Domain Structure of Human Plasma and Cellular Fibronectin. Use of a Monoclonal Antibody and Heparin Affinity To Identify Three Different Subunit Chains†

Eva Marie Click* and Gary Balian

Departments of Orthopedics and Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908

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ABSTRACT: The domain structure of human plasma fibronectin was investigated by using heparin-binding and antibody reactivity of fibronectin and its proteolytically derived fragments. Digestion of human plasma fibronectin with a combination of trypsin and cathepsin D produced six major fragments. Affinity chromatography showed that one fragment (M_r 45 000) binds to gelatin and three fragments (M_r 31 000, 36 000, and 61 000) bind to heparin. The 31K fragment corresponds to NH_2-terminal fragments isolated from other species. The 36K and 61K fragments are derived from a region near the C-terminus of the molecule and appear to be structurally related as demonstrated by two-dimensional peptide maps. A protease-sensitive fragment (M_r 137 000), which binds neither gelatin nor heparin but which has been shown previously to be chemotactic for cells [Postlethwaite, A. E., Keski-Oja, J., Balian, G., & Kang, A. H. (1981) *J. Exp. Med. 153*, 494–499], separates the NH_2-terminal heparin- and gelatin-binding fragments from the C-terminal 36K and 61K heparin-binding fragments. A monoclonal antibody to fibronectin that recognized the 61K heparin-binding fragment was used to isolate a sixth fragment (M_r 34 000) that did not bind to heparin or gelatin and that represents a difference between the 61K and 36K heparin-binding fragments. Cathepsin D digestion produced an 83K heparin-binding, monoclonal antibody reactive fragment that contains the interchain disulfide bond(s) linking the two fibronectin chains at their C-termini. The data indicate that plasma fibronectin is a heterodimeric molecule consisting of two very similar but not identical chains (A and B). In contrast, enzymatic digestion of cellular fibronectin produced a 50K heparin-binding fragment lacking monoclonal antibody reactivity which suggests that the cellular fibronectin subunit is similar to the plasma A chain in enzyme susceptibility but contains a larger heparin-binding domain. A model relating the differences in the three fibronectin polypeptides to differences in published cDNA sequences is presented.

Fibronectin is a large glycoprotein constituent of extracellular matrices [for review, see Yamada (1983)]. It appears to play a role in the organization of other matrix components such as collagens and proteoglycans and may be involved in cell attachment to the matrix, along with cell surface heparan sulfate proteoglycan (Laterra et al., 1982). A soluble form of fibronectin is abundant in plasma, is present in other body fluids, and can form complexes with heparin and fibrin. Fibronectin mediates the uptake of gelatin-coated particles by macrophages (Doran et al., 1981a; Gudewicz et al., 1980), binds to *Staphylococcus aureus*, and may be involved in the clearance of pathogens and debris by the reticuloendothelial system.

In addition to binding to cells, fibronectin is capable of interactions with many different molecules, including collagen or gelatin, fibrin or fibrinogen, heparin or heparan sulfate, hyaluronic acid, gangliosides, actin, DNA, transglutaminase, staphylococci containing protein A, and itself (Engvall & Ruoslahti, 1977; Stathakis & Mosesson, 1977; Yamada et al., 1980; Culp et al., 1979; Kleinman et al., 1979; Mosher, 1975; Keski-Oja et al., 1980; Zardi et al., 1979; Doran & Raynor, 1981). Physicochemical data indicate that the fibronectin molecule consists of globular domains linked by flexible connections (Alexander et al., 1978). These globular domains appear to represent discrete biologically active domains, and protease digestion of fibronectin releases stable fragments which retain biological activity.

Structural studies show that fibronectin is a dimer of two similar subunits, each with a molecular weight of 220 000–250 000. When reduced and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE),[1] human plasma fibronectin migrates as a closely spaced doublet, whereas cellular fibronectin migrates more slowly as a diffuse broad band (Balian et al., 1979a). Limited enzymolysis of fibronectins isolated from different species, using a variety of proteases, has produced fragments that have been identified by their biological activity.

Using cathepsin D, we previously generated and characterized a 70K gelatin binding fragment from human plasma and cellular fibronectins that was localized to the N-terminus of the molecule and that could be cleaved by plasmin to produce an N-terminal 31K fragment and a 45K gelatin-binding fragment (Balian et al., 1979a,b; 1980).

In this study we used a combination of trypsin and cathepsin D to produce discrete fragments from both plasma fibronectin and the fibronectin synthesized by cultured amniotic fluid cells.

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*Address correspondence to this author at the Department of Microbiology.

[1] Abbreviations: M_r, molecular weight; DEAE, diethylaminoethyl; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; TBS, Tris-buffered saline (0.15 M NaCl and 0.05 M Tris-HCl, pH 7.5); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin, ELISA, enzyme-linked immunosorbent assay.

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We used heparin affinity and a monoclonal antibody to examine the C-terminus of each of the molecules. Our results indicate that plasma fibronectin is a dimer of two subunits, A and B, which differ in enzyme susceptibility at a region near the C-terminus. Cellular fibronectin contains a third type of subunit which is similar to the plasma A subunit in enzyme susceptibility, but which contains a larger heparin-binding domain. Models are presented that indicate the relative order of the proteolytic fragments and that suggest ways in which the structural differences are generated at the mRNA level.

Experimental Procedures

Preparation of Fibronectins. Plasma fibronectin was isolated from outdated human plasma by affinity chromatography on gelatin–Sepharose (Engvall & Ruoslahti, 1977) and further purified by chromatography on DEAE-cellulose (Balian et al., 1979a). The fibronectin was stored as a pellet at −70 °C after precipitation with 20% ethanol (v/v) at −4 °C. To prevent proteolytic degradation, all buffers were supplemented with 0.1 mM PMSF, except during trypsin digestions. Fibronectin was redissolved in 0.15 M NaCl and 0.05 M Tris, pH 7.5, (TBS), and protein concentrations, measured by using an extinction coefficient of $E^{1\%}_{280} = 13$, were adjusted to the range 0.25–0.35 mg/mL for all enzyme digestions.

Cellular fibronectin was obtained from amniotic fluid cells, either cloned AF cells (Crouch et. al., 1978) or normal GM957 cells (NIMGS Human Genetic Mutant Repository), after 24 h pulse labeling with 60 μCi/mL L-[3,5-^{3}H]tyrosine (New England Nuclear). Cellular fibronectin was isolated from the dialyzed medium by affinity chromatography on gelatin–Sepharose in the presence of protease inhibitors. Cell-surface fibronectin was obtained by extracting the cell layer at room temperature with 1 M urea in PBS for 15 min and was purified by gelatin–Sepharose chromatography. The labeled fibronectins were dialyzed into TBS containing 0.1 mM PMSF. ^{3}H-Labeled cellular fibronectin and unlabeled plasma fibronectin were mixed to yield ratios of 1000–5000 dpm of ^{3}H/μg of protein. Prior to trypsin digestion, the mixture of fibronectins was dialyzed to remove PMSF.

Preparation of Fragments. Trypsin digestions were performed in TBS plus 1 mM $CaCl_2$ for 5 min at 37 °C by using 1 μg/mL enzyme (TPCK–trypsin; Millipore). Reactions were terminated by rapid addition of excess PMSF. Cathepsin D digestion was performed by adjusting the pH of the sample to 3.4 with 34 mM citrate–phosphate buffer, pH 3.2. Enyzme (Sigma) was added at an E:S ratio of 1:50 or 1:400, and digestion at 30 °C was terminated after time periods of 30 min to 4 h with excess pepstatin A (Protein Research Institute, Osaka, Japan).

Fragments were isolated by affinity chromatography on columns containing gelatin, heparin, or anti-fibronectin antisera. Gelatin–Sepharose was prepared by coupling calf skin gelatin (Sigma) to Sepharose CL-4B with CNBr (March et al., 1974). Chromatography was performed in either PBS or TBS, and bound material was eluted with buffer containing 6 M urea. Heparin–Sepharose (Pierce) chromatography was performed in PBS, and bound material was eluted either with buffer containing 0.65 M NaCl or with a gradient from 0.15 to 0.65 M NaCl.

Anti-fibronectin antibodies were coupled to CNBr-activated Sepharose, and affinity chromatography was performed in TBS at 4 °C. Bound material was eluted with 0.1 M HCl–glycine, pH 2.8. Column fractions were pooled, dialyzed against 1 mM NH_4HCO_3, and lyophilized.

SDS–Polyacrylamide Gel Electrophoresis. Samples were analyzed on 10% acrylamide slab gels containing urea (Laemmli, 1970). Reduced samples contained 50 mM dithiothreitol. Molecular weight standard mixtures were obtained from Pharmacia. Plasma fibronectin and fragments were visualized by Coomassie blue staining. When plasma and ^{3}H-labeled cellular fibronectins were compared, stained gels were photographed and then subsequently processed for fluorography. Photographs of the stained gel and the fluorogram were printed at exactly the same size to allow direct comparison of fragment mobilities.

Two-Dimensional Peptide Mapping. Coomassie-stained bands were cut from polyacrylamide gels and radioiodinated with Na^{125}I (New England Nuclear) by using chloramine T (Elder et al., 1977). The slices were digested with 40 μg/mL proteinase K (E. M. Biochemicals), and the extracted peptides were mapped on thin-layer plates by using electrophoresis in one dimension and chromatography in the second dimension (Sage et al., 1981). The peptide patterns were visualized by autoradiography.

Antibody Recognition. Polyclonal antisera to human plasma fibronectin was obtained from immunized rabbits (Balian et al., 1979a). A monoclonal antibody to human myoblast cells, shown to be an IgM that reacts with fibronectin (Walsh et al., 1981), was purchased from Sera Lab. ELISA was performed essentially according to Engvall & Perlman (1972) by using alkaline phosphatase conjugated second antisera (Cappel). Western blotting was performed according to the methods of Towbin et al. (1979) and Burnette (1981). Fibronectin (0.33 mg/mL) was digested either with 1 μg/mL trypsin for 5 min, with cathepsin D (E:S 1:50) for 4 h, or with both enzymes sequentially. Aliquots of 10, 35, and 45 μg of each digest, respectively, were electrophoresed on 10% SDS–polyacrylamide gels. The peptides were transferred to nitrocellulose overnight at 30 V and 4 °C by using a Hoeffer apparatus. The nitrocellulose was first stained with 0.1% amido black and photographed. Reactive sites on the paper were blocked with 5% BSA, and the nitrocellulose was reacted consecutively with a 1:1000 dilution of monoclonal antibody and with 4×10^6 dpm of ^{125}I-labeled goat IgG second antibody prepared using Iodogen (Pierce). Counts were visualized by autoradiography. Photographs of stained nitrocellulose and autoradiograms were printed to the same size to allow direct comparison of lanes.

Results

Characterization of Plasma Fibronectin Fragments Produced by Combined Digestion with Trypsin and Cathepsin D. Initial studies focused on the characterization of plasma fibronectin. It was found that sequential digestion of plasma fibronectin with trypsin for 5 min followed immediately by cathepsin D digestion produced a 45K fragment (M_r 45 000) which binds to gelatin and three major fragments which bind to heparin (Figure 1). Varying the time of cathepsin D digestion from 30 min to 4 h shows these fragments to be relatively protease resistant when compared with the nonbinding fragments.

Reduction of disulfide bonds (Figure 1, bottom) results in an increased apparent molecular weight of two of the heparin-binding fragments (28K to 31K and 55K to 61K). It should be noted that although there is no apparent change in the relative amount of the 55K heparin-binding fragment with time (Figure 1, top, lanes 7–9), there is a decrease in the amount of the corresponding reduced fragment (61K) with a concomitant appearance of the 45K fragment (Figure 1, bottom, lanes 7–9). This suggests that cleavage has occurred within the 55K fragment and the resulting peptides are held together by disulfide bonds.

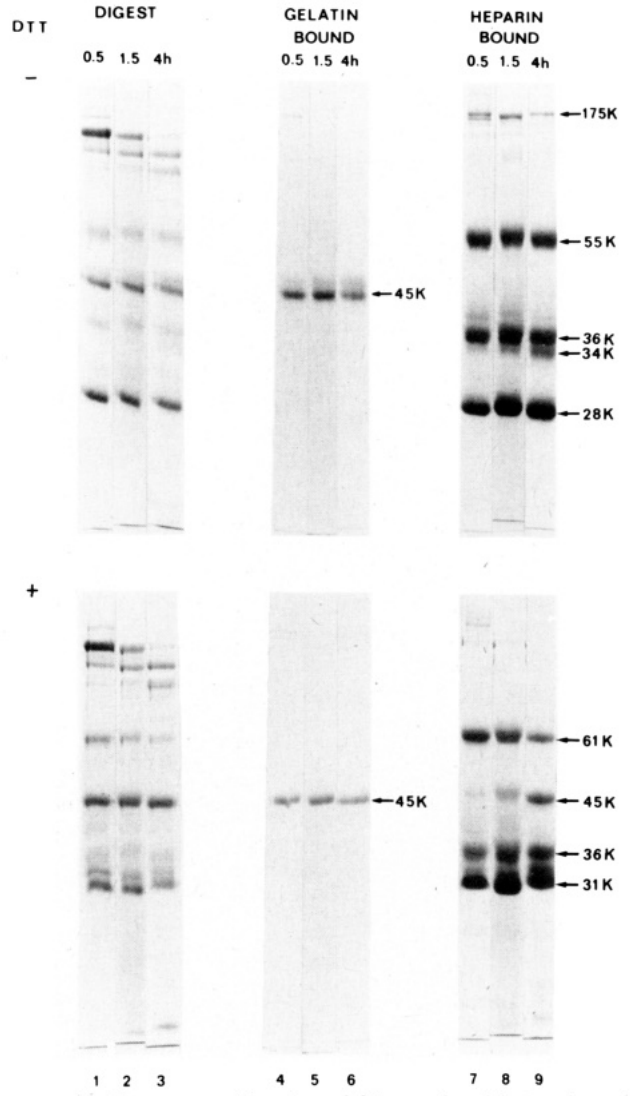

FIGURE 1: Time course digestion of fibronectin with trypsin and cathepin D. Isolation of gelatin- and heparin-binding fragments. Fibronectin was digested for 5 min with trypsin and then with cathepsin D for 0.5, 1.5, or 4 h. Aliquots of the digests (lanes 1–3) were subjected to affinity chromatography to isolate gelatin-binding (lanes 4–6) or heparin-binding (lanes 7–9) fragments. SDS–PAGE was performed in the absence (upper panel) or presence (lower panel) of reducing agent. Apparent molecular weights of the fragments were determined by comparison to mobilities of a standard mixture in the presence of reducing agent: ferritin, 220K; phosphorylase *b*, 94K; albumin, 67K; catalase, 60K; ovalbumin, 43K; lactate dehydrogenase, 36K; carbonic anhydrase, 30K; trypsin inhibitor, 20K.

Reduction of disulfide bonds with 50 mM DTT, followed by affinity chromatography in the presence of 10 mM DTT, did not affect the heparin-binding capacity of plasma fibronectin or its fragments (data not shown). In contrast, the gelatin-binding property of fibronectin and its fragments is abolished when intrachain disulfide bonds are disrupted (Balian et al., 1979b).

The details of isolation and characterization of the fragments are presented in supplementary material (see paragraph at end of paper regarding supplementary material). Amino acid analyses and two-dimensional peptide maps indicate that the 31K heparin-binding fragment, the 45K gelatin-binding fragment, and the 137K fragment, which binds neither heparin nor gelatin, are discrete fragments, distinctly different from each other and from the other heparin-binding fragments.

The 31K (28K unreduced molecular weight) heparin-binding fragment binds weakly at 4 °C. Small increases in ionic strength of buffers or chromatography at room temperature significantly reduce its heparin-binding capacity. The 45K gelatin-binding fragment corresponds to a similar fragment generated by combined digestion with cathepsin D and plasmin (Balian et al., 1979b). The 137K fragment corresponds to the 140 000-dalton cathepsin D fragment shown previously to be chemotactic for fibroblasts (Postlethwaite et al., 1981). This fragment is easily degraded by cathepsin D over a 4-h period (see Figure 1, lanes 1–3).

Further investigation was required to identify the 36K and 61K heparin-binding fragments as discrete entities. The 36K fragment and a 34K fragment shown by peptide mapping to be a proteolytic derivative bind more tightly to heparin than the 31K N-terminal fragment. Amino acid analysis of this fraction shows a low content of cysteine, and the fragments show no change in mobility on SDS–PAGE under conditions that reduce disulfide bonds.

The fraction most tightly bound to heparin contained a 55K fragment and a small amount of a high molecular weight component (175K) not apparent in the original digest (see Figure 1). Identical peptide maps suggest that the 175K fragment is a disulfide-bonded aggregate of 55K. Reduction of the 55K/175K fraction resulted in the appearance of two fragments with molecular weights of 61K and 45K. Peptide mapping of the 45K heparin-binding fragment, which previous time course studies suggested to be a proteolytic derivative of 61K (see Figure 1, lanes 7–9), shows a few peptides in common with the 61K fragment. The map of the 45K heparin-binding fragment is distinctly different from that of the 45K gelatin-binding fragment.

In contrast, there are some similarities between the peptide maps of the 45K heparin-binding fragment (derived from 61K) and the 36K heparin-binding fragment. Likewise, the amino acid composition of the 36K fragment suggests that it is related to a portion of the 61K heparin-binding fragment. However, the 61K fragment contains a significant amount of cysteine, whereas the 36K fragment contains very little cysteine, suggesting that if the 36K and 61K fragments are related, the cysteine residues must be asymmetrically distributed within the 61K fragment.

The fragments of plasma fibronectin generated by sequential digestion with trypsin and cathepsin D were further characterized by their reactivity to various anti-fibronectin antisera. Table I shows the reactivity obtained by ELISA. The 61K and 36K heparin-binding fragments and the 137K chemotactic fragment were recognized by three polyclonal antisera, whereas the N-terminal 31K heparin-binding fragment and the 45K gelatin-binding fragment did not react with any of the antisera. Affinity purification of the antisera on a column of fibronectin–Sepharose selectively decreased the reactivity of the sera to the two heparin-binding fragments, but not to the 137K fragment.

In contrast, the monoclonal antibody used in these studies is seen to react only with the 61K heparin-binding fragment and does not react with the other discrete fragments, including the 36K heparin-binding fragment. Thus, although the 61K fragment displays some similarities to the 36K fragment (heparin binding, polyclonal antibody binding, amino acid composition, and peptide map), it is unique in its reactivity to a monoclonal antibody. Digestion of isolated 61K fragment with various concentrations of either trypsin or cathepsin D failed to separate the heparin and antibody binding domains into discrete fragments (see supplementary material).

By use of affinity chromatography, an additional antibody-binding fragment (M_r 34 000) was isolated from a digest of fibronectin (5-min trypsin/30-min cathepsin D) from which

Table I: Reaction of Fibronectin Fragments with Antibodies[a]

antisera	undigested fibronectin	chemotactic 137K	heparin binding 61K	heparin binding 36K	heparin binding 31K	gelatin binding 45K
M8 serum	1.00	0.67	0.61	0.46	–	–
M9 γg	1.00	0.80	0.56	0.52	–	–
M9 AP	1.00	0.90	0.20	0.17	–	–
CL1 γg	1.00	0.89	0.40	0.23	–	–
CL1 AP	1.00	0.77	0.06	0.07	–	–
monoclonal	1.00	–	0.34	–	–	–

[a] Antibodies were tested on purified peptides by using the ELISA. Polyclonal rabbit antisera were tested as whole serum, γ-globulin fractions (γg), and affinity-purified fractions (AP). Monoclonal antiserum to human plasma fibronectin was derived from mouse ascites fluid. Wells were coated with 1 μg/mL antigen, and antisera were tested at 1:1000 dilution and reacted with alkaline phosphatase conjugated second antibody. Bound second antibody was assayed by hydrolysis of Sigma 104 substrate. The color values obtained in the enzyme reaction were normalized to 1.00 for fibronectin. A dash (–) indicates an optical density of less than 0.05 relative to fibronectin.

FIGURE 2: Affinity chromatography of a 34 000-dalton monoclonal antibody-binding fragment. Plasma fibronectin was digested with trypsin for 5 min followed by cathepsin D for 30 min. Gelatin- and heparin-binding fragments (not shown) were removed by affinity chromatography. The remaining fragments (lane 1) were applied to a 1-mL column of monoclonal antibody–Sepharose in PBS. Bound material (lane 2) was eluted with 0.1 M HCl–glycine, pH 2.8. Aliquots were analyzed by SDS–PAGE in the presence of reducing agent.

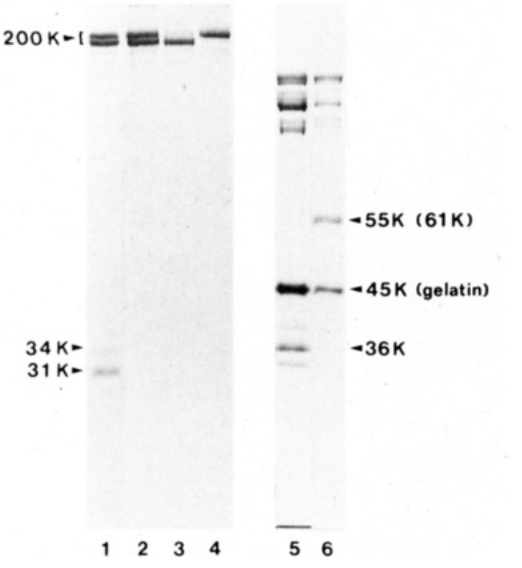

FIGURE 3: Digestion of the 200K trypsin-produced overlap fragments with cathepsin D. Fibronectin was digested for 5 min with trypsin (lane 1), and the 200K overlap fragments were isolated by sequential chromatography on heparin– and gelatin–Sepharose (lane 2). Components of the 200K doublet were separated by affinity chromatography on monoclonal antibody–Sepharose (lane 3, nonbound; lane 4, bound) and then digested with cathepsin D (lanes 5 and 6, respectively). SDS–PAGE in the presence (lanes 1–4) or absence (lanes 5–6) of reducing agent.

gelatin- and heparin-binding fragments had been previously removed. This 34K fragment, which specifically bound to the monoclonal antibody (Figure 2), stains only weakly with Coomassie blue and is relatively sensitive to proteolytic digestion over a time span of 4 h (data not shown).

Characterization of Overlapping Fragments of Plasma Fibronectin. To determine the relationships of the six fragments to each other and to the intact molecule, we examined the fragments produced by digestion with a single enzyme. Trypsin digestion of fibronectin produced two large fragments (about 200K) and two small fragments with apparent molecular weights of 34K and 31K (Figure 3, lane 1). Binding experiments showed that the large fragments bound both gelatin and heparin. The large fragments were purified by sequential affinity binding to heparin and gelatin and then separated from each other by monoclonal antibody–Sepharose. Only the larger of the 200K fragments was retained by the antibody (Figure 3, lane 3). Fibrin–Sepharose also bound only the larger component of the 200K doublet (data not shown). Each isolated component of the 200K doublet was digested with cathepsin D and analyzed by SDS–PAGE under nonreducing conditions (Figure 3, lanes 5 and 6). The results indicate that the 55K (61K reduced molecular weight) heparin-binding fragment is derived from the larger of the 200K components, whereas the 36K heparin-binding fragment is derived from the smaller component.

Cathepsin D digestion of fibronectin produced several fragments (Figure 4) including a 62K (70K reduced molecular weight) fragment, which bound both gelatin and heparin. With an E:S ratio of 1:50, a portion of the 62K fragment was cleaved into a 45K gelatin-binding fragment and a 28K (31K reduced molecular weight) heparin-binding fragment. Cathepsin D digestion also produced a set of heparin-binding fragments (38K/36K/34K) that correspond to the 36K/34K fraction isolated from combined digests with trypsin and cathepsin D (confirmed by peptide mapping; data not shown).

In addition, a new heparin-binding fragment with an apparent molecular weight of 83K (without reduction) was produced. The 83K fragment was purified from a 4-h cathepsin D digest (E:S = 1:50) by chromatography on gelatin–Sepharose, heparin–Sepharose, and Agarose A-0.5m (Figure 4B). Reduction of disulfide bonds resulted in the disappearance of the 83K fragment and the appearance of 61K and 45K fragments, as well as a more weakly staining 38K fragment. Lower enzyme concentrations produced less 45K fragment relative to 61K fragment (data not shown), indicating that the 45K fragment is a proteolytic derivative of the 61K fragment. Two-dimensional peptide mapping of the 61K

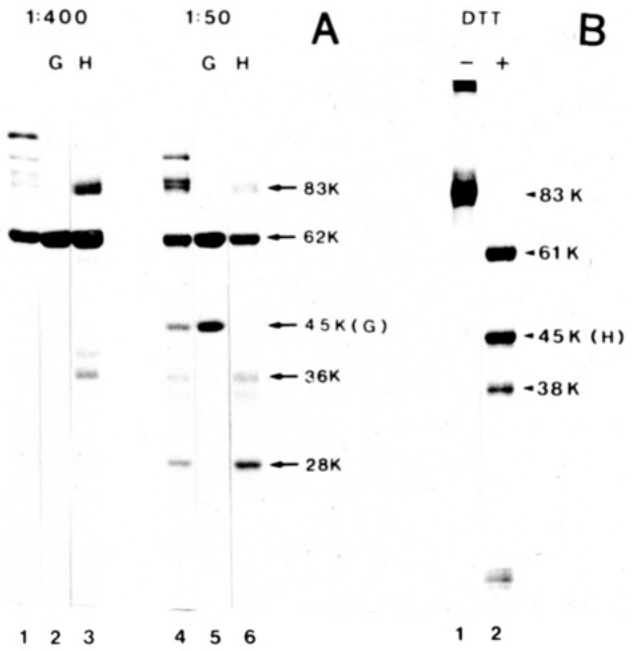

FIGURE 4: Isolation of disulfide-bonded heparin-binding fragment from a cathepsin D digest of fibronectin. (Panel A) Fibronectin was digested for 4 h with cathepsin D at E:S = 1:400 or E:S = 1:50 (lanes 1 and 4, respectively), and aliquots were subjected to affinity chromatography on immobilized gelatin (lanes 2 and 5) or heparin (lanes 3 and 6) followed by SDS–PAGE without DTT. Molecular weight estimates were based on migration of unreduced fragments. (Panel B) Purified 83K fragment analyzed by SDS–PAGE in the absence (lane 1) and presence (lane 2) of DTT. With the exception of 83K, the molecular weights refer to the mobilities of the reduced fragments. 45K(G) and 45K(H) refer to gelatin- and heparin-binding fragments, respectively.

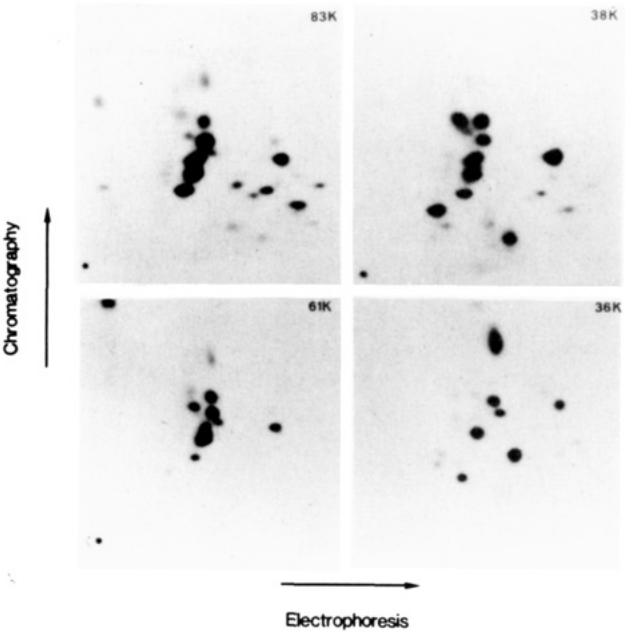

FIGURE 5: Two-dimensional maps of proteinase K digested fragments derived from the C-terminus of fibronectin. Bands from SDS–polyacrylamide gels were excised, labeled with ^{125}I, digested with proteinase K, and then analyzed by two-dimensional peptide mapping using electrophoresis in one dimension followed by chromatography in the second dimension. (Top panels) Fragments 83K and 38K from cathepsin D digests (see Figure 4B). (Bottom panels) Heparin-binding fragments 61K and 36K from combined trypsin and cathepsin D digests (see Figure 1, lane 9).

fragment (not shown) indicates that it corresponds to the heparin-binding 61K fragment previously isolated. As shown in Figure 5, the peptide maps of the 38K and 61K fragments contain spots characteristic of the 83K overlap fragment. Note, however, that the 38K fragment is distinctly different from the 36K heparin-binding fragment previously isolated.

The data indicate that 83K is a disulfide-bonded heterodimer of the two fragments 61K and 38K. The heterodimer does not appear to be produced by partial proteolysis, since we have been unable to show conversion of the 61K fragment to the 38K fragment using increasing concentrations of cathepsin D (data not shown). Also, no fragments corresponding in molecular weight to disulfide-bonded homodimers of either 61K or 38K were observed. Thus, cathepsin D must be cleaving the two chains of the fibronectin dimer at different sites to produce two distinct fragments, 61K and 38K, which are held together by interchain disulfide bonds.

The distribution of monoclonal antibody reactivity among the various fragments was investigated by using Western blotting. Fragments from trypsin, cathepsin D, and combined enzyme digests were separated on SDS–PAGE with and without prior reduction of disulfide bonds. After transfer to nitrocellulose, the fragments were first stained with amido black and then reacted with the monoclonal antibody. The results are shown in Figure 6.

Trypsin digestion of fibronectin produced two fragments that contain monoclonal antibody-binding sites, the larger component of the 200K doublet and a weakly staining 34K fragment (M_r 30K, unreduced). Cathepsin D digestion of fibronectin produced only one major antibody-binding fragment, M_r 83K unreduced. Reduction of disulfide bonds yielded three antibody-reactive fragments, 61K, 45K, and 38K, but of these, the 45K fragment has been identified as a proteolytic derivative

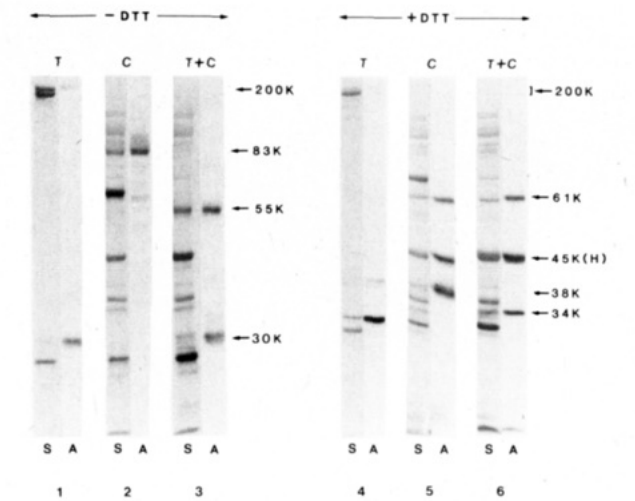

FIGURE 6: Immunoblots of plasma fibronectin peptides with monoclonal antibody. Digests of fibronectin were subjected to SDS–PAGE in the absence (lanes 1–3) or presence (lanes 4–6) of reducing agent, transferred to nitrocellulose, stained with amido black, and photographed. The blots were then reacted sequentially with monoclonal antibody and ^{125}I-labeled second antibody. Immunoreactive peptides were visualized by autoradiography. S, stained gel; A, autoradiogram, T, trypsin; C, cathepsin D; T+C, both enzymes sequentially. 45K(H) is a heparin-binding fragment distinct from the 45K gelatin-binding component. In lane 4, unequal staining of the 200K trypsin doublet with amido black reflects the inefficient transfer of the larger component of this doublet from the gel to the nitrocellulose.

of the 61K fragment. Thus, the 83K fragment consists of two antibody-binding fragments, 61K and 38K, linked by interchain disulfide bonds.

Digestion of fibronectin with a combination of trypsin and cathepsin D also yielded two fragments (M_r 55K and 30K, unreduced) that bind antibody. Reduction of this fraction produced three antibody-reactive fragments. Two of the reduced fragments, 61K and 45K, are comparable to fragments

Table II: Binding Properties of Fibronectin Peptides

digest[a]	fragment M_r[b]	gelatin	heparin	monoclonal antibody	polyclonal antibody	fibrin	cell
T	large 200K	+	+	+		+	
T	small 200K	+	+	–		–	
T	34K (30K)	–	–	+			
T	31K (28K)	–	+	–			
C	137K	–	–	–			+
C	70K (62K)	+	+	–			
C	(83K)	–	+	+			
C	61K[c]			+			
C	38K[c]			+			
C	38K/36K/34K	–	+	–			
T + C	137K	–	–	–	+		
T + C	61K (55K)	–	+	+	+		
T + C	45K heparin[d]	–	+	+			
T + C	45K gelatin	+	–	–	–		
T + C	36K/34K	–	+	–	+		
T + C	34K (30K)	–	–	+			
T + C	31K (28K)	–	+	–	–		
T + C	50K (cellular)	–	+	–	+		

[a] Peptides were obtained by digestion of fibronectin with trypsin (T) or cathepsin D (C) or by combined digestion with trypsin and cathepsin D (T + C). [b] Molecular weight of fragment on SDS–PAGE after reduction. Apparent molecular weights of unreduced peptides, if different from reduced molecular weight, are indicated in parentheses. [c] Obtained upon reduction of 83K fragment. [d] Obtained upon reduction of fractions containing 61K (55K).

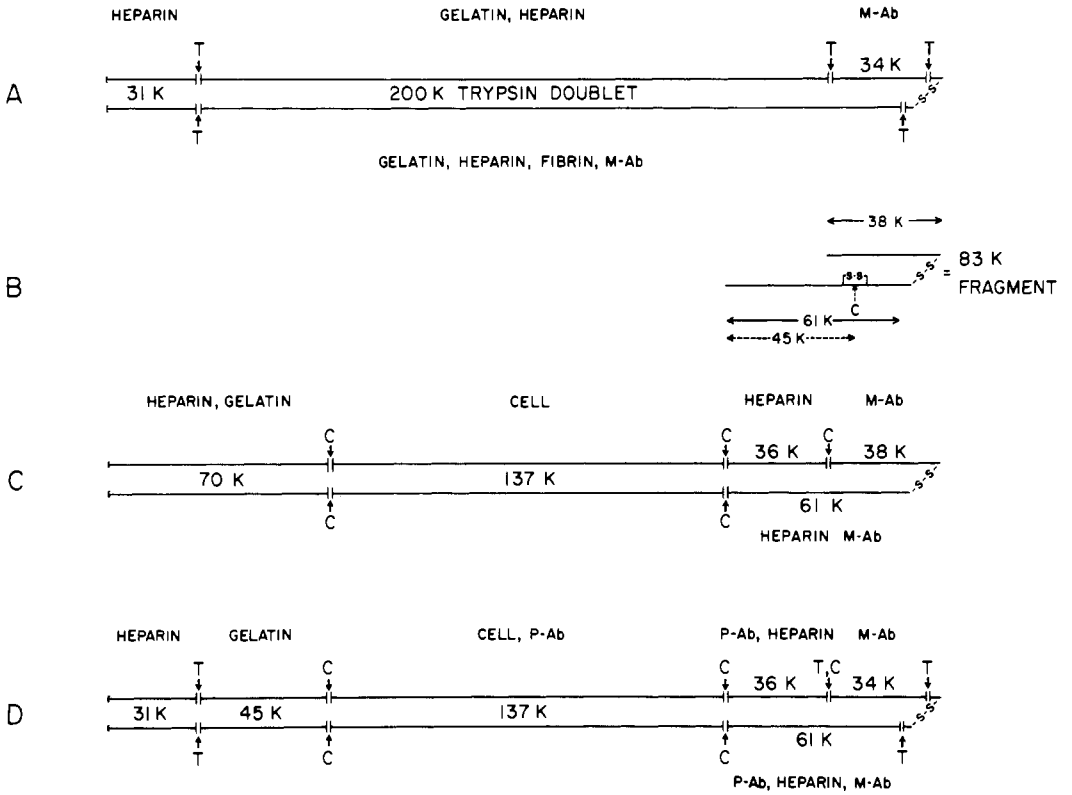

FIGURE 7: Model of heterodimeric structure of plasma fibronectin with location of fragments and domains. (A) Trypsin-digested fibronectin. (B) Detail of 83K cathepsin D derived heparin-binding fragment. (C) Cathepsin D digested fibronectin. (D) Trypsin plus cathepsin D digested fibronectin. Arrows indicate sites of cleavage with trypsin (T) and cathepsin D (C). The relative location of biologically active regions is in agreement with previously published reports (see text). The sum of the molecular weights of the fragments exceeds the apparent molecular weights (220K) of the fibronectin subunits.

obtained by cathepsin D digestion, whereas the third fragment, 34K, corresponds to the 34K trypsin fragment. The results shown in Figure 6 are consistent with the data from ELISA and affinity columns using the monoclonal antibody. The data suggest that an antibody recognition site is located on each of the two subunit chains of fibronectin.

Model: Relationship of Fragments of Plasma Fibronectin. The binding affinities of each fragment are summarized in Table II, and the relative location of the fragments within the fibronectin molecule are illustrated in Figure 7. Figure 7A depicts the location of the trypsin fragments within the fibronectin dimer. Previous studies have located the 31K (heparin binding) fragment at the N-terminus of fibronectin followed by the 200K doublet, which contains the gelatin-binding region at its N-terminus (Furie & Rifkin, 1980; Furie et al., 1980; Wagner & Hynes, 1980). The 34K antibody-

binding fragment, which represents a difference in domains between the two components of the 200K trypsin doublet, must therefore be C-terminal to the smaller component of the 200K doublet. A fragment similar to the 34K fragment was isolated by Atherton & Hynes (1981) and was determined to be from the C-terminus of cellular fibronectin.

Figure 7B illustrates the heterodimeric structure of the 83K cathepsin D fragment and indicates an additional site of proteolysis that generates the 45K heparin binding/monoclonal antibody-binding subfragment of 61K. The relationship of the 83K fragment to the other fragments produced by cathepsin D digestion is shown in Figure 7C. The 70K gelatin- (and heparin-) binding fragment has been previously localized at the N-terminus of fibronectin (Balian et al., 1980; Furie & Rifkin, 1980). The C-terminal location of the 83K heparin-binding fragment, which contains the interchain disulfide bonds linking two nonidentical peptides (Figure 7B), is consistent with observations that place the interchain disulfide bonds at one end of the molecule and with the data of Ruoslahti et al. (1981) that locates heparin-binding domains at both ends of the molecule.

The six fragments generated by combined digestion with trypsin and cathepsin D are represented in the model in Figure 7D. The 31K fragment, which contains intrachain disulfide bonds and binds weakly to heparin, is located at the N-terminus and is adjacent to the 45K gelatin-binding domain. The 36K and 61K heparin-binding regions, which appear to be homologous, have been located on separate chains near the C-terminus of the molecule, separated from the gelatin-binding domain by the 137K chemotactic fragment. The monoclonal antibody-binding 34K fragment is located C-terminal to the 36K heparin-binding fragment. The interchain disulfide bonds link a small trypsin-sensitive region adjacent to the monoclonal antibody-binding domain of each chain.

Characterization of Cellular Fibronectin. In the second part of this study, we investigated the structure of cellular fibronectin, which is known to have a slower mobility on SDS–PAGE than plasma fibronectin. To allow direct comparison of cellular and plasma fibronectin and fragments, ^{3}H-labeled cellular fibronectin with high specific activity was mixed with carrier plasma fibronectin, and all experiments were performed on the mixture of the two proteins.

SDS–PAGE of the mixture of fibronectins that had been digested sequentially with trypsin (5 min) and cathepsin D (4 h) demonstrates a similar overall fragmentation pattern for the two forms of fibronectin (Figure 8, lane 1). Gelatin–Sepharose chromatography revealed a slight difference in mobility between the corresponding cellular and plasma fragments. A similar observation was made in a study of chicken fibronectins (Hayashi & Yamada, 1981). Note that while intact cellular fibronectin appears larger than plasma fibronectin, the cellular gelatin-binding fragment appears smaller than the corresponding plasma fragment.

Heparin affinity chromatography of the digest revealed a major difference between cellular and plasma fibronectins. Lane 3 of Figure 8 demonstrates the presence of a prominent 50K fragment derived from cellular fibronectin that is not seen in digests of plasma fibronectin. Furthermore, in cellular fibronectin, there is a virtual absence of fragments corresponding to the 61K fragment (and its 45K subfragment) of plasma fibronectin. Both cellular and plasma fibronectins contain the 31K N-terminal heparin-binding fragment. Cellular fibronectin also appears to contain some of the 36K heparin-binding fragment (and 34K subfragment) corresponding to the C-terminal region of plasma A chain. Digestion and affinity chromatography of cell surface fibronectin, extracted with urea from the cell monolayer, produced results identical with that for cell medium fibronectin (data not shown).

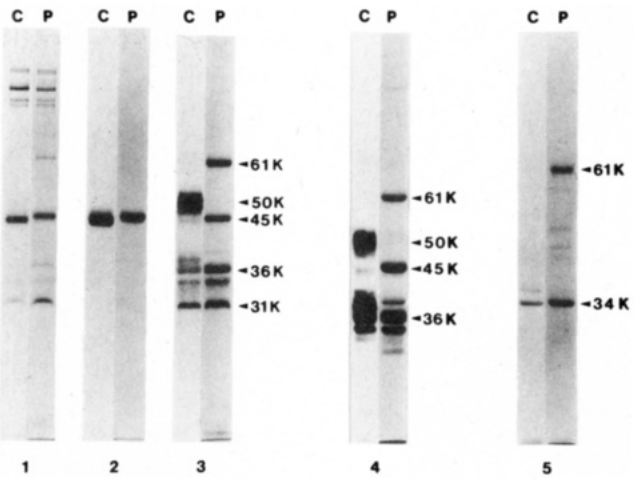

FIGURE 8: Comparison of binding fragments of cellular and plasma fibronectins produced by sequential digestion with trypsin and cathepsin D. Mixture of ^{3}H-labeled cellular fibronectin and carrier plasma fibronectin was digested with trypsin for 5 min and then with cathepsin D for 4 h (lane 1). Gelatin-binding fragments (lane 2) and heparin-binding fragments (lane 3) were isolated from separate aliquots. (Lane 4) Fragments of a digest (5-min trypsin, 2-h cathepsin D) that bind both heparin and polyclonal antibodies. (Lane 5) Fragments of a digest (5-min trypsin, 30-min cathepsin D) that bind a monoclonal antibody. Samples were treated with 50 mM DTT prior to SDS–PAGE. ^{3}H-Labeled cellular fibronectin and fragments (C) were visualized by fluorography after Coomassie staining of the same lanes revealed plasma fibronectin and fragments (P).

In a separate experiment, the binding of fragments to polyclonal antibodies was assessed by affinity chromatography. A mixture of ^{3}H-labeled cellular fibronectin and plasma fibronectin was digested sequentially with trypsin (5 min) and cathepsin D (2 h). The digest was first chromatographed on a column of immobilized polyclonal anti-fibronectin antibodies which bound all fragments except the N-terminal 31K heparin-binding fragment and the 45K gelatin-binding fragment of both fibronectins. The fraction bound to the antibodies was eluted and subsequently chromatographed on heparin–Sepharose. The fraction that bound both polyclonal antibodies and heparin was analyzed by SDS–PAGE (Figure 8, lane 4). The results show that the cellular 50K and 36K heparin-binding fragments also bind polyclonal antibodies as do the 61K/45K and the 36K heparin-binding fragments of the plasma B chain and A chain, respectively.

In contrast, monoclonal antibody reactivity revealed a difference between cellular and plasma fibronectin fragments. A mixture of fibronectins, which had been digested sequentially with trypsin (5 min) and cathepsin D (30 min), was chromatographed on a column of immobilized monoclonal antibody. The results are shown in Figure 8 (lane 5). Whereas two fragments from plasma fibronectin, 61K and 34K, bound to the antibody, only one major cellular fragment, 34K, reacted with the antibody. Subsequent heparin–Sepharose chromatography revealed the presence of the 50K fragment in the fraction that did not bind the monoclonal antibody (data not shown). Thus, although the cellular 50K fragment resembles the plasma 61K fragment in that they both bind heparin and polyclonal antibodies, the 50K fragment differs from 61K in that it does not bind this monoclonal antibody.

The 50K fragment also differs from the plasma 61K fragment in that the 50K fragment does not exhibit a change in mobility on SDS–PAGE with reduction of (intrachain) di-

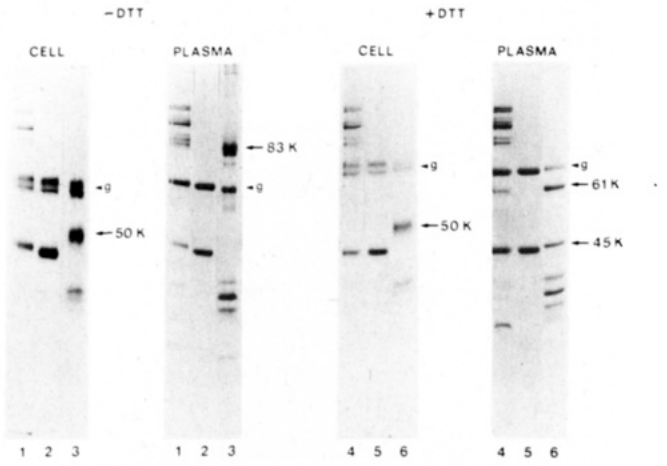

FIGURE 9: SDS–PAGE of gelatin- and heparin-binding fragments isolated from a cathepsin D digest of cellular and plasma fibronectins. Mixture of ^{3}H-labeled cellular fibronectin and carrier plasma fibronectin was digested with cathepsin D for 30 min (lanes 1 and 4). Gelatin-binding fragments (lanes 2 and 5) and heparin-binding fragments (lanes 3 and 6) were isolated from separate aliquots by affinity chromatography. Samples were electrophoresed in the absence (lanes 1–3) or presence (lanes 4–6) of 50 mM DTT. Cellular fibronectin fragments were visualized by fluorography after Coomassie blue staining of plasma fibronectin fragments. Overlap fragments which bind both gelatin and heparin are indicated (g).

sulfide bonds (data not shown). In contrast, the plasma 61K fragment has an apparent molecular weight of 55K in the absence of reduction and has been shown to contain many cysteines by amino acid analysis. Thus, the cellular 50K fragment binds heparin and polyclonal antibodies but does not bind a monoclonal antibody and does not contain significant numbers of intrachain disulfide bonds, in contrast to plasma 61K.

To determine if the cellular 50K fragment was contained in a large C-terminal overlap fragment, a mixture of ^{3}H-labeled cellular and carrier plasma fibronectins was digested with cathepsin D for 30 min. Gelatin- and heparin-binding fragments were isolated from separate aliquots by affinity chromatography and analyzed by SDS–PAGE in the presence and absence of reducing agent (Figure 9).

Cathepsin D digestion of plasma fibronectin produced two overlap fragments described previously. One binds both gelatin and heparin and is derived from the N-terminus of the molecule. A corresponding pair of fragments is present in cellular fibronectin. The second overlap fragment obtained from plasma fibronectin is the 83K heparin-binding fragment, seen in the absence of DTT (Figure 9, lane 3 PLASMA). One of the fragments released from the 83K fragment upon reduction of disulfide bonds is a 61K fragment (and its 45K subfragment). In contrast, in the digests of cellular fibronectin, a 50K fragment appears in both the presence and absence of reducing agent, suggesting the presence of a protease-susceptible site between the heparin-binding domain and the interchain disulfide-bonded region of cellular fibronectin.

The data presented here suggest that cellular fibronectin contains a third type of subunit chain which is similar to the plasma A chain, with an enzyme-susceptible site separating heparin- and antibody-binding domains but which contains a 50K heparin-binding fragment instead of the 36K heparin-binding fragment (see Figure 10). The presence of ^{3}H-labeled 36K heparin-binding fragment in our digests indicates the synthesis of monomers equivalent to the plasma A chain. (A very small amount of 61K fragment, characteristic of plasma B chain, was also detected in some digests.) Since the ^{3}H-labeled heparin-binding fragments 36K and 50K are not contained in interchain disulfide-bonded overlap fragments, we cannot determine from our experiments which subunits are linked together and, thus, whether cellular fibronectin is comprised of homodimers or heterodimers or both.

Discussion

In this study, we used a combination of trypsin and cathepsin D to produce a set of six discrete fragments from human plasma fibronectin. Overlapping fragments produced by digestion with either trypsin or cathepsin D alone allow ordering of the fragments and provide evidence for the existence of a region of difference between the two chains of the plasma fibronectin dimer near the C-terminus of the molecule. The information derived from these studies is depicted in a model (Figure 7) that places the domains in the following order: NH_2-heparin (weak)–gelatin–cell–heparin (strong)–monoclonal antibody–interchain disulfide bonds-COOH. Each of the two chains of plasma fibronectin contain all the domains, in the same relative order. Thus, the two subunits of human plasma fibronectin contain homologous functional domains, but a site of differential susceptibility to proteases is located near the C-terminus of the chains between a (strong) heparin-binding domain and a domain that binds a monoclonal antibody. The larger subunit (A chain) contains the susceptible site and, upon digestion with trypsin, gives rise to the smaller component of the 200K trypsin doublet. Trypsin digestion of the shorter B chain produces the larger component of the 200K trypsin doublet containing a site resistant to cleavage (see Figure 9).

The 31K heparin-binding fragment which binds weakly to heparin corresponds to the N-terminal domain of fibronectin which has been sequenced (Garcia-Pardo et al., 1983). This domain contains many cysteine residues all of which exist as disulfide bonds resulting in a conformation described by Petersen et al. (1983) as five "fingers" (type I homology). It

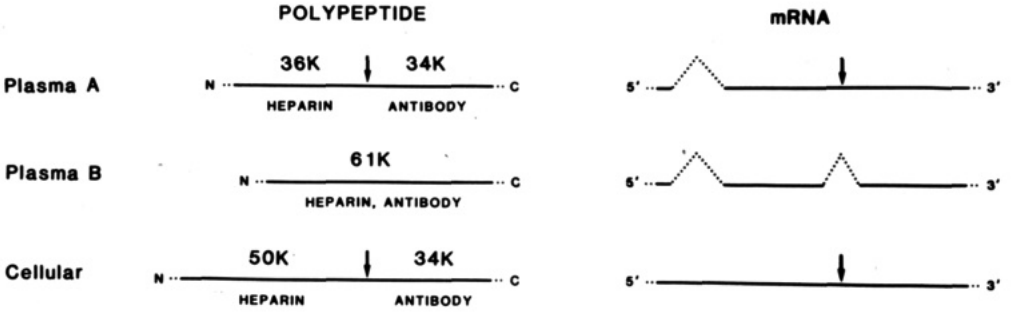

FIGURE 10: Schematic representation of mRNAs that could produce three different fibronectin subunits by alternative splicing. Solid lines indicate C-terminal peptide fragments and corresponding contiguous region of mRNA for each subunit. Dashed lines represent segment of mRNA that is spliced out. Arrows indicate the site of protease sensitivity in the polypeptide and the corresponding site in a segment of mRNA that undergoes alternative splicing.

has an overall basic charge and contains no carbohydrate. This region is rapidly released from fibronectin by serine proteases such as thrombin, plasmin, and trypsin. Binding of this fragment to heparin has been shown to be sensitive to calcium (Hayashi & Yamada, 1982). In addition to binding weakly to heparin, this fragment also binds *S. aureus* and fibrin and can be cross-linked to fibrin or *S. aureus* by transglutaminase (Mosher & Proctor, 1980; Mosher et al., 1980).

Adjacent to the N-terminal heparin-binding domain is a 45K gelatin-binding region which is extremely protease resistant. The bovine fragment generated by plasmin and chymotrypsin digestion has been sequenced (Skorstengaard et al., 1984). It contains numerous disulfide bonds and a significant amount of carbohydrate, which may contribute to its protease resistance (Bernard et al., 1982). The disulfide bonds, which form two different types of homologous loops (Petersen et al., 1983), are necessary for maintaining a conformation essential for gelatin binding (Balian et al., 1979b).

The gelatin-binding domain is separated from the C-terminal heparin-binding domain by a large 137K protease-sensitive fragment which has been shown to be chemotactic for fibroblasts (Postlethwaite et al., 1981) and which reacts with our preparations of polyclonal anti-fibronectin antibodies. It has also been shown to react with two monoclonal antibodies that recognize separate determinants for chemotactic and cell-binding activities (Dziadek et al., 1983) and presumably contains the 15K cell-binding site isolated by Pierschbacher et al. (1981). A monoclonal antibody to the 15K fragment reacts with a set of trypsin-produced heparin-binding fragments (Pierschbacher et al., 1981), indicating the proximity of the cell-binding region to the C-terminal heparin-binding region. The amino acid composition of this 137K fragment agrees with that of Richter & Hormann (1982), revealing little or no cysteine. Richter et al. (1981) found that this fragment bound very weakly to heparin at low ionic strength. In our experiments, we occasionally saw a small proportion of this fragment in heparin-binding fractions. It is likely that this 137K fragment contains a low-affinity heparin-binding region near its N-terminus. Such a domain has been isolated as a discrete 29K fragment (Gold et al., 1983) and shown to be equivalent to the DNA-binding fragment characterized by Pande & Shively (1982). A 16K thermolysin fragment from hamster plasma fibronectin also contains this low-affinity heparin-binding domain (Sekiguchi et al., 1983a). Both groups concluded that this low-affinity domain was located adjacent to the C-terminus of the gelatin-binding domain.

The C-terminal heparin-binding region, contained in our 36K and 61K fragments, corresponds to two heparin-binding subfragments, M_r 38K and 58K, identified by Hayashi & Yamada (1983) in digests of human plasma fibronectin treated extensively with trypsin. These authors also used two-dimensional peptide mapping to demonstrate homology between the two fragments and similarly concluded that they were derived from the A and B chains of plasma fibronectin, respectively. The heparin binding of this region is of high affinity and has been shown to be insensitive to divalent cation concentration (Hayashi & Yamada, 1982).

A related heterogeneous fragment SH29-36K, which binds to heparin with high affinity, was isolated by Gold et al. (1983) from a subtilisin digest of human plasma fibronectin. The amino acid composition of our 36K fragment is comparable to their SH29-36K fraction, and both show little or no cysteine. The heterogeneity in their set of fragments was found to be at the C-terminus, allowing sequencing of the N-terminal portion of the fragment.

Adjacent to the C-terminal heparin-binding domain is a region that binds a monoclonal antibody followed by a small region containing the interchain disulfide bonds, located at the extreme C-terminus of the molecule. Our results show that the monoclonal antibody-binding domain is located at the C-terminus of both chains of the fibronectin dimer but that the domain is released as a discrete fragment from only one of the chains. Thus, trypsin digestion releases a 34K monoclonal antibody-binding fragment from one chain and produces from the other chain a 200K fragment that binds both heparin and monoclonal antibody.

The 34K fragment, which binds the monoclonal antibody used in these studies, corresponds to a fragment identified by Smith & Furcht (1982) using similar techniques. A similar fragment has been identified in digests of hamster cellular fibronectin by Atherton & Hynes (1981) using their monoclonal antibody. Both groups localized the fragment to the C-terminus of the fibronectin molecule. Recent studies of trypsin digests of human plasma fibronectin indicate this fragment also contains the fibrin II binding domain (Hayashi & Yamada, 1983; Sekiguchi & Hakomori, 1983a). It corresponds to the 23K plasmin fragment from bovine fibronectin whose sequence shows that it contains numerous disulfide bonds in three loops of the type I homology (Petersen et al., 1983) as well as one free sulfhydryl (Wagner & Hynes, 1980; Smith & Furcht, 1982).

Monoclonal antibodies are useful tools for structural studies of proteins. The monoclonal antibody used in this study has been identified as an IgM, produced in response to the injection of human myoblast cells in a study of cell surface antigens (Walsh et al., 1981), and is commercially available. Our studies show that this antibody reacts with the C-terminal domain shown by us and others to contain the fibrin II binding region (and perhaps the fibronectin-association domain) of both chains of the human plasma fibronectin dimer and of cellular fibronectin. We have demonstrated that this antibody can be successfully coupled to Sepharose using CNBr and still retain antigenicity. It recognizes native fibronectin and fragments in affinity chromatography and ELISA assay and also recognizes reduced and SDS-denatured fibronectin chains and fragments in a Western blot. Such antibodies may provide a convenient tool for study of this region of both plasma and cellular fibronectin.

Monoclonal antibodies have also been used by others to study the structure of fibronectin. During the course of these studies, Smith et al. (1982) examined the tryptic fragments of human plasma fibronectin with their monoclonal antibody and obtained results similar to our trypsin studies. Smith & Furcht (1982) also investigated the reactivity of cathepsin D produced fragments of fibronectin with monoclonal antibody. However, interpretation of their results was complicated by the fact that the antibody was reacted with native unreduced fragments, but the fragments were identified on SDS–PAGE after reduction of disulfide bonds. Thus, some fragments appeared to be antibody binding but in fact were only disulfide bonded to an antibody-binding fragment.

Differences between the subunit chains of plasma fibronectin have been suspected from the initial observation of a closely spaced doublet on SDS–PAGE after reduction of disulfide bonds. Differential sensitivities to enzymes were suggested by the studies of Isemura et al. (1981), who observed differences in protease sensitivity of the subunit chains of porcine fibronectin using cathepsin B digestion. Also the time course digestion of hamster plasma fibronectin by trypsin and by thermolysin (Sekiguchi et al., 1981) suggested different sus-

ceptibilities of the subunit chains to proteases.

There has been one report of a region of differential enzyme cleavage between the subunits at a region near the N-terminus of the molecule. Vartio et al. (1983) using thrombin and cathepsin G cleavage studied fragments that bound gelatin, heparin, and monoclonal antibody and concluded that there was differential enzyme susceptibility of the two chains of human plasma fibronectin at a region near the gelatin-binding (N-terminal) end of fibronectin. However, their conclusions relied upon published reports of only two heparin-binding domains, one at the N-terminus (in a 29K thrombin fragment) and one at the C-terminus. Under the conditions of heparin binding used in their studies, the low-affinity heparin-binding site located between the gelatin-binding and cell-binding domains would be active and would explain the heparin-binding activities of their various fragments without the need to invoke an N-terminal site of differential enyzme susceptibility. Thus, all the data obtained to date suggest that differences between the two chains of plasma fibronectin are located near the C-terminus of the molecule.

Two studies in other species have suggested that one of the two chains of plasma fibronectin lacks a C-terminal binding domain. In a study of self-association of horse serum fibronectin fragments, Ehrismann et al. (1982) found that only one of the large chymotrypsin fragments was bound by an N-terminal fragment of fibronectin, and they thus concluded that only one chain had the self-association domain. However, it is also possible that a small fragment, corresponding to our 34K monoclonal antibody-binding fragment, was released from the second chain of horse plasma fibronectin and was undetected. In a study of hamster fibronectin, Sekiguchi et al. (1981) used trypsin and thermolysin digestion and found that one subunit lacked a C-terminal fibrin-binding domain. It remains to be demonstrated whether hamster fibronectin mRNA encodes a subunit lacking this domain or whether subsequent proteolytic cleavage removes the fibrin domain from the susceptible subunit.

All other fragments studies (Richter & Hormann, 1982; Hayashi & Yamada, 1983; Sekiguchi & Hakomori, 1983a,b) are consistent with our conclusions and together suggest that human plasma fibronectin consists of two chains, A and B, which have homologous binding domains on each chain but which have a region of differential enzyme suceptibility between the C-terminal heparin-binding domain and the monoclonal antibody/fibrin II/fibronectin-association domain. The presence of two different subunit chains allows for the existence of homodimers and/or heterodimers of fibronectin. The isolation of only one species of disulfide-bonded fragment after cathepsin D digestion (i.e., the 83K fragment), which upon reduction yielded a 38K fragment and a 61K fragment, suggests that plasma fibronectin is a heterodimer. No disulfide-bonded fragments corresponding in size to homodimers of 38K or of 61K were detected. These results are consistent with those of Richter & Hormann (1982).

In addition to the difference between the two chains of plasma fibronectin, the presence of a 50K heparin-binding fragment in digests of cellular (but not plasma) fibronectin suggests a structural difference between human plasma and cellular fibronectin. Differences between the C-terminal regions of cellular and plasma fibronectins have been observed by other investigators. In a study comparing chicken cellular and plasma fibronectins, Hayashi & Yamada (1981) observed an 11 000-dalton difference between comparable large heparin-binding fragments and a 1000-dalton difference between comparable subfragments, using thermolysin digestion. These differences were localized to the C-terminus of the molecule. They also noted a 1000-dalton difference between the gelatin-binding fragments of the two fibronectins (located near the N-terminus). Atherton & Hynes (1981) isolated a monoclonal antibody to hamster cellular fibronectin that does not cross-react with plasma fibronectin. The reactive domain was located in a 40K C-terminal fragment produced by trypsin or chymotrypsin digestion. In a preliminary report, Sekiguchi et al. (1983) compared human cellular and plasma fibronectins using cleavage by S-cyanylation and immunostaining of SDS gels with domain-specific antibodies. They showed a difference in size between large fragments containing the C-terminal heparin- and fibrin-binding domains. More recently, using high-resolution electrophoresis, Paul & Hynes (1984) showed the presence of multiple fibronectin subunits for both cellular and plasma fibronectin obtained from both rat and hamster. Some of the differences were attributable to posttranslational modifications, in particular, the addition of carbohydrate moieties, but some appeared to be due to primary structural differences.

A model relating the peptide differences presented in this study to mRNA differences suggested by recent cDNA cloning studies is depicted in Figure 10. Kornblihtt et al. (1984a) sequenced two cDNA clones of human fibronectin and demonstrated the presence in one clone of a 90 amino acid extra domain (ED) located between the cell-binding and heparin-binding domains. This ED is not seen in the protein sequence of the human plasma fibronectin fragment SH29-36K (Gold et. al., 1983) or in the cDNA sequence of the rat liver clones which span this region (Schwarzbauer et al., 1983). Since liver cells may be synthesizing mainly the plasma form of fibronectin, these results are consistent with the presence of the ED exclusively in the cellular form of fibronectin. The ED represents part of the difference in apparent molecular weight between the cellular 50K and plasma 36K heparin-binding fragments. The remaining difference could be due to posttranslational modifications (including glycosylation), but additional primary sequence differences may also exist.

A second region of difference among the three fibronectin chains lies between the heparin-binding and antibody-binding domains, which includes a site of enzyme sensitivity. Sequencing of rat cDNA clones (Schwarzbauer et al., 1983) and the relevant portion of rat genomic DNA (Tamkun et al., 1984) suggests three transcripts are produced by alternative splicing in this region. One of these transcripts encodes an extra 120 amino acids and another encodes only the last 90 of the extra 120 amino acids. The third and shortest transcript does not encode any part of this segment. Two transcripts corresponding to this region of human fibronectin have been identified. One, obtained from cDNA clones of normal human fibroblasts by Bernard et al. (1985), encodes a 120 amino acid segment homologous to the longest rat transcript. A shorter transcript, obtained from cDNA clones of a human tumor cell line by Kornblihtt et al. (1984b), encodes the first 89 amino acids of this segment. Inspection of the 89 amino acid sequence reveals several arginine and lysine residues that in the linear polypeptide would be susceptible to cleavage by trypsin. Folding of the polypeptide into its native conformation may place one of these residues into an exposed position, generating a site of preferential enzyme susceptibility. Since both of the human transcripts contain this 89 amino acid sequence, we suggest that one or both of these transcripts encode the region of enzyme sensitivity in the plasma A chain and the cellular fibronectin subunit. It seems reasonable to predict that a third mRNA, lacking the 120 amino acid coding segment, would

encode the region of enzyme insensitivity in the human plasma B chain.

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SUPPLEMENTARY MATERIAL AVAILABLE

Four figures and one table showing the isolation, amino acid composition, and two-dimensional peptide maps of plasma fibronectin fragments (8 pages). Ordering information is given on any current masthead page.

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Tyrosine Motions in Relation to the Ferric Spin Equilibrium of Cytochrome P-450$_{cam}$†

Mark T. Fisher and Stephen G. Sligar*
Department of Biochemistry, University of Illinois, Urbana, Illinois 61801
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ABSTRACT: Second derivative spectroscopy was used to determine the percentage of tyrosine residues that are exposed to solvent in cytochrome P-450$_{cam}$ isolated from *Pseudomonas putida*. The ratio between two peak to trough second derivative absorbance differences has been shown to be dependent on the polarity of the microenvironment surrounding tyrosine residues [Ragone, R., Colonana, G., Balestrieri, C., Servillo, L., & Irace, G. (1984) *Biochemistry 23*, 1871]. With a number of camphor analogues that independently vary the spin equilibrium of the ferric cytochrome P-450$_{cam}$, experiments have demonstrated that the percentage of tyrosine residues exposed to solvent is linearly dependent on the percentage of ferric high-spin species present. This is not simply a function of the extent of substrate binding since in all cases the substrate concentration was sufficient to ensure saturation of the cytochrome. The local microenvironment of approximately one tyrosine residue appears to be linearly correlated with the percentage of ferric high-spin cytochrome. Structural studies of cytochrome P-450$_{cam}$ using small-angle X-ray scattering [Lewis, B. A., & Sligar, S. G. (1983) *J. Biol. Chem. 258*, 3599] and high-pressure difference spectroscopy [Fisher, M. T., Scarlata, S. F., & Sligar, S. G. (1985) *Arch. Biochem. Biophys. 240*, 456] imply that global conformational changes linked to the spin equilibria are small. Together with the data reported herein, these results suggest that one tyrosine residue is involved in a conformational change that is directly linked with the spin equilibrium.

Cytochrome P-450$_{cam}$ is a heme monoxygenase from *Pseudomonas putida* that is the ultimate electron acceptor of a short electron-transfer chain consisting of the flavoprotein, putidaredoxin reductase, and an iron–sulfur protein, putidaredoxin. The role of this monoxygenase is to catalyze the specific hydroxylation of the monoterpene camphor in the first metabolic step by which this molecule can provide a sole carbon and energy source for this organism (Hegegaard & Gunsalus, 1965). To hydroxylate camphor, the porphyrin-chelated ferric iron must undergo an initial reduction by putidaredoxin (Peterson, 1971; Gunsalus & Lipscomb, 1972); dioxygen then binds to the ferrous cytochrome P-450$_{cam}$, and a second electron is transferred to the dioxygen-bound ferrous iron, ultimately creating a reactive oxygen species that is responsible for the hydroxylation event. It is at the initial reduction step that the overall activity of this enzyme is thought to be regulated through a shift in the reduction potential of cytochrome P-450$_{cam}$. The observed redox potential shifts from −340 mV in camphor-free cytochrome to −173 mV in the camphor-bound species (Sligar & Gunsalus, 1976). This shift in reduction potential is significant since putidaredoxin bound to cytochrome P-450$_{cam}$ has a redox potential near −196 mV. As a result, the reduction of camphor-bound cytochrome P-450$_{cam}$ is thermodynamically favorable and that of substrate-free protein is thermodynamically unfavorable (Sligar & Gunsalus, 1976). This potential change is reflected in an increase in the first electron-transfer rate from 0.22 s^{-1} to 41 s^{-1} in this dienzyme complex (Pederson et al., 1977; Fisher & Sligar, 1985). The redox potential shift and increased electron-transfer rate provide both thermodynamic and kinetic control for the reduction of cytochrome P-450$_{cam}$, thus preventing the deleterious waste of vital reducing equivalents and concomitant production of reduced oxygen products when substrate is not present (Sligar et al., 1974). This precise control of redox potential is thought to be dictated in part by a shift in the ferric spin equilibrium from a predominantly low-spin ($S = {}^1/_2$) form to a predominantly high-spin ($S = {}^5/_2$) form upon substrate binding (Tsai et al., 1970; Sligar, 1976). Accompanying this change in heme spin state is a blue shift in the wavelength maximum of the Soret region, which has been shown to be directly correlated with magnetic properties of the iron center (Philson, 1977). This spectrally observable change in the heme ligand field conformation provides a means to study the associated structure–function relationship of this cytochrome.

Investigating the source of the spin-state changes occurring in the P-450$_{cam}$ heme protein demands that the various discernible protein structural changes accompanying the two spin states be addressed. Small-angle X-ray scattering (Lewis & Sligar, 1983) and high-pressure UV–visible spectroscopy (Fisher et al., 1985) have been previously employed and strongly suggest that the conformational change involved with the spin equilibria of cytochrome P-450$_{cam}$ is a local rather than global protein conformational change.

Various analogues of the normally metabolized substrate camphor can give rise to varying proportions of low and high ferric spin equilibria upon binding to cytochrome P-450$_{cam}$ (Gould et al., 1981) (Figure 1). Through the use of these

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