AFFINITY OF FIBRONECTIN TO COLLAGENS OF DIFFERENT GENETIC TYPES AND TO FIBRINOGEN*

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Fibronectin is a major cell surface protein of normal fibroblasts (1–4) but is absent from the surface of transformed fibroblasts (2, 5–8). Fibronectin is also present in plasma (1, 9). It has affinity to fibrinogen and fibrin and is identical with cold insoluble globulin (10–12). A recent comparison of fibronectins derived from human plasma and fibroblasts by analysis of polypeptide chains, peptide maps, and amino acid and carbohydrate compositions, strongly suggests that they are the same protein (13). Fibronectin is present in the fibroblasts of different species as immunologically cross-reactive but not identical components (14). Recent results from our laboratory show that human fibronectin, whether derived from cultured fibroblasts or plasma, binds to collagen (15).

During the past several years it has become clear that collagen exists as several distinct types determined by independent structural genes (16, 17). Type I collagen is the most prevalent form of collagen in the mature vertebrate organism and occurs in the arterial walls, bone, dentin, dermis, tendon, and uterine wall. Type II is almost exclusively limited to hyaline cartilages. Type III collagen has a distribution pattern similar to that of type I, but is more prevalent in the distensible soft connective tissue. Recently, additional collagen chains, provisionally designated as the A chain and B chain, have been isolated from extracts of several tissues (18, 19), and it has been suggested that molecules comprising these chains may originate in certain basement membrane structures.

In view of the possible importance of the fibronectin-collagen interaction to the structure of the various connective tissues, we decided to investigate the affinity of fibronectin to the various genetic types of collagen. We show here that all types of collagen tested are active in fibronectin binding, but to a different degree. Further, we demonstrate that the collagen binding activity is shared by fibronectins of a wide variety of species. We also present evidence that the binding to collagen and fibrinogen is mediated by the same binding site in fibronectin.

Materials and Methods

Blood was collected in sodium citrate, and plasma was separated by centrifugation at room temperature. Fresh plasma was used for the purification, or when this was not possible, the plasma was stored frozen at −20°C. Chicken plasma was obtained from Pel-Freez Farms, Inc.,

* Supported by grants CA 22108 and CA 16434 from the National Cancer Institute, Department of Health, Education, and Welfare.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/78/0601-1584$1.00

(Rogers, Ark.) and plasma from *Torpedo californica* from Pacific Bio-Marine Laboratories, Inc. (Venice, Calif.).

Confluent cultures of chicken fibroblasts were kindly provided by Dr. J. Strauss, California Institute of Technology. The cultures were washed three times with phosphate-buffered saline (PBS)[1] and extracted with 8 M urea, 0.05 M Tris-HCl, pH 7.0, containing 10^{-4} M phenylmethanesulphonyl fluoride (PMSF). Five roller bottles were extracted twice with a total of 100 ml of the urea solution. The extracts were combined, centrifuged, and stored frozen. Gelatin (swine skin, 300 Bloom), poly-L-proline (mol wt 10,000–30,000), and cyanogen bromide activated Sepharose were from Sigma Chemical Co. (St. Louis, Mo.), and keratin from ICN, K & K Laboratories, Inc., (Plainview, N. Y.). Pig tropoelastin was a gift from Dr. W. Gray, University of Utah, and a linear polymer of the tripeptide pro-gly-pro was provided by Dr. S. Fuchs, the Weizmann Institute (Rehovot, Israel). Fibrinogen was obtained from Kabi AB (Stockholm, Sweden) and was purified further to remove contaminating fibronectin as described (10).

Purification of Fibronectin. Plasma fibronectin was purified essentially as described earlier (15) using gelatin insolubilized on Sepharose. The modifications were that the plasma sample was passed through a column of plain Sepharose 4B before fractionation on gelatin-Sepharose to remove material binding to Sepharose. The amount of Sepharose was twice the volume of the plasma sample. The gelatin-Sepharose column was washed with PBS containing 10^{-4} PMSF and 1 M urea in 0.05 M Tris buffer, pH 7.5, and the fibronectin was eluted with 4 M urea in 0.05 M Tris buffer. Urea extracts of fibroblasts were diluted 20 times with PBS, and 1 ml of gelatin-Sepharose was added per 200 ml of solution. After incubation for 16 h at 4°C, the particles were packed in a column, washed, and eluted as described above for plasma fibronectin.

Purification of the Collagens. Type I and III collagens were prepared from human placenta after solubilization by limited digestion with pepsin and fractionation by differential salt precipitation at neutral pH as described previously (20). Briefly, these collagens were precipitated from the initial pepsin digest by adding NaCl to a concentration of 0.7 M. The precipitate was collected, redissolved in 1.0 M NaCl, pH 7.5 (0.05 M Tris), and type III collagen was selectively precipitated by increasing the NaCl concentration to 1.5 M. Type I collagen was similarly precipitated at 2.5 M NaCl. After the initial precipitation of type III and I collagens at 1.5 and 2.5 M NaCl, respectively, the precipitation from neutral salt solutions was repeated three times for each collagen.

Collagen molecules comprising the A and B chains were isolated from the supernatant solution of the original pepsin digest after precipitation of the type I and III collagens (18). For this purpose, the NaCl concentration was raised from 0.7 to 1.2 M. The resulting precipitate was collected and redissolved in 1.0 M NaCl, pH 7.5 (0.05 M Tris). The collagen was then selectively precipitated from the latter solution at 4.5 M NaCl (19). The precipitation from the neutral salt solution was repeated three times.

Type II collagen was prepared from infant articular cartilage. This collagen was likewise solubilized by limited digestion with pepsin and purified as previously described (21). The solubilized collagen was precipitated from the pepsin digest by the addition of NaCl to a concentration of 0.9 M. The precipitate was redissolved in 1.0 M NaCl, pH 7.5 (0.05 M Tris), and the collagen was selectively precipitated from this solution by increasing the NaCl concentration to 3.2 M. The latter precipitation was repeated three times.

All of the collagens purified for the present study were ultimately redissolved in 0.5 M acetic acid, dialyzed extensively against the solvent, and lyophilized.

The cyanogen bromide peptides, α1(II)-CB8 and α1(II)-CB12, were isolated from the cleavage products of human α1(II) chains as previously described (22).

Before testing, collagens and peptides were dissolved in 0.2% acetic acid. Denaturation of collagens was obtained by warming to 60°C for 30 min followed by rapid cooling in ice.

Antisera. Antisera were prepared against human and chicken plasma fibronectins. The latter were purified by the gelatin-Sepharose method described above. The antisera were adsorbed with Sepharose-conjugated plasma proteins obtained from plasma passed through gelatin-Sepharose

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[1] *Abbreviations used in this paper:* CAF, cell attachment factor; CAP, cell attachment protein; ELISA, enzyme-linked immunosorbent assays; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate.

(and devoid of fibronectin), bovine plasma proteins (including fibronectin) conjugated to Sepharose, and with gelatin-Sepharose. Antibodies to fibronectin were isolated from the absorbed antisera by absorption to Sepharose-conjugated fibronectin followed by elution with 8 M urea.

Assays for Fibronectin and Collagen Binding. Quantitation of fibronectin was performed by radioimmunoassay as detailed elsewhere (23). Tests for binding and inhibition of binding of fibronectin to various substances were performed using enzyme-linked immunosorbent assays (ELISA) (15, 24) performed in polystyrene microtiter plates (ELISA plates, Cooke Laboratory Products Div., Dynatech Laboratories Inc., Alexandria, Va.). For binding assays, the wells were coated by incubation at room temperature with solutions containing 1 μg/ml of various proteins. Purified fibronectin was incubated in washed, coated wells for 6 h. After washing of the plate, bound fibronectin was quantitated by incubation overnight with excess enzyme-labeled, purified antibodies to fibronectin.

For inhibition assays, the microtiter plate wells were coated with 0.5 μg/ml of gelatin. Human or chicken fibronectin, 0.5 μg/ml, with or without various concentrations of inhibitor was incubated in the wells, and the amount of fibronectin bound to the wells was detected with enzyme conjugated antibodies as described above. All incubations were performed at 4°C. Enzyme activities were measured at room temperature.

Gel electrophoresis was performed in cylindrical (25) gels in the presence of sodium dodecyl sulphate (SDS). Protein was determined according to Lowry et al. (26).

Results

Purification of Fibronectins. We have previously shown that fibronectin from human plasma and spent culture media of human fibroblasts can be isolated on gelatin-Sepharose (15). Similar binding to gelatin-Sepharose was found with fibronectins from chicken plasma and fibroblast extracts. Both preparations showed one major band in electrophoresis (Fig. 1). A slight difference was repeatedly found in the mobilities of the fibronectins from the two sources. The fibroblast fibronectin was slightly slower. A protein with a mobility similar to that of mammalian and chicken fibronectins in SDS gel electrophoresis was obtained from plasma of Torpedo fish (*Torpedo californica*). When run in SDS gel electrophoresis in the absence of reducing agent, it also behaved like fibronectin, giving a band with an apparent molecular weight of about twice the size of the subunit. The yield of this protein, tentatively identified as fibronectin, was comparable to that obtained with mammalian fibronectins.

Binding of Fibronectin to Collagen, Fibrinogen, and Other Proteins and Glycoproteins. Our recent results have established that fibronectin exhibits an affinity for collagen and gelatin (15). The binding of fibronectin to collagen was further characterized using different types of human collagen. It has also been shown that fibronectin has affinity to fibrinogen and fibrin (10). Since fibrinogen and collagen do not have much in common, it was important to compare these binding activities and to test other proteins for binding of fibronectin.

For assays of binding of fibronectin, microtiter plates were coated with various proteins. All four preparations of collagen were found to bind fibronectin. The denatured forms of the collagens had a higher binding capacity than the native collagens. Of the latter, type III collagen was the most efficient (Fig. 2). Fibrinogen bound much less fibronectin than the collagens, and other proteins tested did not show significant binding in this assay. The binding of fibronectin to fibrinogen as well as to collagens was completely inhibited by gelatin, but not with ovalbumin used as a control. Furthermore, the binding of

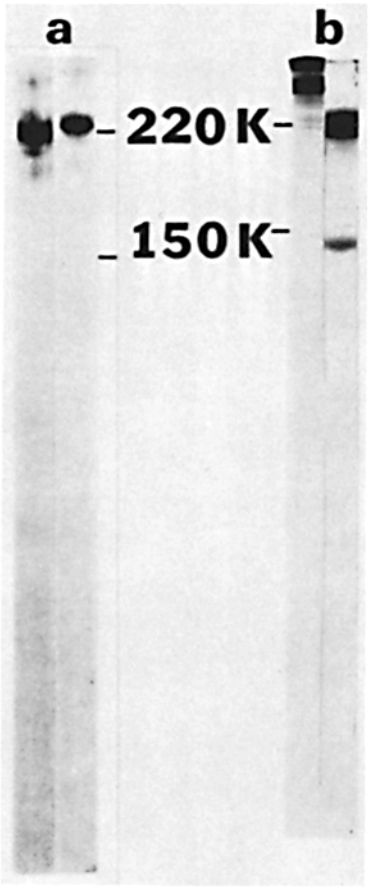

FIG. 1. Gel electrophoresis in the presence of SDS using cylindrical gels with 5% polyacrylamide. a. Chicken plasma fibronectin (left) and chicken fibroblast fibronectin (right). Samples were reduced with 2% 2-mercaptoethanol. b. Fibronectin from *Torpedo californica* plasma. The sample in the left gel was run without reduction, and that in the right gel was reduced. The positions of unreduced IgG (mol wt 150,000) and reduced human plasma fibronectin (mol wt 220,000) are shown.

fibronectin to fibrinogen was inhibited by lower concentrations of gelatin than of fibrinogen itself (not shown).

Lack of Self-Aggregation of Fibronectin. We have noticed that purified fibronectin has a tendency to aggregate in concentrated solutions. Preparations of purified fibronectin eluted from the gelatin-Sepharose columns in concentrations of 1–3 mg/ml have often formed a gel-like precipitate upon freezing and storing, even in the presence of 8 M urea. This prompted us to examine fibronectin for self-associating properties. Fibronectin was coupled to Sepharose, and the binding of ^{125}I-labeled fibronectin to the particles was studied. The radioactivity bound to fibronectin-Sepharose was the same (6%) as binding to particles to which albumin was coupled, while gelatin-Sepharose bound more than 90% of the labeled protein.

Avidity of Binding of Fibronectin to Collagens and Fibrinogen. The binding

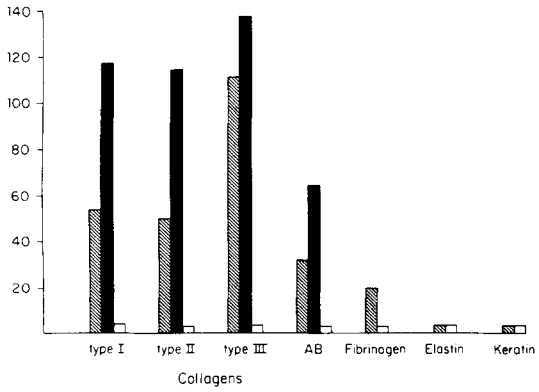

FIG. 2. Binding of fibronectin to microtiter plate wells coated with different proteins as detected by enzyme labeled antifibronectin. Results are expressed as relative enzyme activities (%) compared to that obtained when the wells were coated with gelatin (100%). Hatched bars, binding to wells coated with native proteins, solid bars, binding to wells coated with heat denatured proteins, open bars, binding in presence of 100 μg/ml gelatin.

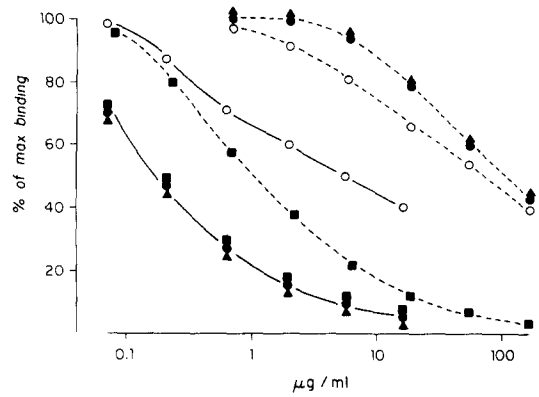

FIG. 3. Inhibition of binding of human fibronectin to microtiter plates coated with gelatin by collagen type I (●), type II (▲), type III (■), and AB chains (○). Hatched lines, native proteins. Solid lines, heat denatured proteins.

of fibronectin to the different collagens was further characterized by testing the capacity of the collagens to inhibit binding of fibronectin to gelatin. Of the native collagens, type III was the most efficient inhibitor (Fig. 3). Heat denaturation of the collagens abolished the differences between the activity of type I, II, and III collagens, all of which became much more efficient inhibitors. Denaturation had less effect on molecules composed of A and B chains, which remained less active than denatured type I, II, and III collagens.

Chicken fibronectin showed a similar preference for denatured collagens and native type III collagen. A slight difference between the assay patterns obtained with chicken and human fibronectins were found, but chicken fibronectins derived from plasma, and fibroblasts, respectively, showed identical specificities (Table I).

Human fibrinogen had some capacity to inhibit fibronectin-gelatin interac-

TABLE I

Inhibition of Binding of Chicken Fibronectin, Isolated from Plasma or Fibroblasts, to Gelatin-Coated Microtiter Plate Wells by Human Collagens

	μg/ml of collagen required for 50% inhibition of	
	Plasma fibronectin	Fibroblast fibronectin
Native collagen type		
I	300	280
II	>100	>100
III	7.5	7.5
AB	>200	>200
Denatured collagen type		
I	1.5	1.7
II	0.8	1.1
III	1.3	1.6
AB	130	150

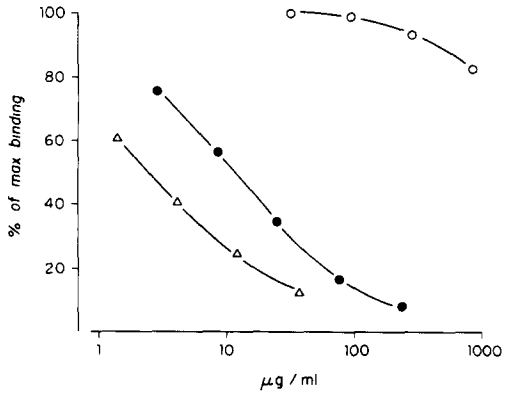

FIG. 4. Inhibition of binding of human fibronectin to gelatin coated plates by fibrinogen (○), *Torpedo californica* fibronectin (●), and rabbit fibronectin (△).

tion at high concentrations (Fig. 4). Serum albumin, ovalbumin, transferrin, and IgG did not inhibit when similarly tested. Fig. 4 also shows a comparison of the gelatin-binding activity of Torpedo fibronectin with rabbit fibronectin. Torpedo fibronectin competed efficiently with human fibronectin for binding to gelatin but was somewhat less active than rabbit fibronectin. The latter was used for comparison because rabbit antibodies to human fibronectin were used to detect the binding, and such antibodies do not react with rabbit (or Torpedo) fibronectin (14, 27). Torpedo fibronectin was also able to inhibit the binding of chicken fibronectin to gelatin and was in this assay more efficient than a preparation of bovine fibronectin (not shown).

Localization of Fibronectin Binding in Collagen Polypeptide Chains. To study the question whether fibronectin binds to a single site or multiple sites on

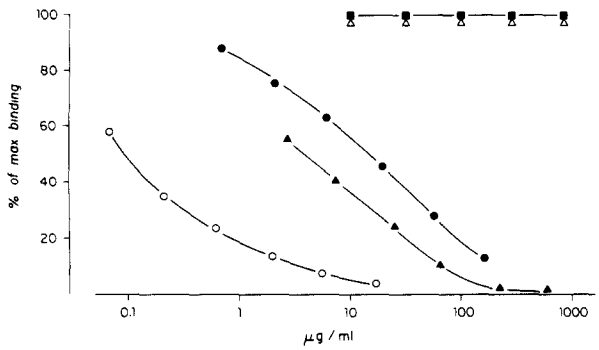

FIG. 5. Inhibition of binding of human fibronectin to gelatin-coated microtiter plate wells by denatured type II collagen (○) and two cyanogen bromide fragments of α1(II) chains, CB8 (●), and CB12 (▲). The synthetic polymer of pro-gly-pro (△), as well as polyproline (■), do not inhibit.

TABLE II

Binding of Fibronectin to Fibrin Clot in the Presence and Absence of Gelatin

	Sample	Temperature	Gelatin	Fibronectin	
				μg/ml	%
Exp. 1	Plasma	+4°C	–	270	100
	Serum	+4°C	–	10	4
	Serum	+4°C	2 mg/ml	110	40
	Serum	+22°C	–	115	42
	Serum	+22°C	2 mg/ml	205	75
Exp. 2	Plasma	+4°C	–	330	100
	Serum	+4°C	–	17	5
	Serum	+4°C	2 mg/ml	218	66
	Serum	+37°C	–	172	52
	Serum	+37°C	2 mg/ml	135	41

the collagen chains, cyanogen bromide peptides from different parts of the α1(II) chain were analyzed in the inhibition assay. Peptides CB8 (13,000 daltons) and CB12 (8,000 daltons) were both able to inhibit completely the binding of fibronectin to gelatin (Fig. 5). Both peptides were more active than native type II collagen but less active than denatured type II collagen, which yields intact α1(II) chains. The smaller of the two peptides, CB12, was more active than the larger, CB8. A polymer of a synthetic tripeptide pro-gly-pro, which has a secondary structure similar to collagen and has been shown to elicit an immune response against collagen (28), did not inhibit the fibronectin-gelatin interaction in concentrations up to 1 mg/ml. Polyproline was also inactive.

Effect of Gelatin on Incorporation of Fibronectin in Blood Clot. Clotting of blood in the cold results in binding of fibronectin to the fibrin clot (10). The addition of gelatin to blood before coagulation partially prevented the incorporation of fibronectin into the blood clot (Table II). Gelatin did not prevent or

slow down coagulation, but rather encouraged it, and no fibrinogen could be detected in serum from gelatin containing plasma as tested by immunodiffusion against antifibrinogen serum.

Discussion

We have previously demonstrated an affinity of the soluble form of human fibronectin from fibroblasts and plasma for collagen and gelatin (15). The present results extend this to chicken fibronectin. A protein of Torpedo fish plasma with molecular properties similar to the mammalian and chicken fibronectins could also be isolated by utilizing the same affinity chromatography procedure that results in isolation of mammalian and chicken fibronectins. The fact that the fish protein could be purified on gelatin-Sepharose implies an affinity for collagen. Moreover, the fish protein competed with human and chicken fibronectins for binding to collagen indicating a similar specificity. Furthermore, it behaved similarly to fibronectin in SDS gel electrophoresis. This evidence allows the tentative conclusion that this protein is a homologue of the mammalian and chicken fibronectins. This, and the fact that we have been able to detect and isolate fibronectin from the plasma of every mammalian species studied (E. Engvall, and E. Ruoslahti, unpublished results), shows that the affinity of fibronectin to collagen is shared by a variety of species ranging from human to fish.

Our results show that fibronectin is the only major component from plasma that binds to gelatin-Sepharose. It also is the only major component of fibroblast extracts that shows this property, as demonstrated by the purification of homogeneous fibronectin from extracts of chicken fibroblasts. These results strongly suggest that the fibronectin-collagen interaction is not fortuitous, but, instead, is likely to have biological significance.

Since fibronectin is a cell surface component, it is possible that fibronectin could attach cells to the extracellular matrix. There is direct evidence from in vitro experiments that this is the case. A plasma component known as cell attachment protein (CAP) and a cell surface component called cell attachment factor (CAF) mediate the attachment of cultured fibroblasts to collagen coated plates (29, 30). CAF and CAP are apparently identical to fibronectin (30, 31). To gain insight into what collagen-fibronectin interactions might occur in vivo, we analyzed the relative capacities of different genetic types of collagens and their unfolded derivatives to bind fibronectin. It was found that fibronectin showed preference to the denatured forms of the collagens. A similar preference is shown by some of the enzymes active in the post-translational modifications of collagen, e.g., the glucosyl and galactosyl transferases as well as the prolyl and lysyl hydroxylases (32). It is unlikely that fibronectin would be identical to any of these enzymes, because they are mainly intracellular and present in lower amounts than fibronectin.

Limited information is available on the nature of the fibronectin binding site in collagen. The fact that commercial gelatin preparations and heat-denatured collagens are active indicates that the binding site does not depend on an intact helical structure of collagen.

We have also found that two peptides representing different parts of the $\alpha 1$(II) chain are both able to inhibit completely the binding of fibronectin to

gelatin. The CAP-mediated attachment of cells to collagen-coated plates has also been shown to be inhibited by several different cyanogen bromide fragments of the α1(I), α2, and α1(II) chains (33). However, the specific peptides we have tested were not highly active in the cell attachment test. This is probably due to lower sensitivity of the latter test as compared to our assay. These results, therefore, permit the tentative conclusion that the binding sites occur at several locations in the collagen sequence. However, it has been shown earlier (15) that free proline, hydroxyproline, lysine, glycine, glucose, and galactose do not inhibit the fibronectin-collagen interactions. Moreover, the present results indicate that the linear polymers [proline-glycine-proline] and polyproline are also inactive.

Immunofluorescence shows that fibronectin is especially abundant in the basement membranes (34). It was somewhat surprising that the A and B chains, which are thought to be derived from certain basement membranes, are only moderately active in fibronectin binding. Accordingly, it will be of great interest to test other collagens derived from basement membranes for fibronectin binding capacity. Since the specialized functions of the different collagens are not completely known, it is difficult to assess the significance of the differences in the activity of the different collagens in fibronectin binding. This may have little influence in vivo. Since all collagens are active to some extent, it may be the types and relative amounts of collagen available at a given location, rather than the relative binding activities, that determines which collagen interacts with fibronectin.

Fibronectin also binds to fibrinogen and fibrin (10, 12). Our results show that this binding was inhibited by gelatin, and fibrinogen showed some inhibitory activity in an assay where fibronectin binds to gelatin. When blood coagulates at low temperature, fibronectin binds to the fibrin clot (10), and it can be covalently linked to fibrin by the fibrin stabilizing factor (11). We found a marked reduction in the incorporation of fibronectin to the blood clot if gelatin was added to the blood before coagulation. These data strongly suggest that the same site on the fibronectin molecule is responsible for binding to collagen and fibrinogen, but that the site has a greater affinity for collagen sequences. We cannot exclude the possibility that small amounts of collagen contaminate the fibrinogen preparations we used. However, it would be difficult to explain the binding of fibronectin to the fibrin clots by assuming that the affinity of fibronectin for fibrinogen is actually due to binding to contaminating collagen. If fibronectin in tissues serves as a mediator of attachment of cells to the collagenous matrix, the significance of the fibrinogen binding could be to allow temporary binding of the cells to a fibrin scaffold in a wound.

Transformed cells lack fibronectin of their surface. This may contribute to their capacity to grow uninhibitedly and metastasize, because they would lack the supportive, and possibly growth inhibitory, attachment to the surrounding extracellular matrix.

Summary

Fibronectin, a fibroblast surface protein, was purified from human and chicken plasma and extracts of cultured chicken fibroblasts with affinity

chromatography on gelatin coupled to Sepharose particles. A fibronectin-like protein was also isolated from the plasma of Torpedo fish.

The collagen binding properties of fibronectin were studied with several genetically distinct collagens. Heat denatured types I, II, and III collagens were equal in their binding capacity and more active than the native collagens or A and B chains. Native type III collagen was more active than the other native collagens. Human and chicken fibronectins showed approximately the same pattern of specificity. Identical specificities were shown by the plasma and fibroblast forms of chicken fibronectin. Two cyanogen bromide peptides of the collagen α1(II) chain, CB8 and CB12, derived from different parts of the chain, were active in fibronectin binding. A polymer of the tripeptide pro-gly-pro, and polyproline were inactive.

Fibronectin also binds to fibrinogen and fibrin. Comparison of this binding to collagen binding showed that fibrinogen inhibited binding of fibronectin to collagen, but was less active than native collagen. Two other fibrous proteins, tropoelastin and keratin, did not bind fibronectin. The binding of fibronectin to fibrinogen was inhibited by collagen and incorporation of fibronectin into blood clot in the cold was inhibited by gelatin. These results suggest that the binding of fibronectin to collagen and fibrinogen depends on the same binding site in the fibronectin molecule.

It is proposed that cell surface fibronectin mediates attachment of cells to the collagenous extracellular matrix and to a temporary fibrin matrix in a wound.

We thank Aulikki Pekkala for technical assistance, Dr. Sara Fuchs for a pro-gly-pro peptide sample, Dr. William Gray for elastin, and Dr. James Strauss for cultured fibroblasts.

Received for publication 13 February 1978.

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