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Model of Fibronectin Tertiary Structure Based on Studies of Interactions between Fragments†

Gene A. Homandberg*,‡ and John W. Erickson‡

Department of Medicine, Hemostasis Research Laboratory, University of Wisconsin Medical School, Milwaukee Clinical Campus, Mount Sinai Medical Center, Milwaukee, Wisconsin 53233

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ABSTRACT: Human plasma fibronectin aggregates in solution and is thought to form fibrils on cell surfaces, perhaps by self-associating and by interacting with other components such as proteoglycans. We have localized the self-association domains by testing the ability of various fragments of fibronectin to interact with each other. Complexation between fluorescamine-labeled fragments and unlabeled fragments or whole molecules was assessed by gel filtration high-performance liquid chromatography. The fragments studied included nonoverlapping fragments that are situated on the fibronectin polypeptide chain in the following order, beginning from the amino terminus: the 29-, 50-, 120-, 35-, and 25-kDa fragments, as well as multiple-domain fragments of 72 kDa containing the 29- and 50-kDa segments, a fragment of 150 kDa containing the 120- and 35-kDa segment, a fragment of 190 kDa containing the 120- and 35-kDa segments, a fragment of 190 kDa containing the 50-, 150-, and 25-kDa segments, and a 45-kDa fragment containing the 35-kDa segment. The amino-terminal 29-kDa fragment bound to the carboxyl-terminal heparin-binding (Hep II) 35-kDa fragment as well as the 150- and 190-kDa fragments that contain the 35-kDa segment. On the other hand, carboxyl-terminal 35- and 45-kDa Hep II containing fragments bound to each other as well as to amino-terminal 29- and 72-kDa fragments and to the 190-kDa fragment. Further, the 25-kDa carboxyl-terminal fibrin-binding fragment bound the 190-kDa fragment, the only fragment containing the 25-kDa segment. We conclude that fibronectin folds in such a way as to allow interaction of the amino-terminal 29-kDa region with the carboxyl-terminal 35-kDa Hep II domains in a fashion that may account for the polymerization of fibronectin. Also, interaction of the carboxyl-terminal 35-kDa heparin-binding and 25-kDa fibrin-binding segments with their like counterparts on adjacent subunits may account for the noncovalent monomer–monomer interactions in the native structure.

Fibronectin is an adhesive glycoprotein consisting of two subunits of 215–250 kDa, which binds a variety of ligands, including fibrin, fibrinogen, collagen, heparin, proteoglycans, actin, and DNA, and cells such as fibroblasts, monocytes, macrophages, and *Staphylococcus aureus* [see reviews by Mosesson and Amrani (1980), Ruoslahti et al. (1981), Hynes and Yamada, (1982), Furcht (1983), Yamada (1983), and Akiyama and Yamada (1983)]. The domains responsible for most of these activities have been identified as proteolytic fragments [see reviews by Mosesson and Amrani (1980), Ruoslahti et al. (1981), Hynes and Yamada (1982), Furcht (1983), Yamada (1983), and Akiyama and Yamada (1983)] and are situated on each subunit of fibronectin in the order shown in Figure 1. There are two sulfhydryls per subunit that are about 30 and 70 kD from the carboxyl terminus (Wagner & Hynes, 1980; Sekiguchi et al., 1981) and that have been located in the amino acid sequence of bovine fibronectin (Petersen & Skorstengaard, 1985).

Electron microscopy of fibronectin molecules deposited on mica from solutions containing 30–40% glycerol suggest an extended V-shaped structure (Engel et al., 1981; Erickson et al., 1981; Erickson & Carrell, 1983) or a rather compact or loosely folded molecule with dimensions varying from 16 × 9 nm to 51 × 32 nm [Koteliansky et al., 1980; Price et al.,

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* Author to whom correspondence should be addressed.
‡ Present address: Physical Biochemistry Lab, Abbott Laboratories, Abbott Park, IL 60064.

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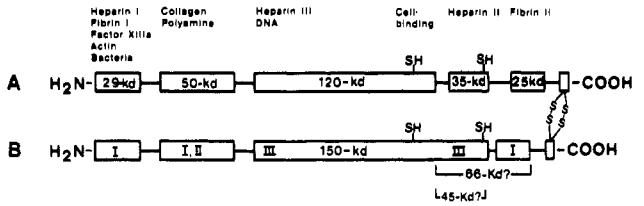

FIGURE 1: Placement of domains in PFn. Location of domains is based on references cited in reviews (Mosesson & Amrani, 1980; Ruoslahti et al., 1981; Hynes & Yamada, 1982; Yamada, 1983; Furcht, 1983; Akiyama & Yamada, 1983). Lines are drawn between domains to emphasize protease susceptibility. The size of the 29-, 50-, 72-, and 190-kDa fragments are based on Furie et al. (1980) and Furie & Rifkin (1980). Our 150-kDa fragment is probably equivalent to the 140-kDa fragment reported by Balian et al., (1979). The size of 150 kDa is based on use, as a $NaDodSO_4$ standard, of reduced PFn with a monomeric molecular weight of 250000. The Roman numerals denote the types of loop structures (Petersen & Skorstengaard, 1985). A 66-kDa fragment derived from the B subunit as reported by Rogers et al. (1985) was not observed under our conditions, although a 45-kDa fragment was, which may be similar to a 43-kDa breakdown product of the 66-kDa fragment as also reported by Rogers et al. (1985). Our assignment of the 150-kDa fragment to the B subunit is based on our observation that the 45-kDa fragment is not present in early digests when the 35-kDa fragment is fully released, but is observed only when no 150-kDa fragment is present.

1982; Tooney et al., 1983; see review by Erickson (1985)]. Most biophysical studies suggest that the structure of fibronectin is pleimorphic and is affected readily by solution and surface conditions (Koteliansky et al., 1980; Engel et al., 1981; Erickson et al., 1981; Price et al., 1982; Erickson & Carrell, 1983; Tooney et al., 1983). Sedimentation velocity studies show that fibronectin can undergo a marked and reversible transition from a compact to a more expanded form (Mosesson et al., 1975; Alexander et al., 1979; Erickson & Carrell, 1983). Fluorescence and electron spin resonance spectroscopy indicate a high degree of chain flexibility for the molecule in both its compact and expanded forms (Williams et al., 1982; Lai & Tooney, 1985). This expansion may be induced by exposing the molecule to high ionic strength or to pH extremes (Alexander et al., 1979; Odermatt et al., 1982; Williams et al., 1982) or to urea (Mosesson et al., 1975). The forces stabilizing the compact form appear to be mediated by salt bridges between acidic and basic residues (Hormann, 1981). A model accounting for the compact form has been proposed in which each subunit of fibronectin is folded over so that its amino- and carboxyl-terminal regions interact to hold the molecule in a compact configuration (Hormann, 1981; Rocco et al., 1983).

The ability of fibronectin to self-associate to form multimers is considered to be of biological significance, since fibronectin is often found in the form of fibrillar structures that are associated with the cell surface and that may contain collagens and proteoglycans (Hynes & Yamada, 1982). These fibrils may align in a parallel fashion with the actin-rich intracellular microfilaments. The conformer of fibronectin required for fibril formation is unknown, and little is understood concerning the mechanism of polymerization. However, fibronectin filaments resembling in vivo fibrils have been produced in vitro by prolonged incubation at low temperatures and by the binding of naturally occurring ligands such as polyamines and heparin (Jilek & Hormann, 1979; Vuento et al., 1980). Also, covalent crosslinking of fibronectin in vitro via the formation of intermolecular disulfide bonds and by transglutaminase (factor XIIIa) has also been observed (Mosher, 1975; Mosher & Johnson, 1983) and may acocunt for in vivo assembly.

The domains of fibronectin that may be responsible for these self-association reactions have been localized to a 60-kDa amino-terminal fragment that binds gelatin and to a carboxyl-terminal 160-kDa fragment (Ehrismann et al., 1981; Ehrismann et al., 1982). The amino-terminal self-association reaction has been related to the gelatin-binding domain since addition of polyamines to fibronectin enhances polymerization (Jilek & Hormann, 1979; Vuento et al., 1980) and polyamines bind the gelatin-binding domain (Vuento & Vaheri, 1978). A carboxyl-terminal self-association domain has been suggested to reside on only one subunit, since a 160-kDa, but not a 140-kDa, chymotryptic produced fragment bound fibronectin Sepharose (Ehrismann et al., 1981; Ehrismann et al., 1982). These carboxyl-terminal regions may mediate noncovalent interchain interactions within individual fibronectin molecules. Sedimentation velocity and molecular-sieving analyses have demonstrated that partially reduced fibronectin, in which the interchain disulfides are cleaved preferentially (Homandberg et al., 1985a), as well as 190-kDa fragments that lack the amino-terminal 29-kDa domain, behave as dimers (Erickson & Carrell, 1983; Robinson & Hermans, 1984; Erickson, Homandberg & Mosesson, unpublished experiments).

We have recently shown that the 29-kDa amino-terminal fragment and 40-kDa carboxyl-terminal heparin-binding fragments, bind native fibronectin (Homandberg et al., 1985b). In order to clarify the placement of the self-association domains and to determine if the monomer–monomer and amino–carboxyl terminal (subunit fold-over) interactions are occurring through the same domains, we have tested the interactions between various fibronectin fragments. We present here a model based on qualitative assessment of these interactions.[1]

Experimental Procedures

All common chemicals and reagents were from Fisher Chemical Co., Pittsburgh, PA. TSK 3000 SW and TSK 6000 PW HPLC[1] sieving columns were from Varian Instruments. Human α-thrombin was a gift of Dr. J. Fenton of the New York State Department of Health, Albany, NY. All electrophoretic supplies were from Bio-Rad Laboratories, Richmond, CA. Cathepsin D, heparin (Grade I, porcine intestinal mucosa, 158 U/mg), pepstatin, and conventional chromatography resins were from Sigma Chemical Co., St. Louis, MO. All sequencing supplies were from Pierce Chemical Co., Rockford, IL.

Generation and Characterization of PFn Fragments and Derivatives. PFn (Engvall & Ruoslahti, 1977) and partially reduced fibronectin (PR-PFn) (Homandberg et al., 1985a) were prepared as described.

Cathepsin D digestion was performed by the method of Balian et al., (1979) for times varying from 3 to 20 h. PFn (0.2 mg/mL) in 0.15 M NaCl and 0.1 M formate buffer, pH 3.7, at 30 °C was adjusted to 5 μg/mL cathepsin D. A 3-h digestion at 30 °C generated an amino-terminal 72-kDa fragment (composed of 29- and 50-kDa fragments; Figure 1), a 150-kDa central fragment, and 45-, 35-, and 25-kDa fragments. The digest was applied to a gelatin–Sepharose column, [prepared as described (Homandberg et al., 1985a)] equilibrated in TBS. The bound 72-kDa fragment was eluted with 3 M urea–PBS. The nonadherent material containing the 150-kDa fragments and small fragments were separated by

[1] Abbreviations: HPLC, high-performance liquid chromatography; PFn, human plasma fibronectin; PR-PFn, partially reduced human plasma fibronectin; kDa, kilodalton; TBS, 0.05 M tris[2-amino-2-(hydroxymethyl)-1,3-propanediol]-buffered (pH 7.4) saline (0.15 M NaCl); PBS, 0.1 M phosphate-buffered (pH 7.4) saline (0.15 M NaCl); $NaDodSO_4$, sodium dodecyl sulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; Fl, fluorescamine labeled; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

gel filtration on Sephacryl S-300 equilibrated in PBS. Digests of up to 20 h produced a 120-kDa fragment, which was separated from the 72-kDa fragment and smaller fragments as above. Amino-terminal analysis showed that the 72-kDa fragment was blocked just as was native PFn. The carboxyl-terminal residue of the 72-kDa fragment was Glx.

In order to generate the 29-kDa amino-terminal fragment and the 50-kDa gelatin-binding fragments, some of the 72-kDa fragment was digested further with thrombin (Furie et al., 1980) in TBS, free of inhibitors (see Figure 1). The digest mixture was diluted with one volume of water and applied to gelatin–Sepharose equilibrated in PBS. The bound 50-kDa fragment was desorbed with 4 M urea–PBS. The 29-kDa fragment pass-through was further purified by adsorption to heparin–Sepharose at 22 °C [prepared as described (Homandberg et al., 1985a)] equilibrated in 50 mM NaCl and 20 mM Tris, pH 7.4, and eluted with buffer adjusted to 300 mM NaCl. The 50-kDa fragment had an amino-terminal sequence of Ala-Ala-Val, which corresponds to residues 260–262 of the native sequence (Kornblihtt et al., 1985) while the 29-kDa fragment was blocked as was the 72-kD fragment and PFn. The carboxyl-terminal residue of the 29-kDa segment was Arg and both the 50- and 72-kDa sequences ended in Glx.

Carboxyl-terminal 170/190-kDa fragments (composed of 50-, 150-, and 25-kDa segments; Figure 1) were generated by thrombin digestion (Furie & Rifkin, 1980) of PFn (2 mg/mL) in 20 mM sodium phosphate, pH 7.4, with 1 U/mL thrombin. The progress of the reaction was monitored by $NaDodSO_4$ gel electrophoresis (Laemmli, 1970). After several days, the digestion was stopped by the addition of (phenylmethyl)-sulfonyl fluoride (final concentration 0.1 mM), and the resulting mixture was diluted with one volume of water and applied to a column of gelatin–Sepharose equilibrated in PBS. Two fragments, 190 and 170 kDa, bound, while two minor fragments, 110 and 140 kDa, as well as smaller fragments, washed through with PBS. The 110- and 140-kDa fragments likely arise by removal of the 50-kDa gelatin-binding segment from the 170- and 190-kDa fragments. The 170- and 190-kDa fragments were desorbed with 3 M urea–PBS and will be referred to as the 190-kDa fragment. These two fragments bound to heparin–Sepharose and fibrin–Sepharose. The 29-kDa fragment was recovered from the wash-through by application of the wash to a heparin–Sepharose column equilibrated in 50 mM NaCl and 20 mM Tris buffer, pH 7.4, and elution of the 29-kDa fragment with 300 mM NaCl and 20 mM Tris buffer, pH 7.4. The 190-kD fragment had an amino-terminal sequence of Ala-Ala-Val, which corresponds to residues 260–262 in the native sequence (Kornblihtt et al., 1985), and therefore has the 50-kDa sequence at its amino terminus.

Concentrations of Fragments. Extinction coefficients for the fragments were estimated from the amino acid compositions by using the method of Edelhoch (1967): The $A^{0.1\%}_{280nm}$ values for the 29-, 50-, 72-, 120-, 150-, and 190-kDa fragments were 2.1, 1.7, 1.8, 1.0, 1.0, and 1.2, respectively. The $A^{0.1\%}_{280nm}$ value used for PFn was 1.2 (Mosesson et al., 1975).

Heparin Affinity Chromatography. Heparin was coupled to CNBr-activated Sepharose 4B as described (Homandberg et al., 1985a). One to five milligrams of fragments were applied to a 5-mL heparin–Sepharose column at 22 °C in 50 mM NaCl and 20 mM Tris buffer, pH 7.4. Columns were eluted with 300 mM NaCl and 20 mM Tris buffer, pH 7.4.

Fibrin Affinity Chromatography. Human fibrinogen was first coupled to CNBr-activated Sepharose 4B and coupled protein subsequently reacted with thrombin as described (Homandberg et al., 1985a). One to five milligrams of fragments in 50 mM NaCl and 20 mM Tris buffer, pH 7.4, were applied to a 5-mL column of fibrin–Sepharose equilibrated at 9 °C. Adherent material was eluted with 8 M urea and 0.1 M sodium acetate, pH 5.2, at 9 °C.

PFn Affinity Chromatography. PFn was concentrated to 4 mg/mL by layering poly(ethylene glycol) (20000 Da) crystals over dialysis tubing filled with protein solution. The concentrated solution was then dialyzed against 0.1 M sodium bicarbonate, pH 9.5, and reacted with one volume of CNBr-activated Sepharose 4B, essentially as described (Homandberg et al., 1985a). Fragments in 150 mM NaCl and 20 mM Tris buffer, pH 7.4 were mixed for 24 h with PFn–Sepharose at 22 °C or with fragment–Sepharoses. After the resin was washed with equilibration buffer, bound material was eluted with 4 M urea, 1 M KBr, and 100 mM acetate, pH 5.2.

Amino-terminal amino acid sequencing was performed by the method of Weiner et al., (1972). Carboxyl-terminal sequencing was performed as described (Homandberg et al., 1985d).

Quantification of sulfhydryl content was performed on PFn or fragments in 6 M urea by using DTNB (Ellman, 1959).

HPLC Assay for Interaction. Interaction was assayed by a method described earlier (Homandberg et al., 1985b), which allowed us to test interaction in the fluid phase as opposed to solid-phase interactions of affinity chromatography. One fragment was labeled with fluorescamine and mixed with unlabeled fragment, and complexation was observed by the appearance of a new higher molecular weight peak on HPLC gel filtration. In this way, assays with microgram quantities of protein could be performed quickly, in the absence of a solid phase, with low fluorescent background, and without removal of reagent. Further, the unlabeled fragment did not interfere with visualization of the complex. Solutions of fragments (0.1 to 1 mg/mL) in 100–500 μL of PBS or TBS adjusted to pH 8.5 were fluorescently labeled by addition at 22 °C of 0.2 volume of a stock fluorescamine solution (0.15 mg/mL in acetone). After 5 min, the solution was adjusted to pH 7.4 and aliquots mixed with aliquots of unlabeled fragments in TBS or PBS at 22 °C. After 24 h at 22 °C, complex formation was monitored by gel filtration HPLC as described (Homandberg et al., 1985b). Gel filtration was performed on a Varian Vista 54 system, controlled by a Varian Model 401 microprocessor. Samples of 50–500 μL were injected onto a TSK 3000 SW column in line with a TSK 6000 PW column. The flow rate was 0.5 mL/min, and the column was equilibrated in TBS. Detection was with a Varian Fluorochrom detector with an excitation filter of 340–380 nm and emission filters of 460 nm.

Various controls were also performed. Fluorescamine decays in aqueous buffer within seconds (Bohlen et al., 1973). In order to verify that the excess fluorescamine from labeling of the first reactant did not label the second reactant, e.g. PFn, an aliquot of fluorescamine was added to buffer and PFn added 5 min later; no labeled PFn was detected. In order to show that unreacted but hydrolyzed fluorescamine or acetone did not account for interactions, we dialyzed solutions of labeled fragment prior to addition to the unlabeled reactant. In this case, the same spectrum of interactions occurred as when solutions were not dialyzed. Where complexation did occur between particular fragment combinations, the complex was isolated by HPLC and subjected to $NaDodSO_4$ electrophoresis under reducing conditions; in each case, the new, higher mo-

lecular weight fluorescent peak observed on HPLC contained the two test fragments. Interaction therefore was not due to aggregation or association of the labeled fragment. As will be discussed, interactions were also confirmed by solid-phase affinity chromatography. Interaction of unlabeled 29- and 35-kDa fragments as well as their fluorescamine-labeled derivatives with PFn was reported earlier (Homandberg et al., 1985).

Sedimentation Velocity Studies of Fragments. The corrected $s_{20,w}$ values for fragments were determined in sedimentation velocity experiments, using a Beckman Model E Analytical Ultracentrifuge equipped with schlieren optics (Erickson, Homandberg, and Mosesson, unpublished experiments).

Results

Isolation of Defined Fragments. The 29-, 50-, 72-, 150-, and 190-kDa fragments were isolated by reported procedures that are based on placement of the gelatin-binding and amino-terminal heparin-binding domains (Figure 1). Plasma fibronectin is a heterodimer of two major types of subunits, termed A and B, which have apparent mobilities of $NaDodSO_4$ gels of 220- and 215-kDa (see Mosesson & Amrani, 1980), respectively, but which may be about 250-kDa each based on other methods (see Hermans, 1985). The model shown in Figure 1 incorporates this aspect of subunit differences, the known placement of the thrombin-generated 29-kDa fragment and 170/190-kDa fragments (Furie & Rifkin, 1980), the thrombin and cathepsin D generated 50-kDa gelatin-binding fragment (Furie et al., 1980), the cathepsin D generated 72- and 140-kDa (150-kDa in this study) fragments (Balian et al., 1979), and on the basis of our studies, placement of carboxyl-terminal cathepsin D generated fragments. Our amino-terminal analysis showed that the 29- and 72-kDa fragments contain the blocked amino terminus at position 1 as does native PFn. The 50- and 170/190-kDa fragments both began with Ala-Ala-Val, which corresponds to positions 260–262 in the native sequence (Kornblihtt et al., 1985). The 150- and 120-kDa fragments both began with Thr-Pro-Ser, corresponding to residues 589–591 in the native sequence (Kornblihtt et al., 1985).

These fragments were subjected to $NaDodSO_4$ gel electrophoresis in a 5–20% linear gradient gel to demonstrate their relative homo- and heterogeneity (Figure 2A). Most of the fragments were homogeneous. The 190-kDa sample shows the 170/190-kDa doublet. The 150-kDa sample showed about 20% contamination with lower molecular weight fragments of 100–120-kDa. The 45/35/25-kDa mixture in lane 8 was purified further as described below.

Isolation and Characterization of Cathepsin D Generated Fragments. Cathepsin D digestion was originally reported to produce major fragments of 140 and 72 kDa (Balian et al., 1979). We have assigned the size of our largest fragment as 150 kDa rather than 140 kDa (see Discussion). A sample of a cathepsin D digestion of PFn which produced 150- and 72-kDa fragments with trace amounts of 45-, 35-, and 25-kDa fragments is shown (Figure 2B, lane 1). Kinetic analysis of digests showed that the amount of 35-kDa fragment increased concomitant with the appearance of a 120-kDa fragment while the 25-kDa fragment was present in early digests and did not increase as the digest continued. The 43-kDa fragment appeared later in the digest. After removal of the 72-kDa fragment by adsorption to gelatin–Sepharose, the nonadherent material (Figure 2B, lane 2) was subjected to gel filtration on Sephacryl S-300. Homogeneous 150-kDa fragment (Figure 2B, lane 3), which bound heparin-Sepharose but not fibrin-

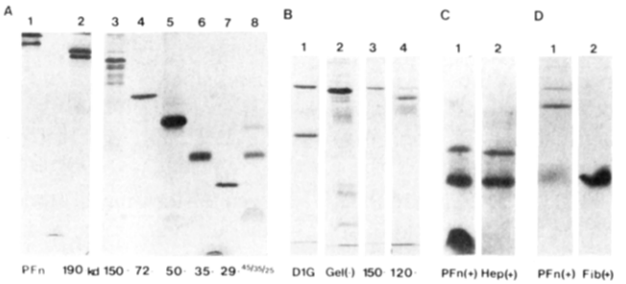

FIGURE 2: $NaDodSO_4$ gel of fragments used in these studies. Gel A is a split 5–20% linear gradient gel with designated fragment (about 40 μg each) subjected to electrophoresis in the Laemmli (1970) system. Gel B represents single lanes from the same 10% gel: lane 1, a cathepsin D digest (3 h); lane 2, the 150-, 45-, 35-, and 25-kDa fragments that did not bind gelatin–Sepharose; lane 3, the non gelatin binding 150-kDa fragment after gel filtration on Sephacryl S-300; lane 4, the 120-kDa product of cathepsin D digestion (24 h) of isolated 150-kDa material from lane 3. Gel C is a 20% gel: lane 1, the 25/35/45-kDa, fragments from gel filtration of material in gel B, lane 2, that bound PFn–Sepharose (PFn^+); lane 2, 35- and 45-kDa fragments from lane 1 that bound heparin–Sepharose (Hep^+). Gel D is a 20% gel: lane 1, PFn–Sepharose binding fragments (PFn^+) (as in gel C, lane 1); lane 2, 25-kDa fragment from lane 1 that bound fibrin–Sepharose (Fib^+).

Sepharose, was isolated and was shown to contain 1.6 SH/mol of protein. This was the same SH content as measured for native PFn or for the 190-kDa fragment, both of which bind heparin–Sepharose and fibrin–Sepharose. Therefore, on the basis of SH content alone, the 150-kDa fragment should bind heparin and does, if the model in Figure 1 is correct. When this 150-kDa fragment was further digested with cathepsin D (20 h), a 120-kDa fragment resulted (Figure 2b, lane 4), which no longer bound heparin–Sepharose under our conditions, although the weak heparin-binding domain (Hep III) should be contained within this fragment (see Figure 1). Both the 150- and 120-kDa fragments had the same amino-terminal sequence, consistent with the difference in size being due to the lack of the carboxyl-terminal Hep II domain in the 120-kDa fragment. The SH content of the 120-kDa fragment isolated either from 4- or 20-h digests was 0.8 SH/mol of protein, again consistent with its lack of the SH-containing Hep II domain. In order to diagram A and B subunits of about 250 kDa each, the 120- and 150-kDa fragments were assigned to the A and B subunits, respectively (Figure 1).

The 45-, 35-, and 25-kDa fragments were isolated by Sephacryl S-300 chromatography of the non gelatin binding fraction, and were tested for affinity to PFn–Sepharose. Solutions of the fragments in TBS were shaken gently with PFn–Sepharose for 24 h. Over 80% of the material bound and was eluted with 4 M urea, 1 M KBr, and 0.05 M sodium acetate, pH 5.2. (Figure 2C, lane 1). A separate fraction of the three fragments was tested for binding to heparin–Sepharose. The 25-kDa fragment did not bind (Figure 2C, lane 2) but did bind fibrin–Sepharose (Figure 2D, lane 2). The 35-kDa fragment was isolated from the 45/35-kDa mixture (Figure 2C, lane 2) by gel filtration on Sephadex G-100, resulting in a homogeneous fraction (Figure 2A, lane 6). Formation of the 45-kDa fragment during cathepsin D digestion could be reduced by using a shorter 3-h digestion. The 25-, 35-, or 45-kDa fragments aggregated on storage as evidenced by elution of higher molecular weight peaks on the HPLC gel filtration column. In this case, the solutions were chromatographed on Sephacryl S-300 immediately prior to use or were applied to PFn–Sepharose and eluted with 4 M urea, 1 M KBr, and 100 mM acetate, pH 5.2.

The SH content of the 35-kDa fragment or of the 45-kDa fragment was 0.8 mol/mol of protein whereas the SH content

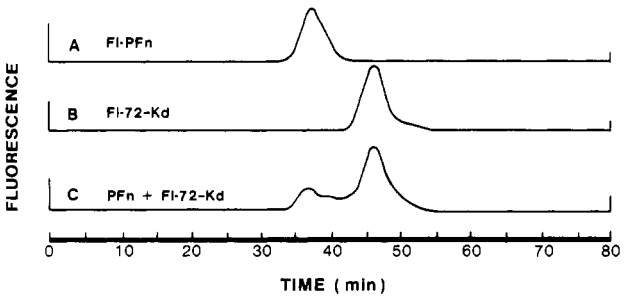

FIGURE 3: Interaction of the 72-kDa amino-terminal fragment with PFn. (A) PFn (0.5 mg/mL) in PBS was adjusted to pH 8.5 by addition of dilute base. A 20% volume of fluorescamine in acetone was added. After 5 min, the pH was lowered to 7.4 by addition of dilute HCl and a 140-μL aliquot subjected to chromatography, as described. (B) a solution of 72-kDa fragment (1 mg/mL) was similarly treated and 20 μL applied to the column. (C) A stoichiometric quantity (calculated per subunit of PFN) of labeled 72-kDa fragment (20 μg in 20 μL) was mixed with unlabeled PFn (70 μg in 140 μL) and subjected to chromatography 24 h later.

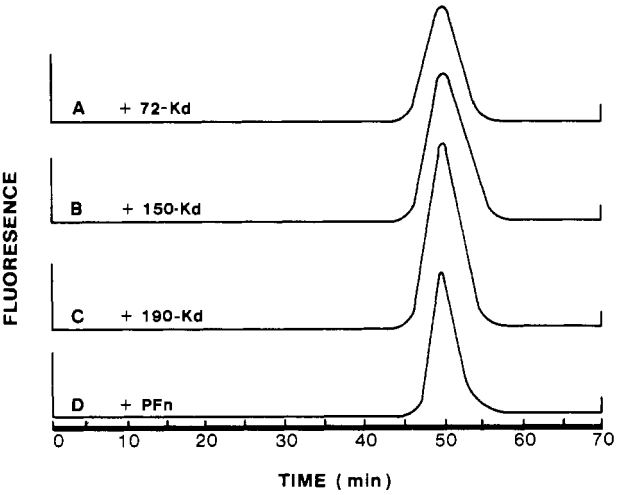

FIGURE 4: Tests of interaction of the 50-kDa gelatin-binding fragment with other fragments. A solution of the 50-kDa fragment (1 mg/mL) was labeled as in Figure 3, and 50-g quantities were mixed with equimolar amounts (in terms of PFn subunit) of other unlabeled fragments as designated. Interaction was tested 24 h later.

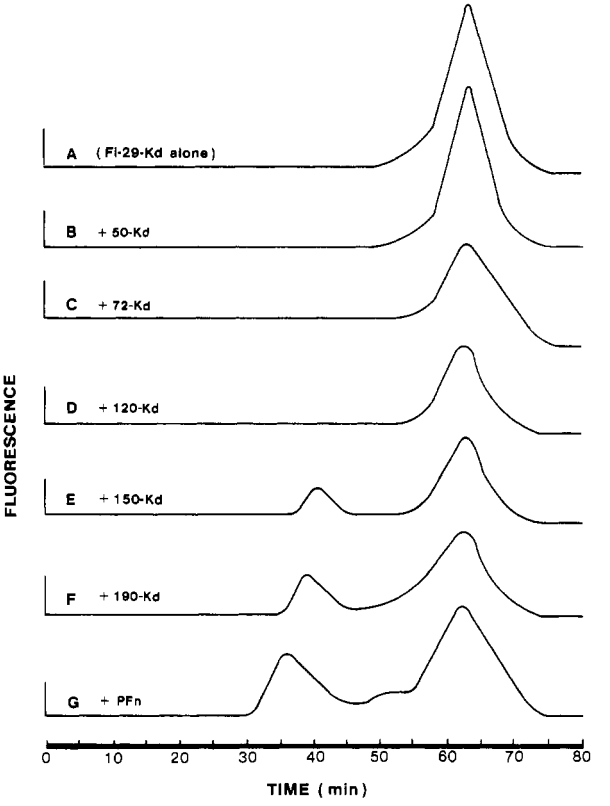

FIGURE 5: Interaction of the 29-kDa amino-terminal fragment with other fragments. A solution of the 29-kDa fragment (1 mg/mL) was labeled as in Figure 3, and 50-μg quantities were mixed with equimolar amounts of other unlabeled fragments as designated and the mixtures chromatographed 24 h later.

of the 25-kDa fragment was less than 0.1 mol/mol of protein. These values are consistent with the assignment of the 35- and 45-kDa fragments as Hep II domains. When the 35- or 45-kDa fragments were exposed to 0.1 M DTT and reacted with DTNB after extensive dialysis, no increase in SH content was observed, consistent with the fragments having a type III disulfide-poor structure. However, the 25-kDa fragment showed an increase to 11.8 SH/mol of protein after reduction, dialysis, and reaction with DTNB, consistent with it having a disulfide-rich type I structure (Figure 1).

The amino-terminal sequence of the 35-kDa fragment was Lys-Thr-Gly-Pro, which is identical with a sequence of the CB10 fragment of the bovine PFn molecule (Skorstengaard et al., 1982) and which is homologous to the 29- and 36-kDa heparin-binding fragments reported by Gold et al. (1983). This sequence corresponds to positions 1731–1734 in the native sequence (Kornblihtt et al., 1985). The residue preceding Lys in the amino acid sequence is Glu (residue no. 1730), an observation consistent with the known specificity of cathepsin D (Mycek, 1970). Amino-terminal heterogeneity was observed with the 25-kDa fragments with a predominant sequence of Asx-Ser, which may correspond to residues 2144 and 2145 in the native sequence (Kornblihtt et al., 1985).

On the basis of these characterizations and on other reports, these carboxyl-terminal fragments have been assigned the relative positions as shown (Figure 1). These positions are consistent with those of cathepsin D generated heparin-binding carboxyl-terminal 33-, 43-, and 66-kDa fragments reported by Rogers et al. (1985). Their 43-kDa fragment may be a breakdown product of the 66-kDa fragment (Rogers et al., 1985), and their 35- and 66-kDa fragments may arise from the A and B subunits, respectively. Our 45- and 35-kDa heparin-binding cathepsin D generated fragments are likely similar to their 43- and 33-kDa fragments, respectively. The presence of a sulfhydryl group in our 35- and 45-kDa fragments is consistent with other reports of a sulfhydryl within the Hep II domain (Wagner & Hynes, 1980; Sekiguchi et al., 1981). Our 25-kDa fragment binds fibrin, is low in SH content, and may arise from breakdown of the 66-kDa fragment of the B subunit as well as by breakdown from a 150-kDa fragment of the A subunit. Since the 25-kDa fragment has six disulfide bridges, it likely has a type I structure known to be contained within the Fib II domain (Petersen & Skorstengaard, 1985).

Localization and Specificity of the Amino-Terminal Self-Association Domain. The work of Ehrismann et al. (1981, 1982) showed that an amino-terminal gelatin-binding 60-kDa chymotryptic PFn fragment also bound PFn itself. We tested, from the same region, the 72-kDa fragment for interaction (Figure 3). Complexation occurred between fluorescamine-labeled 72-kDa fragments and PFn after 24 h when stoichiometric levels were used. The PFn-affinity region was localized further, by using fluorescamine-labeled 50- (Figure 4) and 29-kDa (Figure 5) fragments, which are cleavage products derived from the 72-kDa fragment. The 50-kDa gelatin-binding fragment did not bind to PFn or to any of its fragments. After 24 h, the 29-kDa fragment reacted with the 150- and 190-kDa fragments and native PFn (Figure 5), but

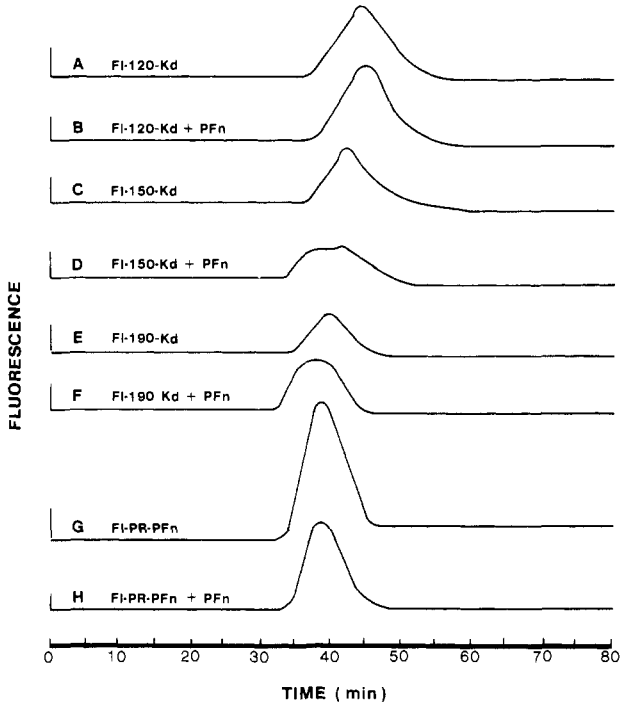

FIGURE 6: Interaction of large carboxyl-terminal fragments with PFn. Solutions of 0.5 to 1 mg/mL of the 120-, 150-, and 190-kDa fragments and of PFn were labeled and chromatographed or 50-μg quantities were mixed with equimolar quantities of unlabeled PFn, as designated, and itneraction was assayed 24 h later.

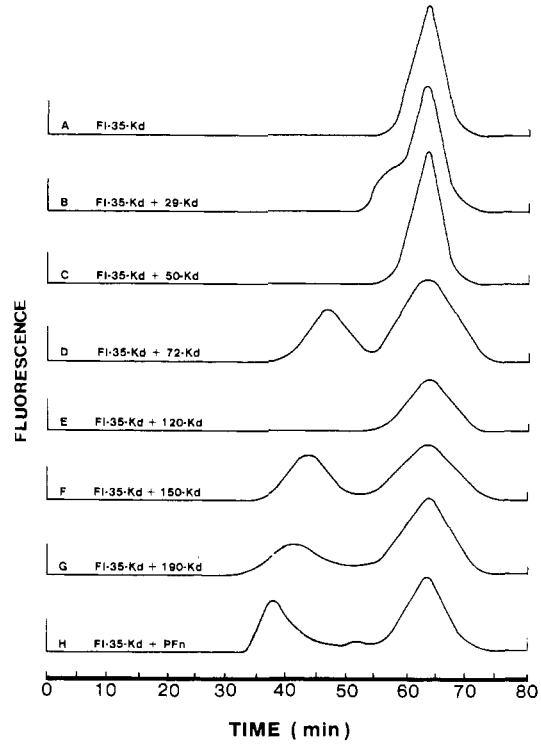

FIGURE 7: Interaction of the 35-kDa carboxyl-terminal fragment with other fragments. A solution of the 35-kDa fragment was labeled as in Figure 3, 50-μg quantities were mixed with equimolar quantities of the unlabeled fragments as designated, and the mixtures were chromatographed 24 h later.

not with the 120-kDa fragment, the 72-kDa fragment, or itself. These interactions were verified by isolation of the complex by HPLC and identification on $NaDodSO_4$ gels. The failure of the 29-kDa fragment to interact with the 120-kDa fragment, the 72-kDa fragment, or itself also argues that the fluorescamine group itself is not responsible for interactions.

The specificity of these interactions was confirmed by testing the ability of soluble 29-kDa fragment to bind to various fragments–Sepharose. When 4 mg quantities of the 29-kDa fragment were applied separately to resins to which 10 mg of the fragments had been coupled, typically ~1 mg of the 29-kDa fragment bound to the 150- or 190-kDa resin or native PFn resin, while less than 100 μg bound to the 29-, 50-, or 72-kDa resin. The amount of the 29-kDa fragment that interacted with the 150- or 190-kDa resin or PFn resin was significant since less than 100 μg of bovine serum albumen bound to any of the resins under similar conditions.

Association of Fragments Lacking the N-Terminal Domains. To localize other self-association domains, we tested interactions between fragments lacking the 29-kDa segment. Figure 6 shows that the 190- and 150-kDa fragments bound to native PFn while the 120-kDa fragment did not. Interaction between labeled PFn and unlabeled PFn could not be demonstrated since PFn eluted near the void volume of the column. Also, for this reason, interaction of Fl-190-kDa fragment with PFn was more weakly suggested than interaction of smaller fragments with PFn. The 35-kDa Hep II domain containing fragment (Figure 2A, lane 6) that had been isolated from the mixture of 45/35/25-kDa fragments (Figure 2A, lane 8) complexed with 29-, 72-, 150-, and 190-kDa fragments as well as with native PFn (Figure 7). In addition, a soluble 35-kDa fragment bound 29-, 72-, 150-, and 190-kDa fragments that were coupled to Sepharose 4B, but did not bind 50- or 120-kDa–Sepharose. We also tested the 35/45-kDa mixture (Figure 2C, lane 3) for interaction in the HPLC assay to determine if both fragments were equally active in binding other fragments. The mixture reacted with the same fragments as did the 35-kDa fraction, and both 35- and 45-kDa peaks decreased in area equally when interactions occurred. These interactions were confirmed by identification of the reactants after $NaDodSO_4$ gel electrophoresis of the complexes isolated by HPLC.

Table I: Measured $s_{20,w}$ Values for Fragments[a]

fragment	$s_{20,w}$	fragment	$s_{20,w}$
29 kDa	*b*	150 kDa	4.4
50 kDa	3.3	170 kDa	10.5
72 kDa	4.1	190 kDa	12.0
120 kDa	5.4	(PFn)	13.0

[a] Measured in 0.05 M NaCl and 0.01 M Tes, pH 7.4; [protein] = 1–2 mg/mL. [b] Not determined.

Interactions of the 25-kDa Fibrin-Binding Fragment. The 25-kDa fragment (Figure 2D, lane 2) isolated as the non heparin binding fraction of the 45/35/25-kDa mixture (Figure 2C, lane 1 and 2D, lane 1) interacted only with native PFn and the 190-kDa fragment, but not with the 29-, 50-, 72-, 120-, or 150-kDa fragments (data not shown), suggesting affinity for the region of the polypeptide chain carboxyl terminal to the Hep II domain. The major differences between the 190- and 150-kDa fragments are the presence of the 50- and the 25-kDa Fib II domain in the 190-kDa fragment. Since the 50-kDa fragment is inactive in self-association, we therefore conclude that the 25-kDa Fib II domain participates in self-association.

Sedimentation Velocity Studies. The corrected $S_{20,w}$ values of the 50-, 72-, 120-, and 150-kDa fragments were consistent with these fragments being monomeric and not associating or aggregating (Table I). However, note the abrupt increase in $S_{20,w}$ value in going from the 150-kDa fragment to the 170- and 190-kDa fragments. These values are consistent with the 170- and 190-kDa fragments being dimeric (Erickson &

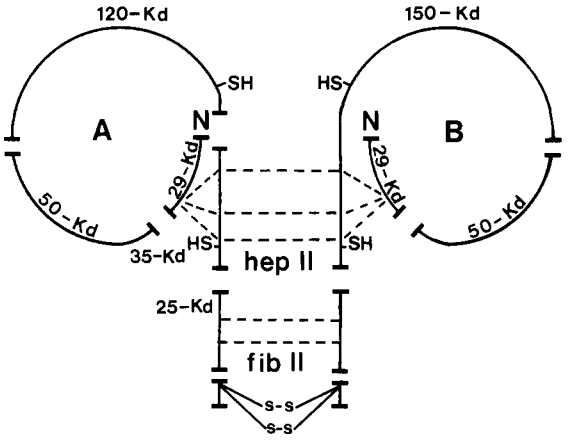

FIGURE 8: Model depicting interactions between PFn domains. The interactions shown here are based on the following observations: (1) The 29-kDa fragment binds the 150- and 190-kDa fragments that contain the Hep II domains but not the 120-kDa fragment that lacks the Hep II domain. (2) The 29-kDa fragment does not bind the 50-kDa fragment, itself, or the 72-kDa fragment. (3) The 35-kDa fragment, containing the Hep II domains, binds not only the amino-terminal 29- and 72-kDa fragments but also the 150- and 190-kDa fragments but not the 120-kDa fragment. (4) The 25-kDa fragment bound native PFn and the 190-kDa fragment but not the 150-, 72-, 50-, or 29-kDa fragments, perhaps due to the absence of Fib II domain.

Carrell, 1983; Robinson & Hermans, 1984; Erickson, Homandberg, & Mosesson, unpublished experiments) and support the conclusion that the Fib II carboxyl-terminal domains contribute to the self-association of PFn.

Discussion

One major assumption of this type of study is that interaction between the fibronectin fragments can be used to derive a model of folding of the native molecule. These interactions likely occur to the same extent between native molecules, since the amino-terminal to carboxyl-terminal and carboxyl-terminal to carboxyl-terminal interactions have been suggested by sedimentation velocity experiments (Robinson & Hermans, 1984; Erickson, Homandberg, and Mosesson, unpublished experiments). Our data support the existence of two types of interaction, an amino-terminal to carboxyl-terminal interaction (foldover interaction) as well as a carboxyl- to carboxyl-terminal interaction (monomer–monomer interaction) as shown in Figure 8. This is a simple unimolecular model; other more complex intermolecular of multimolecular models are possible. For example, the 29-kDa segment of one subunit may bind the Hep II region of the other subunit, thereby imparting intersubunit interactions. In fact, the 190-kDa noncovalently associated dimeric structure is less stable than the noncovalently associated partially reduced fibronectin (Erickson, Homandberg, & Mosesson, unpublished experiments), suggesting that the 29-kDa segment imparts stability to the dimeric structure rather than to only the foldover interaction within a single subunit. Additionally, the 29-kDa segment of one molecule may interact with the Hep II region of a subunit of any other molecule, thereby allowing multimolecular fibril formation of polymerization. This work does not clarify whether or not the interactions between the 29-kDa and Hep-II regions occur exclusively only between the same subunit or between different subunits or between different molecules.

Note in the model (Figure 8) that the 29-kDa segment binds the Hep II region of the 150-kDa segment but cannot bind the smaller 120-kDa fragment since the Hep II region is missing. This result is consistent with the observation that a 160-kDa chymotryptic fragment binds fibronectin, while a 140-kDa fragment does not (Ehrismann et al., 1981, 1982). However, we conclude that our 120-kDa fragment cannot self-associate because it is missing the 35-kDa Hep II domain.

The interactions between the Hep II domains of both subunits may be partially responsible for the noncovalent interactions between partially reduced monomers and between thrombin-generated 190-kDa fragments that behave as dimers (Erickson & Carrell, 1983; Robinson & Hermans, 1984; Hermans, 1985; Erickson, Homandberg, and Mosesson, unpublished experiments).

Our sedimentation velocity constant of 12 reported here for the 190-kDa fragment as well as the work above clearly shows that the 190-kDa fragment can be dimeric under certain conditions. However, other work (Bushuev et al., 1985) suggests a much smaller $S_{20,w}$ value of 6.8 for a 180-kDa fragment prepared by trypsin digestion. This fragment may differ from our 170/190-kD fragment, since tryptic proteolysis is known to remove a 31-kDa carboxyl-terminal fragment, perhaps the Fib II fragment, as well as the amino-terminal 29-kDa fragment (Gold et al., 1983). We have shown here that our 190-kDa fragment that contains the Hep II and Fib II domains and two SH groups has an $S_{20,w}$ value of 12.

Since our 150-kDa fragments do not form dimers, we conclude that the additional presence of the 25-kDa fibrin binding segment is necessary for stable association between monomers. Indeed, this is consistent with the observation that the 25-kDa fragment bound only to native PFn or the 190-kDa segment, which can be dimeric under certain conditions. The observation that the 150-kDa fragment, which is missing the Fib II domains, did not bind the 25-kDa segments suggests that there is no interaction between the Hep II and Fib II domains in the intact fibronectin molecule and that the 25-kDa fragment, like the 35-kDa Hep II fragment, binds its like counterpart in the other subunit. Therefore, in the folded fibronectin molecule, the 29-kDa segment perhaps does not extend to and interact with the Fib II domain. It seems likely that the regions responsible for noncovalent interaction should be very close to the interchain disulfides, since this would allow pairing of the nascent polypeptide chains during biosynthesis. Our work also shows that the isolated gelatin-binding domain of the 50-kDa fragment does not bind fibronectin under our conditions and the sedimentation velocity values also show that amino to amino-terminal interactions, for example aggregation of the 72-kDa fragments, does not occur.

The assignment of the Hep II domain to the 150- and 190-kDa fragments, but not to the 120-kDa fragment, is based on the affinity for heparin and the SH content. One cysteinyl residue is located about 30 kDa from the carboxyl terminus within the Hep II domain, while another sulfhydryl is about 70 kDa from the carboxyl terminus (Wagner & Hynes, 1980; Sekiguchi et al., 1981). Our 120-kDa fragment did not bind heparin under our conditions although it should have retained the weak heparin-binding Hep III domain and contained 0.8 SH group. Therefore, the SH group contained within the 120-kDa fragment is likely the SH described as being 70 kDa from the carboxyl terminus (Wagner & Hynes, 1980; Sekiguchi et al., 1981). The 150- and 190-kDa fragments both bound heparin and contained 1.6 SH groups. Therefore, the 150-kDa fragment differs from the 120-kDa fragment in that the 150-kDa fragment contains the 35-kDa Hep II domain as well as the SH that has been described as being 30 kDa from the carboxyl terminus (Wagner & Hynes, 1980; Sekiguchi et al., 1980). The 190-kDa fragment on the other hand also has the Fib II domain that the 120- and 150-kDa fragments lack. The isolated 35-kDa fragment contained the Hep II domain since it bound heparin and contained 0.8 SH/mol

of protein, the SH that should be 30 kDa from the carboxyl terminus. The 35-kDa fragment is likely released from the A subunit on formation of a 120-kDa fragment, while the 45-kDa fragment may be released from the B subunit at a later stage in the digestion, resulting in a 120-kDa fragment.

These interactins between fragments should make it difficult to isolate homogeneous fragments. We did observe that our 190-kDa preparations were contaminated with 29-kDa fragment after gel filtration in the presence of TBS. In work submitted elsewhere, evidence is presented for the self-association reaction being mainly electrostatic in nature and inhibited by an ionic strength above about 0.6 M (Homandberg, submitted for publication). Consistent with this, we find that when salt concentrations of above 0.6 M NaCl were used during gel filtration, contamination of the 190-kDa population with the 29-kDa fragment was minimized. Also, it was reported that a 27-kDa tryptic fragment of fibronectin, the 29-kDa fragment studied here, was retarded by gelatin-bound 160–180-kDa fragments (Mosher et al., 1980) totally consistent with the interactions shown here.

Another consideration is that the 25-, 35-, and 45-kDa fragments should dimerize and, assuming the interaction is monovalent, should have prevented us from demonstrating interaction with other fragments, unless these interactions are multivalent. We did observe aggregation of these fragments on HPLC gel filtration. The 35-kDa aggregates were often observed as three peaks on HPLC gel filtration of sizes of 35, 80, and 120 kDa, suggesting multivalent interactions. Upon treatment with urea and KBr, this aggregation was reversed. Preliminary work with chemically cross-linked PFn suggests that PFn, with the amino-terminal segment cross-linked to the rest of the structure, can still bind added 29-kDa fragment, an observation also consistent with multivalency of self-association (Homandberg et al., manuscript in preparation).

Others have reported that cathepsin D releases fragments of 23–66 kDa from the carboxyl terminus of fibronectin (Smith & Furcht, 1982). Rogers et al. (1985) isolated 35-, 43-, and 66-kDa fragments from cathepsin D digests, all of which tightly bound heparin. The 43-kDa fragment was presumed to be a breakdown product of the 66-kDa fragment, while the 33- and 66-kDa fragments were thought to originate from the slightly larger A subunit and the B subunit, respectively (Hayashi & Yamada, 1983). Our 45- and 35-kDa fragments are likely the 43- and 33-kDa fragments identified by Rogers et al. (1985). Our 25-kDa fragment may arise by proteolysis of the 66-kDa fragment. Click and Balian (1985) recently presented a model for cathepsin D fragmentation of fibronectin, in which they suggested that a carboxyl-terminal segment of 38 kDa is linked through the carboxyl-terminal interchain disulfides to a 61-kDa fragment from the other subunit. It is possible, therefore, that more extensive degradation by cathepsin D may allow cleavage near the interchain disulfides and release of a fragment of less than 60 kDa and one of less than 38 kDa, which would be similar in size to those of Rogers et al. (1985) and our fragments, characterized here. Our Fib II fragment of 25 kDa would have to be derived from this 38-kDa fragment by further attack by cathepsin D within 10 kDa of the carboxyl terminus in order to make all models consistent.

Our fragments are similar in size to those that have been produced by using other proteases. Hayashi and Yamada (1983) isolated tryptic 38- and 24-kDa Hep II type fragments and 34-kDa Fib II domain fragments. Gold et al. (1983) isolated 36- and 29-kDa subtilisin-generated Hep II type fragments that began about 177 kDa from the amino terminus of the fibronectin molecule. Recently, Zardi et al. (1985) isolated 29- and 38-kDa Hep II fragments as well as a 20-kDa Fib II fragment and suggested that each Hep II fragment arose from a different domain. Our work was not designed to determine the relative subunit origin of the 35- and 45-kDa fragments or, for that matter, the 120- and 150-kDa fragments.

The domains implicated here in self-association are also involved in interactions with cells. For example, the incorporation of fibronectin into fibroblast cell layers may involve disulfide-bonded multimerization of the disulfide-rich amino-terminal third of the molecule (McKeown-Longo & Mosher, 1984). The amino-terminal 70-kDa fragments inhibit binding of fibronectin to the fibroblast cell surface (McKeown-Longo & Mosher, 1985). Incorporation of fibronectin into smooth muscle extracellular matrix in vitro may also involve the amino- and carboxyl-terminal regions (Millis et al., 1985). Further, the carboxyl-terminal heparin-binding region has been shown to supply at least one of the sites involved in the fibronectin cell-mediated attachment and neurite extension (Rogers et al., 1985).

Since these interactions with cells involve the domains that are self-associating, these self-association reactions may have to be broken to allow these cellular interactions. The fact that interaction of fibronectin with monocytes requires prior binding of fibronectin to gelatin or to a matrix (Bevilacqua et al., 1981) may suggest that binding of gelatin disrupts the amino to carboxyl interaction and exposes a monocyte-binding domain. The foldover interactions may explain the inaccessibilities of certain domains in native fibronectin that are fully expressed in fragments of fibronectin. For example, fibronectin fragments have the following properties that are not expressed in native fibronectin: The 29-kDa fragment and the 35-kDa [40 kDa in Homandberg et al. (1985c)] fragments described here are potent inhibitors of endothelial cell growth in vitro, while native fibronectin is not (Homandberg et al., 1985c), a 24-kDa heparin-binding fragment has affinities toward glycosaminoglycans not expressed by native fibronectin (Sekiguchi et al., 1983); a 30-kDa gelatin-binding fragment has a tumor-enhancing activity while native fibronectin does not (DePetro et al., 1981), cathepsin D digests initiate DNA synthesis in serum-derived quiescent cultures of normal hamster fibroblasts, while native fibronectin does not (Humphries & Ayad, 1983), thrombin generated 200-kDa fragments of fibronectin augment the opsonin-independent phagocytosis in human monocyte monolayers (Czop et al., 1981), and large fibronectin fragments have more chemotactic activity for peripheral blood monocytes than does fibronectin (Norris et al., 1982). It will be of interest to determine what ligands, if any, affect the self-association properties of fibronectin and thus some of these cellular interactions.

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