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Domain Structure of Hamster Plasma Fibronectin

ISOLATION AND CHARACTERIZATION OF FOUR FUNCTIONALLY DISTINCT DOMAINS AND THEIR UNEQUAL DISTRIBUTION BETWEEN TWO SUBUNIT POLYPEPTIDES*

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Kiyotoshi Sekiguchi, Minoru Fukuda, and Sen-itiroh Hakomori

From the Program of Biochemical Oncology, Fred Hutchinson Cancer Research Center, Departments of Pathobiology, Microbiology, and Immunology, School of Public Health and School of Medicine, University of Washington 98104

The domain structure of plasma fibronectin was studied by limited proteolysis with trypsin and thermolysin. Intact hamster plasma fibronectin is a disulfide-bonded dimer of two nonidentical subunit polypeptides with M_r = 230,000 (α subunit) and 210,000 (β subunit). Mild trypsin treatment selectively released a small fragment with M_r = 32,000 (designated as 32K fragment, others are designated in the same way) from both subunits, converting α and β subunits into 200K and 180K fragments, respectively. In contrast, thermolysin cleaved intact fibronectin into four fragments, 150K-140K, 40K, 24K, and 21K, which represent distinct constitutive domains of intact fibronectin. Tryptic 200K and 180K fragments gave on thermolysin digestion 150K-140K, 40K, and 21K fragments but not 24K fragment. On the contrary, thermolysin digestion of the 32K tryptic fragment generated only 24K fragment, indicating that both fragments are derived from the same domain. Of the four distinct thermolysin fragments, only 24K and 21K fragments bound to fibrin, whereas 150K-140K (which binds to heparin and promotes cell spreading) and 40K (which binds to gelatin) fragments failed to bind to fibrin. Of three major tryptic fragments, 200K, 180K, and 32K, only 200K and 32K fragments were capable of binding to fibrin. The failure of the 180K fragment to bind to fibrin is due to the absence of the 21K fibrin-binding fragment which was only generated from the 200K fragment by thermolysin treatment. In contrast, 150K-140K and 40K fragments were produced from both 200K and 180K fragments. These results indicate that plasma fibronectin is composed of four structurally and functionally distinct domains, among which one of the fibrin-binding domains represented by 21K thermolysin fragment ("21K" domain) is only present in α subunit while the other three domains, represented by 150K-140K, 40K, and 24K thermolysin fragments, are equally present in both subunits.

The susceptible glutamyl residue(s) for Factor XIIIa-catalyzed transamidation seemed to be located near the NH_2 or COOH terminus of the 24K domain. Half-cystine was enriched in the 40K, 24K, and 21K domains but was scarce in the 150K-140K domain. Intact fibronectin was estimated to contain four carbohydrate units per subunit; three units were attached to the 40K domain and one unit was attached to the 150K-140K domain.

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Fibronectin is a large glycoprotein present at the surface of fibroblasts and other cell types and is greatly decreased after oncogenic transformation (1-9). It is also present in plasma and has long been termed "cold-insoluble globulin" (10, 11). The cell surface and plasma fibronectins are immunologically indistinguishable (12) and share most of their chemical and biological properties (4-9, 13), although they are different in solubility (14), subunit size (13, 15, 16), and the ability to induce morphological change of transformed cells and to agglutinate formalin-fixed sheep erythrocytes (13).

Fibronectin is an adhesive protein which forms *in vivo* pericellular matrix and basement membranes together with collagen, glycosaminoglycan, laminin, and other components (17-19) and *in vitro* mediates cell attachment and spreading onto artificial surfaces (20-23). Plasma fibronectin is incorporated into fibrin clots during blood coagulation and is supposed to play a role in wound healing (12, 24). Opsonic protein in plasma has been shown to be identical with fibronectin (25). Besides its affinities to collagen (21, 26), glycosaminoglycans (27-29), and fibrin (30-32), fibronectin also binds to *Staphylococcus aureus* (33), actin (34), and deoxyribonucleic acid (35). It interacts with blood coagulation Factor XIIIa (36-38), and is thereby cross-linked to fibrin (24, 31, 36), collagen (39), and bacterial cell surface (40).

Several lines of evidence indicate that fibronectin is composed of several domains which are connected by flexible, protease-sensitive polypeptide segments (41-45). Much effort has been made to separate these structural domains with various proteases without losing their biological activities (29, 39, 44-53). One of such domains, the collagen-binding domain, was successfully isolated by using affinity chromatography (45-53). However, the proteolytic conditions employed in most of these studies are either too strong or too mild to simultaneously separate other constitutive domains by retaining their specific biological activities.

Recently, we found that sequential digestion with trypsin and thermolysin or thermolysin digestion alone was suitable to dissect intact plasma fibronectin into its constitutive domains without extensive degradation (51). These domains thus obtained were distinct from each other in the binding specificity to collagen and glycosaminoglycans and ability to promote cell spreading onto artificial surfaces (51). The present paper describes 1) details of the procedures for proteolytic fragmentation of hamster plasma fibronectin with trypsin and thermolysin and purification of the fragments, 2) amino acid and sugar composition of each fragment, 3) the binding capability of each fragment to fibrin, and 4) the interaction with Factor XIIIa. A preliminary note for a part of the isolation of fibrin-binding domains was published elsewhere (72).

MATERIALS AND METHODS

Materials—Thermolysin (protease, type X), gelatin (from swine skin, type I), bovine fibrinogen (type IV), and soybean trypsin inhibitor were obtained from Sigma; trypsin from Worthington; neuraminidase from *Vibrio cholerae* from Behring Diagnostics (Somerville, NJ); galactose oxidase from Kabi (Stockholm, Sweden); [^{3}H]putrescine (24 Ci/mmol) and NaB^3H_4 (9 Ci/mmol) from Amersham (Arlington Heights, IL); Sepharose 4B and Sephacryl S-200 from Pharmacia (Piscataway, NJ); heparin-agarose from Pierce (Rockford, IL); and DEAE-cellulose (DE-32) from Whatman (Kent, England). Purified Factor XIII was a generous gift from Dr. S. I. Chung (Laboratory of Biochemistry, National Institute of Dental Research). Thrombin was kindly donated by Dr. W. Kisiel (Department of Biochemistry, University of Washington). Fibronectin-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 Dr. K. Fujikawa (Department of Biochemistry, University of Washington). Gelatin-Sepharose, soybean trypsin inhibitor-Sepharose, and fibrinogen-Sepharose were prepared according to Cuatrecasas and Anfinsen (54).

Purification of Fibronectin—Fresh citrated blood from 40 hamsters was collected by heart puncture. Plasma was separated by low speed centrifugation and further clarified by high speed centrifugation (12,000 × *g*, 20 min) at 4 °C. The supernatant, to which PMSF[1] (1 mM) was added, was first passed through an underivatized Sepharose 4B column (bed volume, approximately 40 ml) and then applied to a gelatin-Sepharose column (3–4 mg of gelatin/ml of packed gel, bed volume, approximately 40 ml). The gelatin-Sepharose column was extensively washed with 25 mM Tris buffer containing 0.5 mM EDTA, 150 mM NaCl, and 1 mM PMSF, pH 7.6, and then washed with the same Tris buffer containing 1 M urea. Fibronectin was eluted from the column with the Tris buffer containing 4 M urea. Peak fractions (A_{280} > 0.2) were pooled and dialyzed against 10 mM Tris buffer containing 0.5 mM EDTA and 50 mM NaCl (pH 7.6) at 4 °C for 3 days. Fibronectin thus purified was stored at −80 °C. Human plasma fibronectin was purified from the supernatant fraction of glycine precipitation of Factor VIII by affinity chromatography on gelatin-Sepharose as described above. Bovine plasma fibronectin was similarly purified from newborn calf serum (Gibco, Grand Island, NY). Fibronectin from hamster fibroblast (BHK) culture medium was prepared as follows. BHK cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Fetal calf serum was depleted of fibronectin by passing through gelatin-Sepharose in advance. Culture medium was harvested when the cells reached confluency. It was immediately clarified by high speed centrifugation (12,000 × *g*, 20 min) at 4 °C. The resulting supernatant (approximately 1.5 liters), to which was added PMSF (1 mM), EDTA (5 mM), and NaCl (0.5 M), was applied to an underivatized Sepharose 4B column (bed volume, 120 ml) and subsequently to gelatin-Sepharose column (bed volume, 40 ml) at 4 °C. Fibronectin thus bound to gelatin-Sepharose was purified as described above.

Purification of Tryptic Fragments—Intact hamster plasma fibronectin (approximately 30 mg) was digested with trypsin (1 μg/ml) at 22 °C for 15 min in 25 mM Tris buffer containing 0.5 mM EDTA and 50 mM NaCl, pH 7.6. The digest was rapidly passed through soybean trypsin inhibitor-Sepharose (1 to 2 mg of protein/ml of packed gel, bed volume, 20 ml) to remove trypsin. PMSF (1 mM) was added to the digest, which was subsequently fractionated with gelatin-Sepharose (bed volume, approximately 40 ml). The unbound fractions, mainly M_r = 32,000 fragment (32K fragment), were pooled and then applied to a heparin-agarose column (0.4-0.5 mg of heparin/ml of packed gel, bed volume, 20 ml) which was equilibrated with 25 mM Tris buffer (pH 7.6) containing 0.5 mM EDTA and 50 mM NaCl. The column was extensively washed with the 25 mM Tris buffer and bound materials were eluted in a stepwise manner by increasing salt concentration. The 32K fragment was eluted with the 25 mM Tris buffer containing 150 mM NaCl. The peak fractions were pooled and concentrated by vacuum dialysis (MicroProDiCon, Bio-Molecular Dynamics, Beaverton, OR) against 10 mM Tris buffer containing 0.5 mM EDTA and 50 mM NaCl, pH 7.6. The fragments which bound to a gelatin-Sepharose column were eluted with the 25 mM Tris buffer containing 6 M urea. The eluate (which mainly consisted of 200K and 180K fragments) was dialyzed against the 25 mM Tris buffer and then concentrated by vacuum dialysis. The 200K and 180K fragments were further purified on a Sephacryl S-200 column (2.5 × 100 cm) equilibrated with the 25 mM Tris buffer. Peak fractions containing these fragments were pooled and concentrated by vacuum dialysis against the 10 mM Tris buffer. All purified fragments were stored at −80 °C.

Purification of Thermolysin-released Fragments—Intact fibronectin (approximately 20 mg) was digested with thermolysin (2.5 μg/ml) at 22 °C for 4 h in 25 mM Tris buffer containing 0.5 mM EDTA, 50 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.6. The digestion was terminated by adding EDTA (5 mM) to inactivate thermolysin. The whole digest was fractionated with a gelatin-Sepharose column (bed volume, approximately 40 ml). The fractions which passed through the column were pooled and applied to a DEAE-cellulose (DE-32) column (2.5 × 10 cm) equilibrated with 25 mM Tris buffer containing 0.5 mM EDTA and 50 mM NaCl, pH 7.6. The column was extensively washed with 25 mM Tris buffer and then bound fragments were eluted in a stepwise manner by increasing salt concentration. The 21K fragment was eluted with the 25 mM Tris buffer containing 80 mM NaCl and the 150K–140K fragments were eluted with the Tris buffer containing 200 mM NaCl. Both peak fractions were separately pooled, dialyzed against 10 mM Tris buffer containing 0.5 mM EDTA and 50 mM NaCl, pH 7.6, and concentrated by vacuum dialysis. The fragment bound to a gelatin-Sepharose column was eluted with the 25 mM Tris buffer containing 4 M urea, dialyzed against the 10 mM Tris buffer, and concentrated by vacuum dialysis as described. Purified fragments were stored at −80 °C.

Factor XIIIa-catalyzed Incorporation of [^{3}H]Putrescine—Factor XIIIa-dependent amine incorporation was performed essentially according to Mosher *et al.* (39) using [^{3}H]putrescine as a substrate. Briefly, fibronectin (0.75 mg/ml) was dissolved in 25 mM Tris buffer containing 0.5 mM EDTA, 100 mM NaCl, 10 mM $CaCl_2$, 0.57 mM [^{3}H]putrescine, and Factor XIII (50 μg/ml). The reaction was started by adding thrombin (1 unit/ml). After incubation at 22 °C for 1.5 h, EDTA (20 mM) was added to terminate the reaction. The [^{3}H]-putrescine-labeled fibronectin was directly digested by either trypsin (1 μg/ml, 30 min at 37 °C) or thermolysin (2.5 μg/ml, 2 h at 22 °C). In the latter case, EDTA (20 mM) was added only after the digestion was completed.

Labeling of Sugar Residues by Galactose Oxidase—Carbohydrate chains of intact fibronectin were labeled with galactose oxidase according to Gahmberg and Hakomori (55). To fibronectin (1 mg/ml) dissolved in 25 mM Tris buffer containing 0.5 mM EDTA, 100 mM NaCl, 2 mM $CaCl_2$, and 1 mM PMSF, pH 7.6, was added neuraminidase from *V. cholerae* (5 units/ml) and galactose oxidase (10 units/ml) and incubated for 3 h at 22 °C. The oxidized fibronectin was treated with NaB^3H_4 (1 mCi/ml) at 22 °C for 30 min and then with $NaBH_4$ (1 mM) for 30 min. To remove neuraminidase, galactose oxidase, and free NaB^3H_4, labeled fibronectin was first passed through underivatized Sepharose 4B and then adsorbed to a gelatin-Sepharose column. The column was extensively washed with 25 mM Tris buffer containing 0.5 mM EDTA and 100 mM NaCl, pH 7.6. The bound labeled fibronectin was eluted with the 25 mM buffer containing 6 M urea, dialyzed against the 10 mM Tris buffer, and concentrated by vacuum dialysis. The specific activity of the labeled fibronectin thus obtained was 4.5×10^6 cpm/mg of protein.

Amino Acid and Carbohydrate Analysis—Amino acid analysis was performed with a Durrum D-500 amino acid analyzer. Intact fibronectin or its purified proteolytic fragments were hydrolyzed in 6 N HCl at 110 °C for 24 h in evacuated tubes in the presence of 0.2% phenol (56). Half-cystine was determined as cysteic acid after performic acid oxidation. Individual neutral sugars and hexosamines were determined as alditol acetate derivatives by gas chromatography-mass spectrometry on a column of 3% OV-225 on Supelcoport (80–100 mesh, Supelco Inc, PA). The samples for this assay were hydrolyzed in 1 N H_2SO_4 at 100 °C for 8 h in evacuated tubes. For evaluating the efficiency of hydrolysis and the response factor during gas chromatography, purified IgG glycopeptide was hydrolyzed and analyzed simultaneously. Sialic acid was determined by the fluorometric assay according to Hammond and Papermaster (57).

Protein Determination—Protein was determined by the method of Lowry *et al.* (58), using bovine serum albumin as a standard.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (59) with 9.5% polyacrylamide 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; soybean trypsin inhibitor,

[1] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; BHK, baby hamster kidney. The fragment with M_r = 32,000 is described as "32K fragment" in the present paper. Other fragments are described in the same way.

21,000; hemoglobin, 16,000. Fluorography of slab gels were performed according to Bonner and Laskey (60).

RESULTS

Subunit Structure of Hamster Plasma Fibronectin—Intact hamster plasma fibronectin was composed of two different subunit polypeptides with approximately M_r = 230,000 (hereafter referred to as "α subunit") and M_r = 210,000 (hereafter referred to as "β subunit") (Fig. 1, *a* and *b*). The relative intensity of these two subunits on SDS-polyacrylamide gels under reducing conditions was almost equal through 30 different purified batches. Similarly, fibronectins from human and bovine plasma also gave closely spaced bands on SDS-polyacrylamide gels under reducing conditions (31, 53, 63; Fig. 1, *c* and *d*). Thus, such difference in the subunit size seems to be a general characteristic for plasma fibronectins. In contrast, fibronectin obtained from culture medium of BHK cells gave a single broad band which migrated slightly above the α subunit of plasma fibronectins (Fig. 1, *e*).

Hamster plasma fibronectin, as well as fibronectins from human and bovine plasma, gave a single slowly migrating band with approximately M_r = 450,000 under nonreducing conditions (Fig. 1, *f* to *i*). It could be a homogeneous $\alpha\beta$ heterodimer or a mixture of $\alpha\alpha$ and $\beta\beta$ homodimers (see "Discussion"). Fibronectin from BHK culture medium also exists predominantly as a dimer (Fig. 1, *j*).

In the present investigation, hamster plasma fibronectin was used as a model for plasma-derived fibronectins in order to compare its domain structure with that of its cell surface counterpart, whose transformation-dependent changes, sugar structure, and domain structure have been studied in our laboratory (1, 44, 71, 75).

Mild Trypsin Digestion of Plasma Fibronectin; Selective Release of M_r = 32,000 Fragment from Both Subunits—We recently reported that mild trypsin digestion of intact hamster plasma fibronectin generated 200K, 180K, and 32K fragments (51). The result indicates that trypsin released 32K fragment from both α and β subunits when they were, respectively, converted into 200K and 180K fragments. This view was supported by the electrophoretic pattern at different times of trypsin digestion of intact fibronectin. As shown in Fig. 2, α subunit was almost completely converted into 200K and 32K fragments within 1 min, although it took more than 5 min to complete the degradation of β subunit into 180K fragment. No precursor-product relationship between 200K and 180K tryptic fragments, such as an increase of 180K fragment with a concomitant decrease of 200K fragment, was observed. These three tryptic fragments, 200K, 180K, and 32K, seem to have no interchain disulfide bond because they showed almost identical electrophoretic mobility under both reducing and nonreducing conditions except that 32K fragment gave a slightly higher mobility under nonreducing conditions. This is compatible with the fact that this fragment had a high content of intrachain disulfide bonds (see Table I).

The separation of three major tryptic fragments was achieved with a gelatin-Sepharose column. The 200K and 180K fragments bound to gelatin but the 32K fragment failed to bind (Fig. 3*A*, *a* to *d*). The 32K fragment was further

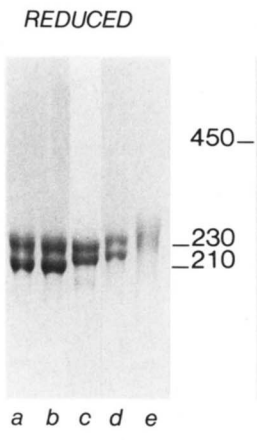


FIG. 1. **Subunit structure of various fibronectins.** Fibronectins purified from plasma and culture medium were subjected to SDS-polyacrylamide gel electrophoresis in a 5% acrylamide gel with (*a* to *e*) or without (*f* to *j*) prior reduction. Electrophoresis was continued for twice as long as the tracking dye reached the front of the gel. *a*, *b*, *f*, and *g*, hamster plasma fibronectin; *a* (or *f*) and *b* (or *g*) were taken from different batches. *c* and *h*, human plasma fibronectin; *d* and *i*, bovine plasma fibronectin; *e* and *j*, fibronectin from BHK culture medium.

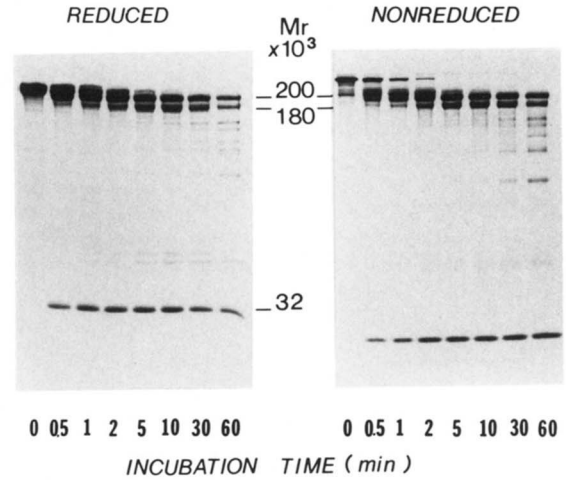


FIG. 2. **Time course of trypsin digestion of plasma fibronectin.** Hamster plasma fibronectin (1 mg/ml) was digested by trypsin (1 μg/ml) at 22 °C for different periods of time as indicated *below* each *lane*. Digestion was terminated by adding soybean trypsin inhibitor (2 μg/ml). The digest was electrophoresed with (*left*) or without (*right*) prior reduction.

TABLE I

Amino acid composition of intact fibronectin and its proteolytic fragments

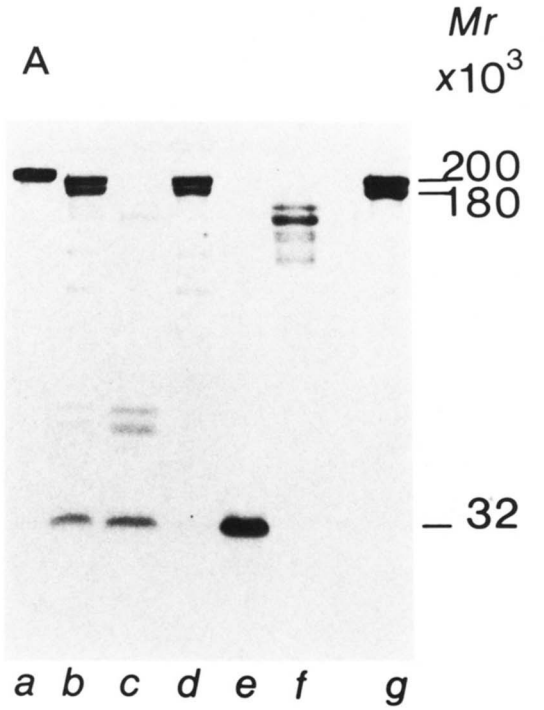
Amino acid	Intact fibronectin	150K–140K thermolysin fragment	40K thermolysin fragment	32K tryptic fragment[a]	21K thermolysin fragment
			M%		
Half-cystine[b]	2.9	0.4	7.2	7.0	5.6
Aspartic acid	9.4	8.7	11.0	10.3	11.4
Threonine	10.4	11.4	6.7	8.1	8.8
Serine	7.3	8.2	5.0	6.9	7.4
Glutamic acid	12.0	11.7	11.6	12.4	12.8
Proline	8.5	11.1	5.7	5.5	5.9
Glycine	8.6	6.5	14.1	13.3	12.1
Alanine	4.4	5.3	3.7	3.7	3.2
Valine	7.2	9.1	4.5	3.2	4.0
Methionine	1.0	1.9	3.3	2.3	1.2
Isoleucine	4.7	5.8	2.4	4.2	3.9
Leucine	5.6	6.7	4.3	3.3	5.9
Tyrosine	4.1	3.7	4.9	3.4	3.1
Phenylalanine	2.4	1.7	3.3	1.6	2.6
Histidine	1.9	0.8	3.4	2.0	3.1
Lysine	3.6	3.0	3.2	6.5	4.6
Arginine	5.3	5.1	5.2	6.3	4.3

[a] The 32K tryptic fragment was analyzed instead of the 24K thermolysin fragment.

[b] Determined as cysteic acid after performic acid oxidation.

purified to homogeneity on a heparin-agarose column (Fig. 3*A*, *e* and Fig. 3*B*). The 200K and 180K fragments, which were eluted from a gelatin-Sepharose column, were further purified by gel filtration with Sephacryl S-200 (Fig. 3*A*, *g*).

Digestion of Intact Hamster Plasma Fibronectin with Thermolysin; Separation of Four Distinct Domains—We previously found that thermolysin was a useful tool to dissect intact fibronectin into its constitutive domains (51). Fig. 4 shows the time course of thermolysin (2.5 μg/ml) digestion of intact fibronectin. The gel profile consists of four distinct



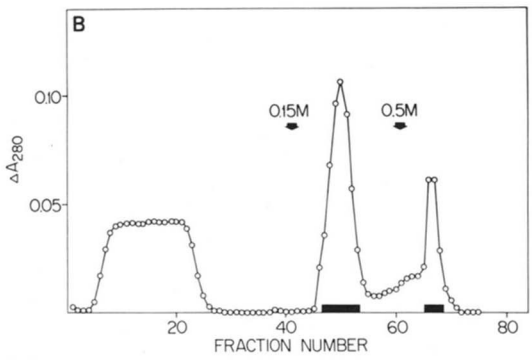


FIG. 3. **Purification of tryptic fragments of fibronectin.** *A*, SDS-polyacrylamide gel electrophoretic patterns of purified tryptic fragments. Hamster plasma fibronectin was digested by trypsin (1 μg/ml) at 22 °C for 15 min and the resulting fragments were purified as described under "Materials and Methods." *a*, intact fibronectin; *b*, the whole trypsin digest; *c*, gelatin-unbound fragments; *d*, gelatin-bound fragments; *e*, purified 32K tryptic fragment eluted from the heparin-agarose column with 150 mM NaCl; *f*, the fragments eluted from the heparin-agarose column with 500 mM NaCl; *g*, the 200K and 180K fragments purified on a Sephacryl S-200 column. *B*, heparin-agarose chromatography of gelatin-unbound tryptic fragments. Detailed conditions were described under "Materials and Methods." Fragments adsorbed on the heparin-agarose column were eluted by increasing salt concentration in a stepwise manner. Fractions of 3.7 ml were collected. The 32K fragment was eluted with 150 mM NaCl. Peak fractions as *underlined* with *solid bars* were pooled and analyzed by SDS-polyacrylamide gel electrophoresis (see Fig. 3*A*, *e* and *f*).

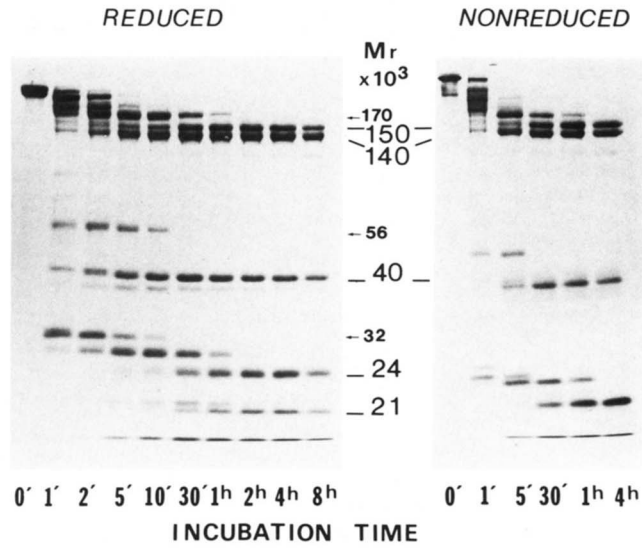


FIG. 4. **Time course of thermolysin digestion of fibronectin.** Hamster plasma fibronectin (1 mg/ml) was digested by thermolysin (2.5 μg/ml) at 22 °C for different periods of time as indicated *below* each *lane*. Digestion was terminated by adding EDTA (5 mM). The digests were analyzed by SDS-polyacrylamide gel electrophoresis with (*left*) or without (*right*) prior reduction.

fragments with M_r = 150,000–140,000, 40,000, 24,000, and 21,000 and it was consistent between 2 h and 8 h of incubation. None of these fragments contained interpolypeptide chain disulfide bridges as judged on electrophoretic mobility with or without prior reduction. The 24K and 21K fragments, however, co-migrated in gel electrophoresis under nonreducing conditions. This was confirmed by the experiment in which both purified 24K and 21K fragments were separately electrophoresed under nonreducing conditions (data not shown). Nonreduced 40K fragment gave a slightly diffuse band on the SDS-polyacrylamide gels. Close examination of the digestion profile at an early stage indicated that (i) α and β subunits were very rapidly converted to 200K and 180K fragments by releasing 32K fragment, as was the case for trypsin digestion (see the profile after 1 min of digestion); (ii) the 200K and 180K fragments were subsequently degraded into 170K and 140K fragments with a concomitant release of 40K (or its possible precursor form, *i.e.* 56K fragment); (iii) 170K fragment was further degraded into 150K fragment with corresponding appearance of 21K fragment; (iv) 32K fragment which was released at a very early stage of digestion was transiently degraded into 27K fragment, and thereafter into 24K fragment. The precursor-product relationships among these various fragments were further investigated in the following sections. Almost the same set of fragments was constantly obtained after 4 h of incubation at an enzyme concentration between 2.5 and 10 μg/ml (data not shown).

To separate these fragments obtained by thermolysin digestion, the whole digest was applied to a gelatin-Sepharose column. Only 40K fragment bound to the column (Fig. 5*A*, *a* to *d*). The unbound fractions were pooled and further fractionated by DEAE-cellulose chromatography (Fig. 5*B*). The 24K fragment did not bind to the column (Fig. 5*A*, *e*). The 21K and 150K–140K fragments were eluted with the Tris buffer containing 80 mM NaCl and 200 mM NaCl, respectively (Fig. 5*A*, *f* and *g*).

Thermolysin Digestion of Purified Tryptic Fragments; Relationships Among Proteolytic Fragments Obtained by Trypsin and Thermolysin Digestion—Purified tryptic fragments, 200K–180K, and 32K fragments, were separately digested by thermolysin in a similar fashion as previously reported (51).

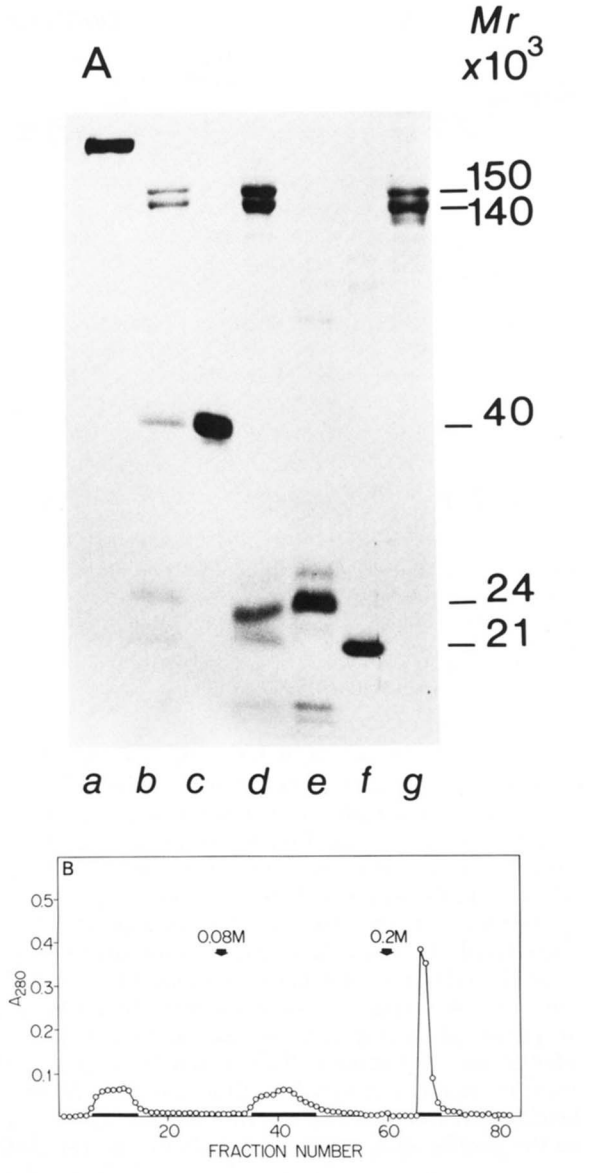


FIG. 5. **Purification of thermolysin-released fragments of fibronectin.** *A*, SDS-polyacrylamide gel electrophoretic patterns of purified thermolysin-released fragments. Hamster plasma fibronectin was digested by thermolysin (2.5 μg/ml) at 22 °C for 4 h and the resulting fragments were separated as described under "Materials and Methods." *a*, intact fibronectin; *b*, the whole thermolysin digest; *c*, gelatin-bound fragment; *d*, gelatin-unbound fragments; *e*, fragments which were not adsorbed on DEAE-cellulose; *f*, 21K fragment eluted from DEAE-cellulose with 80 mM NaCl; *g*, 150K-140K fragments eluted from DEAE-cellulose with 200 mM NaCl. *B*, DEAE-cellulose chromatography of gelatin-unbound thermolysin-released fragments. The thermolysin-released fragments which passed through a gelatin-Sepharose column were fractionated with DEAE-cellulose by increasing salt concentration in a stepwise manner. Fractions of 3.0 ml were collected. Peak fractions as *underlined* with *solid bars* were pooled and analyzed by SDS-polyacrylamide gel electrophoresis (see Fig. 5*A*, *e* to *g*).

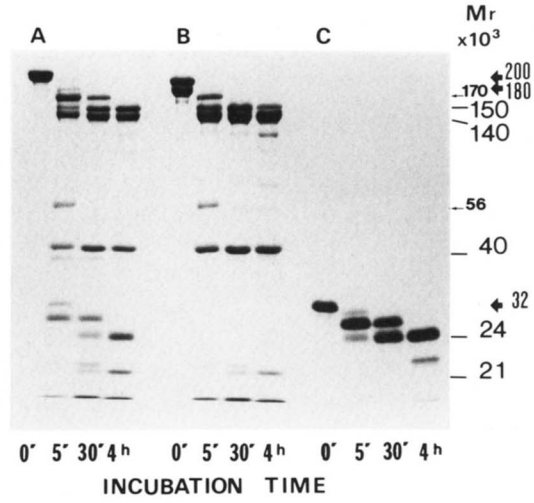


FIG. 6. **Thermolysin digestion of purified tryptic fragments.** Intact fibronectin (*A*), purified 200K and 180K tryptic fragments (*B*), and purified 32K tryptic fragment (*C*) were digested with thermolysin (2.5 μg/ml) at 22 °C for different periods of time as indicated *below* each *lane*.

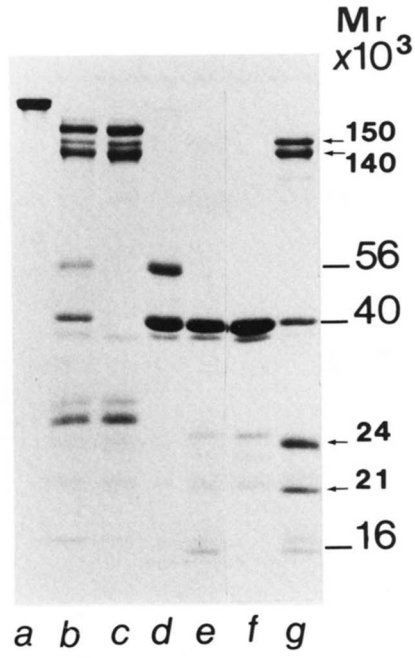


FIG. 7. **Identification of the precursor fragment of 40K gelatin-binding fragment.** Intact fibronectin (*a*) was digested by thermolysin (2.5 μg/ml) at 22 °C for 5 min. The digestion was terminated by adding EDTA (5 mM). The resulting digest (*b*) was fractionated into gelatin-unbound (*c*) and gelatin-bound (*d*) fragments with a gelatin-Sepharose column. It should be noted that the 56K fragment, as well as the 40K fragment, was capable of binding to gelatin. The mixture of 56K and 40K fragments thus obtained was further digested by thermolysin (2.5 μg/ml) at 22 °C for 4 h (*e*). As a control, purified 40K fragment was also digested by thermolysin at 22 °C for 4 h (*f*). *g*, the thermolysin digest (22 °C, 4 h) of intact fibronectin. Only the fragment with M_r = 16,000 was specifically generated from the mixture of 56K and 40K fragments.

The larger fragments produced 150K-140K, 40K, and 21K fragments but not 24K fragment (Fig. 6*B*). Instead, the 32K fragment was transiently converted to 27K fragment and finally to 24K fragment (Fig. 6*C*). No 21K fragment was generated from the tryptic 32K fragment by thermolysin.[2]

These results clearly indicated that (i) the thermolysin-released 24K fragment and the trypsin-released 32K fragment were derived from the same domain in intact fibronectin; (ii) 32K and 27K fragments which appeared transiently in the early stage of thermolysin digestion of intact fibronectin were

[2] Although a faint band was seen below the 24K fragment after 4 h of digestion of tryptic 32K fragment with thermolysin (Fig. 6*C*), this band did not coincide with the 21K fragment. This was further confirmed by a separate experiment in which purified 21K fragment was coelectrophoresed with the thermolysin digest of tryptic 32K fragment in the same lane (data not shown).

precursors of the 24K fragment, thus, all these three fragments were derived from the same domain; (iii) the 24K and 21K fragments were derived from distinctively different domains of intact fibronectin.

Another precursor-product relationship has been suggested between 56K and 40K thermolysin-released fragments by the time course analysis (see Fig. 2). To substantiate this possibility, intact fibronectin was digested by thermolysin for 5 min and the digest was fractionated with a gelatin-Sepharose column. The 56K and 40K fragments bound to gelatin-Sepharose, whereas other fragments did not (Fig. 7, *a* to *d*). The mixture of 56K and 40K fragments thus obtained was further digested with thermolysin at 22 °C for 4 h. The 56K fragment was converted to 40K fragment by releasing a small fragment with M_r = 16,000 (Fig. 7, *e*). The 40K fragment was also slightly degraded into several smaller fragments with molecular weights ranging from 25,000 to 21,000 (Fig. 7, *f*), possibly due to denaturation occurring during the elution from gelatin-Sepharose with urea. These results indicate that 56K fragment is a transient precursor of 40K gelatin-binding fragment.

Identification of Fibrin-binding Fragments; Evidence for Asymmetric Localization of 21K Thermolysin-released Fragments between α and β Subunits—Fibronectin has been shown to bind to fibrin (24, 31, 36). To identify the fibrin-binding fragment(s), intact fibronectin was digested by thermolysin for 4 h and the resulting digest was fractionated with fibrin-Sepharose (Fig. 8*A*). The 24K and 21K fragments bound to fibrin,[3] whereas the 150K–140K and 40K fragments failed to bind (Fig. 8*B*). These results indicated that there were two different fibrin-binding sites on intact fibronectin; one was recovered in the 24K fragment and the other was recovered in the 21K fragment. To confirm this possibility, intact fibronectin was digested by trypsin and the resulting digest was also fractionated with a fibrin-Sepharose column. Of the two large tryptic fragments, only the 200K fragment bound to fibrin whereas the 180K fragment failed to bind (Fig. 9). The 32K tryptic fragment, which shared the same domain with the 24K thermolysin-released fibrin-binding fragment, also bound to fibrin (Fig. 9). These results indicate that the fibrin-binding region recovered as the 21K thermolysin fragment is only present in the 200K tryptic fragment (or α subunit) but absent in the 180K tryptic fragment (or β subunit). The lower intensity of the Coomassie blue-stained band of the 21K fragment than that of the 24K fragment (see Fig. 8*B*, *b*) seems to be in agreement with the asymmetric distribution of the 21K fibrin-binding region between α and β subunits.

To confirm this possibility, purified tryptic 200K and 180K fragments (Fig. 10*A*, *a*) were separated from each other through a fibrin-Sepharose column (Fig. 10*A*, *b* and *c*). Each of these fragments was separately digested with thermolysin. The 200K fragment was first transiently converted to 170K fragment by releasing 40K (or its precursor, 56K) fragment (Fig. 10*B*, *d*). The 170K fragment was subsequently converted to 150K fragment by releasing 21K fragment[4] (or its possible precursor which migrated just above the 21K fragment) (Fig. 10*B*, *e*). The 150K fragment was also partly converted to 140K fragment (Fig. 10*B*, *f*). In contrast, the 180K tryptic fragment

[3] Although more 24K fragment was present than 21K fragment in the unfractionated digest (Fig. 8*B*, *b*), the relative amount of the 24K fragment to 21K fragment was reduced in the fibrin-bound fractions (Fig. 8*B*, *d*). The basis for the low recovery of the former fragment is not clear. The similar results were obtained even when the fibrin-bound fragments were eluted with the Tris buffer containing 6 M urea and 0.5 M NaCl.

[4] It should be noted that a trace amount of 170K fragment was present in the unfractionated thermolysin digest of Fig. 8*B*, and it bound to fibrin-Sepharose (Fig. 8*B*, *d*), suggesting that the 170K fragment was a transient precursor for both 150K and 21K fragments.

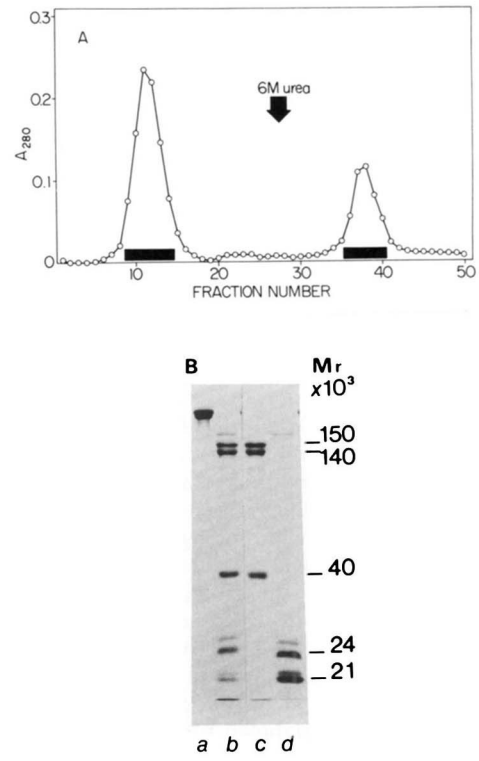


FIG. 8. **Separation of thermolysin-released fragments on a column of fibrin-Sepharose.** *A*, intact fibronectin was digested by thermolysin (2.5 μg/ml) at 22 °C for 2 h and the resulting fragments were fractionated with a fibrin-Sepharose column as described under "Materials and Methods." Unbound and bound fractions were separately pooled and analyzed by SDS-polyacrylamide gel electrophoresis. *B*, *a*, intact fibronectin; *b*, the whole thermolysin digest; *c*, fibrin-unbound fragments; *d*, fibrin-bound fragments.

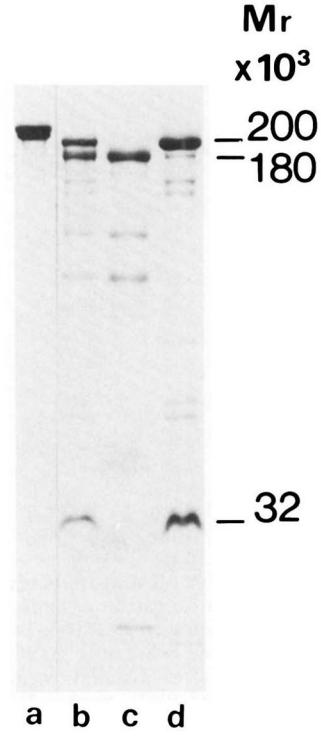


FIG. 9. **Isolation of fibrin-binding tryptic fragments.** Intact fibronectin was digested by trypsin (1 μg/ml) at 22 °C for 15 min and the resulting fragments were fractionated with a fibrin-Sepharose column. *a*, intact fibronectin; *b*, the whole trypsin digest; *c*, fibrin-unbound fragments; *d*, fibrin-bound fragments.

was directly converted to 140K fragment by releasing 56K and 40K gelatin-binding fragments (Fig. 10*B*, *g* to *j*). No 21K fragment was generated from the 180K tryptic fragment. These results clearly indicate that the "21K fibrin-binding domain" was only present in tryptic 200K fragment. The slow conversion of the 150K fragment to the 140K fragment might be due to the denaturation of tryptic 200K fragment during purification steps, especially during repeated elution from the gelatin- and fibrin-Sepharose columns with 6 M urea. Consequently, digestion of intact fibronectin by thermolysin gave almost equal amounts of 150K and 140K fragments (see Fig. 4), while purified 200K and 180K fragments, which were once eluted from gelatin-Sepharose with 6 M urea, gave a much denser amount of 140K fragment than 150K fragment (Fig. 6*B*).

Factor XIIIa-dependent Amine Incorporation into Fibronectin—Since fibronectin has been shown to be incorporated into fibrin clots during blood coagulation and covalently cross-linked to fibrin by Factor XIIIa (12, 24, 36), we examined whether 24K and 21K fibrin-binding fragments could serve as substrates for Factor XIIIa-dependent transamidation. Intact fibronectin was labeled with [^{3}H]putrescine by Factor XIIIa according to Mosher *et al.* (39) and then digested by either trypsin or thermolysin. Among various tryptic fragments, the 32K fragment was predominantly labeled with [^{3}H]putrescine (Fig. 11*B*, *b*), being in good agreement with others (31, 38, 39). Interestingly, when the labeled fibronectin was digested by thermolysin, the 24K fragment, which was derived from the same domain as the 32K tryptic fragment, was not labeled at all (Fig. 11*B*, *c*). These results suggest that the susceptible glutamyl residue(s) in the 32K tryptic fragment is localized near the NH_2 or COOH terminus of the fragment and is not recovered into the 24K thermolysin fragment. Thøgerson *et al.* (62) recently reported that putrescine was incorporated near the NH_2 terminus of the plasmic 29K fragment which corresponds to our 32K tryptic fragment.

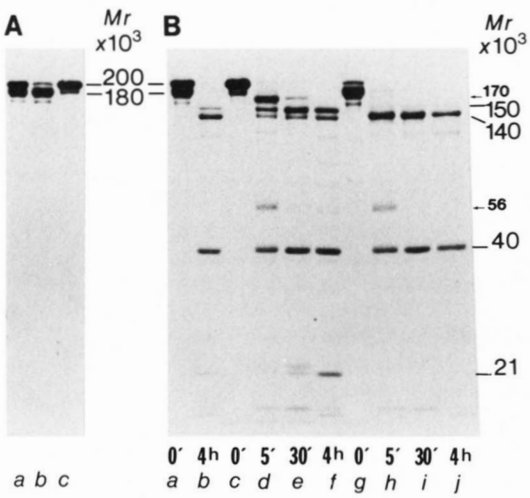


FIG. 10. **Separation of 200K and 180K tryptic fragments and subsequent thermolysin digestion of each of these fragments.** *A*, the 200K and 180K tryptic fragments were separated from each other on a fibrin-Sepharose column. *a*, 200K and 180K fragments before separation; *b*, the fibrin-unbound fragment (180K fragment); *c*, the fibrin-bound fragment (200K fragment). *B*, each of 200K and 180K fragments was separately digested by thermolysin (2.5 μg/ml) at 22 °C for different periods of time as indicated *below* each *lane*. *a* and *b*, intact fibronectin; *c* to *f*, the 200K fragment; *g* to *j*, the 180K fragment. It should be noted that the 21K fragment was only generated from the 200K fragment.

Amino Acid and Sugar Composition of Intact Fibronectin and Its Proteolytic Fragments—Amino acid composition of intact fibronectin and its purified proteolytic fragments is shown in Table I. Overall composition of intact fibronectin was very similar to those obtained from human and bovine plasma (31, 63). Composition of the 150K-140K fragments was similar to that of intact molecules except that the content of half-cystine was markedly low. Instead, the 40K, 32K, and 21K fragments contained most of the half-cystine in intact molecules.

Sugar composition of intact fibronectin and purified fragments is shown in Table II. If we assume that each carbohydrate unit attached to subunit polypeptides contains three mannose residues (71), the present results indicate that intact molecules carry four units per subunit. Similarly, it can be estimated that 150K-140K and 40K fragments contain one and three units, respectively. The 32K fragment did not contain any detectable sugar moieties. The 21K fragment contained a trace amount of carbohydrates, but the number of mannose residues per 1 mol of the fragment was far less than three. Only a minor population of the 21K fragment may contain sugar residue.

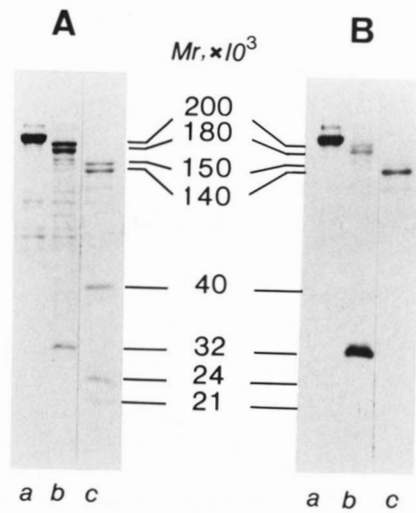


FIG. 11. **Incorporation of [^{3}H]putrescine into fibronectin and its proteolytic fragments by Factor XIIIa.** Detailed conditions are described under "Materials and Methods." Fibronectin was incubated with [^{3}H]putrescine (0.57 mM), Factor XIII (50 μg/ml), and thrombin (1 unit/ml) at 22 °C for 1.5 h. The [^{3}H]putrescine-labeled fibronectin was digested by either trypsin or thermolysin. The resulting digests were directly analyzed by SDS-polyacrylamide gel electrophoresis. The gel was stained with Coomassie blue (*A*) and then subjected to fluorography (*B*). *a*, [^{3}H]putrescine-labeled intact fibronectin; *b*, the trypsin digest of labeled fibronectin; *c*, the thermolysin digest of labeled fibronectin.

TABLE II

Carbohydrate composition of intact fibronectin and its proteolytic fragments

Constituents	Intact fibronectin	150K-140K thermolysin fragment	40K thermolysin fragment	32K tryptic fragment[a]	21K thermolysin fragment
	mol/mol glycoprotein				
Mannose	13.1	2.7	8.2	<0.1	0.4
Galactose	10.4	1.6	5.8	<0.1	0.7
N-Acetylglucosamine	16.1	3.3	10.0	<0.1	0.8
Sialic acid	8.3	2.4	5.0	<0.1	0.4

[a] The 32K tryptic fragment was analyzed instead of the 24K thermolysin fragment.

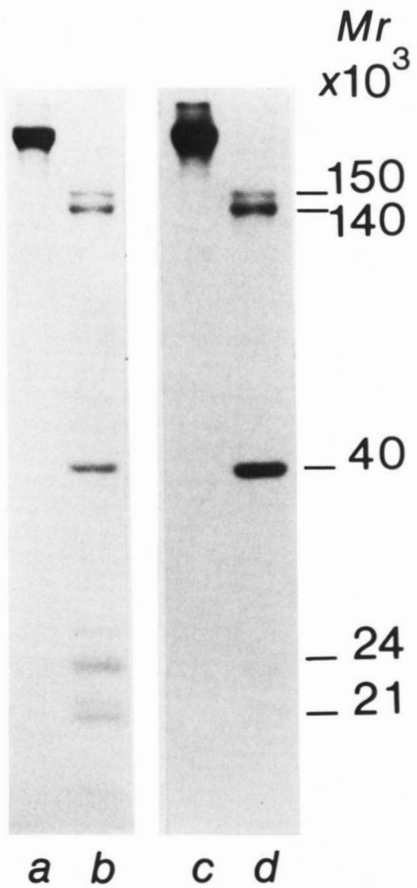


FIG. 12. **Distribution of carbohydrate residues among various thermolysin-released fragments.** Terminal carbohydrate residues were labeled by galactose oxidase/NaB^3H_4 as described under "Materials and Methods." Labeled intact fibronectin was digested by thermolysin (2.5 μg/ml) at 22 °C for 4 h. Both intact fibronectin (*a* and *c*) and the resulting digest (*b* and *d*) were subjected to SDS-polyacrylamide electrophoresis followed by fluorography. *a* and *b*, protein staining with Coomassie blue; *c* and *d*, fluorography.

Localization of sugar residues on 40K and 150K–140K fragments was also confirmed by labeling of sugar units with galactose oxidase and subsequent reduction with NaB^3H_4. After digestion of labeled fibronectin with thermolysin, most of the labels were localized on the 40K fragment while 150K–140K fragments were labeled to a lesser extent and 24K and 21K fragments were almost devoid of labels (Fig. 12).

DISCUSSION

Physicochemical studies on the molecular properties of fibronectin indicate that the protein is composed of several domains connected by flexible polypeptide segments (41–43). A similar domain structure was also proposed from the studies on limited proteolysis and chemical fragmentation of intact fibronectin (44, 45). So far, various proteases, including trypsin (39, 44, 45, 48), plasmin (31, 61, 64), thrombin (39, 52, 65), chymotrypsin (39, 46, 50), cathepsin D (39, 47, 65–67), subtilisin BPN′ (49), mast cell protease (66), thermolysin (51), and leukocyte elastase (53), were used to dissect intact fibronectin to obtain functional fragments. However, the proteolytic conditions employed in these studies were usually too strong to preserve the structure and function of other domains or too mild to separate the rest of the constitutive domains. Recently, we reported that thermolysin dissected plasma fibronectin into (at least) three functionally distinct fragments almost quantitatively (51). This paper described i) the optimal conditions of limited proteolysis to effect maximal yield of fragments, representing the domain structure of fibronectin and ii) the isolation, purification, and characterization of each domain fragment by affinity chromatography.

We found that thermolysin cleaved intact fibronectin into four distinct fragments with M_r = 150,000–140,000, 40,000, 24,000, and 21,000. The 21K fragment was not fully recognized in the previous study (51) because the proteolytic digests were routinely analyzed with 8% acrylamide gels in which 21K fragment usually migrated at the dye front. To obtain better resolution of 21K fragment, 9.5% acrylamide gels were used throughout the present investigation.

Several lines of evidence indicate that these four thermolysin-released fragments represent, respectively, four distinct structural domains of intact molecules. First, time-course analysis of thermolysin digestion of intact fibronectin or its tryptic fragments suggests that there is no precursor-product relationships among these four fragments; instead, each fragment is sequentially released from the intact subunits (see Fig. 13). Second, these four fragments were resistant against further degradation even after prolonged digestion. Third, these four fragments are distinct from each other in their biological activities as discussed in detail later (also see Table III). For convenience, we termed these four distinct domains as 150K–140K, 40K, 24K, and 21K, respectively, according to the molecular weight of representative thermolysin-released fragments. The 24K domain can be obtained as 32K fragment by mild trypsin digestion. Similarly, the 40K domain can be transiently recovered as 56K fragment during thermolysin digestion.

The most important feature of the domain structure of hamster plasma fibronectin is the asymmetric distribution of these four domains between intact α and β subunit polypeptides. The first three domains, *i.e.* 150K–140K, 40K, and 24K, are equally present in both α and β subunits. However, the 21K domain seems to be only present in the larger (α) subunit. This conclusion was drawn from the following observation as schematically summarized in Fig. 13. (i) When purified tryptic 200K and 180K fragments were separately digested by thermolysin, only the 200K fragment gave the 21K fragment together with 150K–140K and 40K fragments. No 21K fragment was, however, released from 180K fragment. Since α and β subunits were respectively converted to 200K and 180K fragments by trypsin by releasing the 32K fragment, the 21K domain must be present only in the α subunit. (ii) Between tryptic 200K and 180K fragments, only the former could bind to fibrin. Since one of the fibrin-binding domains, the 24K domain, was completely absent in both fragments, another fibrin-binding domain, the 21K domain, must be present only in 200K fragment.

The difference in size of subunit polypeptides has been repeatedly reported for plasma fibronectin (31, 52, 61, 63), although the molecular basis for such difference has not been clarified. A recent report by Kurkinen *et al.* (68) that α subunit of intact human plasma fibronectin was more rapidly degraded than β subunit by plasmin, is in good agreement with our present results. However, they failed to find any difference between α and β subunits when they isolated each subunit polypeptide by preparative gel electrophoresis and compared the fragments obtained by *S. aureus* proteinase digestion or cyanogen bromide cleavage. Birdwell *et al.* (73) also failed to detect any structural difference between α and β subunits as they observed an identical peptide map of the electrophoretically separated subunits with two-dimensional electrophoresis. Our present results, however, strongly indicate that the difference of the two subunits of intact plasma fibronectin is mainly due to the asymmetric localization of the 21K domain. In addition, some difference seems to be present in the 150K–

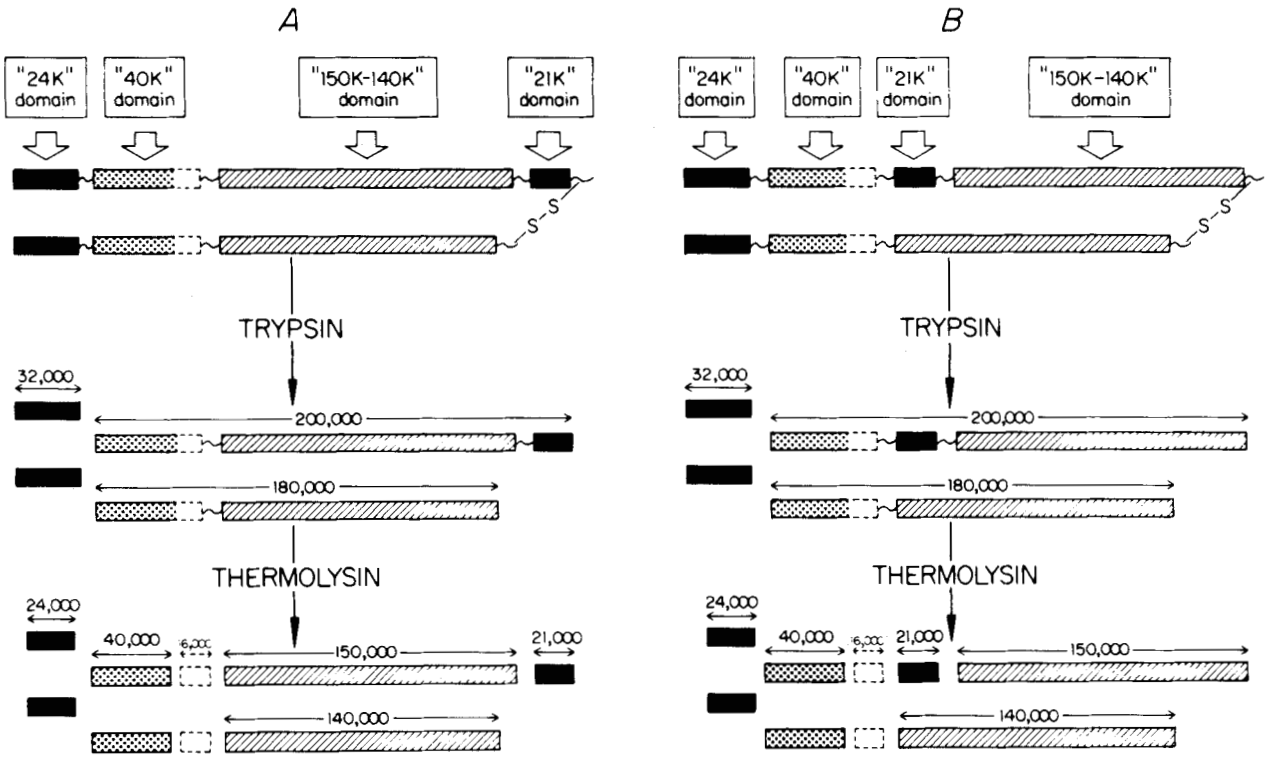


FIG. 13. **Proposed model for sequential digestion of intact fibronectin with trypsin and thermolysin emphasizing fibrin-binding domains (*solid bars*).** Two possible models are presented based upon difference in the alignment of four distinct domains. For details, see the text. Presentation of heterodimer scheme for intact fibronectin is tentative (see "Discussion").

TABLE III

Biological activities of isolated domains

Domains	Binding specificities				Other activities	
	Collagen (gelatin)	Heparin	Fibrin	*S. aureus*[a]	Cell[b] spreading	Factor[c] XIIIa
150K-140K	−	+	−	−	+	−
40K	+	−	−	−	−	−
24K	−	+	+	+	−	+
21K	−	−	+	−	−	−

[a] Binding to *S. aureus* (40; K. Sekiguchi, M. Fukuda, S. Hakomori, unpublished observation).

[b] Activity to promote cell spreading (51).

[c] Susceptibility to Factor XIIIa-catalyzed transamidation.

140K domain, because this domain in α subunit seems to be longer than that of β subunit. However, the basis for the difference remains to be elucidated.

The alignment of these four domains along the subunit polypeptide chain has not yet been studied by ourselves, but several lines of evidence obtained by others indicates that the 24K domain is located at the NH_2 terminus and is followed by the 40K gelatin-binding domain (47, 65, 67, 69). However, the position of the 21K domain is not yet clear. Thus, two possible alignments of these four domains are shown in Fig. 13, depending on the location of the 21K. In model *A*, the 21K domain is located at the COOH terminus of the α subunit. Alternatively, the 21K is inserted between the 40K and the 150K-140K domain of α subunit (model *B*). From model *B*, one might expect to detect the transient fragments with M_r = 77,000 (*i.e.* 56,000 + 21,000) and/or 61,000 (*i.e.* 40,000 + 21,000) at an early stage of thermolysin digestion of intact fibronectin. However, such transient fragments have never been detected. In addition, our preliminary experiments on chemical cleavage of intact fibronectin by *S*-cyanylation showed that the NH_2-terminal two-thirds of α and β subunit polypeptides was released as a single fragment with M_r = 145,000. *S*-cyanylation of the 200K and 180K tryptic fragments gave, instead, a single 115K fragment. Both *S*-cyanylation-released 145K and 115K fragments were heavily labeled by the galactose oxidase/NaB^3H_4 method. These preliminary results taken together suggest that the 21K domain is located in the COOH-terminal one-third because α and β subunits share almost the same primary structure in the NH_2-terminal two thirds. Thus, model *A* (Fig. 13) seems to be more probable than model *B*, although further study is needed to clarify the alignment of the four major domains.

In both models in Fig. 13, we tentatively assumed that intact fibronectin is a heterodimer of α and β subunits. Indeed, intact plasma fibronectin from hamster, human, and bovine apparently gave a single band on SDS-polyacrylamide gels under nonreducing conditions even after prolonged electrophoresis (see Fig. 1), but this does not necessarily exclude an alternative possibility that intact plasma fibronectin is a mixture of homodimers because two possible homodimers with M_r = 460,000 and 420,000 may not be separable on SDS-polyacrylamide gels even under such conditions. One possible approach to solve this problem might be the use of a specific antibody to the 21K domain.

The summation of the molecular weight of the four domains results in 257,000 daltons for α subunit and 226,000 daltons for β subunit instead of 230,000 daltons and 210,000 daltons, respectively. The basis for this apparent discrepancy is not yet well understood. It might be partly due to the inaccuracy in determining the molecular weight of such high molecular weight proteins as intact α and β subunits or tryptic 200K and 180K fragments by SDS-polyacrylamide gel electrophoresis. For example, the molecular weight of subunit polypeptides of human plasma fibronectin has been reported to be from 250,000 to 200,000 daltons (36, 39, 47, 48, 52, 53, 63, 64, 68). These inconsistencies might be due to the lack of a suitable

molecular weight standard just above fibronectin and also due to the difference in acrylamide concentrations and buffer systems. Another possibility is that the molecular weight of the 40K domain may be overestimated because of a high carbohydrate content in this particular domain. Anomalous behavior of highly glycosylated proteins in SDS-acrylamide gels has been reported (70). In addition, physicochemical studies on fibronectin revealed that it has rather unusual secondary and tertiary structures (41-43). Such a unique conformation of fibronectin may also disturb the accurate estimation of molecular weight of intact subunits and its proteolytic fragments.

Another important characteristic of the domain structure of fibronectin is that these four domains are also functionally distinct from each other. Table III summarizes the biological activities of these domains. The 150K-140K domain binds to heparin but not to collagen or fibrin (51). It also promotes cell spreading on an artificial surface. The 40K domain specifically binds to collagen (or gelatin). This domain carries most of the carbohydrate units. The 24K domain exhibits diverse binding specificity; it binds to heparin (51), fibrin, and *S. aureus* (40).[5] This domain also specifically interacts with Factor XIIIa (31, 38, 39). The 21K domain only binds to fibrin. The fact that each domain possesses distinct biological functions excludes any precursor-product relationship among these four domains.

Previously, Engvall *et al.* (16) proposed that the fibrinogen- (or fibrin-) binding site was located on the collagen-binding domain, mainly based upon their finding that gelatin strongly inhibited the binding of fibronectin to fibrinogen. However, the 40K gelatin-binding fragment has never bound to fibrin in repeated experiments. Instead, 24K and 21K fragments consistently bound to fibrin. Thus, this apparent discrepancy seems to be due to the difference in methodology to locate the fibrin-binding site(s). Since the 24K domain has been shown to be in juxtaposition to the 40K gelatin-binding domain (65, 67, 69), the binding of bulky gelatin to the 40K domain may hinder the subsequent binding of fibrinogen to fibronectin. Alternatively, the binding of gelatin to fibronectin may induce a conformational change of fibronectin which prevents subsequent binding of fibrinogen or may lower the affinity of fibronectin to fibrinogen. In support of our results, Hörmann and Seidl (74) recently reported that plasmic 30K fragment (which corresponds to our 32K tryptic fragment) bound to fibrin but 40K gelatin-binding fragment did not bind.

Previously, it was reported that the site interacting with Factor XIIIa and thereby labeled with [^{3}H]putrescine was localized on the plasmic (or tryptic) M_r = approximately 30,000 fragment (38, 39) which seemed to correspond to the 24K domain. Our present data were in good agreement with these data and further clarified that this domain was also capable of binding to fibrin. The coincidence of the sites which interacts with fibrin and Factor XIIIa could well explain the fact that fibronectin is incorporated into fibrin clots and is eventually cross-linked to fibrin by Factor XIIIa. It remains to be solved, however, why another fibrin-binding domain, the 21K domain, could not be a substrate for Factor XIIIa.

Previously, we reported almost all carbohydrate residues of intact fibronectin were exclusively localized on the 40K gelatin-binding fragment (51), based on the labeling of terminal carbohydrate residues by the galactose oxidase/NaB^3H_4 method. However, present data on the carbohydrate composition of various purified fragments indicated that one carbohydrate unit was present on the 150K-140K domain although the other three units were on the 40K domain. Since in the previous experiments the carbohydrate localization was estimated only by labeling carbohydrate residues with galactose oxidase/NaB^3H_4, the detection and evaluation of the faintly-labeled bands was influenced by the extent of development or printing of fluorographs. Indeed, we found that 150K-140K fragments were faintly labeled after prolonged development of the film, being in good agreement to the carbohydrate analysis of these fragments.

[5] K. Sekiguchi, M. Fukuda, and S. Hakomori, unpublished observation.

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