, the 25 kDa fragment has the amino terminal heparin-binding domain (Hap I), the 17 kDa fragment has the fibrin-binding domain and the 14 kDa fragment has the third heparin-binding domain (Hep III). In addition, the 60 kDa fragment composed of 43 kDa and 14 kDa fragments includes the binding activities for both collagen and heparin.

Third, the digestion products were subjected to HP-HIC on TSKgel Phenyl-5PW. Most of the fragments were successfully separated into four fractions (I, II, III and IV) by elution with decreasing ammonium sulfate concentration. These results show that the fibronectin fragments have different surface hydrophobicities, which increase with the order of the elution of the four fractions: I<II<III<IV. Based on these results and the analysis of the four fractions by SDS-PAGE, it was proved that the 140–150 kDa cell-attachment/heparin-binding domain has the lowest surface hydrophobicity, the 25 kDa heparin-binding domain has higher, and the 43

kDa collagen-binding and 17 kDa fibrin binding domains have the highest. Moreover the 60 kDa and 14 kDa fragments seem to have much higher surface hydrophobicities than the collagen-binding domain, because they were not eluted from the hydrophobic column under the conditions adopted in this study.

Concerning the cell-attachment domain, Pierschbacher and Ruoslahti [12] have stated that "the peptide GRGDSP in the cell attachment promoting site of the fibronectin molecule may be a β-turn which probably forms a hydrophilic loop at the surface of the molecule and is thus available to interact with the cell surface". This is consistent with our finding that the 140–150 kDa domain has low surface hydrophobicity.

It was surprising that the 43 kDa collagen-binding domain has the highest surface hydrophobicity among the fragments obtained by HP-HIC, although this domain contains 60% of the hydrophilic carbohydrate chains on the fibronectin molecule. The results indicate that the polypeptide portion is responsible for this high surface hydrophobicity. Collagen binding domains of porcine and human plasma fibronectin have very similar amino acid compositions, and therefore seem to have almost the same amino acid sequences. The hydrophobicity profile of the collagen-binding domain analyzed by the method of Hopp and Woods [37] using the amino acid sequence [5] reported for human plasma fibronectin, consisting of four domains of type I homology and two domains of type II homology [1, 11], was found

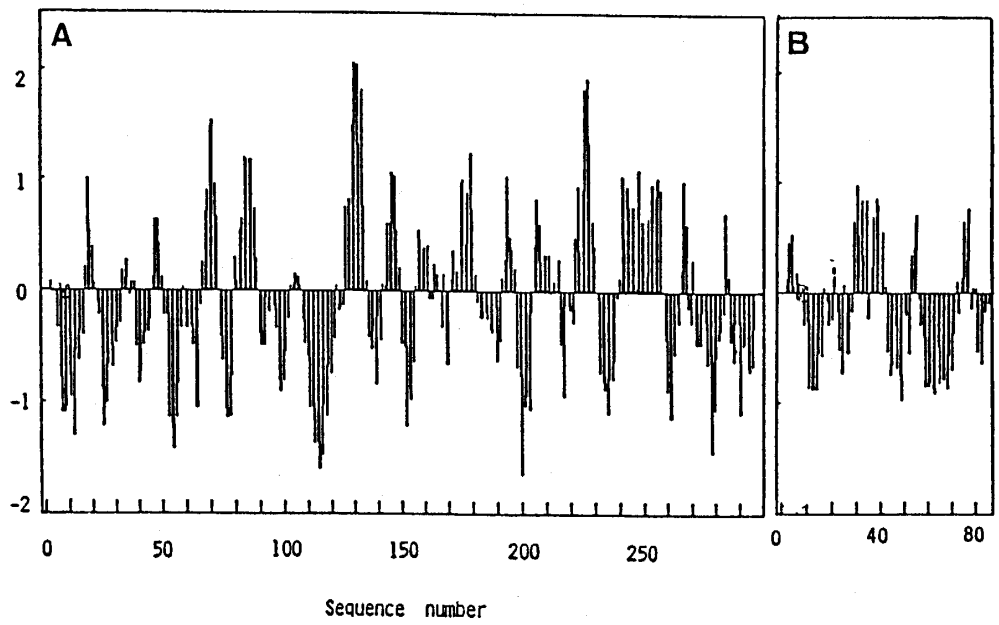

Fig. 5. Hydrophobicity profiles of collagen binding domain (A) and 14 kDa heparin binding domain (B) of human plasma fibronectin. The hydrophobicity was calculated according to the method of Hopp and Woods [37] with an average segment length of six residues. Positive integers represent relative hydrophilicity, and negative values indicate hydrophobicity.

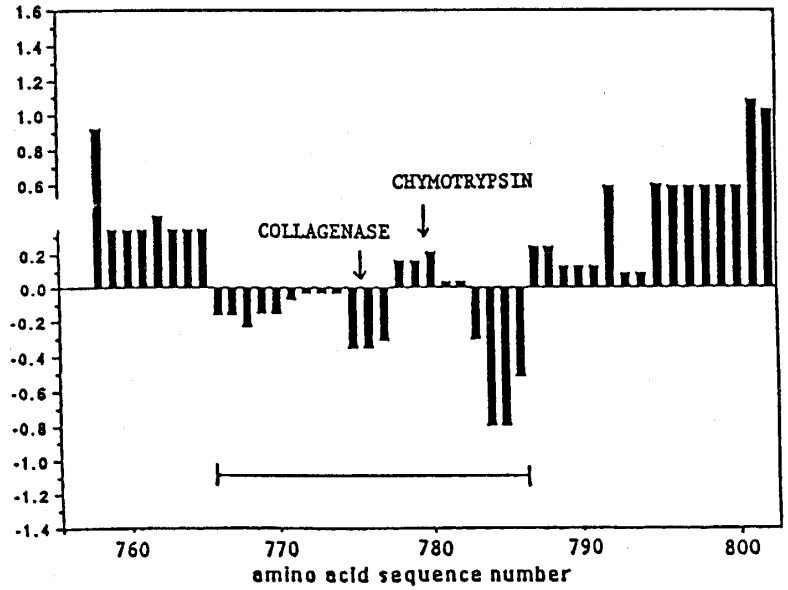

Fig. 6. Hydrophobicity profiles of the fibronectin binding region in collagen. The hydrophobicity was calculated by the same method described Fig. 5.

to be highly hydrophobic as was expected (Fig. 5A). On the other hand, the fibronectin-binding site on type I collagen has an unusual sequence of amino acids [38, 39] (with glycine as every third residue but lacking proline and hydroxyproline) including a bond cleavable by mammalian collagenase [39]; and the hydrophobicity plot analyzed by the same method using the amino acid sequence (775–786) of the fibronectin-binding site on collagen α1(I) chain was strongly hydrophobic (Fig. 6).

The conformational state of fibronectin has been studied by electron microscopy [40, 41], sedimentation velocity studies [42], fluorescence [43] and electron spin resonance spectroscopy [44], light scattering studies [43] and gel filtration high-performance chromatography [45]. The present knowledge suggests that intact fibronectin is not a rigid globular molecule but has a significant degree of intramolecular flexibility under various conditions. In near-physiological conditions, the compact form is favoured, in which the amino-terminal end of one chain is folded over onto its own carboxyl end or that of another chain. Consequently, the collagen-binding domain of fibronectin appears to be exposed to the surface of the molecule. This is consistent with our results on HP-HIC in which intact fibronectin showed high surface hydrophobicity, which was as high as that of its collagen-binding domain. Also the hydrophobic fibronectin-binding site of collagen seems to be exposed to the surface, since it contains the mammalian collagenase [39] and chymotrypsin [38] cleavage sites [46]. In conclusion, our results indicate that the binding of fibronectin with collagen is mainly

based on hydrophobic interaction. Our conclusion is consistent with reports from other laboratories: variation in pH (2.6–10.6) and ionic strength (0.15 M NaCl–4.0 M NaCl) had essentially no effect on fibronectin binding to collagen-coated plastic dishes, while ethylene glycol (a potent hydrophobic solute), several detergents, and decreased temperature inihibited the binding [47]; fibronectin adsorbed on a gelatin-Sepharose column was eluted by ethylene glycol [48] and other chaotropic agents (lithium diiodosalicylic acid, potassium iodide, 8 M urea in 0.1 M citric acid, pH 4.7) [49]. Furthermore, Narasimhan and Lai detected the conformational change of plasma fibronectin upon adsorption to polystylene beads composed of mainly phenyl groups by electron spin resonance spin-label spectroscopy and suggested that plasma fibronectin contains various hydrophobic regions capable of interacting directly with the hydrophobic surfaces [50]. On the other hand, Vuento et al. [51] suggested the presence of essential charged amino acids in the binding of fibronectin to gelatin-Sepharose by chemical modifications, but they also noted that the binding was not completely inhibited even in the presence of 1.0 M NaCl.

Both the 25 kDa Hep I domain and the 14 kDa Hep III domain are cryptic in the intact fibronectin, and therefore their surface hydrophobicities seem to have no effect on that of the intact fibronectin. The hydrophobic value of the 14 kDa Hep III domain calculated by the method of Hoop and Woods using the amino acid sequence reported previously [5] was much higher than that of the collagen-binding domain (as shown in Fig. 5B).

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