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STUDIES ON THE STRUCTURE OF HUMAN FIBRONECTIN

A thesis submitted to the
Faculty of The Rockefeller University
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

by

Martha B. Furie, B.S.

1980

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KEY TO ABBREVIATIONS

Abbreviation	Meaning
dansyl	5-dimethylaminonaphthalene-1-sulfonyl
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
<Glu	pyroglutamic acid
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kd	kilodalton
PBS	phosphate-buffered saline (0.137 M NaCl, 8.8 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.4)
$PhCH_2SO_2F$	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate

SUMMARY

Plasma fibronectin is a glycoprotein that consists of two polypeptide chains of approximately 250,000 and 245,000 daltons, joined by disulfide bonds located near one end of the molecule. Fibronectin was isolated from human plasma by gelatin-agarose affinity chromatography and DEAE-cellulose ion exchange chromatography. The two subunit chains were found to be similar or identical with respect to primary structure, using a one-dimensional peptide mapping technique.

Purified plasma fibronectin was cleaved with human α-thrombin, and three major fragments were recovered: two large ones with molecular weights of approximately 230,000 and 235,000, and a small one with a molecular weight of 29,000. All three fragments appeared to consist of single polypeptide chains. The fragments were separated on a column of gelatin-agarose. The smaller piece was not retained, whereas the larger fragments and remaining undigested molecules were bound and could be eluted with 2 M guanidine HCl. NH_2-terminal analysis was performed on intact plasma fibronectin and on the fragments using the dansyl (5-dimethylaminonaphthalene-1-sulfonyl) chloride technique. No NH_2-terminal residue was detected in either the intact molecule or the small fragment, whereas analysis of the large fragments yielded dansyl-alanine. Treatment of the intact molecule and the small fragment with L-pyroglutamyl-peptide hydrolase resulted in the appearance of NH_2-terminal alanine. These results were confirmed by automated amino acid sequence analysis, which demonstrated

that the intact molecule and the 29,000 dalton fragment contained the same NH_2-terminal sequence, <Glu-Ala-Glx-Glx-Met-Val-. Analysis of the large fragments gave the sequence Ala-Ala-Val-Tyr-. These studies indicate that the small fragment is located at the NH_2-terminus of intact plasma fibronectin, whereas the large fragments constitute the carboxyl portion of the molecule. Analysis of fibronectin isolated from the conditioned medium of human embryonic fibroblast cultures yielded similar results.

A 72,000 dalton, gelatin-binding polypeptide was isolated from cathepsin D digests of plasma fibronectin. This fragment was cleaved further with thrombin to produce a 43,000 dalton fragment which retained the ability to bind to gelatin and a 29,000 dalton fragment which did not. NH_2-terminal analysis of the 72,000 dalton and 29,000 dalton fragments by the dansyl chloride method before and after treatment with L-pyroglutamyl-peptide hydrolase indicated that they shared the same NH_2-terminal sequence, <Glu-Ala-, as intact fibronectin. Automated amino acid sequence analysis of the 72,000 dalton piece confirmed that the first six NH_2-terminal residues of this fragment were identical with those of the intact molecule. The 43,000 dalton fragment contained an NH_2-terminal alanine residue, as monitored by dansylation. These findings indicate that the 43,000 dalton fragment, which contains one or more gelatin-binding sites of plasma fibronectin, is located adjacent to the 29,000 dalton NH_2-terminal region. Both the 29,000 dalton and 43,000 dalton fragments are enriched in cysteine and half-cystine, as demonstrated by amino acid analysis.

The results of this thesis research can be combined with data of other investigators to locate several sites of biological interest within the fibronectin molecule, including binding sites for collagen, fibrinogen, heparin, and cell surfaces.

INTRODUCTION

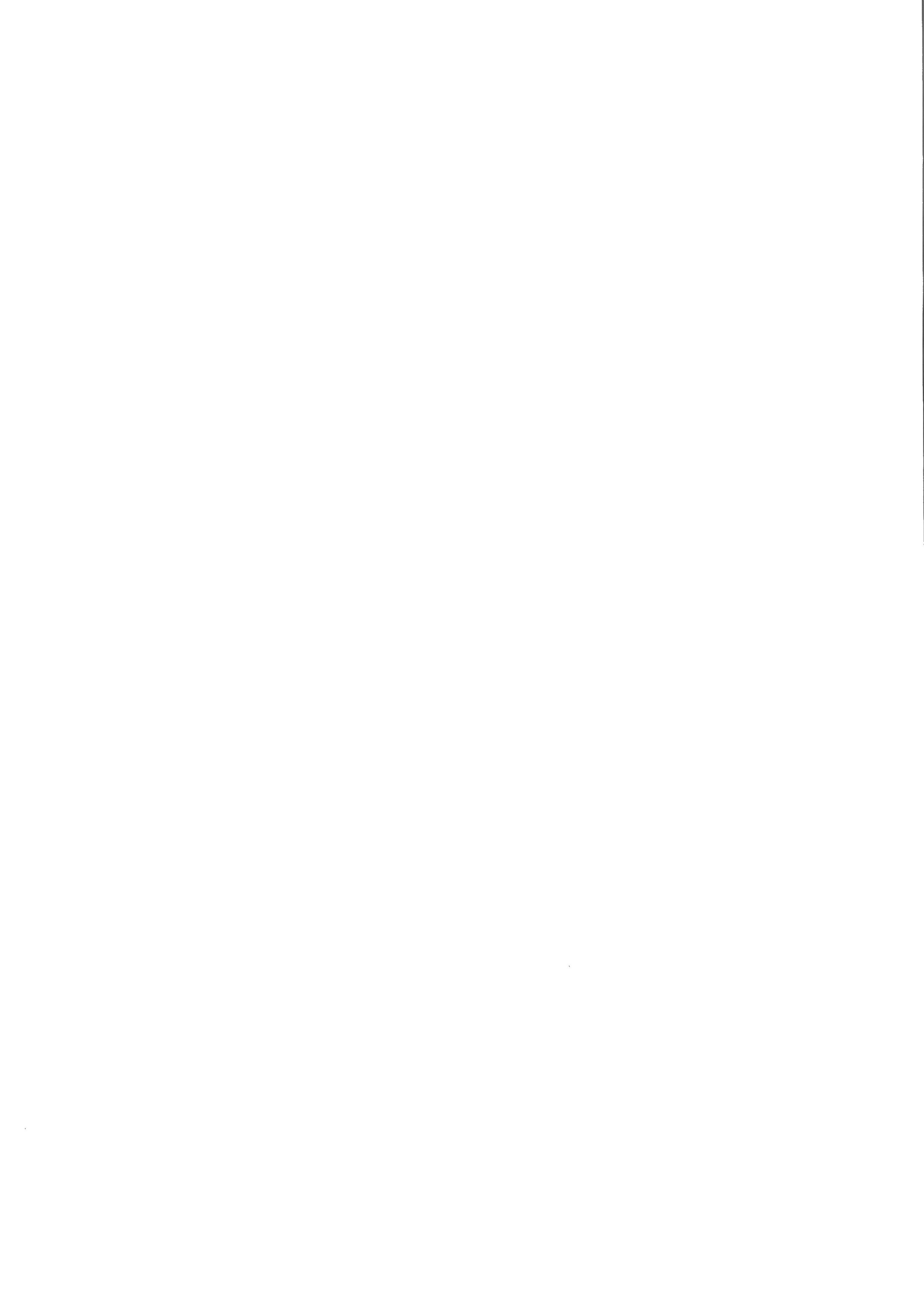
Discovery of fibronectin - Fibronectin is a term that denotes at least two closely related glycoproteins found in the tissues of vertebrates. One form is insoluble and is associated with tissue stroma; the other form is soluble and is found in blood plasma. Fibronectin first became an object of intensive study around 1973, when several laboratories, using immunological or radiochemical techniques to specifically label proteins on outer cell surfaces, discovered a major, high molecular weight glycoprotein which was present on normal fibroblasts but absent on their transformed counterparts (Gahmberg and Hakomori, 1973; Hynes, 1973; Hogg, 1974; Stone *et al.*, 1974; Vaheri and Ruoslahti, 1974). An immunologically indistinguishable protein was detected in blood plasma (Ruoslahti *et al.*, 1973; Ruoslahti and Vaheri, 1974), and it was soon realized that this protein had been known to hematologists for nearly thirty years as "cold-insoluble globulin" (Morrison *et al.*, 1948; Ruoslahti and Vaheri, 1975).

It is only recently that the term "fibronectin" has gained widespread acceptance; both plasma and cellular forms of the molecule have been referred to previously by a number of different names. The cellular form has been called large, external, transformation-sensitive (LETS) protein (Hynes and Bye, 1974), cell surface protein (CSP) (Yamada and Weston, 1974), and fibroblast surface antigen (SFA) (Ruoslahti *et al.*, 1973). The plasma form has been known as cell adhesion factor (CAF) (Pearlstein, 1976), collagen-cell attachment protein (c-CAP) (Klebe *et al.*,

1977), opsonic protein (Allen *et al.*, 1973), and as mentioned, cold-insoluble globulin (CIg) (Morrison *et al.*, 1948).

The literature on fibronectin is vast, and this introduction is intended only to provide a background for discussion of the thesis research. For more comprehensive summaries of the structure and function of fibronectin, the reader is referred to several recent review articles (Vaheri and Mosher, 1978; Yamada and Olden, 1978; Mosher, 1980).

Distribution - Fibronectin is found *in vivo* distributed throughout the body tissues, and it is synthesized and secreted by a number of cell types *in vitro* as well. Immunofluorescent studies of normal adult human tissues show that fibronectin is a component of most basement membranes, including those which underlie the vascular endothelium (Stenman and Vaheri, 1978). It is found as a coating around smooth muscle cells and in the endomysium of striated muscle fibers, as thin strands in loose connective tissue, and as networks and fibrils in the stroma of lymphatic tissue (Stenman and Vaheri, 1978). Fibronectin is present in body fluids as well, including blood plasma (Ruoslahti *et al.*, 1973; Ruoslahti and Vaheri, 1974), amniotic fluid (Crouch *et al.*, 1978), and cerebrospinal fluid (Kuusela *et al.*, 1978), and it is associated with blood platelets (Bensusan *et al.*, 1978; Zucker *et al.*, 1979). In the rat embryo, fibronectin can be detected within the inner cell mass of the blastocyst as early as the third day of development (Zetter and Martin, 1978). In developing chicken embryos, fibronectin is present



in primitive, mesenchymal tissues, but is lost as these differentiate into more specialized structures (Linder et al., 1975).

Fibronectin is the major surface glycoprotein of early-passage cultured fibroblasts and constitutes one to three percent of the total protein of these cells (Yamada et al., 1977b). It is also synthesized by cultured glial cells (Vaheri et al., 1976), endothelial cells (Birdwell et al., 1978; Jaffe and Mosher, 1978), and some other epithelial cell types (L. B. Chen et al., 1977a; Crouch et al., 1978; Quaroni et al., 1978). As fibroblasts attach to a surface and begin to spread, fibronectin is first detected as spots at the cell-substrate interface; at later times, it becomes organized into fibrils (Grinnell and Feld, 1979). As cultures become densely populated, extensive three-dimensional pericellular and intercellular lattices of fibronectin are formed (Bornstein and Ash, 1977; L.B. Chen et al., 1978; Vaheri et al., 1978); these are only loosely associated with the cell membrane (Graham et al., 1975; L.B. Chen et al., 1978). In cultures of other cell types, the pattern of distribution of extracellular fibronectin may vary (L.B. Chen et al., 1977a; Mosher et al., 1977; Birdwell et al., 1978; Jaffe and Mosher, 1978; Quaroni et al., 1978). Fibronectin also occurs in a nonfibrillar, intracellular form (Yamada, 1978) and in a soluble form which is secreted into the culture medium (Vaheri et al., 1976; Mosher et al., 1977; Olden and Yamada, 1977).

Structural features - Both plasma and cellular fibronectins are dimers, composed of polypeptide subunits of approximately 220,000 daltons (Mosesson et al., 1975; Hynes and Destree, 1977; Keski-Oja

et al., 1977). Proteolytic digestion of these proteins suggests that the interchain disulfide bonds which join the monomers lie within a 10,000 to 30,000 dalton region located at one end of the molecule (A.B. Chen *et al.*, 1977; Jilek and Hörmann, 1977a; Wagner and Hynes, 1979). Fibronectin in the extracellular matrix is also found as part of high molecular weight complexes (Hynes and Destree, 1977; Keski-Oja *et al.*, 1977), the components of which have not yet been fully characterized. The NH_2-termini of bovine and human plasma fibronectin are blocked with pyroglutamic acid; treatment with L-pyroglutamyl-peptide hydrolase results in the appearance of NH_2-terminal alanine (Mosesson *et al.*, 1975; Iwanaga *et al.*, 1978). Fibronectin isolated from chick cells also has a blocked NH_2-terminus; however, attempts to remove the blocking residue with L-pyroglutamyl-peptide hydrolase were unsuccessful (Yamada *et al.*, 1977a). Amino acid compositions of cellular and plasma fibronectins isolated from several different species are very similar to one another and show no unusual features (Vuento *et al.*, 1977; Yamada *et al.*, 1977a; Carter *et al.*, 1978; Iwanaga *et al.*, 1978). Antisera to human plasma fibronectin cross-react with plasma and cellular fibronectins from several other mammalian species and from chicken (Kuusela *et al.*, 1976); this result suggests that fibronectin has been highly conserved during evolution.

Cellular and plasma fibronectins are both glycoproteins with similar carbohydrate content (four to five percent) and composition (Vuento *et al.*, 1977). Both forms contain one mole of cysteine per subunit chain (Wagner and Hynes, 1979), and it has been speculated that

these residues may be important in the formation of the high molecular weight, fibronectin-containing complexes that are found in extracellular matrices (Wagner and Hynes, 1979). Cellular fibronectin is both sulfated (Dunham and Hynes, 1978) and phosphorylated (Teng and Rifkin, 1979). Fibronectin secreted by normal chicken embryo fibroblasts contains approximately one mole of phosphate per subunit chain, whereas fibronectin secreted by transformed chicken fibroblasts contains only about one-half of this amount (Teng and Rifkin, 1979).

The physical characteristics of fibronectins isolated from human plasma (Alexander et al., 1979) and from surfaces of chicken fibroblasts (Alexander et al., 1978) have been examined by velocity centrifugation, circular dichroism, and fluorescence spectroscopy under various conditions. Both proteins appear to have a similar, asymmetric tertiary structure, consisting of organized domains connected by random polypeptide chains. The molecules thus have high frictional ratios and considerable flexibility.

Although the studies summarized above indicate that plasma and cellular fibronectins are extremely similar, some differences have been noted. Fibronectin isolated from cell surfaces is considerably less soluble at physiological ionic strength and pH than the plasma form (Yamada and Kennedy, 1979) and, as will be discussed, is more active in certain biological assay systems (Yamada and Kennedy, 1979). Several groups report that fibronectin isolated from cell surfaces and fibronectin secreted into culture media have subunits which migrate identically on SDS-polyacrylamide gels; however, their subunits have a slightly larger apparent molecular weight than those of plasma fibronectin isolated from the same species (Crouch et al., 1978; Quaroni et al., 1978; Yamada and

Kennedy, 1979). Other laboratories report that subunits of plasma and cellular fibronectins have the same mobility (Vuento *et al.*, 1977; Jaffe and Mosher, 1978). Clearly, further work is needed to resolve these discrepancies.

The physical or chemical bases for the observed differences between cellular and plasma fibronectins are uncertain. Some dissimilarities in glycosylation have been reported. For example, human plasma fibronectin contains slightly more sialic acid than fibronectin secreted by human fibroblasts does (Vuento *et al.*, 1977). Detailed structural analyses of the carbohydrate chains of hamster cellular fibronectin (Carter and Hakomori, 1979; Fukuda and Hakomori, 1979) and bovine (Takasaki *et al.*, 1979) and human (Wrann, 1978) plasma fibronectins reveal some differences; however, direct comparisons of cellular and plasma fibronectins derived from a single species have not been made. It has been suggested that fibronectin isolated from cell surfaces is less soluble and more active in some assays than plasma fibronectin because it exists largely in a multimeric, multivalent state (Mosher, 1980). Further comparisons of plasma fibronectin, fibronectin secreted by cells, and fibronectin extracted from cell surfaces, with special regard to the degree of aggregation of each preparation, will be useful in testing this hypothesis. Comparisons of the fibronectins produced by different cell types may also aid in identifying the source or sources of plasma fibronectin, which are presently unknown.

Biological activities - Although the primary biological roles of

fibronectin are unclear, both the plasma and cell surface molecules are involved in a number of processes where they function in an adhesive or binding capacity. Plasma fibronectin mediates the adhesion and spreading of some cell types on plastic tissue culture dishes (Grinnell and Hays, 1978; Hughes et al., 1979) and on surfaces which have been coated with dried collagen films (Klebe, 1974; Pearlstein, 1976). Plasma fibronectin markedly enhances adhesion of baby hamster kidney cells to denatured collagen substrates (Pearlstein, 1976; Grinnell and Minter, 1978); however, it is not required for their attachment and spreading on native collagen gels (Grinnell and Minter, 1978). Plasma fibronectin also promotes the spreading of platelets on collagen surfaces, although it is not necessary for their initial attachment (Hynes et al., 1978).

In addition to its role as a cell adhesion factor, plasma fibronectin has been demonstrated to be identical with α_2-surface binding glycoprotein (Blumenstock et al., 1978b), a serum component which binds to gelatinized lipid emulsions and potentiates their clearance from the blood by the reticuloendothelial system (Allen et al., 1973; Blumenstock et al., 1978a). Some septic surgical and trauma patients have impaired reticuloendothelial system function and abnormally low levels of circulating plasma fibronectin (Saba et al., 1978). In one study, infusion of three such patients with fibronectin-containing cryoprecipitate was associated with improved pulmonary function and lowering of elevated body temperature (Saba et al., 1978). Furthermore, fibronectin promotes binding of fibrin monomer to macrophages (Jilek and Hörmann, 1978b). Macrophages also specifically

bind denatured, but not native, collagen; uptake is mediated by a high molecular weight, gelatin-binding serum component which is probably identical with fibronectin (Hopper *et al.*, 1976). These results indicate that plasma fibronectin may function *in vivo* as an opsonic protein which promotes removal of debris from the circulation following tissue injury.

Like plasma fibronectin, cellular fibronectin mediates attachment of some cell types to denatured collagen films (Pearlstein, 1976). Some cell strains do not require addition of fibronectin for substrate adhesion; however, studies indicate that endogenous, secreted fibronectin plays a role in their attachment and spreading (Grinnell and Feld, 1979). Fibronectin extracted from chicken embryo fibroblasts agglutinates formalinized sheep erythrocytes (Yamada *et al.*, 1975) and promotes aggregation of living mammalian and chicken cells in suspension (Yamada *et al.*, 1978). Fibronectin extracted from cell layers or secreted into the medium enhances the motility of both normal and transformed cells (Ali and Hynes, 1978), possibly by providing cell-substrate anchorage sites.

As mentioned, fibronectin is present in greatly reduced amounts on the surfaces of many transformed cells (Gahmberg and Hakomori, 1973; Hynes, 1973; Hogg, 1974; Stone *et al.*, 1974; Vaheri and Ruoslahti, 1974). The addition of fibronectin extracted from monolayers of normal cells to such transformed cultures causes them to assume a more normal morphology. The cells adhere more tightly to the substrate, become more spread out and elongated, and become more aligned with one another (Yamada *et al.*, 1976b; Ali *et al.*, 1977). Exogenous cell surface fibronectin also decreases the number of plasma membrane microvilli, blebs, and ruffles

(Yamada et al., 1976a) and restores ordered actin microfilament bundles (Ali et al., 1977; Willingham et al., 1977). Added cellular fibronectin does not affect a number of other properties of transformed cells, including rate of division and saturation density (Yamada et al., 1976b), rates of sugar (Ali et al., 1977; Willingham et al., 1977) and amino acid (Willingham et al., 1977) transport, and levels of cAMP (Yamada et al., 1976b). It is possible, then, that all of the effects that exogenously supplied cell surface fibronectin exerts on transformed cells arise from its ability to increase adhesion between cell and substrate.

The various forms of fibronectin differ in activity when tested in some of these in vitro systems. Fibronectins extracted from cell layers, secreted by cells, and isolated from plasma are all equally capable of mediating cell attachment and spreading on collagen (Pearlstein, 1976; Pena and Hughes, 1978; Yamada and Kennedy, 1979). However, plasma fibronectin is one hundred fifty-fold less active than fibronectin extracted from cell layers in hemagglutination assays, and fifty-fold less active in restoring a more normal appearance to transformed cells (Yamada and Kennedy, 1979). As has been discussed, these differences possibly reflect the proportion of molecules in each preparation which are in an aggregated, multivalent state (Mosher, 1980). That the morphology of transformed cells is unaffected by their co-cultivation with normal cells (L.B. Chen et al., 1977b) might be explained similarly; that is, the fibronectin secreted by the normal cells may not be in the proper multimeric state to effectively reconstitute a matrix. However, there is at least one report that fibronectin isolated

from conditioned media is as effective in inducing changes in transformed cells as fibronectin extracted from cell layers (Ali _et al._, 1977). Studies that relate the activites of various fibronectin preparations to their physical state will be required to clarify this point.

Interactions with other macromolecules - Presumably, plasma and cellular fibronectins function _in vivo_ as bridging, adhesive elements by binding to other macromolecules, and indeed, a number of interactions of potential biological interest have been demonstrated _in vitro_. Both forms of fibronectin have strong affinities for collagen when tested in a variety of assay systems (Engvall and Ruoslahti, 1977; Dessau _et al._, 1978; Engvall _et al._, 1978; Jilek and Hörmann, 1978a). In most of these systems, binding of fibronectin to denatured collagen (gelatin) is considerably stronger than to the native molecule (Engvall and Ruoslahti, 1977; Engvall _et al._, 1978; Jilek and Hörmann, 1978a); differences in affinities of one hundred-fold and more have been reported (Engvall and Ruoslahti, 1977; Engvall _et al._, 1978). Fibronectin interacts with both interstitial (Types I, II, and III) (Engvall and Ruoslahti, 1977; Dessau _et al._, 1978; Engvall _et al._, 1978; Jilek and Hörmann, 1978a) and basement membrane (Types IV and AB) (Dessau _et al._, 1978; Engvall _et al._, 1978) collagens. In some assays, differences in affinities of fibronectin for the various types of native collagen are detected; binding to native Type III collagen is strongest (Engvall _et al._, 1978; Jilek and Hörmann, 1978a).

Fibronectin binds most strongly to cyanogen bromide peptide 7 of the $\alpha 1$ (I) collagen chain (Kleinman _et al._, 1976; Dessau _et al._, 1978) and

to homologous peptides derived from the α1 (II) chain (Kleinman et al., 1976; Dessau et al., 1978) and the α2 chain (Dessau et al., 1978). Further digestion of cyanogen bromide peptide 7 with proteases has more precisely located the binding site of fibronectin within residues 757 to 791 of the α1 (I) chain (Kleinman et al., 1978), a region that also contains the peptide bond cleaved by animal collagenases (Gross et al., 1974). However, fibronectin also interacts with other collagen fragments (Kleinman et al., 1976; Dessau et al., 1978; Engvall et al., 1978), suggesting that collagen may contain several binding sites for fibronectin.

In addition to directly binding to collagen, plasma fibronectin can be covalently cross-linked to Types I and III collagens by plasma transglutaminase (coagulation factor $XIII_a$) (Mosher et al., 1979). Cross-linking to intact collagen does not occur at temperatures lower than 37°C, indicating that some local denaturation may be required to expose active residues on the collagen molecule. As has been discussed, fibronectin also mediates attachment of cells to denatured collagen (Klebe, 1974; Pearlstein, 1976; Hynes et al., 1978), increases opsonization of gelatinized particles (Allen et al., 1973; Blumenstock et al., 1978), and may promote binding of denatured collagen to macrophages (Hopper et al., 1976). Furthermore, there is an extensive codistribution of fibronectin and collagen in the extracellular matrices of normal fibroblasts, and both proteins are lost upon transformation (Vaheri et al., 1978).

It has long been known that plasma fibronectin has a tendency to coprecipitate with fibrin and fibrinogen in the cold (Morrison et al., 1948). Plasma fibronectin and fibrinogen are the major components of plasma cryoprecipitate (Mosesson and Umfleet, 1970), and fibronectin,

fibrinogen, and fibrin constitute the pathologic plasma precipitates termed "cryofibrinogens" (Stathakis _et al._, 1978). Plasma fibronectin is included in fibrin clots, particularly those formed at $0^{o}C$, and is adsorbed to fibrin powder in the cold (Ruoslahti and Vaheri, 1975). It also has an affinity for fibrin-Sepharose at low temperatures (Stemberger and Hörmann, 1976; Stathakis _et al._, 1978), but there are conflicting reports with regard to its affinity for fibrinogen-Sepharose (Ruoslahti and Vaheri, 1975; Stemberger and Hörmann, 1976; Stathakis _et al._, 1978). Binding of fibronectin to fibrin is stronger than binding to fibrinogen (Stemberger and Hörmann, 1976; Stathakis _et al._, 1978). The presence of the carboxyl region of the α-chain of fibrin and fibrinogen is essential for interaction with fibronectin (Stathakis and Mosesson, 1977; Stathakis _et al._, 1978). Plasma fibronectin also enhances binding of fibrin monomer to macrophages (Jilek and Hörmann, 1978b). Although plasma fibronectin can be cross-linked by plasma transglutaminase to the α-chain of fibrin (Mosher, 1975), cellular fibronectin cannot (Keski-Oja _et al._, 1976).

Plasma transglutaminase not only cross-links plasma fibronectin to collagen (Mosher _et al._, 1979) and fibrin (Mosher, 1975), but also, in the presence of reducing agent, to other fibronectin molecules (Mosher, 1975). Plasma fibronectin is also polymerized by a cellular transglutaminase isolated from guinea pig liver (Birckbichler and Patterson, 1978). Experiments using (^{14}C)putrescine show that there is probably one transamidation-sensitive glutaminyl residue per 220,000 dalton plasma fibronectin chain (Jilek and Hörmann, 1977b). Treatment of fibroblast cultures with plasma transglutaminase results in the cross-

linking of cell surface fibronectin to a high molecular weight complex (Keski-Oja *et al.*, 1976). The components of this complex have not yet been analyzed; cell surface fibronectin might be cross-linked to itself or to other matrix proteins.

Interactions of fibronectin with glycosaminoglycans and proteoglycans have also been demonstrated. Addition of the anti-coagulant glycosaminoglycan heparin to plasma results in the formation of a cold-precipitable complex composed primarily of plasma fibronectin, fibrinogen, and heparin, and it has been shown that it is the plasma fibronectin which first associates with heparin (Stathakis and Mosesson, 1977). Presumably, fibrinogen is included in this complex by virtue of its affinity for fibronectin. Heparin also enhances the formation of insoluble complexes of plasma fibronectin and native Type III or denatured Types I and III collagens (Jilek and Hörmann, 1979); hyaluronic acid inhibits this complex formation (Jilek and Hörmann, 1979). Fibronectin secreted by fibroblasts has been coupled to photoactivatable chemical cross-linking agents and added back to cell cultures; following exposure of the cultures to ultraviolet light, complexes of fibronectin and sulfated proteoglycans can be isolated from the cell surface (Perkins *et al.*, 1979).

Immunofluorescent staining of cultured cells indicates that arrays of extracellular fibronectin fibrils and intracellular actin microfilaments often coincide (Hynes and Destree, 1978). Electron microscopic studies suggest that fibronectin fibrils and microfilaments may be connected within dense plaques located just beneath the plasma membrane (Singer, 1979). Furthermore, there is a preliminary report that plasma fibronectin and fibronectin secreted by cells bind to immobilized actin (Keski-Oja

et al., 1979). Fibronectin may also interact with DNA. Fibronectin is present in chromatin prepared from cultured human fibroblasts (Zardi et al., 1979). It has been reported that plasma fibronectin binds to DNA-cellulose (Zardi et al., 1979), but not to DNA-Sepharose (Keski-Oja et al., 1979). However, since fibronectin has not been detected in the nucleus by immunofluorescent staining (Yamada, 1978), the in vivo relevance of its association with DNA is unclear.

Fibronectin-mediated cell attachment to collagen is inhibited by a number of gangliosides isolated from brain, especially GT_1 and GD_{1a} (Kleinman et al., 1979). Oligosaccharide chains isolated from gangliosides retain inhibitory activity, whereas the ceramide portions alone do not (Kleinman et al., 1979). These results suggest that gangliosides or other macromolecules containing similar carbohydrate moieties may represent cellular receptors for fibronectin.

Fibronectin thus interacts with a wide variety of different macromolecules in vitro. These associations could be important in a number of in vivo processes. For example, fibronectin might play a role in the organization of extracellular ground substance and basement membranes, mediate adhesion and migration of cells during embryogenesis and wound repair, aid in formation of platelet-fibrin plugs following vascular injury, and enhance clearance of tissue debris from the blood. Clearly, the possible biological functions of fibronectin merit further study.

Aim of the thesis research - At the time this thesis research was undertaken, relatively little was known about the physical and chemical properties of fibronectin. Therefore, it was decided to focus these studies on the structure of fibronectin rather than on its biological characteristics. It was hoped that a fuller understanding of its structural properties would eventually lead to a more detailed picture of the ways in which fibronectin acts as a bridge between macromolecules and between cells and their environment. One impediment to studying fibronectin structure is the extremely large size of the molecule. The approach taken, therefore, was to cleave the molecule with proteases, to isolate and characterize some of the fragments produced, and finally, to locate the fragments within the intact molecule. In this way, it might be possible to construct a map of the molecule relating structure to some of fibronectin's functions, including binding to other macromolecules. The remainder of this thesis will be largely devoted to showing how such an approach has been used to build a detailed model of the fibronectin molecule.

EXPERIMENTAL PROCEDURES

Materials

Purified human α-thrombin (>2300 NIH units/mg) was a gift from Dr. J. W. Fenton II, New York State Department of Health, Albany, New York. Rabbit skeletal muscle myosin was provided by Dr. Richard Peluso, Veterans Administration Hospital, San Francisco, California. Pepstatin was a gift from Dr. Walter Troll, New York University Medical Center, New York, New York, and was supplied by the United States-Japan Cooperative Cancer Research Program. Fresh-frozen acid citrate dextrose human plasma was obtained from the Greater New York Blood Program. Dansyl chloride (1 g/10 ml of acetone), constant-boiling HCl (Sequenal grade), dithiothreitol, and Schleicher and Schuell micropolyamide sheets with aluminum foil support base were all purchased from Pierce Chemical Co. SDS was a specially pure grade manufactured by BDH Chemicals, LTD, Poole, England, and distributed by Gallard-Schlesinger Chemical Mfg. Corp. Other products were obtained from the following sources: $PhCH_2SO_2F$, benzamidine HCl, and 6-aminocaproic acid, Adrich Chemical Co., Inc.; DEAE-cellulose (DE52) and phosphocellulose (P11), Whatman, Inc.; acrylamide (electrophoresis grade) and N, N'-methylene-*bis*-acrylamide, Eastman Kodak Co.; soybean trypsin inhibitor, bovine serum albumin, and *Staphylococcus aureus* V8 protease, Miles Laboratories, Inc.; Sepharose CL-4B, Sephadex G-150, ovalbumin, ribonuclease, and chymotrypsinogen, Pharmacia Fine Chemicals; β-galactosidase, Worthington Biochemical Corp.; cathepsin D (EC 3.4.23.5), gelatin (Type I: 300 bloom), dansyl-amino acid standards, and hirudin (Grade IV), Sigma Chemical Co.; guanidine HCl (extreme purity), Heico, Inc., Delaware Water Gap, Pennsylvania;

$(NH_4)_2SO_4$ (ultra-pure), Schwarz/Mann; L-pyroglutamyl-peptide hydrolase (EC 3.4.11.8), Boehringer Mannheim Biochemicals; streptokinase-streptodornase (Varidase), Lederle Laboratories; L-pyroglutamyl-L-alanine, Vega-Fox Biochemicals, Tucson, Arizona; Trasylol, FBA Pharmaceuticals, New York, New York; ^{125}I (carrier-free, 17 Ci/mg), New England Nuclear; plastic tissue culture roller bottles (490 cm^2), Corning Glass Works; Eagle's medium and calf serum, Flow Laboratories; fetal calf serum, Reheis Chemical Co.; Fungizone, E. R. Squibb and Sons; and incomplete and complete Freund's adjuvant, Grand Island Biological Co. All other chemicals were of reagent grade.

Methods

Purification of human plasma fibronectin - Four to five units of fresh-frozen, acid citrate dextrose plasma were thawed at 37°C and pooled, and soybean trypsin inhibitor (100 mg/liter) was added. Vitamin K-dependent clotting factors were removed by the addition of $BaCl_2$ (15 g/liter) (Aronson and Ménaché, 1966); the resulting suspension was stirred gently at 0°C for 30 min. The precipitate, consisting of barium citrate and adsorbed protein, was removed by centrifugation at 5000 x *g* for 20 min at 4°C. Excess barium was precipitated from the supernatant by adding $(NH_4)_2SO_4$ (9.5 g/liter), followed by centrifugation as above (A.B. Chen and Mosesson, 1977). $PhCH_2SO_2F$ was added to the plasma to a final concentration of 1 mM. The plasma was passed over a 180 ml column (4.0 x 14 cm) of lysine-Sepharose CL-48 (Deutsch and Mertz, 1970) equilibrated at 4°C with PBS containing soybean trypsin inhibitor (100 mg/liter) and 1 mM $PhCH_2SO_2F$. The rate of flow was approximately 200 ml/h. The effluent was collected into a beaker containing sufficient benzamidine HCl to bring the final concentration to approximately 3 mM.

Plasma fibronectin was removed from the plasminogen-depleted plasma by adsorption to a gelatin-agarose column as described by Engvall and Ruoslahti (1977). The plasma was passed over a 100 ml column (2.5 x 20 cm) of gelatin-Sepharose CL-4B equilibrated with PBS containing 3 mM benzamidine HCl and 1 mM $PhCH_2SO_2F$. The rate of flow was 100 to 150 ml/h, and chromatography was carried out at room temperature. The column was washed with 1 liter of the buffer used

for equilibration, followed by 500 ml of 0.5 M guanidine HCl, 0.05 M Hepes, 3 mM benzamidine HCl, pH 7.5. Plasma fibronectin was eluted with 1.0 M guanidine HCl, 0.05 M Hepes, 3 mM benzamidine HCl, pH 7.5. Fractions of 5 ml were collected, and the concentration of protein in the effluent was monitored by absorption at 280 nm. Protein-containing fractions were pooled and dialyzed against 0.2 M phosphate-Tris, 3 mM benzamidine HCl, pH 7.0, at room temperature.

Plasma fibronectin was further purified by ion-exchange chromatography on DEAE-cellulose according to Mosesson and Umfleet (1970). The dialyzed sample was diluted with three volumes of 3 mM benzamidine HCl in water and applied at a rate of 120 ml/h to a 55 ml column (2.0 x 18 cm) of DEAE-cellulose equilibrated with 0.05 M phosphate-Tris, pH 7.0. Chromatography was carried out at room temperature. The column was washed with approximately 75 ml of 0.05 M phosphate-Tris, pH 7.0, and 200 ml of 0.09 M phosphate-Tris, pH 7.0. Plasma fibronectin was eluted with 0.2 M phosphate-Tris, pH 7.0. Fractions of 5 ml were collected. Material representing the peak of absorbance at 280 nm was pooled and dialyzed against PBS at $4^{o}C$. Any precipitate formed during dialysis was redissolved by warming the plasma fibronectin solution to $37^{o}C$. Aliquots were then frozen in a dry ice-methanol bath and stored at $-70^{o}C$.

Purification of human cellular fibronectin - Fibronectin was isolated from the conditioned medium of human embryonic fibroblasts by a modification of the method of Teng and Rifkin (1979). Tertiary cultures of human embryonic fibroblasts were trypsinized, seeded into

plastic roller bottles (490 cm^2), and grown to confluency at 37°C in Eagle's medium containing 10% fetal calf serum. For preparation of conditioned medium, cultures were maintained in Eagle's medium containing soybean trypsin inhibitor (100 μg/ml), 6-aminocaproic acid (500 μg/ml), Fungizone (1 μg/ml), and 2% calf serum which had been depleted of plasminogen (Deutsch and Mertz, 1970) and fibronectin (Engvall and Rouslahti, 1977) by affinity chromatography. Spent medium was collected every 48 h, centrifuged at 5000 x g and 4°C for 20 min to remove cell debris, and stored at -20°C.

To isolate fibronectin, conditioned media were pooled and made 0.5 M in guanidine HCl, 5 mM in Na_2EDTA, and 3 mM in benzamidine HCl. Gelatin-Sepharose (1 ml of Sepharose/100 ml of medium) was added, and the suspension was gently stirred overnight at 4°C. The Sepharose was allowed to settle, the bulk of the medium was decanted, and the remaining slurry was poured into a glass column. The packed gelatin-Sepharose was washed extensively with 0.05 M Hepes, 5 mM Na_2EDTA, 3 mM benzamidine HCl, pH 7.5, containing 0.5 M guanidine HCl. Fibronectin was subsequently eluted with this same buffer containing 1.5 M guanidine HCl. All chromatographic steps were performed at room temperature. Fractions representing the peak of absorbance at 280 nm were pooled and dialyzed against PBS supplemented with additional NaCl (17.5 g/liter) and made 5 mM in Na_2EDTA and 3 mM in benzamidine HCl. Purified material was stored at 4°C in the presence of 0.02% NaN_3.

SDS-polyacrylamide gel electrophoresis - Polyacrylamide gel electrophoresis in the presence of SDS was performed in a slab gel

apparatus (Studier, 1973), using the discontinuous buffer system described by Laemmli (1970). Separating gels consisted of linear 5 to 16% acrylamide gradients; stacking gels were 3% acrylamide. Protein samples were prepared by heating them at 100°C for 2 min in 0.0625 M Tris-HCl, 10% glycerol, 2% SDS, 0.001% Bromphenol Blue, pH 6.8, with or without the addition of 2-mercaptoethanol to 5%. The following proteins were used as molecular weight standards: myosin (M_r=200,000), β-galactosidase (M_r=130,000), bovine serum albumin (M_r=68,000), ovalbumin (M_r=43,000), chymotrypsinogen (M_r=25,700), and ribonuclease (M_r=13,700). Gels were fixed and stained as described by Fairbanks *et al.* (1971) and, in some cases, were scanned using an Ortec model 4310 densitometer.

Peptide mapping of plasma fibronectin subunits - One-dimensional peptide mapping by limited proteolysis in SDS-polyacrylamide gels was performed according to Cleveland *et al.* (1977). Samples of purified plasma fibronectin were iodinated by the chloramine-T method (McConahey and Dixon, 1966) and electrophoresed on an SDS-polyacrylamide gel in the usual manner. The gel was stained and destained only briefly to avoid acid hydrolysis of the protein. Protein bands were cut from the gel, soaked for 30 min in 0.125 M Tris-HCl, 0.1% SDS, 1 mM Na_2EDTA, pH 6.8, trimmed, and placed at the bottoms of the sample wells of a second SDS-polyacrylamide gel. This gel was also prepared in the normal manner, except that the stacking gel was longer than usual, and Na_2EDTA was added to the polyacrylamide solutions to a final concentration of 1 mM prior to polymerization. The separating gel

consisted of a linear 8 to 18% acrylamide gradient; the stacking gel contained 4% acrylamide. The fibronectin-containing slices were overlaid with 10 µl of 0.125 M Tris-HCl, 0.1% SDS, 1 mM Na_2EDTA, 0.001% Bromphenol Blue, pH 6.8, containing 20% glycerol. Next, 10 µl of this same buffer containing 10% glycerol and an appropriate amount of Staphylococcus aureus V8 protease were added to each well. Electrophoresis was carried out as usual, except that the current was turned off for 30 min when the Bromphenol Blue neared the bottom of the stacking gel. Autoradiography of the fixed, dried gel was performed using DuPont Cronex medical x-ray film.

Digestion of fibronectin with thrombin and separation of fragments - Purified human α-thrombin (75 µg) was added to 10 mg of plasma fibronectin in 2 to 3 ml of PBS. The mixture was incubated for 1 h at 37°C. Thrombin was removed by passing the digest over a 0.5 ml column of phosphocellulose equilibrated with PBS. The digest was then applied to a 7.5 ml column (1.2 cm x 6.5 cm) of gelatin-Sepharose CL-4B equilibrated with PBS, and the column was washed with this same buffer. Both chromatographic steps were performed at room temperature. Fractions of 0.5 to 1.0 ml were collected and their protein content determined by the method of Lowry et al. (1951). A small plasma fibronectin fragment which was generated was not retained by the gelatin-Sepharose and was collected in these fractions. When examined by SDS-polyacrylamide gel electrophoresis, the first several protein-containing fractions were seen to contain a relatively large proportion of high molecular weight contaminant fragments and were discarded. The remainder of the protein

was pooled; solid $(NH_4)_2SO_4$ was then added to 90% saturation at 0^oC. The precipitate was collected by centrifugation at 12,000 x g and 4^oC for 30 min, redissolved in a small amount of PBS, and dialyzed against this buffer at 4^oC. The large proteolytically-derived fragments and remaining intact molecules were eluted from the column with 2.0 M guanidine HCl, 0.05 M Hepes, pH 7.5, and dialyzed against PBS at 4^oC.

Fibronectin isolated from conditioned media of human embryonic fibroblast cultures was prepared for digestion as follows. Purified material was dialyzed against PBS at room temperature; the dialysis buffer was then changed twice at hourly intervals. Purified human α-thrombin was added to the dialyzed fibronectin solution (1:100, w/w), and the mixture was incubated for 1.5 h at 37^oC. The digest was then treated as described above for plasma fibronectin.

Digestion of plasma fibronectin with cathepsin D and separation of fragments - Cleavage of fibronectin with cathepsin D and isolation of a collagen-binding fragment were carried out essentially according to Balian et al. (1979). Fibronectin (8 to 10 mg in 2 ml of PBS) was diluted with 0.1 M sodium formate, 0.2 mM $PhCH_2SO_2F$, pH 3.5, to a final concentration of 200 μg/ml. Cathepsin D (40 μg) was added, and the mixture was incubated at 30^oC for 4 h. The reaction was stopped by the addition of 12 μg of pepstatin, and the solution was brought to neutrality with 1.5 M Tris-HCl, pH 8.8. Digested protein was precipitated by the addition of an equal volume of saturated $(NH_4)_2SO_4$

at $0^{o}C$ and sedimented by centrifugation at 12,000 x g for 20 min at $4^{o}C$. The pellet was resuspended in 1.5 ml of 0.15 M NaCl, 0.05 M Tris-HCl, 3 mM benzamidine HCl, 0.2 mM $PhCH_2SO_2F$, pH 7.5. The suspension was incubated at $37^{o}C$ for 10 to 15 min with occasional shaking; any undissolved protein was then removed by brief sedimentation on a clinical centrifuge at room temperature. The supernatant was applied to a column (1.2 x 95 cm) of Sephadex G-150 equilibrated with 0.15 M NaCl, 0.05 M Tris-HCl, 3 mM benzamidine HCl, 0.2 mM $PhCH_2SO_2F$, pH 7.5, at $4^{o}C$. The column was eluted with this same buffer at a flow rate of approximately 3 ml/h. Two protein-containing peaks were seen as monitored by absorbance at 280 nm; material from the more slowly eluting peak was pooled and stored at $-20^{o}C$.

A 72,000 dalton gelatin-binding fragment was isolated from this material by affinity chromatography. Pooled protein solutions were passed over a column of gelatin-Sepharose equilibrated with 0.15 M NaCl, 0.05 M Tris-HCl, 3 mM benzamidine HCl, 0.2 mM $PhCH_2SO_2F$, pH 7.5, at room temperature. Approximately 0.5 ml of resin was used for every 5 ml of protein solution. The column was then washed extensively with the buffer used for equilibration. Gelatin-binding fragments were eluted with this same buffer containing 2.0 M guanidine HCl. Protein-containing fractions were pooled, dialyzed at $4^{o}C$ against 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5, and stored at $-20^{o}C$.

<u>Digestion of the gelatin-binding fragment with thrombin and separation of fragments</u> - The 72,000 dalton gelatin-binding fragment

(1.0 mg in 1.0 ml of 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.5) was digested further with 40 μg of purified human α-thrombin for 7 h at 37^oC. Thrombin was removed from the digest by passage over a column (0.4 ml) of phosphocellulose equilibrated with PBS at room temperature. The thrombin-depleted digest was applied to a column (1.0 ml) of gelatin-Sepharose equilibrated with PBS at room temperature. The column was washed with PBS, and a 29,000 dalton fragment which was not retained by the gelatin was collected in the effluent. A 43,000 dalton gelatin-binding fragment was then eluted from the column with 2.0 M guanidine HCl in PBS and was dialyzed at 4^oC against an appropriate buffer solution.

Digestion of fibronectin and its fragments with L-pyroglutamyl-peptide hydrolase - Enzymatic removal of pyroglutamic acid was performed essentially according to Podell and Abraham (1978). Native or reduced and alkylated proteins were dialyzed at 4^oC against buffer prepared as by Podell and Abraham (1978), but without dithiothreitol. The buffer was made by bringing 0.1 M Na_2HPO_4 to pH 8.0 with 0.1 M NaH_2PO_4; this solution was then made 10 mM in Na_2EDTA and 5% in glycerol (v/v), and readjusted to pH 8.0 with 1 N NaOH. The dialyzed protein solution was made 5 mM in dithiothreitol, and one-tenth as much lyophilized crude L-pyroglutamyl-peptide hydrolase as protein was added on a weight basis. After an 8 h incubation at room temperature under N_2, another portion of enzyme equal to the first was added, and incubation was continued as before for an additional 16 h. The samples were then made 1% in SDS, heated for 3 min at 100^oC, and dialyzed

against 0.2 M $NaHCO_3$, 1% SDS, pH 8.5, at room temperature in preparation for dansylation. Alternatively, samples were prepared for automated sequence analysis by dialysis against 0.2 M ammonium acetate at 4°C and lyophilization. L-pyroglutamyl-peptide hydrolase activity was quantitated using L-pyroglutamyl-L-alanine as a substrate (Doolittle, 1972).

NH_2-terminal amino acid analysis - A modification of the dansyl chloride method of Weiner et al. (1972) was used to determine the NH_2-terminal amino acid residues of intact fibronectin and the various proteolytically-derived fragments. Protein solutions were mixed with 10% SDS (10:1, v/v), immediately heated at 100°C for 3 min, and then dialyzed against 0.2 M $NaHCO_3$, 1% SDS, pH 8.5, at room temperature. Aliquots (20 to 200 μl) containing 1 to 5 nmol of protein were removed and mixed with an equal volume of dansyl chloride solution (5 mg/ml of acetone) at 37°C for 2 h. Protein was precipitated by addition of ice-cold 20% trichloroacetic acid, sedimented in a clinical centrifuge, and washed once or twice with 200 μl of 1 N HCl. The protein was hydrolyzed in 100 μl of constant-boiling HCl for 4 to 6 h at 108°C in sealed tubes. The hydrolysate was dried under vacuum and extracted twice with 100 μl aliquots of water-saturated ethyl acetate (Gray, 1972). The ethyl acetate extract was dried by heating in an 80°C water bath. Both ethyl acetate extractable and nonextractable residues were dissolved in 5 μl of 50% aqueous pyridine and analyzed by thin-layer chromatography on polyamide sheets (5 x 5 cm) (Woods and Wang, 1967), using the solvents described by Hartley (1970). The chromatographic system clearly resolved all components of a standard mixture containing twenty dansyl-amino acids and dansyl-amide.

Automated amino acid sequence analysis - Reduced and alkylated, lyophilized samples were dissolved in 0.5 to 1.0 ml of heptafluorobutyric acid or 1% SDS and analyzed on a Beckman 890C Sequencer run with a 0.1 M Quadrol program modified from Brauer *et al.* (1975). The phenylthiohydantoin derivatives were identified on a Hewlett-Packard 1084 A liquid chromatograph equipped with either a DuPont Zorbax ODS 4.6 mm column or an Altex Ultrasphere C18 column and a 254 nm detector.

Amino acid analysis - Protein samples were prepared for amino acid analysis by dialysis against 0.2 M acetic acid and lyophilization. For determination of cysteine content, some samples were oxidized with performic acid according to Moore (1963). The oxidation reaction was quenched by the addition of HBr (Moore, 1963), and the samples were dried on a vacuum centrifuge. Remaining traces of Br_2 were removed by adding a small amount of 88% formic acid and drying under vacuum once again. Untreated and oxidized samples were hydrolyzed in 250 µl of 6 N HCl at 110°C for 20 to 72 h in sealed tubes evacuated to below 25 millitorr. Hydrolysates were analyzed on a Durrum D-500 automatic amino acid analyzer.

Preparation of gelatin- and lysine-Sepharose CL-4B - Sepharose CL-4B was activated by cyanogen bromide in a high capacity buffer (Porath *et al.*, 1973). Swollen Sepharose CL-4B (500 ml) was washed with 2 liters of 1.0 M potassium phosphate, pH 12.1, by filtering under vacuum on a coarse sintered glass funnel. The moist cake was suspended to a volume of 800 ml with 1.0 M potassium phosphate, pH 12.1, and cooled to 5 to 10°C by gently

stirring in an ice bath. Cyanogen bromide (100 g) was dissolved in approximately 100 ml of N,N-dimethylformamide (Blackburn et al., 1977) and added to the Sepharose with stirring over a period of 2 min. The reaction mixture was then stirred for an additional 30 min. The temperature was maintained at 5 to 10°C by addition of ice. The activated Sepharose was washed with approximately 4 liters of cold distilled water and either used immediately for coupling or stored in dry acetone at 4°C.

The Sepharose was prepared for coupling by washing it with 2 liters of 0.1 M $NaHCO_3$, pH 8.9. (Deutsch and Mertz, 1970). Gelatin (5 g) was dissolved in 500 ml of warm 0.1 M $NaHCO_3$, pH 8.9. The solution was allowed to cool, mixed with the moist cake of Sepharose, and placed on a rotary shaker at room temperature for several hours. The gelatin-Sepharose was then washed with 2 liters of 2.0 M NaCl, 0.1 M $NaHCO_3$, pH 8.9, to remove unbound ligand, and with 2 liters of 0.1 M $NaHCO_3$, pH 8.9, to remove salt. Any unsubstituted groups which remained were quenched by mixing the washed Sepharose with 250 ml of 0.1 M ethanolamine-HCl, pH 9.0, on a rotary shaker for 2 h at room temperature. The gelatin-Sepharose was then washed with 2 liters of PBS and stored suspended in PBS containing 0.02% NaN_3 at 4°C. Lysine-Sepharose was prepared similarly, except that 100 g of lysine were dissolved in 500 ml of 0.1 M $NaHCO_3$, pH 8.9, and the coupling reaction was carried out overnight at 4°C (Deutsch and Mertz, 1970).

Reduction and alkylation - Proteins were exhaustively reduced and alkylated essentially according to Konigsberg (1972). Samples

were dialyzed against 0.5 Tris-HCl, 5 mM Na_2EDTA, pH 8.5. Dialyzed solutions were then made approximately 6 M in guanidine HCl and heated for 3 to 5 min at 100°C. Samples were flushed with N_2 and incubated at 50°C for 30 min. A 20- to 50-fold molar excess of dithiothreitol (compared with moles of cysteine in the protein) was added. The solution was again flushed with N_2, and incubation at 50°C was continued for an additional 4 h. The solution was cooled to room temperature, a slight molar excess (compared with moles of dithiothreitol) of iodoacetic acid was added, and the sample was incubated in the dark for 5 min. Two more aliquots of iodoacetic acid equal to the first were added, followed by incubations in the dark of 5 min and 20 min, respectively. The solution was then dialyzed in the dark at 4°C against an appropriate buffer containing 0.1% 2-mercaptoethanol (v/v). Subsequent changes of dialysis buffer contained no 2-mercaptoethanol.

Immunological techniques - For preparation of antiserum, 100 to 250 μg of purified plasma fibronectin were electrophoresed on a 5% acrylamide gel in the presence of SDS and 2-mercaptoethanol. Following electrophoresis, vertical strips were sliced from the gel, fixed and stained, and used as guides to determine the position of the fibronectin band. Fibronectin-containing regions of the gel were excised, ground with a mortar and pestle, mixed with complete Freund's adjuvant, and injected into an adult New Zealand white rabbit. Injections prepared similarly, but with incomplete rather than complete adjuvant, were repeated at weekly intervals for three weeks. Beginning three weeks after the last injection, antiserum was collected and prepared by

standard techniques and stored at -20°C. Double immunodiffusion was performed by the method of Ouchterlony (1958).

Preparation of human plasmin - Plasminogen was isolated from human acid citrate dextrose plasma by lysine-Sepharose affinity chromatography (Deutsch and Mertz, 1970) and activated by streptokinase as described by Robbins and Summaria (1970).

Estimation of protein concentration - Protein concentrations were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard, or by absorbance at 280 nm using an $E^{1\ cm}_{mg/ml}$ value of 1.28 for plasma fibronectin (Mosesson and Umfleet, 1970).

RESULTS AND DISCUSSION

Purification of Fibronectin

Plasma fibronectin - Fibronectin was isolated from human plasma by affinity chromatography on gelatin-Sepharose. A first attempt at affinity purification, using the method of Engvall and Ruoslahti (1977), yielded fibronectin which was obviously degraded; therefore, this procedure was modified to minimize the possibility of proteolytic digestion of the molecule during processing. Protease inhibitors were present throughout the early steps of purification, and vitamin K-dependent proteases of the clotting cascade were removed from the plasma by adsorption to barium (Aronson and Ménaché, 1966). Furthermore, since plasma fibronectin is susceptible to cleavage by plasmin (A.B. Chen et al., 1977; Jilek and Hörmann, 1977a; Iwanaga et al., 1978), plasminogen was removed from the plasma by lysine-Sepharose affinity chromatography (Deutsch and Mertz, 1970). Plasma fibronectin purified with these precautions showed no evidence of proteolytic degradation as monitored by SDS-polyacrylamide gel electrophoresis even after incubation at 37°C for 24 h (not shown). Carbamylation of amino groups in the protein was avoided by using guanidine HCl rather than urea (Engvall and Ruoslahti, 1977).to elute fibronectin from the gelatin-Sepharose.

Fibronectin samples prepared by gelatin affinity chromatography were seen to contain three prominent contaminants, with apparent molecular weights of 69,000, 53,000 and 49,000, when examined by SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol (Fig. 1D). It is likely that these bands represented the three subunit chains of

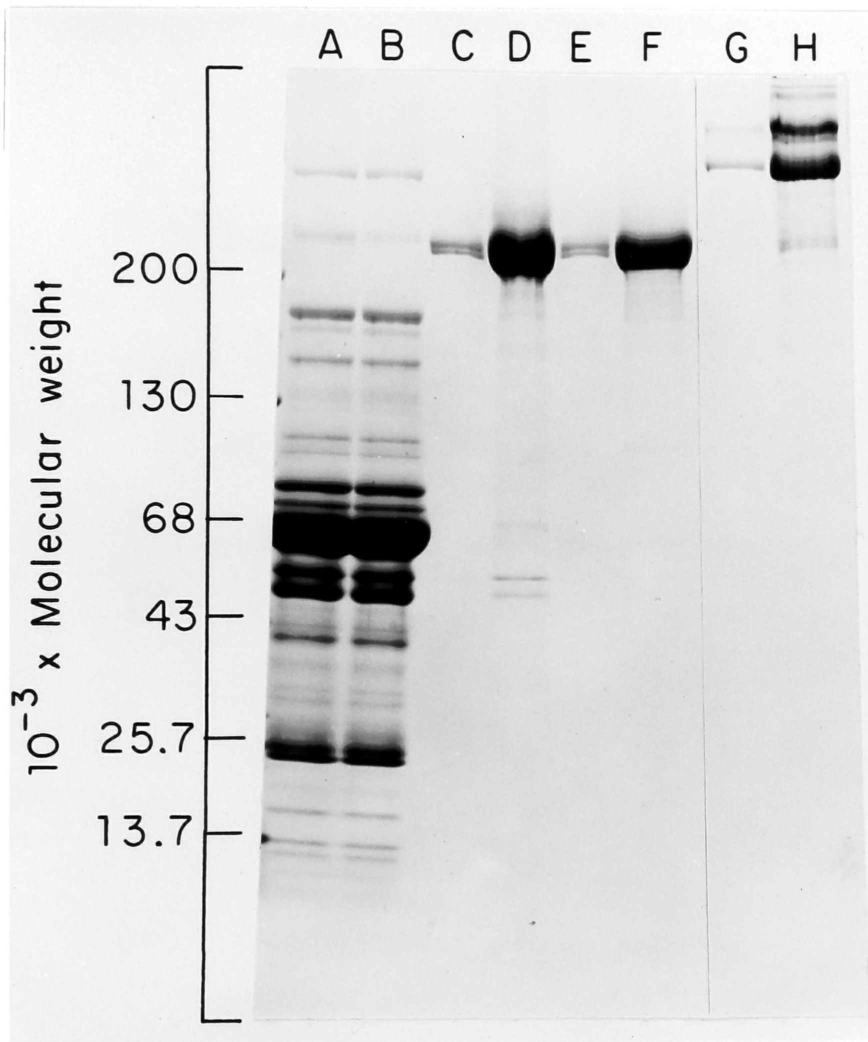


Figure 1. PURIFICATION OF HUMAN PLASMA FIBRONECTIN

Details of the experimental procedure are given under "Methods". Protein samples were applied to a 5 to 16% gradient polyacrylamide gel containing SDS with (A to F) or without (G and H) prior reduction. A, human plasma (70 μg); B, human plasma following gelatin-Sepharose chromatography (70 μg); C, protein eluted from gelatin-Sepharose with 1 M guanidine HCl (2 μg); D, same as C (20 μg); E and G, protein eluted from gelatin-Sepharose and further purified by DEAE-cellulose chromatography (2 μg); F and H, same as E and G (20 μg). The scale indicates the positions of molecular weight standards listed under "Methods".

fibrinogen, which have molecular weights of approximately 71,000, 60,000, and 49,000 (Mosesson *et al.*, 1972, 1973). Therefore, the fibronectin preparation was chromatographed on DEAE-cellulose, using a procedure specifically designed to separate fibronectin and fibrinogen (Mosesson and Umfleet, 1970). As seen in Fig. 1F, material purified on DEAE-cellulose contained no trace of the suspected fibrinogen contaminants.

Yields of 55 to 120 mg of purified fibronectin were obtained routinely from 900 to 1200 ml of pooled plasma. It has been determined, using immunochemical techniques, that normal adult human plasma contains from 150 to 720 mg of fibronectin per liter (Mosher and Williams, 1978). Since very little protein was lost during DEAE-cellulose chromatography, it would seem that gelatin-Sepharose affinity chromatography did not completely remove fibronectin from plasma. However, plasma that had been passed over a gelatin-Sepharose column contained no fibronectin detectable by SDS-polyacrylamide gel electrophoresis or by double immunodiffusion using antiserum to human plasma fibronectin (not shown). The reasons for this discrepancy remain unclear.

SDS-polyacrylamide gel electrophoresis of isolated plasma fibronectin under reducing conditions revealed two closely spaced bands with apparent molecular weights of approximately 250,000 and 245,000 (Fig. 1E). Other laboratories have also reported that the subunits of plasma fibronectin migrate as a closely spaced doublet of bands; however, their estimates of the subunit molecular weights were lower, ranging from 200,000 to 220,000 (Mosesson *et al.*, 1975;

Iwanaga *et al.*, 1978; Yamada and Kennedy, 1979). This discrepancy is probably a reflection of the different gel systems used, and emphasizes the inaccuracy inherent in the determination of molecular weights of large molecules by SDS-polyacrylamide gel electrophoresis.

Smaller polypeptides were also seen in the fibronectin sample, most notably with molecular weights of 150,000, 95,000, and 60,000. Several faint bands representing proteins with molecular weights ranging from 210,000 to 170,000 were detected as well. These components may be just visible in Fig. 1F. It is likely that some or all of these contaminants represent fragments of plasma fibronectin generated *in vivo*. A. B. Chen *et al.* (1977) have reported that similar proteins were present in material precipitated from freshly prepared plasma by anti-serum to plasma fibronectin. Furthermore, these same proteins were seen in samples of fibronectin isolated by gelatin-Sepharose affinity chromatography from plasma collected directly into a mixture of protease inhibitors, including soybean trypsin inhibitor, N-ethylmaleimide, pepstatin, benzamidine HCl, $PhCH_2SO_2F$, and Na_2EDTA (not shown).

Analysis of nonreduced, purified plasma fibronectin by poly-acrylamide gel electrophoresis showed the molecule to exist primarily as a species with an apparent molecular weight of approximately 400,000, although some higher molecular weight aggregates and monomers of 245,000 and 250,000 daltons were also present (Fig. 1, G and H). The 400,000 dalton band undoubtedly represents dimers of 245,000 and 250,000 dalton subunits, since Mosesson *et al.* (1975) have demonstrated that native plasma fibronectin occurs largely in this form.

Cellular fibronectin - Cellular fibronectin was purified from the conditioned media of human embryonic fibroblast cultures by gelatin-Sepharose affinity chromatography according to Teng and Rifkin (1979). As seen in Fig. 7A, the subunits of the cellular form of the molecule did not migrate as a doublet, but as a broad, diffuse band. In the polyacrylamide gel system employed, cellular fibronectin subunits migrated slightly more slowly than the subunits of plasma fibronectin (not shown). Samples of cellular fibronectin prepared in this manner were not as pure as corresponding samples of the plasma form. However, because of the relative insolubility of cellular fibronectin under physiological conditions and the difficulty of obtaining large amounts of material, no attempt was made to purify the molecule further.

Peptide Mapping of Plasma Fibronectin Subunits

The relationship between the 245,000 dalton and 250,000 dalton subunits of plasma fibronectin was examined using a one-dimensional peptide mapping technique developed by Cleveland _et al._ (1977). Subunit bands, labeled with ^{125}I (McConahey and Dixon, 1966), were sliced from an SDS-polyacrylamide gel and placed in the sample wells of a second polyacrylamide gel. _Staphylococcus aureus_ V8 protease, which is active in SDS, was then added to each well to generate an array of fragments. In Fig. 2, B and C, gel slices containing either of the subunits were electrophoresed in the absence of protease to demonstrate that these two polypeptides could be separated cleanly from one another. The peptide maps derived from the two subunits were identical (compare Fig. 2E with Fig. 2F, and Fig. 2H with Fig. 2I). As a control, the three subunit chains of bovine fibrinogen, which are known to be dissimilar, were analyzed in a similar manner. As expected, the peptide maps of these chains were clearly different from one another (not shown).

These experiments indicate that the 245,000 dalton and 250,000 dalton subunits of plasma fibronectin are very similar. The difference in molecular weights of the chains must arise from only a slight difference in the primary structure of the two polypeptides. Alternatively, the two subunits may have identical polypeptide backbones, but migrate differently because they are glycosylated to different extents. This hypothesis is supported by results of Wagner and Hynes (1979), who metabolically labeled cellular fibronectin with (^{3}H)glucosamine and

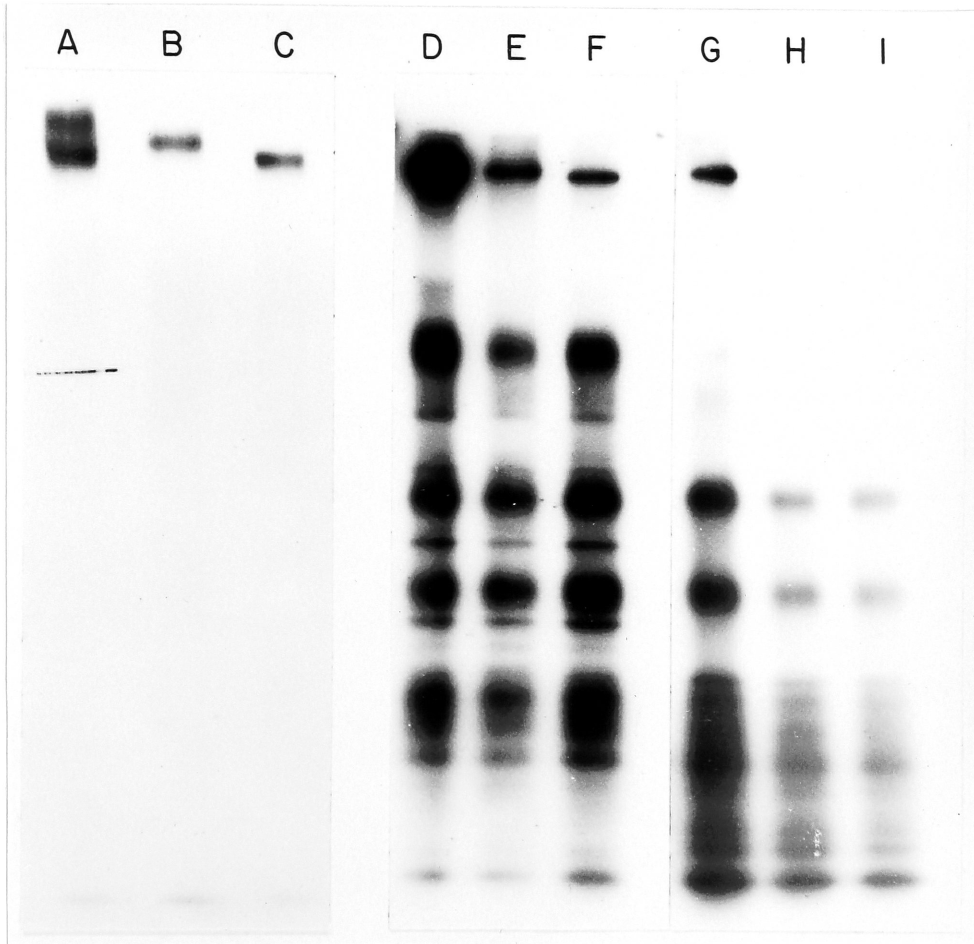


Figure 2. PEPTIDE MAPPING OF PLASMA FIBRONECTIN SUBUNITS

Details of the experimental procedure are given under "Methods". Gel slices containing one or both bands of the reduced fibronectin doublet were placed in the sample wells of a 5% (A to C) or an 8 to 18% gradient (D to I) polyacrylamide gel containing SDS. The slices were then overlaid with the indicated amounts of S. aureus V8 protease, and electrophoresis was carried out as indicated in the text. A to C, no protease: A, 245 and 250 kd bands; B, 250 kd band; C, 245 kd band. D to F, 0.05 μg of protease: D, 245 and 250 kd bands; E, 250 kd band; F, 245 kd band. G to I, 0.5 μg of protease: G, 245 and 250 kd bands; H, 250 kd band; I, 245 kd band.

(^{14}C)amino acids. This material migrated as a broad band on SDS-polyacrylamide gels. The ratio of ^{3}H to ^{14}C was higher in slices cut from the upper part of the band than in slices cut from the lower portion; this finding indicates that the more slowly migrating fractions of fibronectin contained a proportionately greater amount of glucosamine than the more rapidly migrating fractions did. In any event, the close similarity of the subunits to one another simplified the design and interpretation of the proteolytic digestion experiments that follow, since cleavage of either chain could be expected to yield similar fragments.

It is not known whether plasma fibronectin molecules are heterodimers or homodimers. That is, a single dimeric fibronectin molecule may be composed of one 245,000 dalton subunit and one 250,000 dalton subunit, or there may be two different classes of fibronectin dimers, each composed of identical subunits. However, observations that dimeric plasma fibronectin appeared as a single band in gels (Fig. 1, G and H) and that the two monomer bands always stained with approximately equal intensity tend to support the former possibility.

Analysis of Fibronectin Structure with Thrombin

The first protease used for the analytical dissection of fibronectin was thrombin. Thrombin was chosen for two reasons: first, it is quite specific in its action and might be expected to introduce only a limited number of cleavages in the fibronectin chain; second, fibronectin is a potential substrate for thrombin *in vivo*, although there is no evidence to date which indicates that such an interaction occurs.

Cleavage of fibronectin with thrombin - Incubation of plasma fibronectin with human α-thrombin generated three major fragments: two large ones with molecular weights, as determined by SDS-polyacrylamide gel electrophoresis under nonreducing conditions, of approximately 235,000 and 230,000, and a small one with a molecular weight of 26,000 (Fig. 3, E to H). Reduction of the digest with 2-mercaptoethanol prior to electrophoresis caused no change in the apparent molecular weights of the large fragments, but that of the small fragment rose to 29,000 (Fig. 3, A to D). This unexpected rise in molecular weight suggests that the small fragment contains a large number of intrachain disulfide bonds. The presence of such bonds may prevent the nonreduced molecule from assuming the extended, rod-like configuration typical of SDS-protein complexes (Reynolds and Tanford, 1970), thus resulting in aberrant migration. Similar atypical electrophoretic behavior has been described for fibronectin fragments produced by trypsin (Wagner and Hynes, 1979) and cathepsin D (Balian *et al.*, 1979) digestion. Other proteases also yield patterns of digestion similar to that obtained with thrombin; both

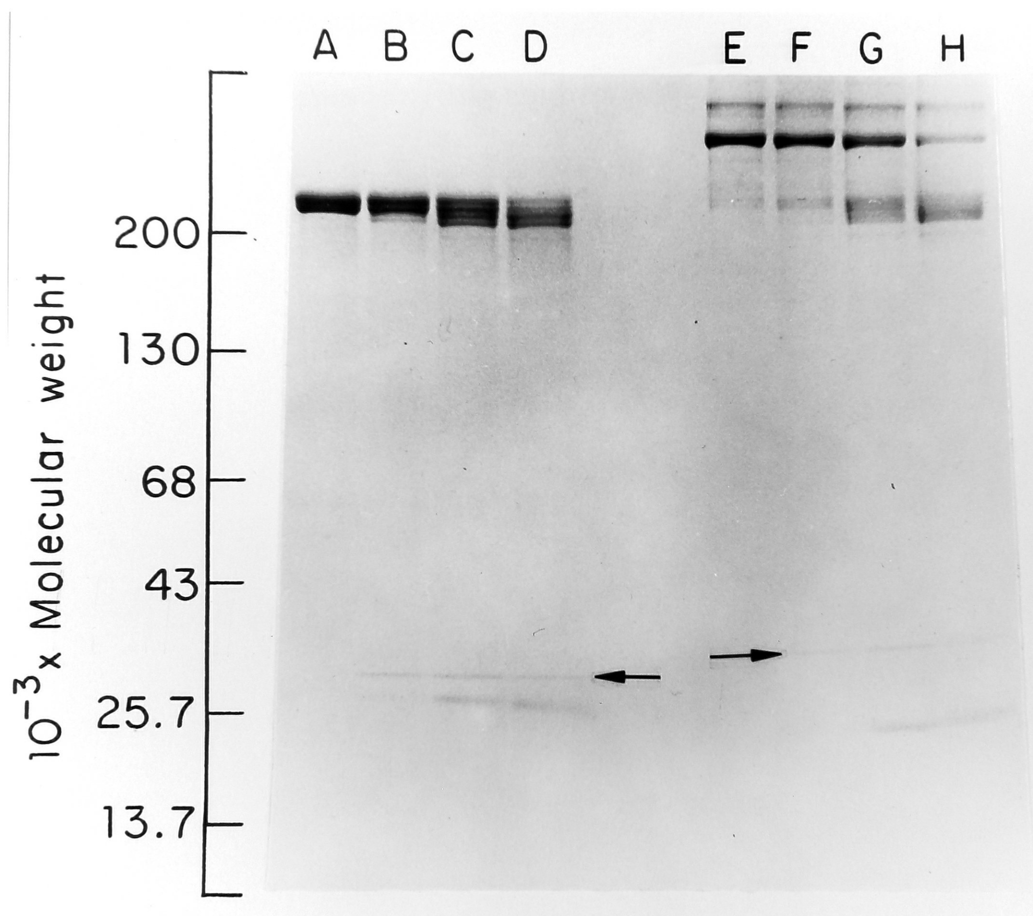


Figure 3. DIGESTION OF PLASMA FIBRONECTIN WITH THROMBIN

Plasma fibronectin (1 mg/ml of PBS) was mixed with human α-thrombin (50:1, w/w) and incubated at 37°C. Protein samples were applied to a 5 to 16% gradient polyacrylamide gel containing SDS with (A to D) or without (E to H) prior reduction. A and E, plasma fibronectin alone; B and F, mixture incubated 15 min; C and G, mixture incubated 1 h; D and H, mixture incubated 2 h. Each lane contains 6 μg of protein. Arrows indicate the α-thrombin bands. The scale indicates the positions of molecular weight standards listed under "Methods".

plasmin cleavage of plasma fibronectin (A.B. Chen *et al.*, 1977; Jilek and Hörmann, 1977a; Iwanaga *et al.*, 1978) and trypsin cleavage of cellular fibronectin (Wagner and Hynes, 1979) produce large fragments of approximately 200,000 daltons and small fragments of 23,000 to 29,000 daltons. These results indicate that fibronectin may contain a region that is highly susceptible to cleavage by serine proteases.

Since none of the thrombin-derived fragments showed a decrease in apparent molecular weight upon reduction, it is likely that all three are composed of single polypeptide chains. It is possible that the nonreduced small fragment is actually composed of two 29,000 dalton subunits and migrated anomalously as a 26,000 dalton species due to extensive disulfide bonding. However, results of the experiment depicted in Figure 4 suggest that the interchain disulfide bond(s) of fibronectin are located not within the small fragment, but within another peptide situated close to one end of the molecule. At early times of digestion by thrombin, plasma fibronectin fragments with molecular weights nearly identical with those of intact monomers were liberated (compare Fig. 4, B to D, with Fig. 4J). This observation suggests that removal of a small fragment, probably weighing no more than several thousand daltons, from one end of the molecule results in the concomitant removal of all linkages which join the subunits of this dimer. The monomer-length fragments appeared only transiently; fragments of 230,000 and 235,000 daltons accumulated at later times (Fig. 4, G to I). Minor components which migrated just ahead of and behind the large fragments were also observed after prolonged digestion. Whereas the former species is probably derived from further cleavage of the large fragments, the origin of the latter is at present unknown.

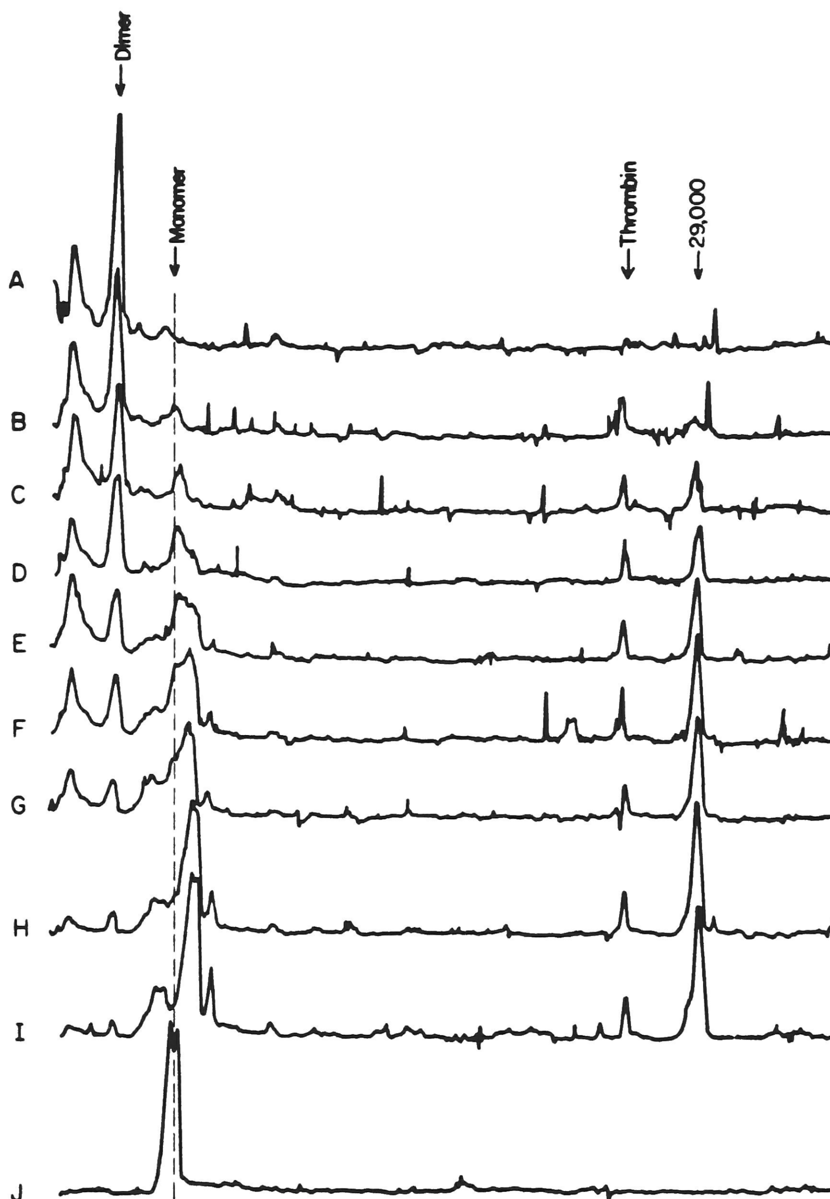


Figure 4. TIME-COURSE OF DIGESTION OF PLASMA FIBRONECTIN WITH THROMBIN

Plasma fibronectin (1 mg/ml of PBS) was mixed with human α-thrombin (50:1, w/w) and incubated at 37°C. Protein samples were applied to a 5 to 16% gradient polyacrylamide gel containing SDS with (J) or without (A to I) prior reduction. Fixed, stained gels were then scanned with a densitometer. A and J, plasma fibronectin alone; B to I, mixture incubated for the following times: B, 10 min; C, 20 min; D, 40 min; E, 60 min; F, 90 min; G, 2 h; H, 4 h; I, 6 h. Each lane contains 8 μg of fibronectin. The arrows indicate the positions of migration of the fibronectin dimer and monomer, α-thrombin, and a 29 kd fibronectin fragment.

It thus appears that thrombin cleaves fibronectin at a minimum of two sites. One cleavage removes a small fragment, which contains all of the interchain disulfide linkages, from one end of the molecule. A second cleavage produces the 235,000, 230,000, and 29,000 dalton fragments. It should be emphasized, however, that fragments containing the interchain bonds were not detected in the thrombin digests. Furthermore, the presence of aggregated material at the top of the gel, as well as the lack of a clear separation between some of the peaks, makes a detailed, quantitative analysis of the scans shown in Figure 4 difficult. Further research will be required to locate the position of the interchain bonds with certainty.

As mentioned, the pattern of thrombin digestion seen in these experiments is quite similar to that reported for plasmin degradation of plasma fibronectin (A.B. Chen _et al_., 1977; Jilek and Hörmann, 1977a; Iwanaga _et al_., 1978). Furthermore, fibronectin has previously been reported to be resistant to thrombin attack (Mosesson and Umfleet, 1970; Zetter _et al_., 1976). To ensure that the observed degradation was indeed due to thrombin and not to small amounts of contaminating plasmin, thrombin digestions were performed in the presence of the protease inhibitors Trasylol and hirudin. Trasylol is an efficient inhibitor of plasmin (Feeney _et al_., 1969) but not of thrombin, whereas hirudin specifically blocks thrombin (Markwardt, 1970). As seen in Figure 5, hirudin completely inhibited digestion of plasma fibronectin by the thrombin preparation (Fig. 5D); in contrast, Trasylol had no effect (Fig. 5C). As expected, the converse result was obtained when digestion of fibronectin by purified human plasmin was examined. Addition of Trasylol completely blocked degradation (Fig. 5G); addition

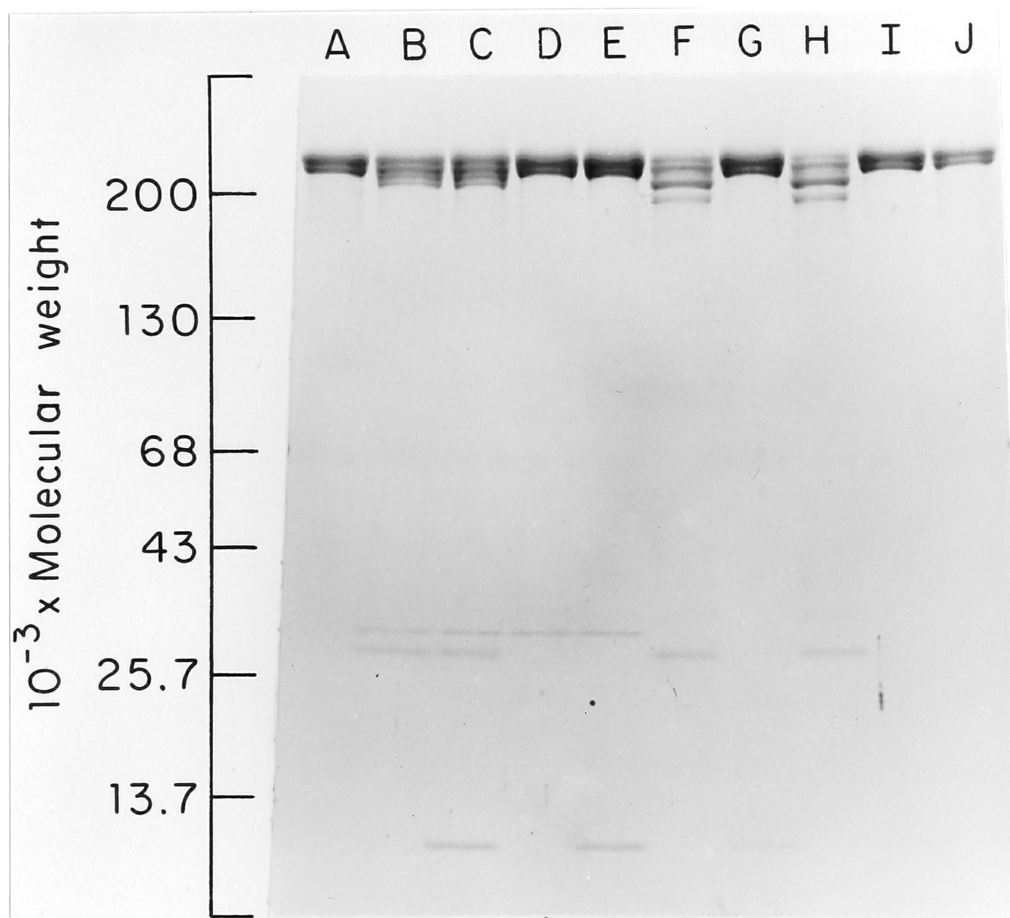


Figure 5. EFFECT OF PROTEASE INHIBITORS ON DIGESTION OF PLASMA FIBRONECTIN WITH THROMBIN AND PLASMIN

Thrombin and plasmin were incubated for 0.5 h at room temperature with or without the addition of Trasylol or hirudin (10 U of inhibitor/μg of protease). Plasma fibronectin was then mixed with the thrombin (35:1, w/w) or plasmin (75:1, w/w) preparations and incubated at 37°C for an additional 2 h. Reduced samples were applied to a 5 to 16% gradient polyacrylamide gel containing SDS. A and J, plasma fibronectin alone; B, fibronectin plus thrombin; C, fibronectin plus thrombin and Trasylol; D, fibronectin plus thrombin and hirudin; E, fibronectin plus thrombin, Trasylol, and hirudin; F, fibronectin plus plasmin; G, fibronectin plus plasmin and Trasylol; H, fibronectin plus plasmin and hirudin; I, fibronectin plus plasmin, Trasylol, and hirudin. Each lane contains 6 μg of fibronectin. The scale indicates the position of molecular weight standards listed under "Methods".

of hirudin had no effect (Fig. 5H). Cleavage of fibronectin by the human thrombin preparation thus appears to result from the action of thrombin alone and not from that of any contaminating proteases.

The results of this experiment also demonstrate that plasmin and thrombin do not cleave plasma fibronectin identically. Although both proteases produced small fragments of 29,000 daltons, plasmin yielded relatively greater amounts of the 230,000 dalton fragment and lesser amounts of the 235,000 dalton fragment than did thrombin (compare Fig. 5B with Fig. 5F). An additional fragment of approximately 200,000 daltons was seen in the plasmin digests (Fig. 5F), but also appeared after prolonged digestion of fibronectin with thrombin (Fig. 4I).

When a thrombin digest of plasma fibronectin was passed over a column of gelatin-Sepharose, it was discovered that the small, 29,000 dalton fragment was not retained. The large fragments, however, were still capable of binding to gelatin and could be eluted from the column with 2.0 M guanidine HCl. Thus, the digest could be separated into two fractions for further analysis: one containing the large, 230,000 and 235,000 dalton pieces as well as remaining undigested fibronectin (Fig. 6D), and one containing primarily the small, 29,000 dalton fragment (Fig. 6E). The small fragment preparation contained traces of higher molecular weight polypeptides, the major contaminant having a molecular weight of about 30,000. However, densitometric scans of Fig. 6E indicated that the 29,000 dalton fragment was more than 95% pure (not shown).

Thrombin cleavage of fibronectin isolated from the culture medium of human embryonic fibroblasts produced fragments similar to those obtained from the plasma form. SDS-polyacrylamide gel electrophoresis

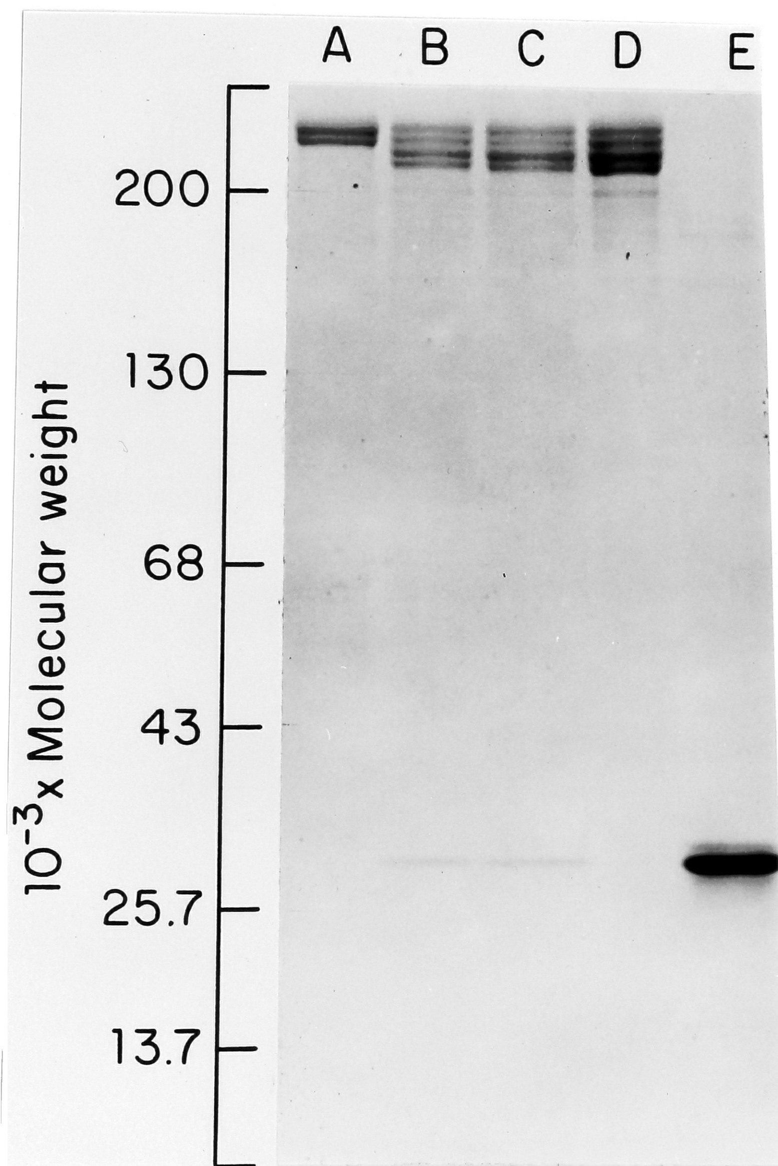


Figure 6. SEPARATION OF THROMBIN-DERIVED PLASMA FIBRONECTIN FRAGMENTS

Human plasma fibronectin (3.3 to 5 mg/ml of PBS) was mixed with human α-thrombin (133:1, w/w) and incubated at 37°C for 1 h. Fragments were then separated by gelatin-Sepharose affinity chromatography as discussed under "Methods". Reduced protein samples were applied to a 5 to 16% gradient polyacrylamide gel containing SDS. A, plasma fibronectin plus thrombin prior to incubation (2.5 μg); B, fibronectin plus thrombin after incubation (2.5 μg); C, fibronectin plus thrombin incubated and passed over phosphocellulose (2.5 μg); D, fraction of thrombin digest bound to gelatin-Sepharose (3.3 μg); E, fraction of thrombin digest not retained by gelatin-Sepharose (7 μg). The amount of thrombin in the digestion mixture is too small to be seen on this gel. The scale indicates the positions of molecular weight standards listed under "Methods".

of the cellular fibronectin digests in the presence of 2-mercaptoethanol revealed two major components: a diffuse band or group of bands migrating just ahead of intact cellular fibronectin, and a band migrating with an apparent molecular weight of 29,000 (Fig. 7, B and C). Less prominent bands with molecular weights of 28,000 and 30,000 were also detected. Like the 230,000 and 235,000 dalton plasma fibronectin fragments, the large cellular fibronectin fragments retained the ability to bind to gelatin-Sepharose (Fig. 7I); the small, 29,000 dalton fragment did not bind (Fig. 7, D to H). Although it may not be evident from Figure 7, samples of intact cellular fibronectin and its thrombin-derived fragments were considerably less pure than corresponding samples of plasma fibronectin and its fragments.

NH_2-terminal analysis of intact fibronectin and thrombin-derived fragments - When intact plasma fibronectin or the thrombin-generated small fragment was analyzed by the dansyl chloride technique, no NH_2-terminal amino acids were detected.. Exhaustive reduction and alkylation of the samples prior to analysis did not affect these results, indicating that failure to see an NH_2-terminal residue was probably not due to its inaccessibility to the dansyl reagent. Treatment of the pooled large fragments, on the other hand, yielded a single dansyl-amino acid which co-chromatographed with standard dansyl-alanine.

Since plasma fibronectin has been reported to contain NH_2-terminal pyroglutamic acid (Mosesson *et al.*, 1975), an attempt was made to remove the blocking residue with L-pyroglutamyl-peptide hydrolase and

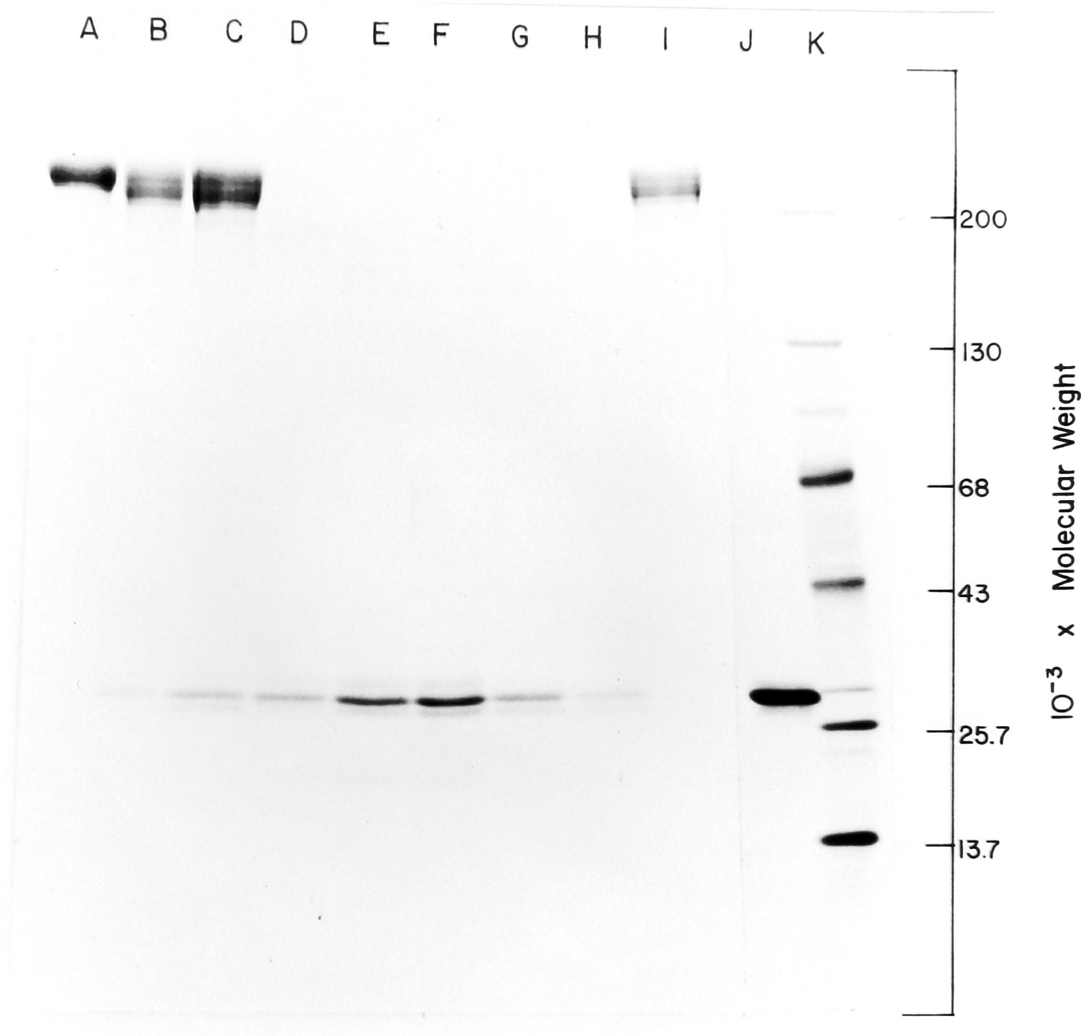


Figure 7. DIGESTION OF CELLULAR FIBRONECTIN WITH THROMBIN AND SEPARATION OF FRAGMENTS

Human cellular fibronectin (2.9 mg/ml of PBS) was mixed with human α-thrombin (100:1, w/w) and incubated at 37°C for 1.5 h. Fragments were then separated by gelatin-Sepharose affinity chromatography as discussed under "Methods". A, cellular fibronectin plus thrombin prior to incubation (7 μg); B, fibronectin plus thrombin after incubation (7 μg); C, fibronectin plus thrombin incubated and passed over phosphocellulose (12 μg); D to H, fractions containing digest components not bound by gelatin-Sepharose (1.5 μg, 4.6 μg, 5.3 μg, 1.7 μg, and 0.8 μg, respectively); I, digest components bound to gelatin-Sepharose (5 μg); J, 29 kd fragment produced by thrombin digestion of human plasma fibronectin (8 μg); K, molecular weight standards. The scale indicates the molecular weights of the standard proteins, which are listed under "Methods".

thus expose the next amino acid in the sequence. The enzyme had a specific activity of 50 units/mg using L-pyroglutamyl-L-alanine as a substrate.[1] After treatment with the L-pyroglutamyl-peptide hydrolase, both the intact molecule and the small fragment had an NH_2-terminal alanine residue, as monitored by dansylation. As a control, proteins and enzyme were incubated separately and mixed together only immediately prior to boiling with SDS. No NH_2-terminal amino acids were detected in these preparations. Neither whole plasma fibronectin nor the small fragment appeared degraded after enzyme treatment when examined by SDS-polyacrylamide gel electrophoresis (compare Fig. 8A with Fig. 8B, and Fig. 8F with Fig. 8G), although small changes in molecular weight would not be detectable by this technique.

A similar analysis was performed on intact cellular fibronectin and its thrombin-derived fragments. However, the results of these experiments were less clear-cut because of the relative impurity of the cellular fibronectin preparations. Chromatography of hydrolysates of dansylated, intact cellular fibronectin revealed three faint spots. Two of these were identified as dansyl-alanine and dansyl-glycine. The third spot did not co-migrate with any of twenty dansyl-amino acid standards. This spot might represent a hydrolysis-resistant dansyl-dipeptide; however, the reported chromatographic behaviors of such dipeptides are not consistent with the behavior of the unidentified derivative (Sutton and Bradshaw, 1978). Identical results were

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[1] A unit is defined as the amount of enzyme which will release 1.0 nmol of alanine per min (Doolittle, 1972) under the assay conditions described in "Methods".

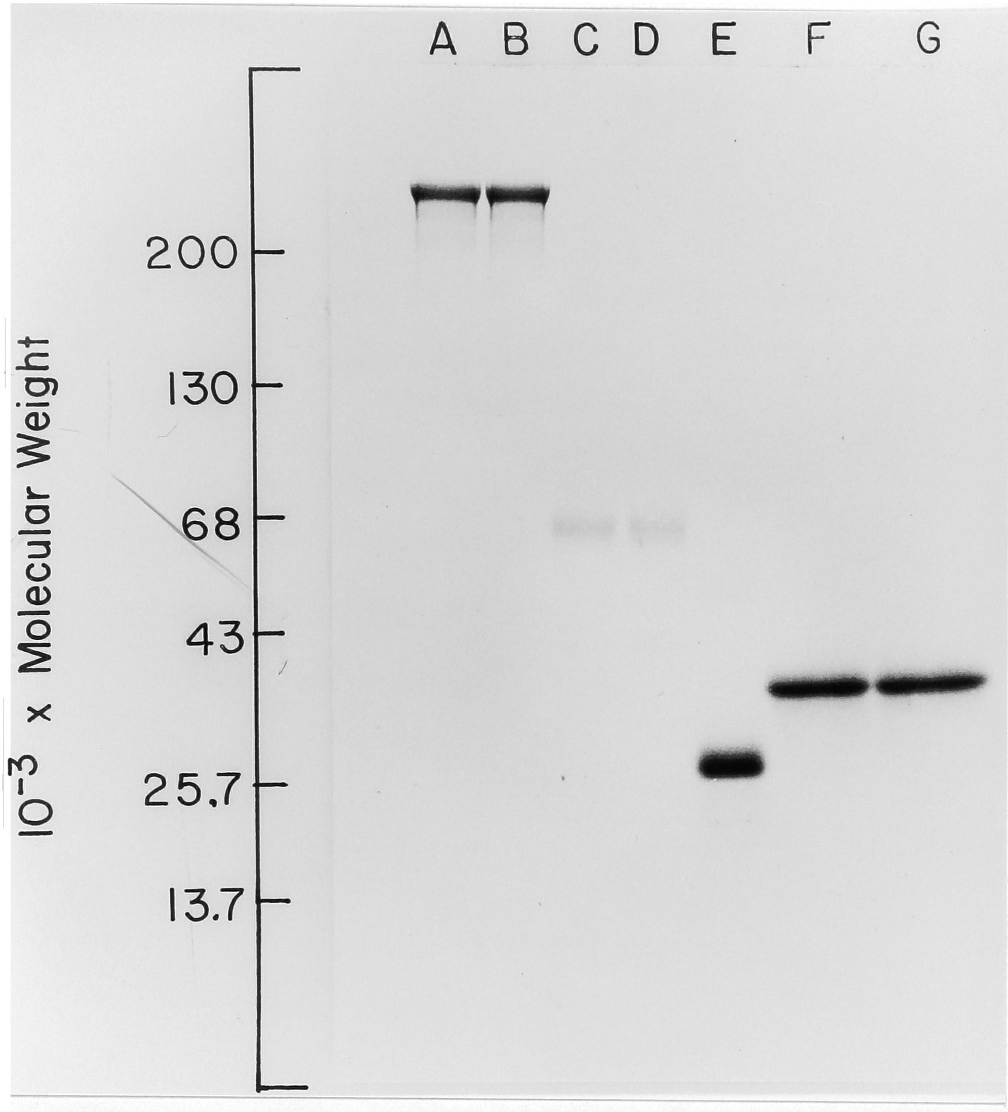

Figure 8. EFFECT OF L-PYROGLUTAMYL-PEPTIDE HYDROLASE TREATMENT ON ELECTROPHORETIC MOBILITY OF INTACT PLASMA FIBRONECTIN AND SOME OF ITS FRAGMENTS

Intact fibronectin, a 29 kd fragment produced by thrombin digestion, and a 72 kd gelatin-binding fragment produced by cathepsin D digestion were isolated and digested with L-pyroglutamyl-peptide hydrolase as discussed under "Methods". Nonreduced samples were applied to a 5 to 16% gradient polyacrylamide gel containing SDS. A and B, reduced and alkylated intact fibronectin before (A) and after (B) L-pyroglutamyl-peptide hydrolase digestion (5.5 μg); C and D, 72 kd fragment before (C) and after (D) digestion (1 μg); E, 29 kd fragment (10 μg); F and G, reduced and alkylated 29 kd fragment before (F) and after (G) digestion (7 μg). The scale indicates the positions of molecular weight standards listed under "Methods".

obtained from dansyl analysis of the 29,000 dalton cellular fibronectin fragment. Treatment of the intact molecule with L-pyroglutamyl-peptide hydrolase led to a marked increase in fluorescence at the dansyl-alanine position; the other two spots remained faint. Analysis of the large cellular fibronectin fragments yielded a single spot, which was identified as dansyl-alanine.

Although these results are not conclusive, it appears that human cellular fibronectin, like the plasma form, contains an NH_2-terminal pyroglutamic acid residue and alanine as the penultimate residue. Furthermore, cleavage of cellular fibronectin with thrombin produced a small fragment with a blocked NH_2-terminus and large fragments with NH_2-terminal alanine. It has been reported that chicken cellular fibronectin also has a blocked NH_2-terminus; however, the blocking residue was not removed by treatment with L-pyroglutamyl-peptide hydrolase (Yamada et al., 1977a). Therefore, the NH_2-terminus of fibronectin from this species may consist of a residue with an acylated α-amino group, rather than pyroglutamic acid.

Automated sequence analysis of intact plasma fibronectin and thrombin-derived fragments - Analysis of whole plasma fibronectin and its thrombin-derived fragments by an automated amino acid sequencer confirmed the results of the dansylation study. Intact plasma fibronectin and the small fragment yielded no phenylthiohydantoin derivatives, indicating NH_2-terminal blockage. The pooled large fragments gave the sequence Ala-Ala-Val-Tyr-. After treatment with L-pyroglutamyl-peptide

hydrolase, both the intact molecule and the small fragment yielded the sequence Ala-Glx-Glx-Met-Val-.

The yield of alanine at the first step of sequencing, expressed as a percentage of the number of nanomoles of protein applied to the sequencer, was approximately 52% for the large fragments, 42% for the intact molecule, and 52% for the small fragment. It should be noted that the large fragment sample contained a considerable proportion of undigested, blocked molecules; it has been estimated from densitometric scans of SDS-polyacrylamide gels that the fragments constituted only 60 to 75% of the protein applied (not shown). The corrected yield of alanine, then, was approximately 70 to 87%. There are several steps in the automated sequencing process where protein can be lost, including transfer of the protein to the sample cup, drying of the protein onto the cup as a film, and initial washing of the protein film. In fact, it has been reported that a yield of only 50 to 70% can be expected at the first step of automated sequencing under usual conditions (Niall, 1973). Although the yields obtained from the intact molecule and the small fragment clearly lie near the lower end of this range, it may be that the conditions employed did not permit complete enzymatic removal of NH_2-terminal pyroglutamic acid. In any case, it is evident that in all three instances, the observed sequences were derived from the major portion of the protein samples applied and did not arise from minor contaminants. Because of the difficulty in obtaining sufficient quantities of highly purified material, automated sequence analysis of cellular fibronectin was not performed.

Summary - Human plasma fibronectin was cleaved by thrombin, and three major fragments were recovered: two large ones with molecular weights of 230,000 and 235,000, and a small one with a molecular weight of 29,000. No NH_2-terminal residue was detected, using the dansyl chloride method, in either the intact molecule or the small fragment. Analysis of the large fragments revealed NH_2-terminal alanine. Treatment of both the intact molecule and the small fragment with L-pyroglutamyl-peptide hydrolase resulted in the appearance of NH_2-terminal alanine. Automated sequence analysis confirmed these findings and demonstrated that the intact molecule and the small fragment have identical NH_2-terminal sequences. From these results, it is concluded that the small 29,000 dalton fragment is located at the NH_2-terminus of the parental molecule, whereas the large fragments constitute the carboxyl portion, as shown in Figure 9. Preliminary analysis of human cellular fibronectin indicates that it contains a homologous thrombin-sensitive site.

The location of the interchain disulfide linkage(s) cannot be determined with certainty from these data. However, as previously discussed, results of the preliminary experiment shown in Figure 4 suggest that these bonds may be located no more than several thousand daltons from one end of the molecule. Wagner and Hynes (1979) have similarly concluded, from results of digestion of cellular fibronectin with trypsin, that the interchain disulfide bonds are located less than 10,000 daltons from one end. Since it has been established that the 29,000 dalton thrombin-derived fragment constitutes the amino end of

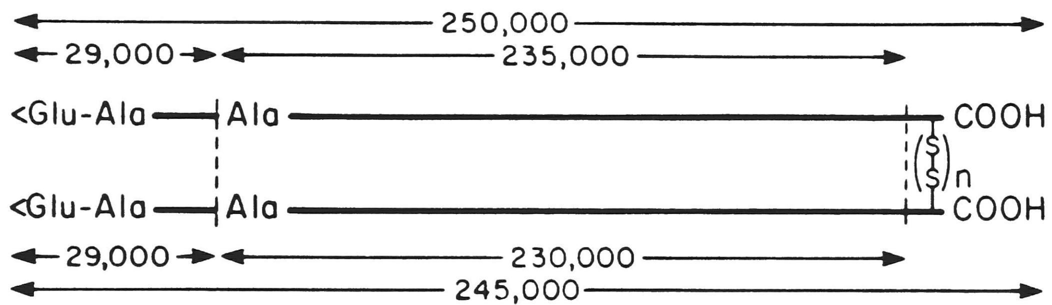

Figure 9. LOCATION OF THROMBIN-DERIVED FRAGMENTS WITHIN INTACT FIBRONECTIN

The small fragment is located at the NH_2-terminus; the large fragments constitute the carboxyl portion. Dashed lines denote proposed sites of thrombin cleavage. Apparent molecular weights of the intact polypeptide chains and thrombin-derived fragments, as determined by SDS-polyacrylamide gel electrophoresis under reducing conditions, are indicated by numbers between horizontal arrows. As discussed in the text, placement of the interchain disulfide bonds near the COOH-terminus is tentative.

fibronectin, it is therefore likely that the interchain bonds are located near the COOH-terminus (Figure 9). Experiments designed to test this hypothesis are in progress.

Wagner and Hynes (1980) have obtained other evidence which also suggests that the interchain disulfide bonds are located near the carboxyl, rather than the amino, end of fibronectin. Hamster cellular fibronectin was treated with DTNB, which reacts with cysteine residues. The fibronectin was then incubated with $Na^{14}CN$, which cleaves peptide bonds rapidly at modified cysteine groups and, more slowly, at cystine residues. The first fragments to appear in the cyanide digests had apparent molecular weights of 170,000 and 33,000, as determined by SDS-polyacrylamide gel electrophoresis under reducing conditions. The nonreduced 33,000 dalton fragment was involved in a disulfide-bonded complex, the components of which were not characterized. In contrast, the 170,000 dalton fragment contained no interchain disulfide bonds. Only the 33,000 dalton fragment was labeled with ^{14}C. Since the (^{14}C)cyanide is incorporated into peptides formed to the carboxyl side of the cleaved bond, it follows that the 170,000 dalton fragment represents the amino end of fibronectin. The interchain disulfide bonds, therefore, must be situated near the COOH-terminus.

Iwanaga _et al_. (1978) have described experiments in which they digested bovine plasma fibronectin with plasmin and separated the resulting fragments by gel filtration. They, too, obtained a small fragment of 29,000 daltons and a large one of about 200,000 daltons. However, in this case, NH_2-terminal analysis of the fragments led to

the conclusion that the large fragment is located at the amino end of fibronectin and the small one at the COOH-terminus. The first two NH_2-terminal residues which Iwanaga _et al_. (1978) found in the intact bovine molecule, <Glu-Ala-, are the same as reported here and by Mosesson _et al_. (1975) for human fibronectin. The third residue, threonine, is different, but this variation may represent heterogeneity between the two species. The NH_2-terminal sequence that they reported for the 29,000 dalton fragment, Val-Val-Gln-, shares no obvious similarities with the sequence obtained from the thrombin-derived small fragment. Furthermore, careful examination of the automated sequence data for the thrombin-derived small fragment gave no indication that a protein with this sequence was present as a minor component of the preparation.

The explanation for the differences in results of these two sets of experiments is unclear. It is unlikely that the bovine and human proteins differ greatly in structure, since fibronectin has been highly conserved during evolution (Kuusela _et al_., 1976). It is evident from Figure 5 that plasmin and thrombin cleave fibronectin in a similar, but not identical, manner. Since Iwanaga _et al_. (1978) did not report any quantitative data, it is conceivable that the NH_2-terminal sequence which they detected in the 29,000 dalton fragment sample was derived from a minor component that is present in plasmin, but not thrombin, digests. It is more difficult to explain why their large fragment preparation was found to contain the same NH_2-terminal sequence as the intact molecule.

Although the data reported by Iwanaga _et al_. (1978) contradict the

conclusions presented in this thesis, results of experiments by others are in complete agreement. Wagner and Hynes (1980) have mapped tryptic fragments of cellular fibronectin, using a procedure modified from Dintzis (1961). Hamster fibroblasts were incubated with (^{35}S)methionine and lysed; newly synthesized, labeled fibronectin was isolated from the lysates by gelatin-Sepharose affinity chromatography. The periods of labeling were shorter than the time required to synthesize a complete fibronectin molecule. Therefore, since protein synthesis proceeds from the NH_2-terminus toward the carboxyl end, newly synthesized molecules contained the greatest density of label in their carboxyl portions. Cleavage of the pulse-labeled molecules with trypsin produced fragments of 25,000 daltons and 200,000 daltons. The 200,000 dalton fragment was labeled preferentially during very short incubations with (^{35}S)methionine; as the length of the labeling period increased, the numbers of counts in the two fragments reached a constant ratio. These results indicate that the 25,000 dalton trypsin-generated fragment is located at the NH_2-terminus of cellular fibronectin. It is likely that this fragment is equivalent to the 29,000 dalton, thrombin-derived plasma fibronectin fragment. Both lack the ability to bind to gelatin, show similar anomalous migration in SDS-polyacrylamide gels, and as will be discussed, contain a high proportion of cysteine (Wagner and Hynes, 1979). Thus, the findings of Wagner and Hynes independently corroborate the model of fibronectin presented in Figure 9.

Location of a Gelatin-binding Region of Plasma Fibronectin

Both plasma and cellular fibronectins specifically interact with collagen in a variety of *in vitro* assays (reviewed in Vaheri and Mosher, 1978; Yamada and Olden, 1978; Mosher, 1980). Recently, several laboratories have isolated proteolytically-derived fragments of fibronectin that retain gelatin- or collagen-binding activity (Balian *et al*., 1979; Gold *et al*., 1979; Hahn and Yamada, 1979a, 1979b; Ruoslahti and Hayman, 1979; Ruoslahti *et al*., 1979). For example, Balian *et al*. (1979) have used cathepsin D to produce a gelatin-binding fragment of plasma fibronectin with a molecular weight of 72,000. This fragment could be cleaved further with plasmin to yield a 42,000 dalton fragment which retained the ability to bind to gelatin and a 30,000 dalton fragment which did not. Since it has been demonstrated that plasmin and thrombin treatment of fibronectin result in similar patterns of digestion (Fig. 5, B and F), it was reasoned that the 30,000 dalton fragment described by Balian *et al*. might be similar or identical to the 29,000 dalton fragment generated by thrombin digestion of intact fibronectin. This hypothesis predicts that the 72,000 dalton gelatin-binding fragment produced by cathepsin D digestion is located at the NH_2-terminus of fibronectin. Thrombin treatment of this fragment should produce a 29,000 dalton, NH_2-terminal fragment which cannot bind to gelatin and a 43,000 dalton fragment which retains gelatin-binding activity. The experiments described below confirm these suppositions and demonstrate conclusively that a gelatin-binding site of plasma fibronectin is located within a 43,000 dalton region, adjacent to the 29,000 dalton, NH_2-terminal peptide.

Production and isolation of gelatin-binding fragments - Treatment of human plasma fibronectin with cathepsin D as described by Balian et al. (1979) resulted in a complex mixture of fragments (Fig. 10B). The two major components had molecular weights of approximately 150,000 and 72,000, as determined by SDS-polyacrylamide gel electrophoresis in the presence of a reducing agent. When the digest was passed over a gelatin-Sepharose column, only three fragments were retained: the 72,000 dalton fragment, and two minor components with molecular weights of approximately 200,000 and 180,000 (not shown). Since these higher molecular weight components were more prominent at early times of digestion and disappeared as the 72,000 dalton fragment accumulated (not shown), it is likely that they represent precursors to the 72,000 dalton piece.

In order to purify the 72,000 dalton fragment, the digest was first depleted of the higher molecular weight gelatin-binding fragments by gel filtration on a column of Sephadex G-150. Two overlapping peaks of protein were eluted from the column (Fig. 11); material from the more slowly eluting peak (Fig. 10C) was subjected to gelatin-Sepharose affinity chromatography. Most of the fragments passed through the gelatin-Sepharose column (Fig. 10D); the material that was bound and subsequently eluted with 2.0 M guanidine HCl consisted of nearly pure 72,000 dalton fragment (Fig. 10E). Several minor contaminants remained, including fragments of 200,000 and 180,000 daltons, as well as several pieces with molecular weights ranging from approximately 55,000 to 68,000.

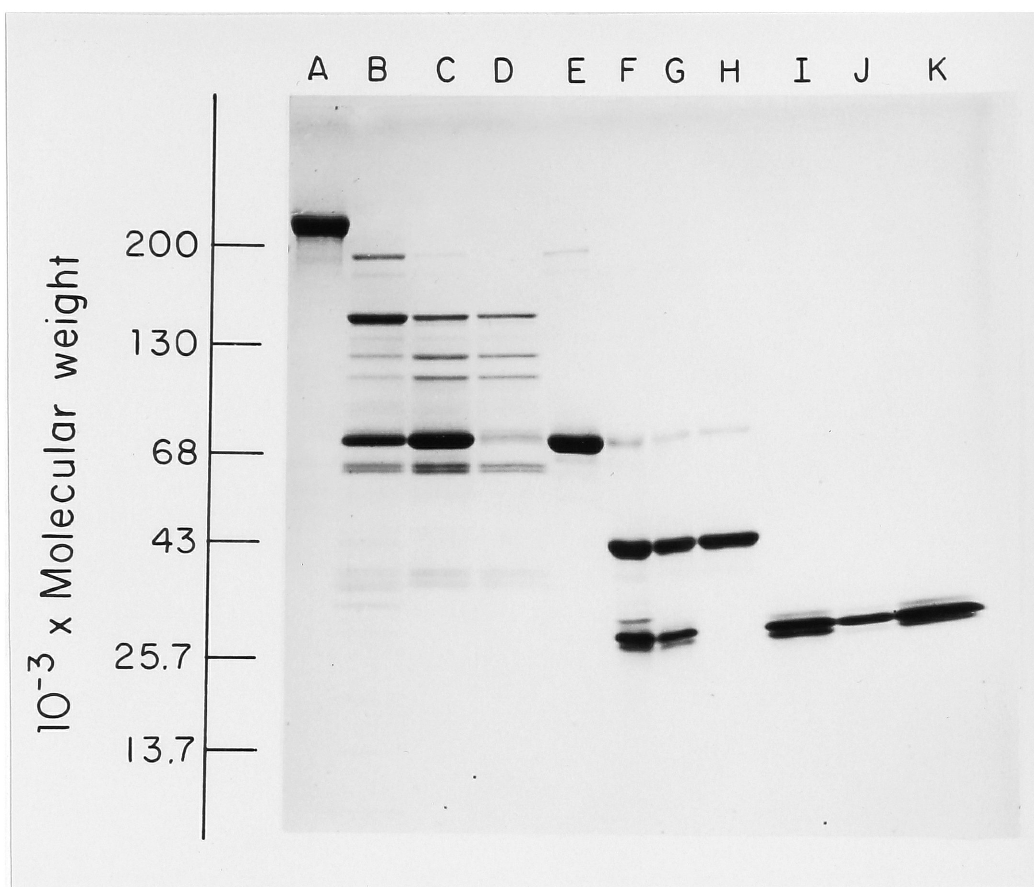

Figure 10. PRODUCTION AND ISOLATION OF GELATIN-BINDING FRAGMENTS OF PLASMA FIBRONECTIN

Samples prepared as described under "Methods" were reduced and applied to a 5 to 16% gradient polyacrylamide gel containing SDS. Sample A, intact fibronectin (7 μg); Sample B, fibronectin digested with cathepsin D (11 μg); Sample C, Sample B following Sephadex G-150 chromatography to remove higher molecular weight components (13 μg); Sample D, fraction of Sample C which did not bind to gelatin (3 μg); Sample E, gelatin-binding fraction of Sample C (8 μg); Sample F, Sample E digested with thrombin (10 μg); Sample G, Sample F following phosphocellulose chromatography to remove thrombin (8 μg); Sample H, gelatin-binding fraction of Sample G (5 μg); Sample I, fraction of Sample G which did not bind to gelatin (5 μg); Sample J, 29 kd fragment isolated from thrombin digests of intact fibronectin (5 μg); Sample K, mixture of Samples I and J (10 μg). The scale indicates the positions of molecular weight standards listed under "Methods".

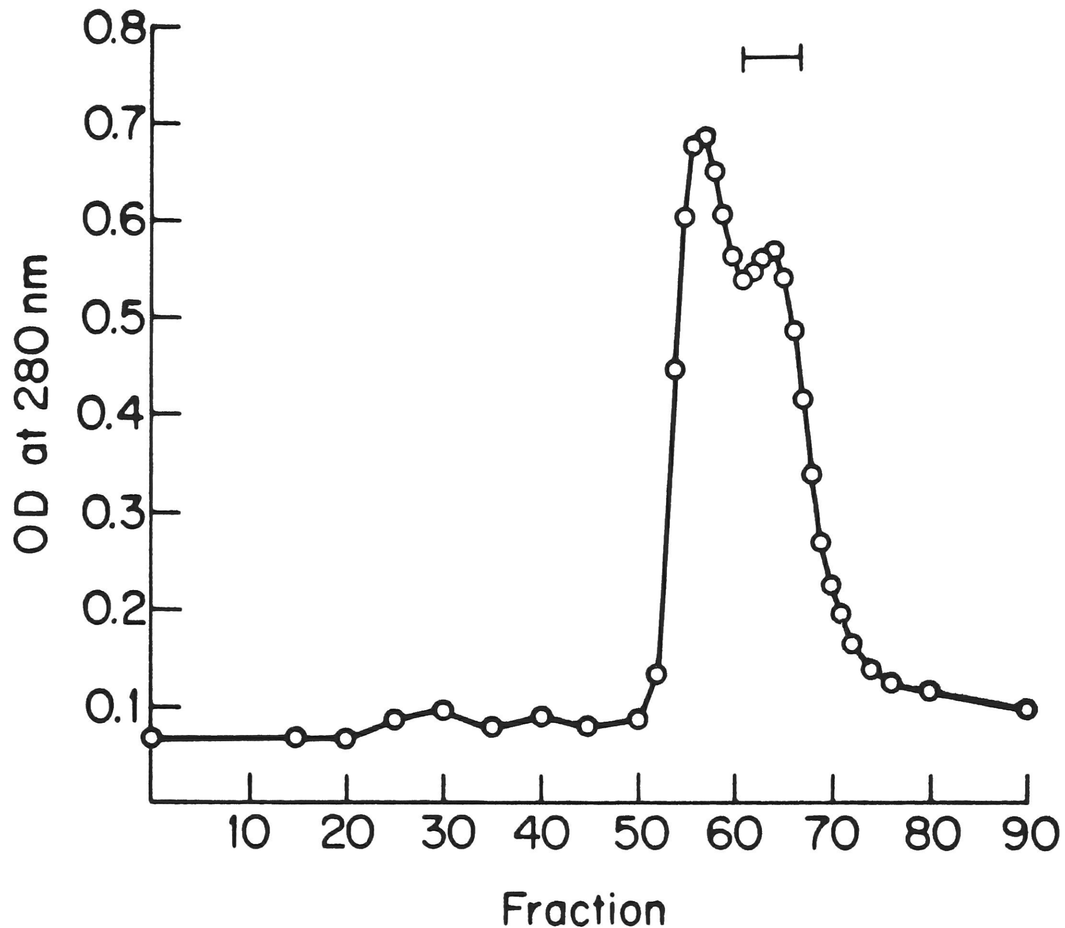

Figure 11. GEL FILTRATION OF PLASMA FIBRONECTIN-CATHEPSIN D DIGEST

Human plasma fibronectin was digested with cathepsin D, added to a column of Sephadex G-150, and eluted. Details of the experimental procedure are given under "Methods". Protein content of the effluent was monitored by its absorption at 280 nm. A 72 kd gelatin-binding fragment was obtained free of higher molecular weight gelatin-binding components by pooling fractions 61 to 67 (indicated by the horizontal bar).

As predicted, treatment of the purified 72,000 dalton fragment with thrombin produced two major fragments which migrated, when reduced, with apparent molecular weights of approximately 43,000 and 29,000 (Fig. 10, F and G). Passage of the digest over a gelatin-Sepharose column demonstrated that the 43,000 dalton fragment retained the ability to bind to gelatin (Fig. 10H), whereas the 29,000 dalton fragment did not (Fig. 10I). The 29,000 dalton fragment prepared in this manner had an electrophoretic mobility identical with that of the 29,000 dalton fragment prepared by thrombin digestion of intact fibronectin (compare Fig. 10I with Fig. 10J; see also Fig. 10K).

Preparations of both the 43,000 dalton and 29,000 dalton fragments contained some impurities. The principal contaminant in samples of the 43,000 dalton fragment consisted of a small amount of undigested 72,000 dalton fragment (Fig. 10H). The 29,000 dalton fragment preparation contained rather large amounts of impurities migrating with molecular weights of 28,000 and 30,000 (Fig. 10I). The 29,000 dalton fragment sample prepared by thrombin digestion of intact fibronectin contained only traces of such contaminants (Fig. 10J).

Results of SDS-polyacrylamide gel electrophoresis in the absence of reducing agent suggest that the 72,000 dalton, 43,000 dalton, and 29,000 dalton fragments all consist of single polypeptide chains. The nonreduced 72,000 dalton and 29,000 dalton fragments migrated more rapidly than in the presence of a reducing agent (compare Fig. 12B with Fig. 12F, and Fig. 12D with Fig. 12H), indicating that they

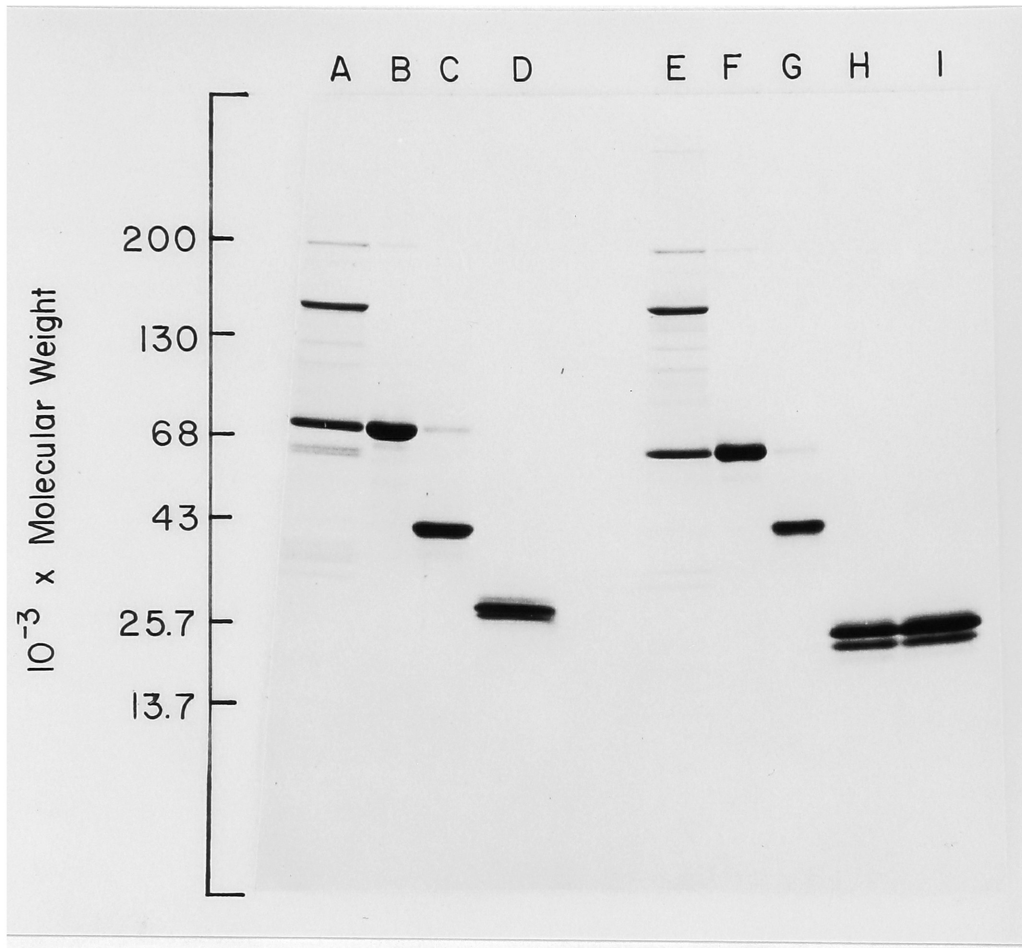

Figure 12. ELECTROPHORETIC MOBILITY OF SOME CATHEPSIN D- AND THROMBIN-DERIVED PLASMA FIBRONECTIN FRAGMENTS UNDER REDUCING AND NONREDUCING CONDITIONS

Human plasma fibronectin was digested with cathepsin D, and a 72 kd gelatin-binding fragment was isolated. The 72 kd fragment was cleaved further with thrombin, and 29 kd and 43 kd fragments were isolated. Details of the experimental procedure are given under "Methods". Samples were applied to a 5 to 16% gradient polyacrylamide gel containing SDS under reducing (A to D) or nonreducing (E to I) conditions. A and E, plasma fibronectin digested with cathepsin D (11 μg); B and F, 72 kd fragment (8 μg); C and G, 43 kd fragment (5 μg); D and H, 29 kd fragment (5 μg); I, 29 kd fragment isolated from thrombin digests of intact fibronectin (5 μg). The scale indicates the positions of molecular weight standards listed under "Methods".

contain intrachain, but not interchain, disulfide bonds. The 43,000 dalton fragment had similar mobilities under both reducing and nonreducing conditions (compare Fig. 12C with Fig. 12G).

NH_2-terminal analysis of the fragments - The 72,000 dalton gelatin-binding fragment and its 43,000 dalton and 29,000 dalton thrombin-generated derivatives were each subjected to NH_2-terminal analysis, using the dansyl chloride technique. No NH_2-terminal amino acids were detected in either the 72,000 dalton or 29,000 dalton fragments, indicating NH_2-terminal blockage. Analysis of the 43,000 dalton fragment, by contrast, revealed an NH_2-terminal alanine residue. Treatment of both the 72,000 dalton and 29,000 dalton fragments with L-pyroglutamyl-peptide hydrolase resulted in the appearance of NH_2-terminal alanine. Neither the 29,000 dalton (not shown) nor the 72,000 dalton (Fig. 8, C and D) fragment appeared degraded following enzyme digestion, as monitored by SDS-polyacrylamide gel electrophoresis.

Since all of the fragment preparations contained some impurities, a quantitative confirmation of these results was obtained as follows. The 72,000 dalton fragment was reduced and alkylated, digested with L-pyroglutamyl-peptide hydrolase, and analyzed on an automated sequencer. The NH_2-terminal sequence obtained, Ala-Glx-Glx-Met-Val-, is identical with that of L-pyroglutamyl-peptide hydrolase-treated intact fibronectin. The yield of alanine at the first step of sequencing was 59%, indicating that the observed sequence was not derived from a minor contaminant.

Summary - Treatment of human plasma fibronectin with cathepsin D produced a gelatin-binding fragment with a molecular weight of 72,000. This fragment could be cleaved further with thrombin to yield a 43,000 dalton fragment which bound to gelatin and a 29,000 dalton fragment which did not. Results of NH_2-terminal analysis of intact fibronectin and the various proteolytically-derived fragments are summarized in Table 1. Both the 72,000 dalton fragment and the 29,000 dalton fragment derived from it by thrombin digestion have the same NH_2-terminal sequence, <Glu-Ala-, as intact fibronectin. In contrast, the 43,000 dalton gelatin-binding fragment has an NH_2-terminal alanine residue. Automated amino acid sequence analysis of the 72,000 dalton piece confirmed that this fragment contains the same NH_2-terminal sequence as intact fibronectin.

The model of human plasma fibronectin that can be constructed from these data is shown in Figure 13. The 72,000 dalton gelatin-binding fragment produced by cathepsin D digestion is located at the NH_2-terminus of the parental molecule. Further cleavage with thrombin yields a 29,000 dalton fragment which is also located at the NH_2-terminus, and a 43,000 dalton fragment which constitutes the carboxyl portion of the 72,000 dalton piece. Since the 43,000 dalton fragment retains the ability to bind to gelatin, one or more gelatin-binding sites of fibronectin must be located within this region.

This model is consistent with the results that were described for thrombin digestion of intact fibronectin. It is likely that thrombin cleaves both the intact molecule and the 72,000 dalton fragment at the same site, since the 29,000 dalton fragments produced in either case

Table 1

NH_2-TERMINAL ANALYSIS OF INTACT PLASMA FIBRONECTIN AND SOME PROTEOLYTICALLY-DERIVED FRAGMENTS

Substrate	Treatment with L-Pyroglutamyl-peptide Hydrolase	NH_2-terminal Residue by Dansylation	NH_2-terminal Sequence on Automated Sequencer
Intact plasma fibronectin	-	None	None
	+	Ala	Ala-Glx-Glx-Met-Val- (42%)[a]
230 and 235 kd thrombin fragments	-	Ala	Ala-Ala-Val-Tyr- (52%)
	+	Not Done	Not Done
29 kd thrombin fragment	-	None	None
	+	Ala	Ala-Glx-Glx-Met-Val- (52%)
72 kd cathepsin D fragment	-	None	Not Done
	+	Ala	Ala-Glx-Glx-Met-Val- (59%)
43 kd cathepsin D/ thrombin fragment	-	Ala	Not Done
	+	Ala	Not Done
29 kd cathepsin D/ thrombin fragment	-	None	Not Done
	+	Ala	Not Done

[a] The values in parentheses represent the yields of alanine at the first step of sequencing, expressed as percentages of the number of mol of protein applied to the sequencer.

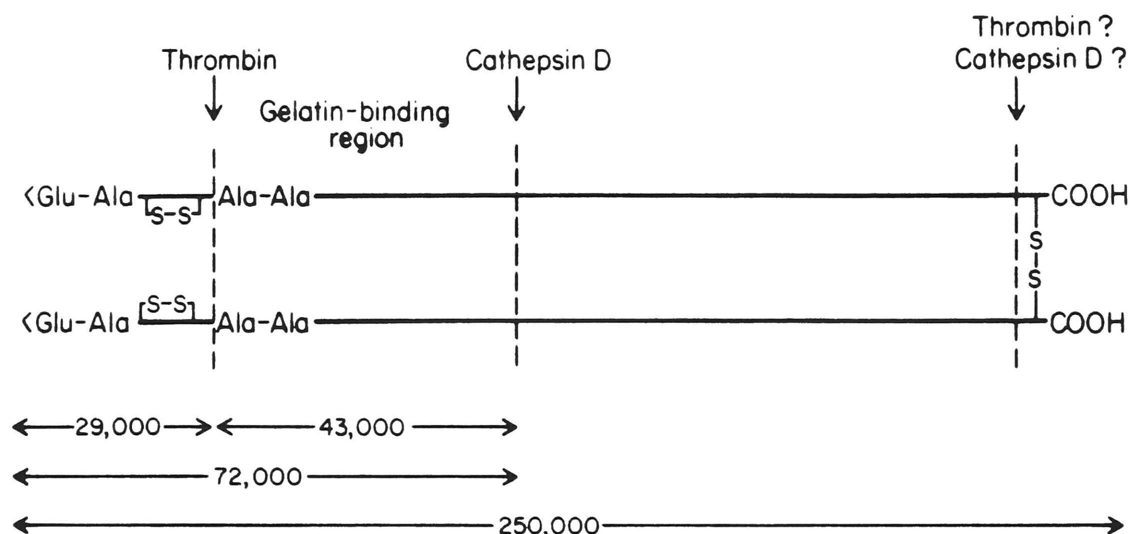

Figure 13. LOCATION OF THE GELATIN-BINDING REGION WITHIN INTACT FIBRONECTIN

Note the relative positions of the 29 kd disulfide-enriched fragment and the 43 kd region which contains one or more gelatin-binding sites. Dashed lines indicate some of the sites of fibronectin that are sensitive to cleavage by thrombin or cathepsin D. Apparent molecular weights of some of the fragments, as determined by SDS-polyacrylamide gel electrophoresis under reducing conditions, are indicated by numbers between horizontal arrows. As discussed in the text, placement of the interchain disulfide bonds near the COOH-terminus is tentative.

have identical electrophoretic mobilities (Fig. 10, I to K). Furthermore, the 230,000 and 235,000 dalton fragments and the 43,000 dalton fragment all bind to gelatin, and all have the same NH_2-terminal residue, alanine. However, the presence of relatively large amounts of contaminants in the 29,000 dalton fragment sample prepared by thrombin digestion of the 72,000 dalton piece (Fig. 10I) indicates that the 72,000 dalton fragment may contain some thrombin-sensitive sites that are not exposed in the intact molecule.

Gold *et al*. (1979) have isolated a 50,000 dalton gelatin-binding fragment from subtilisin digests of plasma fibronectin. The NH_2-terminal sequence of this fragment is Val-Tyr-Gln-Pro-Gln-Pro-His-Pro-Gln-Pro-(Pro)-(Gly)-Tyr-Gly-His-(   )-Val-. It is possible that this sequence overlaps with that of the thrombin-derived 230,000 and 235,000 dalton fragments, Ala-Ala-Val-Tyr-. If so, the peptide bonds cleaved by subtilisin and thrombin are separated by only two amino acid residues.

It should be noted that the 43,000 dalton fragment described above may not represent the only binding region for gelatin that fibronectin contains. It is possible that there are additional sites which are destroyed by protease digestion. However, gelatin-binding fragments have been isolated from fibronectin following treatment with a variety of proteases, including chymotrypsin (Hahn and Yamada, 1979a, 1979b), trypsin (Ruoslahti and Hayman, 1979; Ruoslahti *et al*., 1979), and subtilisin (Gold *et al*., 1979); in each of these cases, only a single binding region was demonstrated.

Analyses similar to those reported herein were not performed on cellular fibronectin. However, Balian *et al*. (1979) have produced a

gelatin-binding fragment by cathepsin D digestion of fibronectin secreted by cultured amniotic fluid cells. This fragment had a slightly higher apparent molecular weight than the 72,000 dalton fragment derived from plasma fibronectin and migrated as a more diffuse band on SDS-polyacrylamide gels (Balian _et al_., 1979). In addition, Hahn and Yamada (1979a) have found that chymotrypsin digestion of both plasma and cellular fibronectins from chicken gave rise to collagen-binding fragments of similar electrophoretic mobilities. It seems likely, then, that cellular fibronectin has a gelatin-binding region comparable to that of plasma fibronectin. Therefore, the model depicted in Figure 13 is probably representative of both forms of the molecule.

Amino Acid Compositions of Intact Plasma Fibronectin and Some Proteolytically-derived Fragments

The amino acid composition of human plasma fibronectin was determined by standard techniques and is given in Table 2. The composition shows no remarkable features and, as seen in Table 2, is quite similar to those obtained by other laboratories (Mosesson *et al.*, 1975; Vuento *et al.*, 1977; Iwanaga *et al.*, 1978; Balian *et al.*, 1979; Gold *et al.*, 1979). There are, however, two exceptions. First, the content of cysteine is higher than previously reported. Cysteine was quantitated following its conversion to cysteic acid by performic acid oxidation of the protein. In the experiments described herein, the oxidation reaction was quenched with HBr (Moore, 1963), a procedure which prevents loss of cysteic acid by further oxidative degradation. None of the other groups reported including such a quenching step, which may account for their comparatively low yields of cysteic acid. Second, the methionine content is lower than others have found; this result will be discussed more fully below.

The amino acid compositions of the 43,000 dalton gelatin-binding fragment and the 29,000 dalton fragment produced by thrombin cleavage of intact fibronectin are given in Tables 3 and 4. When these compositions, expressed as mole percentages of amino acids, are compared with that of intact fibronectin, several differences are apparent (Table 3). The 43,000 dalton fragment is relatively enriched in glycine, cysteine, methionine, phenylalanine, and histidine. The 29,000 dalton fragment is also enriched in glycine, cysteine, and

Table 2

AMINO ACID COMPOSITION OF PLASMA FIBRONECTIN

Amino Acid	Human[a,b] mol/mol[h]	Human[a,b] mol %	Other Investigators: Bovine[c] (mol %)	Human[d]	Human[e]	Human[f]	Human[g]
Aspartic Acid	164	9.4	9.1	9.3	9.2	10.0	10.1
Threonine	195[i]	11.1[i]	9.8	9.7	10.7	10.0	10.4
Serine	142[i]	8.1[i]	8.0	6.8	7.8	8.4	8.6
Glutamic Acid	206	11.8	11.4	11.6	11.8	11.6	11.8
Proline	136	7.8	7.9	7.6	8.0	8.0	7.0
Glycine	148	8.5	9.1	8.0	9.1	8.8	8.3
Alanine	73	4.2	4.6	4.3	4.5	4.5	5.1
Cysteine + Half-Cystine	55[j]	3.1[j]	2.0	2.6	2.2	2.0	2.1
Valine	138	7.9	7.6	8.1	7.8	7.9	6.0
Methionine	11	0.6	1.0	1.1	1.0	1.1	1.1
Isoleucine	79	4.5	4.3	4.4	4.4	4.6	3.1
Leucine	94	5.4	5.6	5.7	5.5	5.7	6.2
Tyrosine	76	4.3	4.0	4.5	4.1	4.8	4.3
Phenylalanine	43	2.5	2.2	2.7	2.5	2.9	2.6
Histidine	37	2.1	1.8	2.1	2.4	2.2	1.8
Lysine	59	3.4	3.9	3.6	3.6	3.8	4.4
Arginine	95	5.4	5.4	5.2	5.4	6.2	4.5

[a] Tryptophan was not determined

[b] Values are means of triplicate analyses

[c] Iwanaga *et al.*, 1978

[d] Calculated from data of Mosesson *et al.*, 1975

[e] Balian *et al.*, 1979

[f] Gold *et al.*, 1979

[g] Vuento *et al.*, 1977

[h] Calculated assuming M_r=250,000

[i] Calculated by extrapolation of values at 24 h, 48 h, and 72 h of hydrolysis to 0 h

[j] Determined as cysteic acid following performic acid oxidation of samples (Moore, 1963)

Table 3

AMINO ACID COMPOSITIONS (mol %) OF INTACT PLASMA FIBRONECTIN AND SOME PROTEOLYTICALLY-DERIVED FRAGMENTS[a]

Amino Acid	Intact Fibronectin[b]	43 kd Cathepsin D/Thrombin Fragment[c]	29 kd Thrombin Fragment[b]
		(mol %)	
Aspartic Acid	9.4	11.4 (+21)[d]	10.0 (+ 6)[d]
Threonine	11.1[e]	9.0[f] (-19)	8.8[e] (-21)
Serine	8.1[e]	5.3[g] (-35)	6.5[e] (-20)
Glutamic Acid	11.8	12.9 (+ 9)	12.3 (+ 4)
Proline	7.8	4.9 (-37)	4.1 (-47)
Glycine	8.5	13.0 (+53)	12.6 (+48)
Alanine	4.2	3.4 (-19)	3.8 (- 9)
Cysteine + Half-Cystine[h]	3.1	7.0 (+126)	8.8 (+184)
Valine	7.9	4.9 (-38)	4.4 (-44)
Methionine	0.6	2.4 (+300)	1.2 (+100)
Isoleucine	4.5	2.2 (-51)	4.1 (- 9)
Leucine	5.4	3.7 (-31)	3.2 (-41)
Tyrosine	4.3	3.3 (-23)	4.7 (+ 9)
Phenylalanine	2.5	4.2 (+68)	1.8 (-28)
Histidine	2.1	4.8 (+129)	2.1 (0)
Lysine	3.4	2.8 (-18)	4.7 (+38)
Arginine	5.4	4.6 (-15)	7.0 (+30)

[a] Tryptophan was not determined

[b] Values are the means of triplicate analyses

[c] Values are the means of duplicate analyses

[d] Values in parentheses indicate the percentage of increase or decrease in amino acid content when compared with the content in intact fibronectin

[e] Determined by extrapolation of values at 24 h, 48 h, and 72 h of hydrolysis to 0 h

[f] Corrected for 5% loss during 20 h hydrolysis (Moore and Stein, 1963)

[g] Corrected for 10% loss during 20 h hydrolysis (Moore and Stein, 1963)

[h] Determined as cysteic acid following performic acid oxidation of samples (Moore, 1963)

Table 4

AMINO ACID COMPOSITIONS (mol/mol of protein) OF INTACT PLASMA FIBRONECTIN AND SOME PROTEOLYTICALLY-DERIVED FRAGMENTS[a]

Amino Acid	Intact Fibronectin[b]	43 kd Cathepsin D/Thrombin Fragment[c] (mol/mol of protein)	29 kd Thrombin Fragment[d]
Aspartic Acid	164	34	17
Threonine	195[e]	27[f]	15[e]
Serine	142[e]	16[g]	11[e]
Glutamic Acid	206	39	21
Proline	136	15	7
Glycine	148	39	21.5
Alanine	73	10	6.5
Cysteine + Half-Cystine[h]	55	21	15
Valine	138	15	7.5
Methionine	11	7	2
Isoleucine	79	7	7
Leucine	94	11	5.5
Tyrosine	76	10	8
Phenylalanine	43	13	3
Histidine	37	14.5	3.5
Lysine	59	8	8
Arginine	95	14	12

[a] Tryptophan was not determined

[b] Values are means of triplicate analyses and are calculated assuming $\underline{M}_r$=250,000

[c] Values are means of duplicate analyses and are calculated assuming $\underline{M}_r$= 43,000

[d] Values are means of triplicate analyses and are calculated assuming $\underline{M}_r$= 29,000

[e] Determined by extrapolation of values at 24 h, 48 h, and 72 h of hydrolysis to 0 h

[f] Corrected for 5% loss during 20 h hydrolysis (Moore and Stein, 1963)

[g] Corrected for 10% loss during 20 h hydrolysis (Moore and Stein, 1963)

[h] Determined as cysteic acid following performic acid oxidation of samples (Moore, 1963)

methionine, with lesser increases in lysine and arginine content. The 43,000 dalton fragment is relatively poor in serine, proline, valine, leucine, and isoleucine, whereas the 29,000 dalton fragment is poor in proline, valine, leucine, and phenylalanine. For the 43,000 dalton fragment, the most striking changes are 2.3-fold enrichments in histidine and cysteine, and a 4-fold increase in methionine content. The 29,000 dalton fragment shows 2.8-fold and 2-fold enrichments in cysteine and methionine, respectively.

Both fragments, then, appear to be greatly enriched in methionine. In fact, the two fragments combined contain almost all of the methionine in the intact molecule (9 of 11 residues) (Table 4). However, a closer examination of the data indicates that this result may be an artifact. Methionine can be quantitated not only directly, but also as the more stable methionine sulfone derivative following performic acid oxidation. In the experiments reported herein, methionine sulfone was eluted from the automated amino acid analyzer column as a shoulder on the aspartic acid peak. By taking the difference in areas under aspartic acid peaks from comparable oxidized and untreated protein samples, an estimate of the number of nanomoles of methionine sulfone can be obtained. In this way, it was estimated that the intact molecule contains about 35 residues of methionine per subunit chain. The 43,000 dalton and 29,000 dalton fragments contain approximately 9 residues and 1 residue, respectively. For the fragments, these estimates of methionine content are about the same as obtained directly by analysis of unoxidized protein samples. For the intact molecule, however,

there is a 3-fold difference in the two sets of figures (35 residues compared with 11 residues). Apparently, there was a variable loss of methionine upon acid hydrolysis which was proportionately greater for the intact molecule than for the fragments. Therefore, the fragments are probably not enriched in methionine.

Both the 29,000 dalton and 43,000 dalton fragments contain proportionately more cysteine than intact plasma fibronectin. Balian et al. (1979) have reported that the 72,000 dalton cathepsin D-generated fragment contains nearly all of the cysteine in the human plasma fibronectin molecule. The data in Table 4, on the other hand, indicate that the 43,000 dalton and 29,000 dalton fragments together contain only about 65% of the total cysteine (36 of 55 residues). However, these calculations are based on data expressed as moles of amino acid per mole of polypeptide chain; as such, they reflect errors both in the measurement of amounts of protein analyzed and in the estimation of molecular weights of the proteins by SDS-polyacrylamide gel electrophoresis. Therefore, although it is clear that the fragments are enriched in cysteine, the fraction of the total cysteine found in fibronectin that they contain is uncertain. Gold et al. (1979) have reported similar enrichments in cysteine for 30,000 dalton and 50,000 dalton gelatin-binding fragments produced by subtilisin digestion of human plasma fibronectin. Two fragments that are probably comparable to the 29,000 dalton thrombin-derived fragment are also cysteine-enriched: a 29,000 dalton fragment produced by plasmin cleavage of bovine plasma fibronectin (Iwanaga et al., 1978), and a 25,000 dalton tryptic fragment of hamster cellular fibronectin (Wagner and Hynes, 1979).

The complete amino acid compositions of some of these fragments are listed in Table 5.

Wagner and Hynes (1979) have demonstrated that cellular and plasma fibronectins have only one or, at most, two free sulfhydryl groups on each subunit chain; all other cysteine residues are involved in disulfide linkages. Therefore, the 43,000 dalton and 29,000 dalton fragments must contain a large number of intrachain disulfide bonds. As previously discussed, the presence of such bonds in the 29,000 dalton fragment is also indicated by its atypical migration in SDS-polyacrylamide gels under nonreducing conditions. Although the 43,000 dalton fragment is almost as rich in cysteine as the 29,000 dalton piece (7.0 mol % compared with 8.8 mol %), it has similar electrophoretic mobilities in both the presence and absence of a reducing agent. Presumably, the nonreduced 43,000 dalton fragment can assume a more extended conformation in the presence of SDS than the nonreduced 29,000 dalton fragment can.

Table 5

AMINO ACID COMPOSITIONS OF SOME PROTEOLYTICALLY-DERIVED PLASMA FIBRONECTIN FRAGMENTS ISOLATED BY OTHER INVESTIGATORS

Amino Acid	29 kd Plasmin Fragment[a] (mol %)	Gelatin-binding fragments (mol %): 72 kd Cathepsin D Fragment[b]	50 kd Subtilisin Fragment[c]	30 kd Subtilisin Fragment[c]
Aspartic Acid	10.0	11.1	13.0	13.7
Threonine	8.9	8.7	7.7	12.2
Serine	6.6	5.2	5.6	4.8
Glutamic Acid	12.8	13.6	13.6	12.6
Proline	4.4	4.6	4.4	4.1
Glycine	12.8	11.6	12.0	14.8
Alanine	4.2	3.2	3.5	2.9
Cysteine + Half-Cystine	5.0	7.8	5.3	6.2
Valine	4.1	5.1	6.1	5.7
Methionine	1.9	2.5	2.3	2.8
Isoleucine	4.4	3.0	2.4	1.5
Leucine	4.2	3.1	4.8	4.3
Tyrosine	5.0	5.6	6.1	4.1
Phenylalanine	2.1	3.0	3.3	3.2
Histidine	2.0	3.6	4.0	3.8
Lysine	4.9	3.1	3.6	2.8
Arginine	6.7	5.0	4.0	3.8

[a] Prepared from bovine plasma fibronectin by Iwanaga _et al._, 1978

[b] Prepared from human plasma fibronectin by Balian _et al._, 1979

[c] Prepared from human plasma fibronectin by Gold _et al._, 1979

A Possible Model of Fibronectin Structure

The results presented in this thesis determine the positions of several proteolytic fragments within the intact plasma fibronectin molecule. These data can be combined with results of other investigators to construct a more detailed model of fibronectin.

Engvall et al. (1978) have investigated the ability of various macromolecules to inhibit binding of fibronectin to gelatin. Collagen and fibrinogen were able to interfere with this interaction, suggesting that they bind to the same site on fibronectin as gelatin does. Therefore, these molecules must also attach to fibronectin within the 43,000 dalton region.

Mosher et al. (1980) have used plasma transglutaminase (factor $XIII_a$) to label human plasma fibronectin with a fluorescent substrate, dansylcadaverine. The labeled molecule was then treated with various proteases, and the digests were electrophoresed on SDS-polyacrylamide gels to identify fragments containing the dansyl group. Following cleavage of labeled fibronectin with thrombin, fluorescence was seen only in the small fragment. Similar results have been obtained by Jilek and Hörmann (1977b), using trypsin digestion of fibronectin labeled with (^{14}C)putrescine and plasma transglutaminase. The transglutaminase-sensitive glutamine residue of fibronectin must therefore be located within the 29,000 dalton NH_2-terminal region. As previously discussed, plasma transglutaminase covalently cross-links plasma fibronectin to collagen (Mosher et al., 1979), fibrin (Mosher, 1975), and other plasma fibronectin molecules (Mosher, 1975); cellular fibronectin can be cross-linked to high molecular weight cell surface complexes (Keski-Oja

et al., 1976). It is noteworthy that covalent binding of fibrinogen and collagen to fibronectin takes place within the 29,000 dalton NH_2-terminal peptide, but that noncovalent binding occurs within the adjacent 43,000 dalton region. Mosher (1980) has speculated that noncovalent interactions between these molecules and fibronectin may be required to bring transglutaminase-reactive residues into apposition.

Ruoslahti and Hayman (1979) have produced a group of gelatin-binding fragments with molecular weights of approximately 70,000 by trypsin digestion of human plasma fibronectin. More extensive digestion yielded a 30,000 dalton gelatin-binding piece. These gelatin-binding fragments did not promote attachment and spreading of cells on plastic dishes, whereas fragments which lacked the ability to bind to gelatin retained this activity. These results suggest that gelatin-binding and cell-attachment sites are located in different regions of fibronectin. Results of experiments by Hahn and Yamada (1979b) lead to a similar conclusion and, furthermore, allow the mapping of a cell attachment site. Hahn and Yamada have isolated 160,000 dalton and 40,000 dalton fragments from chymotryptic digests of chicken cellular fibronectin. The 160,000 dalton piece did not bind to gelatin, but was able to promote hemagglutination and cell spreading. In contrast, the 40,000 dalton fragment bound to gelatin, but was inactive in the other two assays. The 160,000 dalton fragment inhibited fibronectin-mediated cell attachment to collagen when preincubated with the cells, but not when preincubated with the collagen-coated substrate. Conversely, the 40,000 dalton fragment

inhibited cell attachment only when preincubated with the collagen. It appears, then, that the 160,000 dalton fragment binds to cells but not to gelatin, whereas the 40,000 dalton gelatin-binding fragment has no cell-binding activity. Since it has been established that the 43,000 dalton gelatin-binding region is separated from the NH_2-terminus of intact fibronectin by a peptide of only 29,000 daltons, the 160,000 dalton fragment must be derived from a region located to the carboxyl side of the gelatin-binding site. It is likely, therefore, that a cell surface-binding site of fibronectin is located farther from the NH_2-terminus of the molecule than is the gelatin-binding site.

Gold et al. (1979) have isolated a 50,000 dalton subtilisin-generated fragment of plasma fibronectin which both binds to gelatin and promotes attachment of fibroblasts to collagen. As previously discussed, NH_2-terminal analysis of the 50,000 dalton and 43,000 dalton gelatin-binding fragments suggests that they may be generated by cleavage of peptide bonds separated by only two amino acid residues. If so, it follows that a cell attachment site must be located either within the 43,000 dalton gelatin-binding region or within a 7,000 dalton region adjacent to its carboxyl end.

Heparin does not inhibit binding of fibronectin to gelatin, indicating that heparin and gelatin interact with different sites on the molecule (Engvall and Ruoslahti, 1977). In accordance with this finding, Yamada et al. (1980) have discovered that only the 160,000 dalton chymotryptic cellular fibronectin fragment binds to heparin-Sepharose. In a preliminary experiment, both the small and the large thrombin-generated fragments were bound by heparin-Sepharose.

Further experimentation will be required to resolve this discrepancy.

Wagner and Hynes (1979) have labeled hamster cellular fibronectin with (^{3}H)glucosamine. Subsequent digestion with trypsin produced an unlabeled, disulfide-enriched 25,000 dalton fragment and a labeled 200,000 dalton fragment. Since this 25,000 dalton fragment is probably comparable to the 29,000 dalton thrombin-generated fragment, it appears that none of the carbohydrate chains of fibronectin is located in the NH_2-terminal region. Takasaki et al. (1979) have shown that bovine plasma fibronectin contains an average of three asparagine-linked sugar chains per subunit; hamster cellular fibronectin contains five or six chains per subunit (Carter and Hakomori, 1979). However, the distribution of these chains between the subunits and among the various proteolytically-derived fragments is not known. The gelatin-binding fragments isolated by Ruoslahti et al. (1979) are probably glycosylated, since they were bound by concanavalin A-Sepharose. Preliminary reports of experiments involving proteolytic cleavage of fibronectin labeled with radioactive sugars also indicate that the gelatin-binding region contains at least one carbohydrate chain (Balian et al., 1979; Hahn and Yamada, 1979a).

Wagner and Hynes (1980) have determined, using DTNB, that plasma and cellular fibronectins contain one or two cysteine residues in each subunit chain. As previously described, treatment of cellular fibronectin with DTNB and subsequent cleavage at modified cysteine residues with (^{14}C)cyanide yielded fragments of 170,000

daltons and 33,000 daltons (Wagner and Hynes, 1980). The 170,000 dalton fragment was not labeled with ^{14}C, and is therefore located at the NH_2-terminal end of the intact molecule. Thus, a sulfhydryl group is located immediately adjacent to the carboxyl end of the 170,000 dalton region, approximately two-thirds of the linear distance from the NH_2-terminus of intact fibronectin. Since the 170,000 dalton and 33,000 dalton fragments do not account for the entire fibronectin molecule, Wagner and Hynes (1980) have suggested that there may be a second cysteine residue, or perhaps a particularly labile cystine group, located somewhat farther from the NH_2-terminus. Cleavage at this site would give rise to the 33,000 dalton fragment, as well as another peptide which was not detected in these experiments.

Treatment of cellular fibronectin with N-ethylmaleimide or iodoacetic acid, in the absence of a reducing agent, destroys its ability to bind to monolayers of hamster cells (Wagner and Hynes, 1979), suggesting that the sulfhydryl groups play a role in interaction with cell surface components. However, as mentioned, analysis of a subtilisin fragment of plasma fibronectin (Gold et al., 1979) indicates that a site which interacts with cells to promote their attachment to collagen is located closer to the NH_2-terminus, perhaps within the 43,000 dalton gelatin-binding region or an adjacent 7,000 dalton region. It may be, then, that cells interact with different sites on the fibronectin molecule in these two assays. However, it should be noted that the fragment described by Gold et al. (1979) is much less active than intact fibronectin in promoting cell attachment. In contrast, the 160,000 dalton fragment described by Hahn and Yamada

(1979b), which must contain at least one of the sulfhydryl groups, is nearly as active as the whole molecule when tested in two different cell adhesion assays.

The results of this thesis research thus allow the positioning of several sites of biological interest within the fibronectin molecule, as summarized in Figure 14. Although some findings have not been confirmed for both cellular and plasma fibronectins, all available evidence indicates that the two forms are quite similar with respect to the structural features that have been discussed. Clearly, many important questions regarding the structure of fibronectin remain unanswered. For instance, the location of the interchain disulfide bonds has not been demonstrated conclusively, and the structural basis for the functional differences between cellular and plasma fibronectins is still uncertain. Studies involving further fragmentation of fibronectin may answer these and similar questions and will lead to more detailed models of the molecule. Such models will serve as a basis for further experimentation, and may eventually contribute to a better understanding of how plasma and cellular fibronectins act *in vivo* as bridges between macromolecules and between cells and their environment.

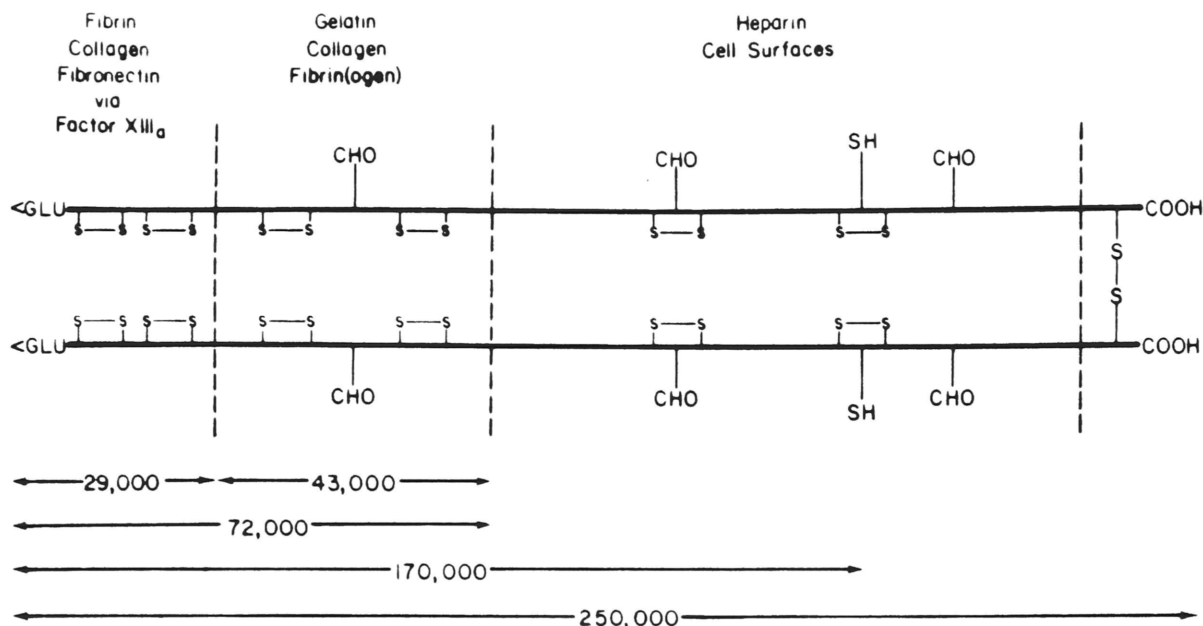

Figure 14. A POSSIBLE MODEL OF FIBRONECTIN STRUCTURE

As discussed in detail in the text, results of this thesis research were combined with those of experiments by others to determine the approximate locations of several features of interest within the intact fibronectin molecule, including binding sites for other macromolecules and cell surfaces. Dashed lines denote approximate sites of cleavage by various proteases; approximate molecular weights of some fragments so produced are indicated by numbers between horizontal arrows. The 29 kd and 43 kd regions are enriched in intrachain disulfide bonds, but the numbers and locations of these bonds within each fragment are not known. The assignment of the interchain disulfide bonds to a COOH-terminal region is tentative. A second cysteine residue may be located to the carboxyl side of the residue which is depicted. Plasma fibronectin contains an average of 3 carbohydrate chains per subunit; cellular fibronectin may contain 5 or 6 chains per subunit. The 29 kd region probably contains no carbohydrate, whereas the 43 kd region probably contains at least one chain. The distribution of carbohydrate chains between the subunits and among the various fragments is otherwise unknown. Binding sites for heparin and cell surfaces are located to the carboxyl side of the gelatin-binding site, but the possibility that they lie within the 43 kd region has not been ruled out. There is some evidence that suggests that there are multiple binding sites for heparin and cell surfaces; another heparin-binding site may be located within the 29 kd region.

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