Communication

Location of a Gelatin-binding Region of Human Plasma Fibronectin*

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Plasma fibronectin, which is also known as cold-insoluble globulin, consists of two polypeptide chains of approximately 250,000 daltons joined near one end of the molecule. Proteolytic digestion of human plasma fibronectin and NH2-terminal sequence analysis of some of the fragments produced were used to locate the gelatin-binding region of fibronectin within the intact molecule. Fibronectin was cleaved with cathepsin D, and a 72,000-dalton gelatin-binding fragment was isolated. This fragment could be cleaved further with thrombin to produce a 43,000-dalton fragment which retained the ability to bind to gelatin, and a 29,000-dalton disulfide-enriched fragment which did not. All three fragments appeared to consist of single polypeptide chains. NH2-terminal analysis of the 72,000-dalton and 29,000-dalton fragments by the 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) chloride method before and after treatment with L-pyroglutamyl-peptide hydrolase indicated that they shared the same NH2-terminal sequence, pyroglutamic acid (<Glu-Ala-), as intact fibronectin. Automated amino acid sequence analysis of the 72,000-dalton piece following L-pyroglutamyl-peptide hydrolysis digestion confirmed that the first 6 residues of this fragment, <Glu-Ala-Glu-Glu-Met-Val-, were identical with those of intact fibronectin. The 43,000-dalton gelatin-binding fragment contained an NH2-terminal alanine residue as monitored by dansylation. These results indicate that the 29,000-dalton fragment is located at the NH2 terminus of fibronectin and that a gelatin-binding site lies within an adjacent 43,000-dalton region.

Fibronectin is a high molecular weight glycoprotein which is found on the surfaces of fibroblasts and some other cell types as well as in blood plasma. Although the in vitro functions of fibronectin have not yet been clearly demonstrated, in vivo studies suggest that this molecule plays an important role in the adhesion of cells to their substrates (for reviews, see Refs. 1 and 2). Both cellular and plasma fibronectins consist of two subunits of approximately 220,000 to 250,000 daltons (3-6) joined by disulfide bonds located near one end of the molecule (7-9). The cellular form exists as part of high molecular weight complexes as well (4, 5). Although plasma and cellular fibronectins are quite similar, some differences have been noted and are discussed in Refs. 1 and 10.

Both plasma and cellular fibronectins interact with collagen in several ways. Both fibronectins bind strongly to collagen, especially in its denatured form (gelatin) (11, 12). Plasma fibronectin can also be covalently cross-linked to types I and III collagen by the action of plasma transglutaminase (Factor XIIIa) (13). Plasma fibronectin is required for the spreading of platelets on collagen surfaces (14) and promotes the clearance of gelatinized lipid emulsions from the blood by the reticuloendothelial system (15). Both plasma and cellular fibronectins mediate the attachment of some normal and transformed cells to collagen-coated substrates (16, 17). Furthermore, immunofluorescence studies indicate that there is an extensive co-distribution of fibronectin and collagen in cultures of normal fibroblasts (18).

Recently, several laboratories have isolated proteolytically-derived fragments of fibronectin which retain gelatin- or collagen-binding activity (19-24). In this study, we use NH2-terminal sequence analysis of some of these fragments to determine the location of a gelatin-binding site within the intact human plasma fibronectin molecule.

**EXPERIMENTAL PROCEDURES**

**Preparation of Fibronectin Fragments—**Fibronectin was isolated from human plasma by gelatin-Sepharose affinity chromatography (11) and ion exchange chromatography on DEAE-cellulose (25) as previously described (9). Digestion of fibronectin with cathepsin D and isolation of collagen-binding fragments were carried out essentially according to Balian et al. (19). Fibronectin (8 to 10 mg in 2 ml of NaCl/Pi) was diluted with 0.1 mM sodium formate, 0.2 mM PhCH2SO2F, pH 3.5, to a final concentration of 200 μg/ml. Cathepsin D (40 μg) was added, and the mixture was incubated at 30°C for 4 h. The reaction was stopped by the addition of 12 μg of pepstatin, and the solution was brought to neutrality by the addition of 1.5 M Tris-HCl, pH 8.8. Digested protein was precipitated by the addition of an equal volume of saturated (NH4)2SO4 at 0°C and sedimented by centrifugation at 12,000 × g for 20 min at 4°C. The pellet was resuspended in 1.5 ml of 0.15 M NaCl, 0.05 M Tris-HCl, 3 mM benzamidine HCl, 0.2 mM PhCH2SO2F, pH 7.5, and applied to a column (12 × 95 cm) of Sephadex G-150 equilibrated with this same buffer at 4°C. Two protein-containing peaks were eluted; material from the more slowly eluting peak was pooled and stored at -20°C.

A 72,000-dalton gelatin-binding fragment was isolated from this material by gelatin-Sepharose affinity chromatography. Pooled protein was passed over a column of gelatin-Sepharose CL-4B (prepared as in Ref. 6) equilibrated with 0.15 M NaCl, 0.05 M Tris-HCl, 3 mM benzamidine HCl, 0.2 mM PhCH2SO2F, pH 7.5, at room temperature. Approximately 0.5 ml of resin was used for every 5 ml of protein solution. The column was then washed extensively with the buffer used for equilibration. Gelatin-binding fragments were eluted with this same buffer containing 2.0 M guanidine HCl. Protein-containing fractions were pooled and dialyzed against 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5, at 4°C.

The 72,000-dalton gelatin-binding fragment (1.0 mg in 1.0 ml of 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5) was digested further with 40

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1 The abbreviations used are: NaCl/Pi, phosphate-buffered saline (0.13 M NaCl, 8.8 mM NaH2PO4, 2.7 mM KH2PO4, pH 7.4); PhCH2SO2F, phenylmethylsulfonyl fluoride; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; SDS, sodium dodecyl sulfate; <Glu pyroglutamic acid.
µg of purified human α-thrombin for 7 h at 37°C. Thrombin was removed from the digest by passage over a column (0.4 ml) of phosphocellulose equilibrated with NaCl/P, at room temperature. The thrombin-depleted digest was applied to a column (1.0 ml) of gelatin-Sepharose equilibrated with NaCl/P, at room temperature. The column was washed with NaCl/P, and a 29,000-dalton fragment was not retained by the gel. The material collected in the first elution of fragments (Fig. 1B) was then eluted from the column with 2.0 M guanidine HCl in NaCl/P, and was dialyzed at 4°C against an appropriate buffer solution.

**NH₂-terminal Analysis**—Digestion of intact plasma fibronectin and the various proteolytically-derived fragments with L-pyroglutamyl-peptide hydrolase was carried out using a modification (6) of the procedure of Podell and Abraham (26). NH₂-terminal amino acid analysis with dansyl-chloride was performed according to Weiner et al. (27) with modifications as previously described (6). Dansyl-amino acids were identified by chromatography on polyamide sheets (5 x 5 cm) (28). Automated amino acid sequence analysis was performed on a Beckman 890C Sequencer run with a 0.1 M Quadrol program modified from Brauer et al. (29). Phenylthiohydantoin derivatives were identified on a Hewlett-Packard 1084 A liquid chromatograph equipped with an Altex Ultrasphere C18 column and a 254 nm detector. In most cases, polypeptides were exhaustively reduced with diithothreitol and alkylated with iodoacetic acid according to Konigsberg (30) prior to L-pyroglutamyl-peptide hydrolase digestion and NH₂-terminal analysis.

**Gel Electrophoresis**—Discontinuous slab polyacrylamide gel electrophoresis in the presence of SDS was carried out according to Laemmli (31). The separating gel consisted of a linear 5 to 10% acrylamide gradient; the stacking gel contained 3% acrylamide. The gel was fixed and stained with Coomassie blue as described by Fairbanks et al. (32). Molecular weights were estimated using a mixture of proteins of known molecular weights.

**Materials**—Purified human α-thrombin (>2000 National Institutes of Health units/mg) was kindly provided by Dr. J. W. Fenton II, New York State Department of Health, Albany, N. Y. Pepstatin was a gift from Dr. Walter Toll, New York University School of Medicine, New York, N. Y., and was supplied by the United States-Japan Cooperative Cancer Research Program. Other products were obtained from the following sources: P-11 phosphocellulose, Whatman, Inc.; Sephadex G-150 and Sepharose CL-4B, Pharmacia Fine Chemicals; cathepsin D (EC 3.4.23.3), Sigma Chemical Co.; L-pyroglutamyl-peptide hydrolase (EC 3.4.11.8), Boehringer Mannheim Biochemicals; dansyl-chloride (1 g/10 ml of acetone) and Schueler & Schuell micropolyamide sheets with aluminum foil support base, Pierce Chemical Co. All other reagents were of the best grade available.

**RESULTS**

Recently, we reported the results of experiments involving digestion of human plasma fibronectin with purified human α-thrombin (6). Intact fibronectin, composed of two subunit chains of approximately 245,000 and 250,000 daltons, was cleaved with thrombin to produce three major fragments: two large ones with molecular weights of about 230,000 and 235,000, and a small one with a molecular weight of 29,000. All three fragments appeared to consist of single polypeptide chains. Automated NH₂-terminal sequence analysis of the intact molecule and the small fragment showed that they contained identical amino acid sequences of Glu-Ala-Glx-Met-Val. The large fragments, on the other hand, yielded the sequence Ala-Ala-Val-Tyr. These results indicate that the 29,000-dalton fragment is located at the NH₂ terminus of the parental molecule, while the larger fragments constitute the carboxyl portion.

Balian et al. (19) have employed cathepsin D to produce a 72,000-dalton gelatin-binding fragment of fibronectin. This fragment could be cleaved further with plasmin to yield a 42,000-dalton fragment which retained the ability to bind to gelatin and a 30,000-dalton fragment which did not. Since we have demonstrated that plasmin and thrombin treatment of fibronectin result in similar patterns of digestion (6), we reasoned that the 30,000-dalton fragment which Balian et al. described might be similar to or identical with the 29,000-dalton fragment produced by thrombin treatment of intact fibronectin. This hypothesis predicts that the 72,000-dalton gelatin-binding fragment produced by cathepsin D digestion is located at the NH₂ terminus of fibronectin. Thrombin treatment of this fragment should produce a 29,000-dalton NH₂-terminal fragment which cannot bind to gelatin and a 43,000-dalton fragment which retains gelatin-binding activity. The experiments described below confirm these suppositions and allow us to demonstrate conclusively the location of a gelatin-binding region within the fibronectin molecule.

Digestion of human plasma fibronectin with cathepsin D as described by Balian et al. (19) resulted in a complex mixture of fragments (Fig. 1B). The two major components had molecular weights of approximately 150,000 and 72,000, as determined by SDS-polyacrylamide gel electrophoresis in the presence of a reducing agent. When the digest was passed over a gelatin-Sepharose column, only three fragments were retained: the 72,000-dalton fragment, and two minor components with molecular weights of approximately 200,000 and 180,000 (not shown). Since these higher molecular weight components are more prominent at early times of digestion and disappear as the 72,000-dalton fragment accumulates (not shown), it is likely that they are precursors to the 72,000-dalton piece.

In order to purify the 72,000-dalton fragment, the digest was first depleted of the higher molecular weight gelatin-binding fragments by gel chromatography on a column of Sephadex G-150. Two peaks of material were eluted from the column; material from the more slowly eluting peak (Fig. 1C) was subjected to gelatin-Sepharose affinity chromatography. Most of the fragments passed through the gelatin-Sepharose column (Fig. 1D); material which was bound and subsequently eluted with 2.0 M guanidine HCl consisted of a nearly pure 72,000-dalton fragment (Fig. 1E). Several minor contaminants remained, including fragments of approximately 200,000 and 180,000 daltons, as well as several pieces with molecular weights ranging from approximately 55,000 to 68,000.

**Fig. 1.** Proteolytic digestion of fibronectin and isolation of several fragments. Samples prepared as described under "Experimental Procedures" were reduced and applied to a 5 to 15% gradient SDS-polyacrylamide gel. A, intact fibronectin (7 µg); B, fibronectin digested with cathepsin D (11 µg); C, B following Sephadex G-150 chromatography to remove higher molecular weight components (13 µg); D, fraction of C which does not bind to gelatin (3 µg); E, gelatin-binding fraction of C (8 µg); F, E digested with thrombin (10 µg); G, F following phosphocellulose chromatography to remove thrombin (6 µg); H, gelatin-binding fraction of G (5 µg); I, fraction of G which does not bind to gelatin (5 µg); J, fragment which does not bind to gelatin isolated from thrombin digests of intact fibronectin (5 µg); K, mixture of I and J (10 µg). The scale indicates the positions of migration of molecular weight standards.
As predicted, treatment of the purified 72,000-dalton fragment with thrombin produced two major fragments which migrated, when reduced, with apparent molecular weights of approximately 43,000 and 29,000 (Fig. 1, F and G). Passage of the digest over a gelatin-Sepharose column demonstrated that the 43,000-dalton fragment retained the ability to bind to gelatin (Fig. 1H), whereas the 29,000-dalton fragment did not (Fig. 1I). The 29,000-dalton fragment prepared in this manner had an electrophoretic mobility identical with that of the 29,000-dalton fragment prepared by thrombin digestion of intact fibronectin (compare Fig. 1, I and J; see also Fig. 1K).

Preparations of both the 43,000-dalton and 29,000-dalton fragments contained some impurities. The principal contaminant in samples of the 43,000-dalton fragment consisted of a small amount of undigested 72,000-dalton fragment (Fig. 1H). The 29,000-dalton fragment preparation contained rather large amounts of impurities migrating with molecular weights of 28,000 and 30,000 (Fig. 1I). The 29,000-dalton fragment sample prepared by thrombin digestion of intact fibronectin contained only traces of such contaminants (Fig. 1F).

Results of SDS-polyacrylamide gel electrophoresis in the absence of reducing agent (not shown) suggest that the 72,000-dalton, 43,000-dalton, and 29,000-dalton fragments all consist of single polypeptide chains. The nonreduced 72,000-dalton and 29,000-dalton fragments migrated more rapidly than in the presence of a reducing agent, indicating that they contain intrachain but not interchain disulfide bonds. The 43,000-dalton fragment had the same mobility under both reducing and nonreducing conditions.

The 72,000-dalton gelatin-binding fragment and its 43,000-dalton and 29,000-dalton thrombin-generated derivatives were each subjected to NH2-terminal analysis using the dansyl-chloride technique. No NH2-terminal amino acids were detected in either the 72,000-dalton or 29,000-dalton fragments, indicating NH2-terminal blockage. Analysis of the 43,000-dalton fragment, on the other hand, revealed an NH2-terminal alanine residue. Treatment of both the 72,000-dalton and 29,000-dalton fragments with L-pyroglutamyl-peptide hydrolase resulted in the appearance of NH2-terminal alanine. These results indicate that the 72,000-dalton and 29,000-dalton fragments are located at the NH2 terminus of fibronectin, since they contain the same NH2-terminal sequence, <Glu-Ala-, as intact fibronectin (3, 6).

Since all of the fragment preparations contained some impurities, a quantitative confirmation of these results was obtained as follows. The 72,000-dalton fragment was reduced and alkylated, digested with L-pyroglutamyl-peptide hydrolase, and analyzed on an automated sequencer. The NH2-terminal sequence obtained, Ala-Glx-Glx-Met-Val-, was identical with that of L-pyroglutamyl-peptide hydrolase-treated intact fibronectin (6). The yield of alanine at the first step of sequencing, expressed as a percentage of the number of nanomoles of protein applied to the sequencer, was 55%. Since, under usual conditions, the yield at the first step of sequencing ranges from 50 to 70% (33), it is clear that the observed sequence was not derived from a minor contaminant. The results thus indicate that the 72,000-dalton fragment contains the same NH2-terminal sequence as intact fibronectin and must, therefore, be located at the amino end of the parental molecule.

**DISCUSSION**

These experiments show that treatment of human plasma fibronectin with cathepsin D produces a gelatin-binding fragment with a molecular weight of 72,000. This fragment can be cleaved further with thrombin to yield a 43,000-dalton fragment which binds to gelatin and a 29,000-dalton fragment which does not. NH2-terminal analysis of the 72,000-dalton and 29,000-dalton fragments by the dansyl-chloride method before and after treatment with L-pyroglutamyl-peptide hydrolase indicates that these fragments have the same NH2-terminal sequence, <Glu-Ala-, as intact fibronectin (3, 6). In contrast, the 43,000-dalton gelatin-binding fragment has an NH2-terminal alanine residue. Automated amino acid sequence analysis of the 72,000-dalton piece confirms that this fragment contains the same NH2-terminal sequence as intact fibronectin.

The model of human plasma fibronectin that can be constructed from these data is shown in Fig. 2. The 72,000-dalton gelatin-binding fragment produced by cathepsin D digestion of fibronectin is located at the NH2 terminus of the parental molecule. Further cleavage with thrombin yields a 29,000-dalton fragment which is also located at the NH2 terminus, and a 43,000-dalton fragment which constitutes the carboxyl portion of the 72,000-dalton piece. Since the 43,000-dalton fragment retains the ability to bind to gelatin, one or more gelatin-binding sites of fibronectin must be located within this region. This model conflicts with that of Balian et al. (19). These investigators, on the basis of results reported by Iwagama et al. (34), placed the 72,000-dalton cathepsin D-derived fragment near the carboxyl end of fibronectin.

Our model is consistent with the results that we reported for thrombin digestion of intact fibronectin (6). In this case, thrombin cleavage yielded a 29,000-dalton NH2-terminal fragment and 230,000-dalton and 235,000-dalton fragments which were shown to constitute the carboxyl portion of the molecule. Only the larger fragments bound to gelatin. It is likely that thrombin cleaves both the intact molecule and the 72,000-dalton fragment at the same site, since the 29,000-dalton fragments produced in either case have identical electrophoretic mobilities (Fig. 1, I, J, and K). Furthermore, both the 230,000- and 235,000-dalton fragments (6) and the 43,000-dalton fragment have the same NH2-terminal residue, alanine. However, the presence of relatively large amounts of contaminants in the 29,000-dalton fragment sample prepared by thrombin digestion of the 72,000-dalton piece (Fig. 1I) indicates that the 72,000-dalton fragment also may contain some thrombin-sensitive sites which are not exposed in the intact molecule.

The aberrant migration of the 29,000-dalton NH2-terminal fragment on SDS-polyacrylamide gels in the absence of a reducing agent, as well as its relatively high cysteine content.
suggests that this fragment is enriched in intrachain disulfide bonds. The data reported here do not indicate the position of the interchain disulfide bond(s) which join the subunits of fibronectin; however, there is some preliminary evidence which suggests that they may be located close to the carboxyl end, as depicted in Fig. 2 (6). Experiments designed to confirm this hypothesis are in progress.

It should be noted that the 43,000-dalton fragment described above may not represent the only binding region for gelatin which fibronectin contains. It is possible that there are additional sites which are destroyed by protease digestion. However, gelatin-binding fragments have been isolated from fibronectin following treatment with a variety of different proteases including chymotrypsin (20, 21), trypsin (22, 23), and subtilisin (24); in each of these cases, only a single binding region was demonstrated.

Our data can be combined with that of others to build a more detailed model of the fibronectin molecule. Using a competitive binding assay, Engvall et al. (12) have demonstrated that native collagen and fibrinogen bind to the same site on fibronectin as gelatin does. Therefore, these molecules must also attach to fibronectin within the 43,000-dalton region. Mosher et al. (35) have employed thrombin digestion of plasma fibronectin labeled with dansyl-cadaverine and plasma transglutaminase to demonstrate that a transamidation-sensitive site is located in the 29,000-dalton NH₂-terminal fragment. Similar results have been obtained by Jilek and Hor- 

Our results thus allow the positioning of several sites of biological interest within the fibronectin molecule. A clearer picture of the ways in which these sites relate to one another may eventually lead to a better understanding of how plasma and cellular fibronectins function in vivo as bridges between macromolecules and between cells and their environment.

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REFERENCES