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Domain Structure of Human Plasma Fibronectin

DIFFERENCES AND SIMILARITIES BETWEEN HUMAN AND HAMSTER FIBRONECTINS*

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The domain structure of human plasma fibronectin was investigated by limited proteolysis with trypsin and thermolysin with special emphasis on the comparison with hamster plasma fibronectin. Both human and hamster plasma fibronectins gave closely spaced doublet bands on NaDodSO₄-polyacrylamide gels after reduction, but the spacing between these two bands was significantly smaller in human plasma fibronectin. Thermolysin digestion of human plasma fibronectin produced a similar set of fragments as hamster plasma fibronectin, *i.e.* $M_r = 155,000$ (155K), 145,000 (145K), 44,000 (44K), 24,000 (24K), and 22,000 (22K) fragments. The binding properties of these fragments are also identical with those from hamster plasma fibronectin. However, tryptic digestion of human plasma fibronectin gave a slightly different fragmentation profile than that of hamster plasma fibronectin. Human plasma fibronectin was cleaved into 215K, 185K, 32K, and 37K fragments, of which the first three fragments were also generated from hamster plasma fibronectin but the 37K fragment was not. Time course analysis of tryptic digestion of human plasma fibronectin suggested that the 37K fragment was derived from large (α) subunit together with the 32K and the 185K fragments whereas small (β) subunit was cleaved into the 215K and the 32K fragments. The human plasma fibronectin-specific 37K fragment was shown to be composed of the "Fib-2" domain which is represented by the 22K thermolysin fragment, because (i) the 37K tryptic fragment was degraded to the 22K fragment by thermolysin, (ii) the binding properties to fibrin, heparin, and gelatin of the 37K fragment were identical with those of the 22K thermolysin fragment, and (iii) affinity-purified anti-37K antibodies specifically reacted with the 22K fragment as well as the 37K tryptic fragment. In addition, the anti-37K antibodies also reacted with the tryptic 215K fragment but not with the tryptic 185K fragment, suggesting that only the 215K fragment contains the Fib-2 domain of the β subunit, while the 185K fragment was lacking the Fib-2 domain of the α subunit which was identified as the 37K fragment. In support of this view, only the 215K fragment could bind to fibrin. Furthermore, the antibodies also reacted with the 155K thermolysin fragment but not with the 145K. This indicates that the 37K tryptic fragment contains not only the Fib-2 domain but also the COOH-terminal 10K region of the 155K thermolysin fragment which is not present in the 145K fragment. In conclusion, the Fib-2 domain of human plasma fibronectin is, unlike hamster

plasma fibronectin, equally present in both α and β subunits, but only the domain present in the α subunit is released as the 37K fragment upon tryptic digestion, possibly because of the different susceptibility of the hinge region between the "cell/Hep-2" (which corresponds to the 155K-145K thermolysin fragments) and Fib-2 domains.

FNs¹ are a class of high molecular weight glycoproteins abundantly present in pericellular matrices and in various body fluids, and their expression on the cell surface is greatly affected by oncogenic transformation (1-9). FN can be characterized by sharing a number of common properties. First, it exists as a disulfide-bonded dimer or multimer of identical or nonidentical subunit chains with $M_r = 230,000$ -210,000. Second, it binds to collagen (or gelatin), fibrin, heparin, and some other glycosaminoglycans, actin, and *Staphylococcus aureus*, and thereby mediates cell attachment and spreading onto various substrates. Third, antibodies raised against any types of FN cross-react with other types within the same species. However, despite these common properties, FN also shows molecular heterogeneity in terms of subunit size and composition, solubility, and structure of carbohydrate units (9, 10). Thus, FNs from different sources are similar but not identical.

Several lines of evidence indicate that FN is composed of several structural domains which are connected by flexible peptide segments (11, 12). Such domains can be separated by mild protease treatment (13-17). Recently, we have shown that sequential digestion with trypsin and thermolysin or single thermolysin digestion can cleave hamster pFN into four distinct domains almost quantitatively (14, 16). These four domains, represented by thermolysin fragments with $M_r = 150,000$ -140,000, 40,000, 24,000, and 21,000 (150K-140K, 40K,² 24K, and 21K fragments), are also distinct from each other in their biological functions. These domains are referred to as the "Hep-1/Fib-1," "Gel," "Cell/Hep-2," and "Fib-2" domain in this paper based upon their biological activities.

Hamster pFN consists of two nonidentical subunits with $M_r = 230,000$ (α subunit) and 210,000 (β subunit). Both subunits equally contain the Hep-1/Fib-1, Gel, and Cell/Hep-2 domains, but the Fib-2 domain has been shown to be present only in the α subunit (16). Thus, the difference in molecular

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¹ The abbreviations used are: FN, fibronectin; pFN, plasma fibronectin; NaDodSO₄, sodium dodecyl sulfate; DBM paper, diazobenzyl-oxyethyl paper; PBS, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4; BSA, bovine serum albumin. 155K is used to indicate the fragment of $M_r = 155,000$, for example.

² The molecular weight of the '40K' fragment was tentatively determined to be 40,000 in the previous paper (14, 16), but it has now been re-estimated to be approximately 43,000 in the recent investigation.

weights between α and β subunits is considered to be due to the asymmetric distribution of the Fib-2 domain.

Human pFN also consists of two nonidentical subunit chains, but the apparent difference in molecular weight between the two subunits is much smaller than that for hamster pFN. This raises a question of whether human pFN consists of the same set of the domains as hamster pFN, and if so, whether the Fib-2 domain is also present only in the large subunit. In the present investigation, we analyzed the domain structure of human pFN by limited proteolysis with trypsin and thermolysin and compared it with that of hamster pFN. It was found that the basic domain composition was similar between human and hamster pFNs but the Fib-2 domain was, unlike hamster pFN, present in both large and small subunits of human pFN.

EXPERIMENTAL PROCEDURES

Materials—Thermolysin (protease, type X), gelatin (from swine skin, type I), bovine fibrinogen (type IV), and soybean trypsin inhibitor were obtained from Sigma; trypsin was from Worthington; bovine thrombin was from Dade Diagnostics, Inc. (Miami, FL); Sepharose 4B and Sephacryl S-200 were from Pharmacia; heparin-agarose was from Pierce; DEAE-cellulose was from Whatman (Kent, England). Ultrapure urea was obtained from Schwarz/Mann and was used without further purification. FN-rich supernatant of glycine precipitation of Fraction I-1, obtained as a by-product during purification of blood coagulation Factor VIII, was kindly provided by Drs. M. W. Chopek and K. Fujikawa (Department of Biochemistry, University of Washington). Gelatin-Sepharose, soybean trypsin inhibitor-Sepharose, and fibrinogen-Sepharose were prepared according to Cuatrecasas and Anfinsen (18). Fibrin-Sepharose was prepared by incubating fibrinogen-Sepharose (5 mg of protein/ml of packed gel) with bovine thrombin (1 NIH unit/ml) at 22 °C for 30 min. The fibrin-Sepharose was washed once with 25 mM Tris-HCl (pH 7.6) containing 8 M urea and 0.5 mM EDTA. Nitrocellulose and aminobenzyloxymethyl paper were purchased from Bio-Rad.

Purification of pFNs—Human and hamster pFNs were purified on gelatin-Sepharose from the supernatant fraction of glycine precipitation of Factor VIII and from fresh citrated hamster plasma as described previously (16). FN was eluted from a gelatin-Sepharose column with 25 mM Tris-HCl (pH 7.6) containing 4 M urea and 0.5 mM EDTA and dialyzed against 10 mM Tris-HCl containing 0.5 mM EDTA and 50 mM NaCl, pH 7.6 ("10 mM Tris buffer"). Human pFN thus purified often contains a small amount of a fragment which migrates slightly faster than the β subunit (see Fig. 1B, lanes b and c).

Purification of Tryptic Fragments—Human pFN (33 mg), dissolved in 10 ml of 10 mM Tris buffer, was digested by trypsin (1 μ g/ml) at 22 °C for 15 min. The digest was quickly passed through a soybean trypsin inhibitor-Sepharose column (2.5 \times 8 cm) to remove trypsin. The digest was then fractionated on gelatin-Sepharose (2.5 \times 9 cm). The bound fragments (215K and 185K fragments) were eluted from the column with 25 mM Tris-HCl (pH 7.6) containing 4 M urea and 0.5 mM EDTA, dialyzed against 10 mM Tris buffer, and concentrated by vacuum dialysis with a Micro-ProDiCon (Bio-Molecular Diagnostics, Beaverton, OR). The unbound fragments were pooled and applied to a heparin-agarose column (2.5 \times 8 cm) equilibrated with 10 mM Tris buffer. The bound fragment (32K fragment) was eluted with 10 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 0.5 mM EDTA, dialyzed against 10 mM Tris buffer, and concentrated by vacuum dialysis. The unbound fragment (37K fragment) was again pooled and applied to a DEAE-cellulose column (2.5 \times 10 cm). The column was washed with 10 mM Tris buffer and then the bound fragment (37K fragment) was eluted from the column with 10 mM Tris-HCl (pH 7.6) containing 150 mM NaCl and 0.5 mM EDTA.

Anti-37K Fragment Antibodies—The antibodies against purified 37K fragment were raised in New Zealand White rabbits. Before immunization, the 37K fragment, purified by the method described above, was further purified by preparative NaDodSO₄-acrylamide gel electrophoresis after reduction with 2% 2-mercaptoethanol. After electrophoresis, the gel was briefly stained with 0.25% Coomassie blue dissolved in 25% isopropyl alcohol and 10% acetic acid. The 37K band was excised and placed in a dialysis tube filled with electrode buffer (i.e. 20 mM Tris, 150 mM glycine, pH 8.3). The fragment was eluted from the gel by electrophoresis and dialyzed against PBS containing

Bio-Beads SM-2 to remove NaDodSO₄. The 37K fragment thus purified was emulsified with Freund's complete adjuvant and injected subcutaneously into rabbits at multiple sites. Rabbits were immunized three times with 100–200 μ g of purified fragment at 2-week intervals. The antibodies were affinity-purified on 37K fragment-adsorbed DBM paper according to Olmsted (19) and also as follows. The 37K fragment was first separated on preparative NaDodSO₄-polyacrylamide gel electrophoresis and the 37K band was excised after brief staining with Coomassie blue. The fragment was blotted onto DBM paper by electrophoresis according to Towbin *et al.* (20). The DBM paper was incubated with 0.1 M Tris-HCl (pH 9.0) containing 10% ethanolamine and 2.5% BSA to block unbound diazonium groups. For purification of anti-37K antibodies, the thin strip of DBM paper was incubated with 200-fold-diluted antiserum for 2 h at 22 °C in the presence of 5% BSA. The DBM paper was washed with PBS containing 0.05% Triton X-100 four times and then briefly with PBS. The DBM paper was incubated with 10 ml of ice-cold 0.2 M glycine-HCl, pH 2.8, for 2 min. The eluted antibodies were quickly neutralized with 1 M Tris and then mixed with 40 ml of PBS containing 5% BSA.

Immunostaining with Anti-37K Antibodies—Intact pFN and its proteolytic fragments were separated by NaDodSO₄-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose sheet by electrophoresis. The nitrocellulose sheet was incubated with PBS containing 5% BSA, and then with affinity-purified antibodies in PBS containing 5% BSA for 1 h at 22 °C. The nitrocellulose was washed with PBS containing 0.05% Triton X-100 four times. The washed nitrocellulose was incubated with ¹²⁵I-protein A in PBS containing 5% BSA for 30 min at 22 °C. The nitrocellulose was again washed, dried on filter paper, and exposed to Kodak XAR-2 film with an intensifying screen.

NaDodSO₄-Polyacrylamide Gel Electrophoresis—NaDodSO₄-polyacrylamide gel electrophoresis was performed according to Laemmli (21) with 9% gels. Samples were reduced with 2% (v/v) 2-mercaptoethanol. The apparent molecular weight was estimated by using the following proteins as standards: skeletal muscle myosin, 200,000; β -galactosidase, 116,000; phosphorylase b, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400.

RESULTS

Domain Analysis of Human pFN—Human pFN consists of two nonidentical subunits as is the case for hamster pFN, but the size difference between large (α) and small (β) subunits is apparently less pronounced than that of hamster pFN. When analyzed on 8% polyacrylamide gels after prior reduction, human pFN gave a single band whereas hamster pFN gave clear doublet bands (Fig. 1A). Prolonged electrophoresis on 5% gels, however, could resolve human pFN into closely spaced doublet bands (Fig. 1B) as previously observed (16, 22, 23). Under the same condition, hamster pFN was separated

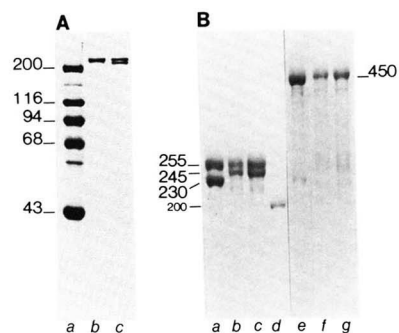


FIG. 1. NaDodSO₄-polyacrylamide gel electrophoresis of human and hamster pFNs on 8% (A) and 5% (B) gels. In A, lane a, molecular weight standards; lane b, human pFN (5 μ g); lane c, hamster pFN (5 μ g). All samples were reduced by 2-mercaptoethanol. In B, lanes a and e, hamster pFN (10 μ g); lanes b and f, human pFN (5 μ g); lanes c and g, human pFN (10 μ g); lane d, skeletal muscle myosin. Samples were either reduced (lanes a–d) or not reduced (lanes e–g) by 2-mercaptoethanol. The molecular weights of the subunit polypeptides were tentatively estimated as shown at the left margin.

in two discrete bands with greater separation between the two bands than those from human pFN (Fig. 1B).

The smaller difference in the molecular weight between α and β subunits of human pFN than that of hamster pFN raised a question whether the domain structure of human pFN was the same as that of hamster pFN. Previously, we found that thermolysin was a good tool to dissect hamster pFN into its constitutive domains (14). Thermolysin digestion of human pFN revealed that its domain structure was basically the same as that of hamster pFN (Fig. 2). It was cleaved into five distinct fragments, each of which corresponded to 150K, 140K, 40K, 24K, and 21K thermolysin fragments of hamster pFN. The molecular weights of each of these human fragments were slightly different from those of corresponding hamster fragments, and were tentatively estimated to be 155,000, 145,000, 44,000, 24,000, and 22,000. In addition, human pFN was slightly more resistant to thermolysin than hamster pFN, because it required twice as much of the protease to give a comparable digestion profile in the same time period. The 155K fragment was apparently produced in much lesser amounts than the 145K fragment, whereas corresponding fragments from hamster pFN were produced in almost equal amounts. This could be due to the partial degradation of 155K fragment into 145K and possibly a fragment with $M_r = 105,000$ under the condition employed.

To further confirm that these thermolysin fragments represent the same functional domains as represented by corresponding hamster fragments, the binding properties of these fragments were examined. Among them, only 44K fragment bound to gelatin while other fragments failed to bind (Fig. 3, lanes c and d). In contrast, 155K, 145K, and 24K fragments were capable of binding to heparin (Fig. 3, lanes e and f), and 24K and 22K fragments were capable of binding to fibrin while 155K, 145K, and 44K fragments did not bind to fibrin (Fig. 4). All these results were in good agreement with that of

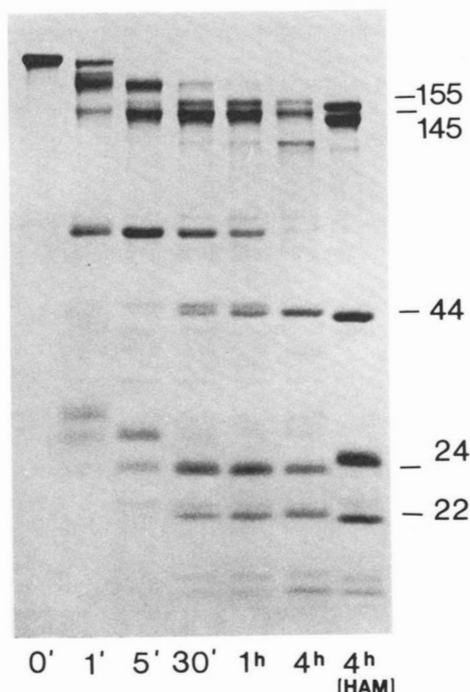


FIG. 2. Time course of thermolysin digestion of human pFN. Human pFN (1 mg/ml) was digested by thermolysin (5 μ g/ml) at 22 $^{\circ}$ C for different periods of time as indicated below each lane. Digestion was terminated by adding EDTA (5 mM). The digest was analyzed by NaDodSO₄-polyacrylamide gel electrophoresis with prior reduction. Right lane is the thermolysin digest of hamster (HAM) pFN.

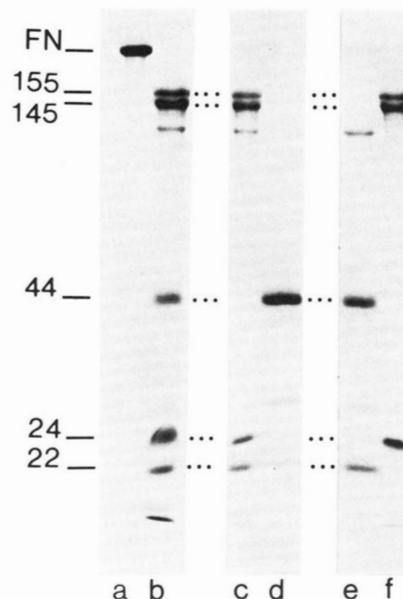


FIG. 3. Binding of thermolysin fragments to gelatin and heparin. Intact pFN (lane a) was digested by thermolysin (5 μ g/ml) at 22 $^{\circ}$ C for 4 h and the digest (lane b) was fractionated on gelatin-Sepharose (lanes c and d) or on heparin-agarose (lanes e and f); lane c, gelatin-unbound fragments; lane d, gelatin-bound fragment; lane e, heparin-unbound fragments; lane f, heparin-bound fragments.

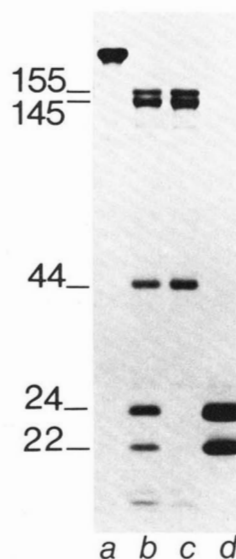


FIG. 4. Binding of thermolysin fragments to fibrin-Sepharose. Intact pFN (4.5 mg) was digested by thermolysin (5 μ g/ml) at 22 $^{\circ}$ C for 4 h and the digest was fractionated on a fibrin-Sepharose column (2.5 \times 8 cm). Lane a, intact pFN; lane b, the thermolysin digest; lane c, fibrin-unbound fragments; lane d, fibrin-bound fragments.

hamster pFN (16), confirming that both hamster and human pFNs were composed of the same four sets of functional domains, *i.e.* the Hep-1/Fib-1, Gel, Cell/Hep-2, and Fib-2 domains which correspond to the 24K, 44K, 155K-145K, and 22K fragments, respectively.

Tryptic Digestion of Human pFN: Detection of a Unique 37K Fragment—Although the basic domain composition was identical between human and hamster pFNs as judged by the thermolysin digestion pattern, human pFN gave upon tryptic digestion a significantly different fragment from hamster pFN (Fig. 5). A mild trypsin treatment of human pFN produced maximal quantities of the 215K, 185K, 37K, and 32K frag-

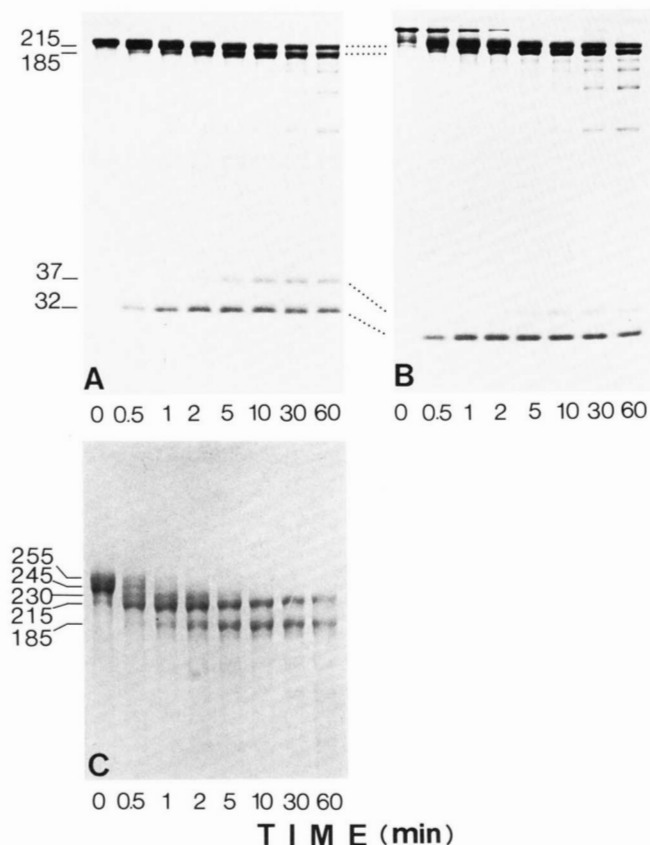
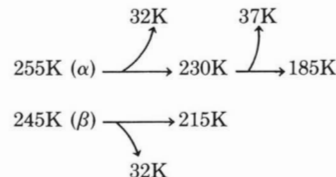


FIG. 5. Time course of trypsin digestion of human pFN. Human pFN (1 mg/ml) was digested by trypsin (1 μ g/ml) at 22 $^{\circ}$ C for different periods of time as indicated below each lane. Digestion was terminated by adding soybean trypsin inhibitor (2 μ g/ml). The digest was analyzed by NaDodSO₄-polyacrylamide gel electrophoresis on 9% (A and B) or 5% (C) gels with (A and C) or without (B) prior reduction. For each lane, 10 μ g of protein were applied. The apparent molecular weight of major fragments is shown at the left margin.

ments after 10-min digestion, although prolonged treatment resulted in the nonstoichiometric degradation of the 215K and 185K fragments. This profile was very similar to that of hamster pFN except that the 37K fragment was not detected in the latter (16). The time course profile shown in Fig. 5A also suggested that a release of the 32K fragment was observed as early as within 30 s and almost completed after 1 min, whereas the release of the 37K fragment was much delayed. The 37K was only weakly detected after 1 min, and its production reached a plateau after 5–10 min (Fig. 5A). Further analysis of the same sample on a 5% gel (Fig. 5C) also revealed that a high molecular weight fragment with $M_r = 230,000$ was transiently generated beside 215K and 185K fragments in the early stage of the trypsin digestion. The 230K and 215K fragments were produced as early as after 30 s with concomitant decrease of intact α and β subunit chains. Since the release of 32K fragment was completed within 1 min, this suggests that α and β subunits were first converted into 230K and 215K fragments, respectively, by releasing 32K fragment. The 230K fragment persisted for 1–2 min and then disappeared after 5–10 min of digestion, while 215K fragment appeared to be resistant against further degradation. In accordance with the decrease of the 230K transient fragment, the 185K fragment gradually increased after 1 min of digestion and reached plateau after 5–10 min, suggesting a possible precursor-product relationship between 230K and 185K fragments. Furthermore, this apparent conversion took place slowly from 1 min of digestion and was completed after 5–10

min. This timing apparently coincided with the appearance of the 37K fragment. All these observations, taken together, suggest that the degradation of α and β subunits of human pFN occurred as the following sequence.



Another possibility is that the 230K fragment was sequentially degraded into 215K and 185K fragments. However, such sequential degradation should accompany the release of smaller fragments, $\sim 15\text{K}$ and $\sim 30\text{K}$, associated with the conversion of 230K to 215K and 215K to 185K, respectively. This possibility is, however, less likely, since the 37K fragment was the only fragment detectable in the small molecular weight region in addition to a well established major tryptic 32K fragment (16) which is produced by rapid degradation of large and small subunit chains into 230K and 215K fragments.

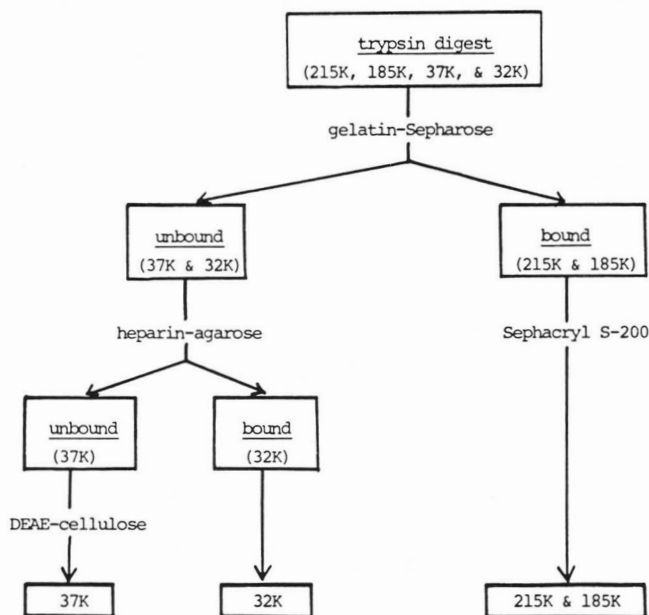
These trypsin fragments appeared to be devoid of interchain disulfide bridge(s), because they exhibited almost the same mobility in the acrylamide gels under both reducing and nonreducing conditions (Fig. 5B). Since interchain disulfide bond(s) were shown to be located at the COOH-terminal ends of both subunits, a small COOH-terminal segment which contained interchain disulfides must be released during the course of the trypsin digestion. Release of the small segment appears to be completed within 5 min (Fig. 5B).

Purification and Characterization of the Tryptic Fragments—The tryptic fragments were separated from each other and purified as illustrated in Fig. 6A. The 215K and 185K fragments were separated from the 37K and 32K fragments on gelatin-Sepharose and further purified by gel filtration on Sephacryl S-200. The 32K fragment was separated from the 37K fragment on heparin-agarose to which only the former could bind. The 37K fragment, which passed through heparin-agarose, was absorbed on DEAE-cellulose and eluted with increasing salt concentration. The relative yield of the 37K fragment to the 32K fragment was 0.65:1, based upon the absorbance at 280 nm, being almost in agreement with their relative intensities on the gel (see Fig. 5A). The binding properties of the 215K-185K and 32K fragments were identical with the 200K-180K and 32K hamster tryptic fragments, respectively (16).

The purified trypsin fragments were digested by thermolysin to elucidate their domain composition (Fig. 7). The 215K and 185K fragments were predominantly cleaved into 155K-145K, 44K, and 22K fragments, although a part of the 155K-145K fragments were further degraded into a fragment with $M_r = 105,000$ (Fig. 7B). A fragment which migrated at the 24K fragment position was also faintly detectable (Fig. 7B), but it may not be derived from the Hep-1/Fib-1 domain, because this domain of hamster pFN was quantitatively recovered in the 32K tryptic fragment (16). Indeed, the human 32K tryptic fragment was sequentially converted to 27K and then 24K fragments (Fig. 7D). A fragment which migrated below the 24K and just above the 22K fragment could be a further degradation product of the 24K fragment (Fig. 7D). The degradation profiles of the 215K-185K and 32K tryptic fragments was essentially identical with that of hamster 200K-180K and 32K tryptic fragments (16). The 37K fragment, which was specific to human pFN, was clearly degraded into the 22K fragment by thermolysin (Fig. 7C), suggesting that it contains the Fib-2 domain.

The possibility that the 37K fragment contains the Fib-2

A



B

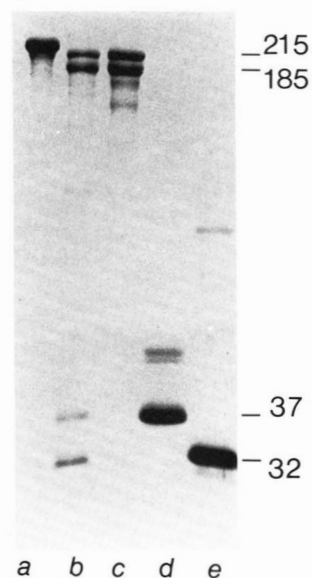


FIG. 6. Purification of tryptic fragments. A, diagram of purification of tryptic fragments. B, electrophoretic pattern of purified fragments. Lane a, intact human pFN; lane b, tryptic digest; lane c, 215K-185K fragments; lane d, 37K fragment; lane e, 32K fragment.

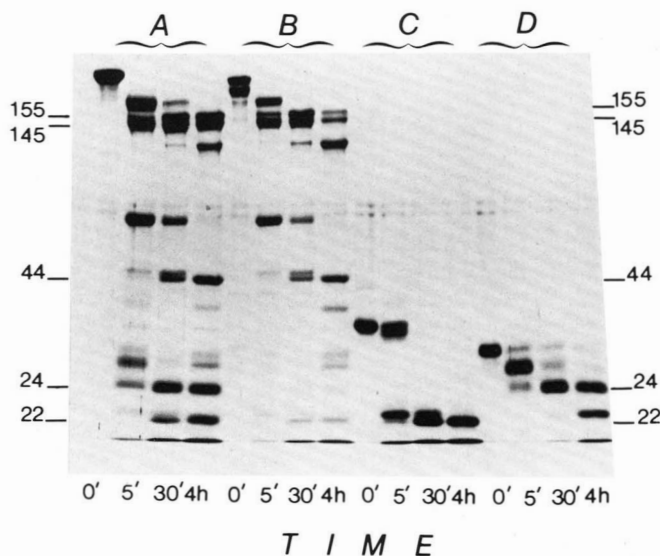


FIG. 7. Thermolysin digestion of purified tryptic fragments. Intact pFN and its purified tryptic fragments were digested by thermolysin (5 $\mu\text{g}/\text{ml}$) at 22 $^{\circ}\text{C}$ for different period of time as indicated below each lane. A, intact FN; B, 215K-185K fragments; C, 37K fragment; D, 32K fragment.

domain was tested by fractionating the whole trypsin digest on fibrin-Sepharose, because the Fib-2 domain is capable of binding to fibrin. As expected, the 37K fragment, as well as the 215K and 32K fragments, bound to fibrin (Fig. 8). Only the 215K, but not the 185K, fragment could bind to fibrin. This indicates that only the 215K fragment contains the Fib-2 domain as was also the case for 200K-180K hamster fragments (16). The 32K tryptic fragment which contained the Hep-1/Fib-1 domain, another fibrin-binding domain present at the NH_2 terminus, also could bind to fibrin.

Immunostaining of the Proteolytic Fragments with Anti-37K Antibodies—The origin of the 37K fragment was further

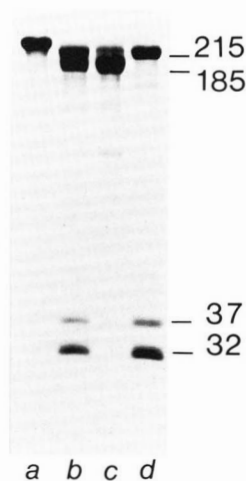


FIG. 8. Binding of tryptic fragments to fibrin-Sepharose. Intact pFN (4.5 mg) was digested by trypsin (1 $\mu\text{g}/\text{ml}$) at 22 $^{\circ}\text{C}$ for 15 min. The digestion was terminated by adding soybean trypsin inhibitor (2 $\mu\text{g}/\text{ml}$). The digest was fractionated on fibrin-Sepharose (2.5 \times 8 cm) at 4 $^{\circ}\text{C}$. The flow rate was 13.5 ml/h. Lane a, intact pFN; lane b, the tryptic digest; lane c, fibrin-unbound fragments; lane d, fibrin-bound fragments.

studied using specific antibodies. Since the 37K fragment, which was purified according to the procedure shown in Fig. 6A, contained a small amount of other fragments, it was further purified by preparative gel electrophoresis, and antibodies against the 37K fragment were raised in rabbits according to the method as described under "Experimental Procedures." The antibodies were affinity-purified on 37K-blotted and cross-linked DBM paper and then used for immunostaining of various proteolytic fragments transferred to nitrocellulose (Fig. 9). Among the tryptic fragments, the antibodies strongly stained the 215K and 37K fragments, whereas the 185K region was only faintly stained (Fig. 9, lane b). A fragment which migrated at the 43K region was also weakly stained by the antibodies, although it was barely

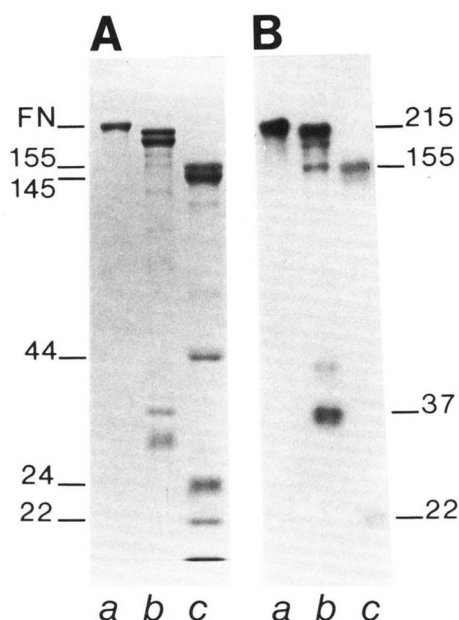


FIG. 9. Immunostaining of proteolytic fragments by anti-37K antibodies. Intact human pFN (lane a), tryptic digest (lane b), and thermolysin digest (lane c) were separately electrophoresed on a 9% gel under reducing conditions in duplicate. Gels were either stained by Coomassie blue (A) or subjected to electrophoretic transfer of proteins onto nitrocellulose followed by immunostaining with anti-37K antibodies (B). For the details, see "Experimental Procedures."

visible upon protein staining. This fragment may correspond to a contaminant fragment in the purified 37K preparation which migrated at the same molecular weight region (see Fig. 6B, lane d). The antibodies did not stain the 32K fragment (Fig. 9, lane b). These results indicate that part of the 215K fragment overlapped with the 37K fragment, while the 185K fragment did not overlap. In addition, immunostaining of thermolysin fragments showed that the 155K and 22K fragments were specifically stained by the anti-37K antibodies (Fig. 9, lane c). The fact that the 155K fragment reacted with the anti-37K antibodies but 145K did not react suggests that the antigenic sites include not only the region within the Fib-2 domain but also a small polypeptide region of approximately 10K. This extra 10K region which is strongly antigenic must be present only in the 155K fragment but not in the 145K.

DISCUSSION

It has been shown that pFNs of any species so far investigated consist of two nonidentical subunit chains which usually give closely spaced doublet bands on NaDodSO₄-polyacrylamide gels, whereas FNs isolated from the cell surface or conditioned medium of fibroblasts appear to be composed of identical subunit chains (10, 24, 25). The basis of subunit heterogeneity of pFN, however, has not been well understood. Recently, we studied the domain structure of hamster pFN by limited proteolysis with trypsin and thermolysin (14, 16). We found that hamster pFN is composed of at least four distinct domains, *i.e.* 150K-140K, 40K, 24K, and 21K domains, which are now termed Cell/Hep-2, Gel, Hep-1/Fib-1, and Fib-2 domains, respectively. The first three domains are equally present in both large and small subunits, but the Fib-2 domain appears to be present only in the large subunit chain. Thus, the subunit heterogeneity of hamster pFN is considered to be due to the asymmetric distribution of the Fib-2 domain.

In the present investigation, we studied the domain structure of human pFN to see whether such asymmetry in domain structure in two subunit chains is a general characteristic for

other pFNs. In fact, human pFN consists of two nonidentical subunit chains, but the apparent difference in molecular weight between two subunits is much smaller than that for hamster pFN. Limited proteolysis of human pFN with thermolysin showed that the basic domain composition was the same as hamster pFN. However, mild tryptic treatment gave a slightly different profile between these pFNs. Besides the 215K-185K and 32K fragments which were also produced from hamster pFN, human pFN gave an additional 37K fragment which was not generated from hamster pFN.

Several lines of evidence indicated that the 37K fragment contains the Fib-2 domain. First, the 37K fragment was converted to the 22K fragment by thermolysin. Second, both the 37K fragment and the Fib-2 domain were capable of binding to fibrin but not to heparin or gelatin. Since the NH₂-terminal Hep-1/Fib-1 domain, one of the fibrin-binding domains, was almost quantitatively released as a 32K fragment upon mild trypsin treatment, the binding of the 37K fragment to fibrin indicates that it contained another fibrin-binding domain, *i.e.* the Fib-2 domain. Third, affinity-purified anti-37K antibodies reacted with the 37K as well as the 22K thermolysin fragment. We also tried to produce specific antibodies against the 22K fragment which would be obviously preferable to anti-37K antibodies, but we have not yet obtained good antibodies. The Fib-2 domain seems to be barely immunogenic under conventional immunization protocols.

The alignment of the tryptic fragments was determined as schematically illustrated in Fig. 10, by the following reasons. First, time course analysis of tryptic digestion of human pFN suggested that α and β subunits were first rapidly cleaved into the 230K and 215K fragments, respectively, by releasing the Hep-1/Fib-1 domain as a 32K fragment. The 230K fragment was further degraded into the 185K fragment upon the release of the 37K fragment. There is extensive evidence indicating that the 32K fragment containing the Hep-1/Fib-1 domain is derived from the NH₂-terminal region of both subunits (22, 26, 27). In contrast, the 37K fragment contained the Fib-2 domain which was recently located at the COOH-terminal region of intact subunit chains (28). Thus, the 185K tryptic fragment must be flanked by the 32K and 37K fragments at NH₂- and COOH-terminal ends, respectively, in the large subunit. Both the 215K and 185K fragments contain Gel and Cell/Hep-2 domains as shown by thermolysin digestion of these fragments.³ This was further confirmed with monoclonal antibodies specific to 44K and 155K-145K fragments (29).

The 37K fragment appears to contain not only the Fib-2 domain but also part of the 155K thermolysin fragment, because anti-37K antibodies reacted with the 155K thermolysin fragment as well. Since the antibodies did not react with the 145K fragment, the extra 10K segment present only in the 155K fragment should share the same antigenic site(s) with the 37K fragment. This indicates that the order of the four domains in α and β subunits should be NH₂-24K-44K-145K-tryptic 37K(22K)-COOH and NH₂-24K-44K-155K-22K-COOH, respectively (Fig. 10). It should be noted that the Fib-2 domain of human pFN is, unlike hamster pFN, present in both large and small subunit chains. In support of this, both α and β subunits which were separated on 5% gels were almost equally reactive with anti-37K antibodies.⁴

³ Preliminary experiments suggest that the 155K and 145K fragments are generated, respectively, from the 215K and 185K fragments upon thermolysin digestion. The purified 185K fragment was cleaved into only 145K and 44K fragments by thermolysin, whereas the partially purified 215K fragment predominantly gave 155K, 44K, and 22K fragments (K. Sekiguchi and S. Hakomori, unpublished observation). This is in good agreement with results obtained with hamster 200K-180K fragments (16).

⁴ K. Sekiguchi and S. Hakomori, unpublished observation.

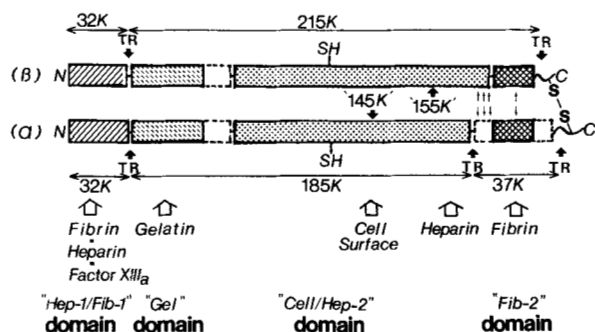


FIG. 10. Proposed model for the domain structure of human pFN. TR indicates the trypsin-susceptible sites. ↑ indicates possible antigenic sites for anti-37K antibodies.

Previously, homology between large and small subunits of human pFN was studied by one-dimensional (23) or two-dimensional (25) peptide mapping, although no significant difference has been observed between the subunits. However, this technique may not be sensitive enough to detect a subtle difference in local amino acid sequence. Indeed, the α subunit is apparently 10,000 daltons larger than the β subunit, but this 10,000-dalton extra peptide did not result in any significant difference in peptide-mapping pattern. Furthermore, these two subunit chains of human pFN showed different susceptibility to trypsin. Both subunits contain at least two common susceptible sites; one is 32,000 daltons from the NH₂ terminus and the other is near the COOH-terminal end whose cleavage probably results in the release of a small peptide segment containing interchain disulfide bond(s). In addition, α subunit contains another susceptible site which is approximately 37,000 daltons in distance from the COOH terminus. Since time course analysis of trypsin digestion showed that the release of the 37K fragment was slower than that of the NH₂-terminal 32K fragment, the additional cleavage site in the large subunits to give the 37K fragment appears to be less susceptible to trypsin than other trypsin-sensitive sites.

Mild trypsin digestion of human pFN has been reported by several other groups and very similar fragmentation patterns were obtained (30, 31), although a slightly different molecular weight was assigned for each tryptic fragment. Smith *et al.* (31) studied the topological arrangement of various tryptic fragments of human pFN by affinity chromatography on gelatin-Sepharose and heparin-Sepharose as well as immunoaffinity chromatography with monoclonal antibodies with different specificities. They suggested that a 31K fragment (which appears to correspond to the 37K fragment in the present study) was derived from the COOH-terminal region of subunit chains, while a 27K fragment (which seems to correspond to the 32K fragment of this report) was derived from the NH₂ terminus. Although they did not assign the origin of the 31K fragment to either the large or small subunit chain, these results are in good agreement with our proposed model shown in Fig. 10.

Recently, Richter and Hörmann (32) obtained a 140K fragment which consists of disulfide-linked 75K and 65K fragments by cathepsin D digestion. Subsequent plasmin digestion of the 140K fragment suggests that only the 75K fragment which seems to be derived from the large subunit contains an additional cleavage site at $M_r = 32,000$ –36,000 from the

COOH-terminal end besides another site at the extreme COOH-terminal region. This finding further supports our proposed model for domain structure of human pFN.

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