Substrate Adhesion of Rat Hepatocytes: on the Mechanism of Attachment to Fibronectin

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ABSTRACT We examined the mechanisms of cell attachment to fibronectin-coated substrates. Inhibition of cell attachment was obtained by species-specific antifibronectin antibodies, which presumably recognize a distinct antigenic structure in the protein located at, or in the immediate vicinity of, the cell-binding site. The inhibiting antibodies could be adsorbed on a column of Sepharose substituted with plasma fibronectin. The initial phase of cell attachment was also inhibited by addition of soluble fibronectin to the incubation medium in a reaction that exhibited specificity and concentration dependence. These data suggest that cell-binding sites are available in an active form on the surface of soluble fibronectin. However, the inhibitory effect of fibronectin was greatly enhanced by adding the protein together with heparin, heparan sulfate, collagen, or a fibronectin-binding collagen peptide (CB-7), which is consistent with an "activation" of fibronectin on binding to these matrix components. A similar activation of fibronectin was obtained by cleaving the protein with trypsin. We discuss these findings in relation to conformational rearrangements in the fibronectin molecule.

Data is presented supporting a mechanism of cell attachment to fibronectin involving multiple weak interactions between cellular receptors and substrate molecules, although some steps in the attachment process appear to disobey the requirements for such a mechanism.

Fibronectin is a polymorphous glycoprotein, found in plasma and loose connective tissue in vivo and synthesized by a number of different cells grown in vitro (for recent reviews, see references 1–3).

The extracellular matrix produced by cultured fibroblasts has been shown to contain fibronectin, procollagens type I and III (4, 5), and heparan sulfate proteoglycans (6), co-distributing in a fibrillar network. Furthermore, a direct binding of fibronectin to collagen (7, 8) and heparan sulfate (9, 10) has been demonstrated. Distinct binding sites for cells (11), collagen (12, 13) and heparan sulfate–related polysaccharides (14) have been localized to different domains in the fibronectin molecule.

The most notable activity of fibronectin is its ability to mediate the adhesion of cells to supporting substrates such as collagen, fibrin clots, or plastic culture dishes (15–17). Presumably, fibronectin interacts with specific receptors located at the cell surface. A direct binding of soluble fibronectin to the postulated cellular receptors has, however, been difficult to demonstrate (15, 18). Pearlstein (18) has suggested that active cell-binding sites are not present on the surface of soluble fibronectin molecules and that binding of fibronectin to the substrate is accompanied by a conformational change that is required for the exposure of active cell-binding sites on the protein. Although this hypothesis of "substrate activation" of fibronectin is supported by many investigators (for a recent review, see reference 19), its validity has never been proven. Recently, the attachment of hepatocytes to a collagen substrate, which proceeds by a fibronectin-independent mechanism, was proposed to involve a clustering of appropriate cell surface receptors at the site of the membrane in contact with the immobilized collagen (20). Interactions between individual receptors and soluble collagen molecules do occur, but only weak bonds are formed. The present investigation was undertaken to analyze the mechanism of substrate attachment to fibronectin-coated substrates with respect to the proposed models of substrate activation and clustering of low affinity receptors.

MATERIALS AND METHODS

Fibronectin was purified from human or rat plasma according to the method of Vuento and Vaheri (21). The preparations of heparin and heparan sulfate

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used have previously been described (22). Trypsin (TPCK treated, type XIII) and soybean trypsin inhibitor (type 1-5) were purchased from Sigma Chemical Co., St. Louis, MO. Heparin-Sepharose was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Laminin, lathyritic collagen type I, and cyanogen bromide fragment 7 of collagen type I (CB-7) were kind gifts from Dr. R. Timpl (Max-Planck-Institute). Proteins were coupled to CNBr-activated Sepharose at 22°C according to the recommendations of the manufacturer.

Antibodies: Antisera against rat and human fibronectin, respectively, were raised in rabbits. 100 μg of fibronectin in Freund's complete adjuvant was injected intramuscularly into the animals, followed by two injections of 100 μg of fibronectin emulsified in Freund's incomplete adjuvant. The injections were given in 2-wk intervals. 10, 20, and 30 d after the third injections, rabbits were bled and IgG was subsequently purified from complement-inactivated sera by chromatography on a column of protein A-Sepharose (23). The IgG eluted from the column, was dialyzed against buffer 3 (see reference 23: 8.0 g NaCl, 0.35 g KCl, 0.16 g $MgSO_4 \times 7\ H_2O$, 0.18 g $CaCl_2 \times 2\ H_2O$, 2.4 g HEPES, H_2O to 1 liter, pH 7.4). Antibodies raised against rat or human fibronectin reacted with fibronectin from both species, as determined by immunodiffusion and enzyme-linked (11) immunoassays. Samples of the anti-rat fibronectin IgG were passed through columns (2 ml) of Sepharose substitute with human and rat fibronectin, respectively. Anti-rat fibronectin antibodies that did not bind to the immobilized human fibronectin still reacted with rat, but not with human fibronectin, as shown by immunodiffusion. Antibodies passed through a column of Sepharose substituted with rat fibronectin did not react with fibronectin isolated from either species.

Preparation and Fractionation of Human Fibronectin Fragments: Human fibronectin (1-2 mg/ml) in buffer 3 was digested with 1 μg/ml of trypsin at 37°C for the indicated periods of time. The digestions were terminated by addition of soybean trypsin inhibitor (final concentration of 2 μg/ml). The fibronectin fragments (generally 10 mg) were fractionated on a 2-ml column of heparin-Sepharose equilibrated with buffer 3. Peptides washed through the column or bound to the column and eluted with 1 M NaCl in buffer 3 were collected. The eluted peptides were dialyzed against buffer 3. The concentration of fibronectin or its peptides were determined from the absorbance of 280 nm using an absorption coefficient of 1.28 mg^{-1} ml/cm (24).

Electrophoresis: Electrophoresis was performed on polyacrylamide gradient gels in SDS according to the method of Blobel and Dobberstein (25).

Preparation of Cell Attachment Substrates: Plastic petri dishes (35 mm in diameter, bacteriological grade; cat. no. 1008; Falcon Labware, Oxnard, CA.) were coated by adding 20 μg of fibronectin (or as otherwise indicated) in 2 ml of water. The dishes were then air dried at 22°C and washed extensively with water. To block protein binding sites on the plastic surface not occupied by substrate molecules, we subsequently incubated the dishes with 1 ml of buffer 3, containing 1.5% bovine serum albumin for 30 min at 22°C. This solution was removed before the cells were seeded in the dishes.

Cell Attachment Assay: Hepatocytes were isolated from male Sprague-Dawley rats after perfusion of the liver in situ with collagenase, as previously described (23). Cells (2.5×10^6; or as indicated) in 2 ml of buffer 3 were seeded in the protein-coated dishes and incubated at 37°C in humidified air or at 4°C in the cold room. After incubations for the indicated times the dishes were washed with 2×1 ml of buffer 3. The number of cells attached to the substrate was determined as described (23). In short, the cells were lysed in a solution of Triton X-100, and the activity in the lysate of the enzyme lactate dehydrogenase was determined. The activity of this enzyme has been shown to be proportional to the cell number. The values shown represent averages of two parallel incubations.

When 2.5×10^6 cells were seeded on a substrate made from 20 μg of fibronectin, 80% of the cells were found to attach to the dish in a confluent layer. However, small variations in the number of attached cells were obesrved from one cell batch to another. Therefore, in each experiment, 2.5×10^6 cells were incubated for 60 min on dishes coated with 20 μg of fibronectin. The number of cells attached under these conditions was set at 100%, unless otherwise indicated.

When inhibition of cell attachment to fibronectin-coated dishes by soluble molecules was studied, the cells (1×10^6) were preincubated for 30 min either at 37°C or on ice in 1 ml of buffer 3 containing the indicated additions and 1.5% bovine serum albumin. The preincubation mixtures were kept on a rotary shaker with a speed of 60 rpm. The cell suspensions were transferred to precoated dishes and incubated at 37°C or 4°C for the indicated times. Percent inhibition was calculated as 100-100 × No. of cells attached in the presence of inhibitors/No. of cells attached in the absence of inhibitors.

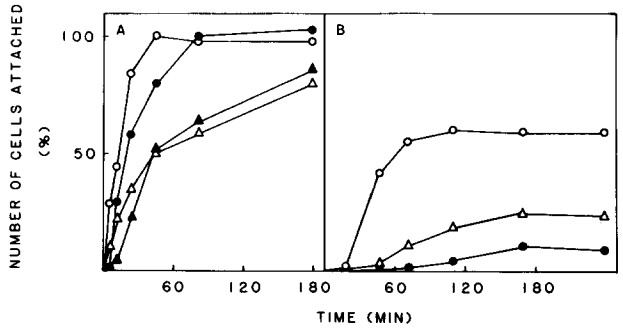

FIGURE 1 Time course of cell attachment to fibronectin and laminin substrates. Cells (2.35×10^6) in 2 ml buffer 3 were incubated at 37°C (*A*) or 4°C (*B*) in plastic petri dishes coated with 20 μg fibronectin (○), 6 μg fibronectin (△), 20 μg laminin (●), and 6 μg laminin (▲), respectively, for the indicated periods of time. The numbers of cells attached were determined as described in Materials and Methods. The number of cells attached to dishes coated with 20 μg fibronectin after incubation at 37°C, for 45 min was set to 100%.

RESULTS

Attachment of Rat Hepatocytes to Fibronectin and Laminin at Different Temperatures

To study the temperature dependence of cell attachment, we seeded rat hepatocytes at 37°C or 4°C in dishes coated with varying amounts of fibronectin and laminin. In accordance with previous results (26), rat hepatocytes were found to attach to culture dishes coated with 20 μg of fibronectin or laminin when incubated at 37°C (Fig. 1*A*). After 40–90 min of incubation the number of cells attached had reached a maximum. Reducing the amounts of adhesion proteins used for coating resulted in a decreased rate of cell attachment. However, after prolonged incubations (>180 min) the number of cells that attached to dishes coated with 6 μg of protein approached the number attached to dishes coated with 20 μg of protein (Fig. 1*A*). With one exception, attachment of cells to fibronectin and laminin proceeded by the same kinetics; an initial lag phase was observed before cells started to attach to laminin. The length of this phase increased as the density of laminin in the substrate decreased. In contrast, attachment of rat hepatocytes to fibronectin proceeded without detectable lag phase even when the substrate was made with a small amount (6 μg) of fibronectin (Fig. 1*A*).

Incubation of rat hepatocytes at 4°C in petri dishes coated with 20 μg of laminin or fibronectin resulted in attachment of a significant number of cells to the fibronectin substrate, while the number of cells attached to the laminin coated dishes was negligible (Fig. 1*B*). A reduced number of cells attached to the fibronectin substrate, and the attachment proceeded at a lower rate when the incubation was carried out at 4°C compared with 37°C. To allow a maximum number of cells to attach at 4°C, we had to make the substrate from >20 μg of fibronectin, whereas at 37°C 6 μg of fibronectin was sufficient (Fig. 2). The number of cells attaching at 4°C was not increased by extending the incubation time beyond 60 min (Fig. 1*B*) or by adding >20 μg of fibronectin to the dish (Fig. 2).

Since a lower number of cells attached to a fibronectin substrate when incubated at 4°C compared with 37°C, the possibility had to be considered that only a subpopulation of the hepatocytes are capable of attaching at the lower temperature. To explore this possibility, we incubated cells at 4°C in fibronectin-coated dishes for 160 min and nonattaching cells were subsequently transferred to new dishes coated with fi-

bronectin and the incubation was continued at 4°C. Hepatocytes also attached to the fibronectin substrate in the second round of incubation. The extent and time course of cell attachment was essentially identical for cells seeded in the first and the second rounds of incubations (data now shown), indicating that all of the hepatocytes had the ability to attach to fibronectin at 4°C. Furthermore, when the number of cells seeded in the fibronectin-coated dishes was reduced, the difference between the numbers of cells attaching to the substrate at 37°C and 4°C was narrowed (Fig. 3). When $<10^6$ cells were incubated per dish, essentially the same number of cells attached at 4°C as at 37°C.

Inhibition of Cell Attachment by Antibodies Directed against Fibronectin

Attachment of rat hepatocytes to plastic culture dishes coated with rat fibronectin was inhibited by antibodies raised against rat fibronectin, but not by antibodies directed against human fibronectin (Fig. 4*A*). When attachment of cells to dishes coated with human fibronectin was examined, the reverse was observed; i.e., antibodies raised against human fibronectin inhibited cell attachment, whereas anti-rat fibronectin was without effect (Fig. 4*B*). These results suggest that the inhibitory activity of the antibodies is species-specific,

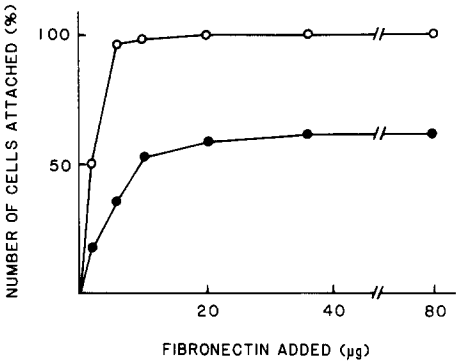

FIGURE 2 Attachment of cells to dishes coated with various amounts of fibronectin. Cells (2.5×10^6) in 2 ml buffer 3 were incubated for 70 min at 37°C (○) or 160 min at 4°C (●) in plastic petri dishes coated by addition of indicated amounts of fibronectin. The numbers of cells attached were determined as described in Materials and Methods. The number of cells attached at 37°C to dishes coated with 20 μg was set to 100%.

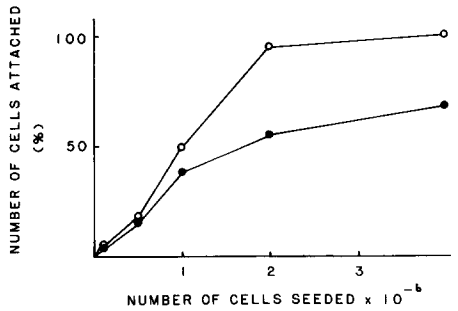

FIGURE 3 Number of cells attached as a function of number of cells seeded. Indicated numbers of cells in 2 ml of buffer 3 were incubated for 70 min at 37°C (○) or 160 min at 4°C (●) in plastic petri dishes coated by addition of 20 μg fibronectin. The number of cells attached were determined as described in Materials and Methods. The number of cells attached when 4×10^6 cells were incubated for 1 h at 37°C (1.8×10^6) was set to 100%.

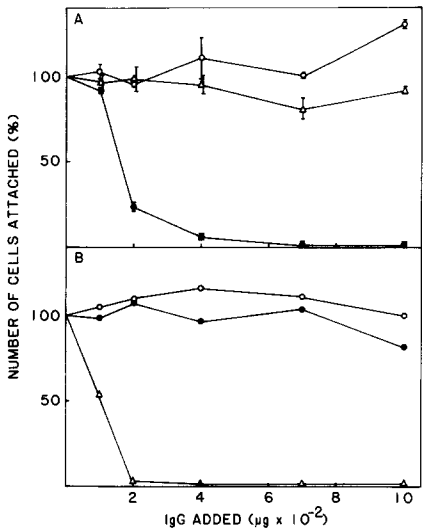

FIGURE 4 Inhibition of attachment of cells to fibronectin substrates by antifibronectin antibodies. Dishes coated with 20 μg of human (*A*) or rat (*B*) fibronectin were preincubated for 30 min at 22°C with the indicated amounts of anti-rat fibronectin IgG (△), anti-human fibronectin IgG (●), or IgG from nonimmunized rabbits (○) in 0.5 ml of buffer 3 supplemented with 1.5% BSA. Cells (2.5×10^6) and buffer 3 were added to a total volume of 2 ml and incubated for 60 min at 37°C. The numbers of cells attached were determined as described in Materials and Methods. The bars indicate the results of two parallel samples. The number of cells attached in the absence of IgG was set to 100%.

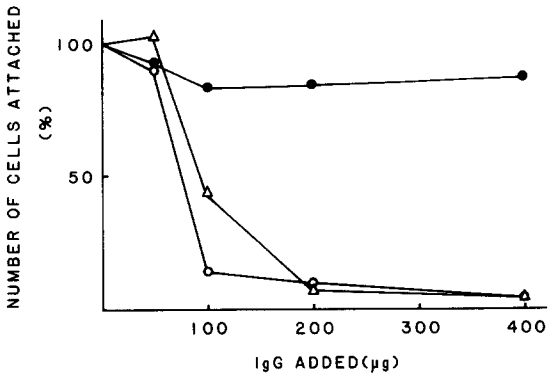

FIGURE 5 Inhibition of attachment of cells to fibronectin substrates by antifibronectin antibodies. Dishes coated with 20 μg of rat fibronectin were preincubated for 30 min at 22°C with 0.5 ml of buffer 3 supplemented with 0.5% BSA and the indicated amounts of anti-rat fibronectin IgG (○), anti-rat fibronectin IgG not binding to a column of Sepharose substituted with human (△), and rat (●) fibronectin, respectively. Cells (2.5×10^6) in buffer 3 were added to a total volume of 2 ml and incubated for 60 min at 37°C. The numbers of cells attached were determined as described in Materials and Methods. The number of cells attached in the absence of IgG was set to 100%.

although both anti-rat and anti-human fibronectin cross-react with fibronectin from the other species.

The species specificity of the inhibiting antibodies was further analyzed. Antibodies raised against rat fibronectin were passed through a column of Sepharose substituted with human fibronectin. Antibodies that did bind to the column retained virtually all inhibitory activity (Fig. 5). When tested in immunodiffusion, these antibodies reacted with rat fibronectin but not with human fibronectin. On the other hand, antibodies that bound to the column and were eluted with

0.1 M glycine, pH 3.0, reacted with both human and rat fibronectin. These antibodies did not markedly affect cell attachment to dishes coated with rat fibronectin (data not shown). In a parallel experiment, antibodies raised against rat fibronectin were passed through a Sepharose column substituted with rat fibronectin. The IgG that did not bind to this column did not inhibit cell attachment to rat fibronectin (Fig. 5).

Inhibition of Cell Attachment by Fibronectin in Solution

The adsorption of cell attachment inhibiting antibodies onto "soluble" fibronectin coupled to Sepharose suggests that the cell-binding site may be exposed in "nonactivated" fibronectin. Therefore we tried to demonstrate a binding of fibronectin to cells in suspension in an assay in which soluble and substrate adsorbed fibronectin competed for binding to cell surface receptors. By including soluble fibronectin in the incubation medium the initial attachment of rat hepatocytes to fibronectin-coated dishes was inhibited (Fig. 6).

The inhibitory effect of the soluble fibronectin was reproducible, but the extent of inhibition varied somewhat from one experiment to another.

The incubation temperature influenced the inhibition obtained. When the experiment was carried out at 37°C, the inhibitory effect lasted for only 5–10 min (Fig. 6*A*), whereas at 4°C inhibition of cell attachment persisted for more than 1 h (Fig. 6*B*). Therefore, most experiments were performed at 4°C.

The added fibronectin was not endocytosed to a great extent. Incubation of cells with ^{125}I-labeled fibronectin (5×10^5 cpm corresponding to 0.2 μg) at 37°C for 40 min resulted in a total binding of <0.5% of the added radioactivity to the cells. The labeled protein remaining in the supernatant was undergraded as indicated by gel chromatography or PAGE analyses (data not shown).

The inhibitory effect was specific for soluble fibronectin; other proteins, i.e., laminin (26), collagen (Table I), or albumin (data not shown) did not cause a similar inhibition. Furthermore, incubation of fibronectin at 70°C for 5 min, which destroys the cell attachment-mediating activity of the molecule (26), also abolished the inhibitory activity of the soluble molecule (Fig. 6). If the inhibitory effect of the soluble fibronectin is caused by an interaction between the soluble protein and fibronectin receptors on the cell surface, we would expect the magnitude of the effect to depend on the concentration of fibronectin in the media. Such a concentration dependence could also be demonstrated (Fig. 7) and the addition of 1 mg/ml of fibronectin caused a 35% reduction in the number of cells attaching during a 45-min incubation at 4°C.

These findings suggest that soluble fibronectin specifically and reversibly interacts with a cell surface receptor. However, the life span of this ligand-receptor complex is presumably not long enough to have a lasting inhibitory effect on cell substrate attachment.

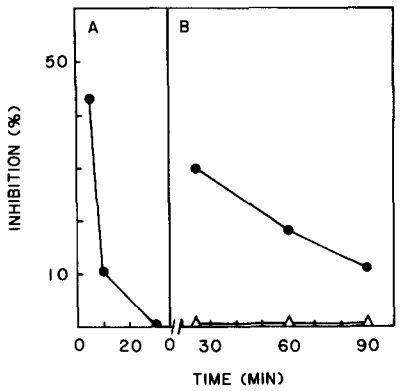

FIGURE 6 Inhibition of cell attachment by soluble fibronectin. Cells were preincubated with soluble fibronectin as described in Materials and Methods and the time course of attachment to fibronectin substrates in the presence of 0.1 mg fibronectin/ml (●) or 0.1 mg heat denatured fibronectin/ml (Δ) at 37°C (*A*) and 4°C (*B*), were followed. The numbers of cells attached and percent inhibition were determined as described in Materials and Methods.

Inhibition of Cell Attachment by Fibronectin Fragments

The inhibitory effect of soluble fibronectin was enhanced more than 10-fold after a brief trypsin digestion of the molecule (Fig. 7), and the addition of 1 mg/ml of fibronectin fragments almost completely blocked cell attachment during a 45-min incubation at 4°C. When cells were incubated at 37°C the inhibitory effect of the degraded fibronectin was more pronounced, but did not last for a markedly longer time than that of the intact fibronectin (Fig. 8*A*). At 4°C the inhibitory effect of the trypsin-generated fragments persisted during the entire incubation period (200 min) at a high but declining level (Fig. 8*B*). Addition of soybean trypsin inhibitor did not enhance the inhibitory effect of intact fibronectin (data not shown).

TABLE I

Fibronectin-dependent Inhibition of Cell Attachment: Effect of Collagen

	Additions to 1 ml medium	Inhibition
		%
Expt. 1, 4°C	0.2 mg collagen	0
	0.1 mg FN	25
	0.1 mg FN + 0.2 mg collagen	64
	0.1 mg FN + 0.1 mg collagen	78
	0.1 mg FN + 0.05 mg collagen	76
Expt. 2, 37°C	0.1 mg FN	32
	0.1 mg FN + 0.05 mg collagen	65
	0.1 mg FN + 0.05 mg CB 7	65

Cells were incubated as described in Materials and Methods, in the presence of the indicated proteins. The numbers of cells attached 45 min after seeding at 4°C (Expt. 1) or 8 min after seeding at 37°C (Expt. 2) were determined, and percent inhibition calculated as described in Materials and Methods. *FN*, fibronectin.

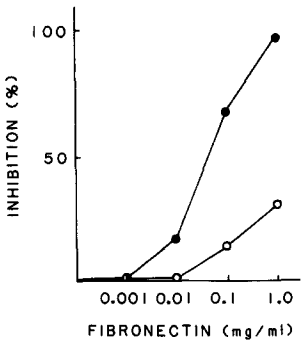

FIGURE 7 Concentration dependence of fibronectin/fibronectin-peptides dependent inhibition of cell attachment. Cells were incubated at 4°C with indicated concentrations of intact fibronectin (○) or fibronectin digested with trypsin for 10 min (●). The numbers of cells attached 45 min after seeding were determined and percent inhibition calculated as described in Materials and Methods.

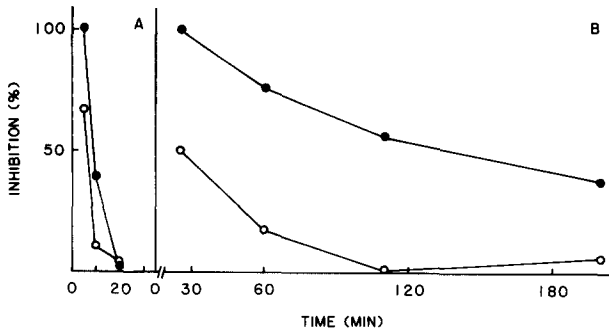

FIGURE 8 Inhibition of cell attachment by fibronectin and fibronectin-peptides at 37°C (*A*) and 4°C (*B*). Cells were incubated for the indicated periods of time as described in Materials and Methods in the presence of 0.1 mg fibronectin/ml (○) or 0.1 mg fibronectin/ml trypsinized for 10 min (●). The amounts of cells attached and percent inhibition were determined as described in Materials and Methods.

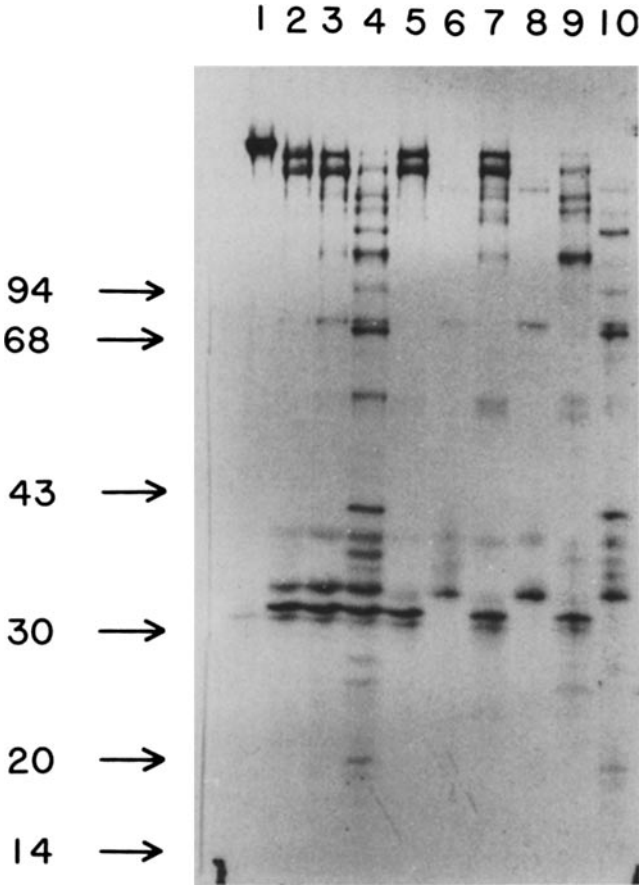

FIGURE 9 SDS electrophoresis of trypsin digested fibronectin. Fibronectin (2 mg/ml in buffer 3) were digested with trypsin as described in Materials and Methods. At various times samples were removed, mixed with soybean-trypsin inhibitor and fractionated on columns of heparin-Sepharose as described in Materials and Methods. Samples (10 μg) were reduced and electrophoresed on a 5–10% polyacrylamide gradient gel as described in Materials and Methods. Lanes: (*1*) fibronectin digested for 0 min; (*2*) fibronectin digested for 3 min; (*3*) fibronectin digested for 10 min; (*4*) fibronectin digested for 90 min; (*5*) heparin binding peptides of 2; (*6*) peptides of 2 not binding to heparin; (*7*) heparin binding peptides of 3; (*8*) peptides of 3 not binding to heparin; (*9*) heparin binding peptides of 4; and (*10*) peptides of 4 not binding to heparin.

The incubation of fibronectin with trypsin for increasing periods of time resulted in the progressive degradation of the protein as shown by PAGE (Fig. 9). The inhibitory effect of soluble fibronectin on cell attachment increased during the period under which degradation of the protein was followed (90 min), although 75% of the optimal activity was reached after a 5-min incubation (Fig. 10).

Activation of Fibronectin by Other Matrix Components

To test the hypothesis of activation of fibronectin on binding to collagen, we added a mixture of soluble fibronectin and collagen together with cells in fibronectin-coated dishes. The inhibitory effect of the fibronectin-collagen mixture on cell attachment was more pronounced than that observed for fibronectin alone. The enhanced inhibitory effect obtained by including 0.05 mg of collagen in the incubation mixture was not further stimulated by increasing the concentration of added collagen (Table I).

The same stimulation of fibronectin-dependent inhibition of cell attachment observed for collagen was also obtained with the CB-7 peptide (Table I). These data suggest that binding of fibronectin to collagen (or CB-7) affects the conformation of the fibronectin molecule and induces a form that shows a higher affinity for the cell surface receptors than noncomplexed fibronectin.

The effects of glycosaminoglycans on fibronectin dependent inhibition of cell attachment were also studied. As shown in Table II, the inhibitory effect of soluble fibronectin was enhanced by the presence of heparin or heparan sulfate, to a level that was comparable to that reached when fibronectin was added together with collagen. Hyaluronic acid, chondroitin sulfate, and dermatan sulfate had no similar effect (data not shown). The most pronounced inhibition of cell attachment was obtained when soluble fibronectin was added together with both heparan sulfate and collagen, whereas addition of heparan sulfate and collagen in the absence of fibronectin had no effect on cell attachment (Table II).

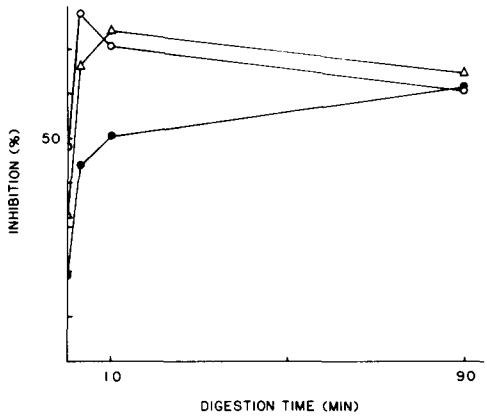

FIGURE 10 Effect of heparin and collagen on inhibition of cell attachment by fibronectin-peptides. Samples of fibronectin were removed at the indicated times from the trypsin digestion shown in Fig. 10 and soybean-trypsin inhibitor was added to a concentration of 2 μg/ml. Cells were incubated as described in Materials and Methods in the presence of 0.1 mg fibronectin-peptides/ml alone (●), together with 50 μg heparin/ml (○), or together with 0.1 mg collagen/ml (△). The amounts of cells attached 45 min after seeding at 4°C were determined and percent inhibition calculated as described in Materials and Methods.

The ability of collagen and heparin to enhance the inhibitory effect of soluble, partially degraded fibronectin was also studied. As shown in Fig. 10, the inhibitory effect of soluble fibronectin, which is stimulated by trypsin digestion, could be further enhanced by adding collagen or heparin to the culture medium. However, the effect of fibronectin fragments generated after a 90-min incubation with trypsin was not significantly stimulated by the presence of either collagen or heparin. Assuming that binding of heparin or collagen to their binding sites results in a conformational change in the cell-binding domain of fibronectin, these ligands can assert their stimulatory effect only when their binding sites reside in a peptide that also contains the cell-binding site. As the degradation of fibronectin proceeds, the chance that the molecule is cleaved between the cell-binding site and the heparin or collagen-binding sites increases. Binding of heparin or collagen to fibronectin peptides that do not contain the cell-binding site should not affect the interaction between the cell-binding fragment and the cellular receptor. This was confirmed in experiments in which fibronectin fragments were fractionated on heparin-Sepharose and then tested for inhibitory activity in the presence and absence of heparin (Fig. 11). Only the effect of the fragments binding to heparin-Sepharose could be further stimulated by the presence of the polysaccharide, whereas no further inhibition of cell attachment was observed by adding heparin to fragments that did not bind to the affinity matrix. Incubation of cells or dishes with heparin followed by washing had no effect on cell attachment (data not shown).

TABLE II

Fibronectin-dependent Inhibition of Cell Attachment: Effect of Heparan Sulfate and Collagen

Additions to 1 ml medium	Inhibition
	%
0.1 mg FN	50
0.1 mg FN + 0.05 mg heparin	78
0.1 mg FN + 0.05 mg HS	68
0.1 mg FN + 0.1 mg collagen	76
0.1 mg FN + 0.05 mg HS + 0.1 mg collagen	94
0.05 mg HS + 0.1 mg collagen	0

Cells were incubated as described in Materials and Methods, in the presence of the indicated compounds. The numbers of cells attached 8 min after seeding at 37°C were determined, and percent inhibition were calculated as described in Materials and Methods. *FN*, fibronectin; *HS*, heparan sulfate.

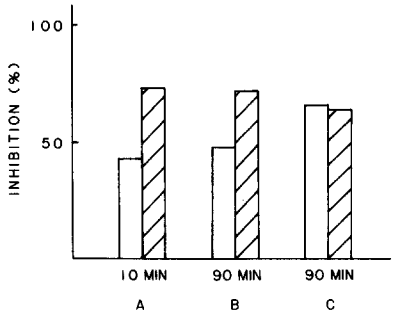

FIGURE 11 Effect of heparin on inhibition of cell attachment by fibronectin fragments fractionated on heparin-Sepharose. The peptides generated by trypsin digestion of fibronectin for various times were fractionated on columns of heparin-Sepharose as described in Materials and Methods. Cells were incubated for 45 min at 4°C in the presence of fibronectin-peptides with (hatched bars) or without (open bars) addition of 50 μg heparin/ml. (*A*) Peptides from a 10 min digest adsorbed on and eluted from heparin-Sepharose (lane *7* in Fig. 10). (*B*) Peptides from a 90 min digest adsorbed on and eluted from heparin-Sepharose (lane *9* in Fig. 10). (*C*) Peptides from a 90-min digestion not binding to heparin-Sepharose (lane *10* in Fig. 10). The numbers of cells attached were determined and percent inhibition calculated as described in Materials and Methods.

DISCUSSION

In this study we examined the mechanism of cell attachment to fibronectin substrates. In accordance with previous reports, antibodies raised against fibronectin inhibited cell substrate attachment. However, the inhibiting antibodies were found to be species specific, i.e., antibodies raised against human fibronectin inhibited cell attachment to human fibronectin, but not to dishes coated with rat fibronectin and vice versa. Apparently the inhibitory antibodies bound to a distinct antigenic site(s) in the fibronectin molecule located at or close to the cell-binding site(s). These data are consistent with the results of Pierschbacher et al. (11), who identified a monoclonal antibody that effectively inhibited attachment of cells to fibronectin-coated dishes by binding to the cell-binding site in the protein. Since the inhibiting antibodies (both the monoclonal and the polyclonal antibodies) could be absorbed with soluble fibronectin, the corresponding antigenic site is exposed on the surface of soluble fibronectin without prior activation of the molecule.

Addition of soluble fibronectin to the incubation medium inhibited initial cell attachment, which not only indicates that the cell-binding site(s) is exposed on soluble fibronectin but that it is functional. The concentration dependence and the specificity of this effect (i.e., other proteins and heat-inactivated fibronectin did not inhibit attachment of cells to fibronectin-coated substrate) suggest that the soluble fibronectin does in fact interact with the appropriate receptors. In a previous study Hahn and Yamada (27) found a reduced attachment of Chinese hamster ovary cells to a fibronectin substrate when intact fibronectin or cell-binding fragments were present in the culture medium. Grinnell et al. (28) also demonstrated a binding of soluble fibronectin to baby hamster kidney cells in suspension, although this binding could be detected only at 4°C.

Soluble fibronectin inhibits cell attachment reversibly. When the incubations were carried out for more than 20 min at 37°C, the inhibitory effect of the soluble fibronectin could no longer be detected. At 4°C, where the attachment process is slower, the inhibitory effect of the fibronectin in the medium persisted longer.

The inhibitory effect of soluble fibronectin on initial cell attachment was enhanced when the fibronectin was allowed to complex with collagen or heparan sulfate. A combination of fibronectin, collagen, and heparan sulfate had a greater inhibitory effect than fibronectin together with either collagen or heparan sulfate. These results can be due either to conformational changes in the fibronectin molecule induced by binding to heparan sulfate and collagen or to formation of aggregates containing several fibronectin molecules and acting as multivalent ligands. Support for a conformational change in the fibronectin molecule to a form for which the cellular receptors have an increased affinity is given by the following observations. Data which suggest that collagen (29) and heparin (30) induce conformational changes in fibronectin, have been reported. The CB-7 fragment of collagen that stimulated the inhibitory effect of soluble fibronectin on cell attachment

to the same extent as collagen, does not induce aggregation of fibronectin (E. C. Williams, P. A. Janney, J. D. Ferry, and D. F. Mosher; personal communications). Furthermore, trypsin degradation of fibronectin generated peptides that exhibited a stronger inhibitory activity on cell attachment than did intact fibronectin. Gel chromatography of trypsin-digested fibronectin demonstrated that generated peptides did not form aggregates (data not shown). These observations suggest that the cell-binding site is somewhat masked in intact fibronectin and more readily exposed in the trypsin-cleaved molecule or after binding of fibronectin to collagen or heparin/heparan sulfate (see the model in Fig. 12). Also in other systems, an increase in the biological activity of fibronectin has been observed after partial degradation of the molecule; e.g., trypsin-degraded fibronectin showed a higher affinity for staphylococci and streptococci than did the intact protein (31). Interactions of fibronectin with macrophages were also more pronounced with fibronectin fragments than with the intact molecule (32).

But even after "activation" of soluble fibronectin by proteolysis or binding to collagen and/or heparan sulfate, the inhibitory effect on cell attachment was transient. Apparently the fibronectin molecules adsorbed on the plastic dish (>20 μg) competed efficiently with the soluble fibronectin (100 μg) for the cellular receptors. These data are consistent with a model of cell attachment to fibronectin substrates involving multiple weak interactions between the ligands in the substrate and the corresponding cellular receptors. Both activated and nonactivated fibronectin in the media interact with the cell surface receptor, but the receptor-ligand complex is not held together by strong forces and therefore dissociates readily. Reassociation is statistically favored if the receptor and the ligand after dissociation remain close to each other, which is the case for cellular receptors binding to fibronectin coated on a plastic surface. As a consequence, only the initial attachment is inhibited by soluble fibronectin. During this phase of attachment cellular receptors will migrate from the upper surface of the cell to the site of substrate contact, provided that the fluidity of the membrane allows movement of membrane proteins (see also reference 20 on the attachment of cells to collagen substrates, which appears to follow a similar mechanism). In support for this aspect of the model, Grinnell et al. (33) found that latex beads coated with fibronectin bound to cells in suspension but once the cells had attached to, and spread on a fibronectin-coated substrate, the fibronectin receptors had apparently disappeared from the exposed upper surface of the cells and the latex beads could no longer bind to the cells.

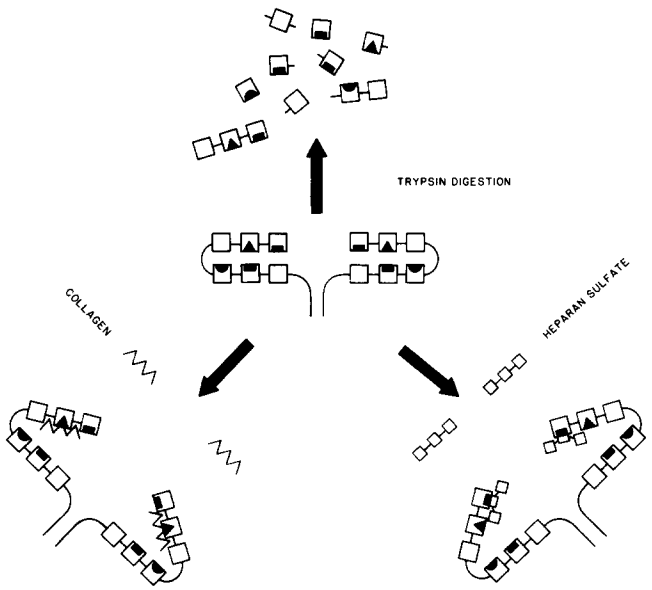

FIGURE 12 Model of conformational changes in fibronectin induced by collagen, heparin or trypsin digestion. This model is a modified version of that presented by Williams et al. (see Fig. 4 in reference 29). The two polypeptide chains are kept together by disulfide linkages in the "stalk." Binding sites in the fibronectin molecule for heparin (▬), collagen (▲), and cells (◡) are as indicated.

Further support for the model of "multiple weak interactions" is provided by the finding that it takes a comparably longer time for the maximum number of cells to attach to a substrate made from small amounts of fibronectin. This could reflect an increasing difficulty for a sufficient number of membrane receptors to find matching ligands, when the number of substrate molecules is reduced. On the other hand, the finding that cells attach to fibronectin substrates at 4°C (or at 0°C; data not shown) when the bilateral diffusion of membrane components are markedly reduced indicates that extensive protein migration in the membrane is not necessary for cell attachment to fibronectin. Furthermore, attachment of cells to fibronectin showed no initial lag phase at 37°C, which was observed for cells attaching to laminin.

In conclusion, binding of cells to fibronectin is enhanced by (but does not require) "activation" of the protein. Cell attachment to a fibronectin substrate appears to involve multiple weak interactions with the ligands, although additional yet unknown steps are probably involved in the cell attachment process.

In the extracellular matrix, fibronectin occurs in close association with collagen and heparan sulfate, and the conformation of the protein is presumably affected by the presence of these other matrix components. Our data suggest that cells in vivo may have a higher affinity for a matrix form of fibronectin than for the soluble fibronectin found in plasma and other body fluids. This may at least partly explain why cells attach to a fibronectin matrix in the presence of a high concentration of soluble fibronectin in the surrounding fluid.

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