DIRECT ASSOCIATION OF FIBRONECTIN AND ACTIN MOLECULES IN VITRO

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ABSTRACT

Affinity chromatography with actin-Sepharose conjugates of purified human fibronectin, normal human plasma, or serum-free culture fluid from human fibroblasts showed that fibronectin molecules can directly bind to actin. A quantitative recovery of soluble human fibronectin was accomplished by chromatography on actin immobilized on Sepharose beads. Human fibronectin molecules bound to actin-Sepharose were eluted with 0.25–0.35 M potassium bromide, and these molecules competed in a species-specific radioimmunoassay for human fibronectin. The subunits of fibronectin isolated by actin-Sepharose chromatography comigrated in SDS polyacrylamide gel electrophoresis with those of electrophoretically homogeneous fibronectin purified by conventional procedures. The efficient direct binding of fibronectin to actin suggests that interactions between these proteins might also take place in vivo but further studies are needed to elucidate the biological significance of this affinity.

Fibronectin is a major fibroblast surface glycoprotein consisting of two disulfide-linked subunits of 220,000 M_r each and is found in vertebrate plasma and tissues as well as in cultures of adherent vertebrate cells (12, 35, 37). Fibronectins are capable of interacting with a number of molecules, and recent studies of its domain structure have revealed different binding sites for collagen, heparin, and cell surfaces (28).

Fibronectin is not a conventional membrane protein, and it is generally considered not to be a transmembrane protein. However, a high molecular weight transmembrane glycoprotein, probably identical with fibronectin, has been described in fibroblastic mouse L cells (10). Cytochalasin B dissociates microfilament bundles and releases fibronectin from cultured cells (1, 18); treatment of cells with the protease, plasmin, releases fibronectin and causes a loss of actin microfilament bundles (26). The treatment of fibroblasts with biolog-ically active phorbol esters releases cellular fibronectin into the medium (15). It is possible to extract cellular fibronectin from cells by low concentrations of urea (38). Selective cytotoxity of antifibronectin antiserum to cells containing fibronectin has suggested that at least some of the cellular fibronectin molecules are closely associated with the cell membrane (29). The addition of fibronectin to the culture medium of transformed cells causes a partial restoration of cytoskeletal microfilament organization and cell morphology (2, 36). Double-label immunofluorescence analysis demonstrating a codistribution of actin bundles and fibronectin during the spreading of cultured cells has suggested an in vivo relationship between actin and fibronectin (9, 12). Transmission electron microscope studies have also suggested the possibility of a transmembrane association between fibronectin and actin (32).

In this report of our studies in which total

human plasma and serum-free supernates of cultured human fibroblasts are used, we present biochemical evidence of an affinity between actin and fibronectin molecules. We were able to demonstrate the reversible in vitro binding of fibronectin to actin using total plasma, serum-free cell culture medium, or purified fibronectin obtained by conventional approaches. The authenticity of the actin-bound fibronectin molecules was shown by a sensitive radioimmunoassay specific for human fibronectin and by analysis of reduced, denatured polypeptides by polyacrylamide gel electrophoresis. A preliminary report of this work has been published elsewhere (14).

Our in vitro binding study provides biochemical evidence that direct interactions between a major intracellular cytoskeletal protein, actin, and a major cell surface protein, fibronectin, are possible in the plasma membrane, provided that either of the proteins is transmembrane. The ability of fibronectin to bind to actin might also play a role in a variety of in vivo mechanisms in which their interaction becomes possible as the result of a disturbance in normal metabolism or physiology. Further studies are, however, needed to demonstrate the biological significance of the affinity between these conserved molecules.

MATERIALS AND METHODS

Cell Cultures

The human fibroblast line CCL 137 (ATCC) was maintained in Dulbecco's modification of Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (Colorado Serum Co., Denver, Colo.).

To metabolically label cell fibronectin, monolayer cultures at ~70% confluence were shifted down to methionine-free medium (Grand Island Biological Co.) for 3 h and then labeled for 20 h with [35S]methionine (New England Nuclear, Boston, Mass.) at a final concentration of 50 μCi/ml. At the end of the labeling period, free methionine was removed by washing the cells with phosphate-buffered saline.

Preparation of Cellular and Plasma Fibronectins

Human diploid fibroblasts labeled with [35S]methionine were washed with serum-free medium for 2 h. The cells were then incubated with fresh Eagle's medium (10 ml/10^7 cells) for 20 h and the supernate was collected. The cell-free supernate (15 ml) was dialyzed extensively against phosphate-buffered saline, then clarified by centrifugation (20,000 g for 30 min). The crude supernate was used as a source of cell-derived fibronectin.

Normal human citrate plasma was clarified by centrifugation at 10,000 g for 30 min and used as a source of plasma fibronectin for affinity chromatography.

Affinity Chromatography of Plasma and Cell Culture Fluid

Purified rabbit muscle actin (Sigma Chemical Co., St. Louis, Mo.) was dissolved at a concentration of 2-3 mg/ml at 4°C by extensive dialysis in 0.01 M Tris (pH 8.0)–50 mM KCl–0.2 mM ATP (27, 32). The solution was clarified by centrifugation at 10,000 g for 30 min. The purity of commercial actin was analyzed by SDS polyacrylamide gels, which indicated that the preparations were >95% pure. Purified rat skin collagen type I (17) was dissolved in phosphate-buffered saline, incubated at 37°C for 15 min, and clarified by centrifugation at 10,000 g for 30 min. Bovine serum albumin and ovalbumin were obtained from Sigma Chemical Co. The proteins were dissolved in phosphate-buffered saline (2 mg/ml) and conjugated to Sepharose by a method previously described in detail (5). Sepharose 2B was activated with cyanogen bromide (200 mg/ml of slurry) at room temperature at pH 10.0–10.5. Conjugation of proteins to Sepharose beads was performed by mixing them at room temperature for 1 h, followed by 4 h at 4°C. Unbound proteins were removed by washing with 1 M KBr and 4 M urea. The yield of actin-Sepharose in this conjugation procedure was about 0.5 mg of actin/ml of packed Sepharose as estimated by analyzing the amount of unbound protein after the conjugation (21). No major differences in the activities were found between samples from different conjugations.

Calf thymus DNA was purchased from Worthington Biochemical Co. (Freehold, N. J.). It was dissolved in phosphate-buffered saline and coupled to activated Sepharose as described above. To obtain single-stranded DNA, the dissolved DNA was incubated at 95°C for 15 min and rapidly chilled on ice before being coupled to Sepharose.

Sepharose conjugate columns (~3.0 ml bed vol) were equilibrated with 20 mM Tris buffer (pH 8.0) containing 50 mM KCl and 5 mM MgCl$_2$. Plasma samples (5 ml) or culture supernates (2.5 ml) were dialyzed extensively against the column buffer, applied to the columns at room temperature and recycled twice. The unbound proteins were removed by washing with 5–10 vol of the loading buffer until no A$_{280}$ was detectable in the effluent. Bound proteins were eluted with increasing concentrations of KBr. Protein in the effluent fractions was determined by the method of Lowry et al. (21).

Radioimmunoassay

Human plasma fibronectin was purified by gelatin-Sepharose affinity chromatography and diethylaminoethyl cellulose chromatography, as previously described (11, 15), and antibodies were raised in rabbits.

Competition radioimmunoassay was performed by a double antibody precipitation method (15, 24). This radioimmunoassay using immune sera preabsorbed with calf serum and ^{125}I-labeled human plasma fibronectin is specific for human fibronectin (15). Plasma from mouse and rat and tissue culture medium from cell lines of heterologous species failed to compete in the assay. Culture medium that contained 10% fetal calf serum caused no inhibition of binding. The appropriate dilution of rabbit antifibronectin was chosen for the precipitation of ~50% of input radiolabeled fibronectin. The antisera were first incubated with serial dilutions of unlabeled competing test antigen samples at 37°C for 2 h. Approximately 20,000 dpm of ^{125}I-fibronectin was then added and incubated for an additional 2 h at 37°C. The immune complex was incubated at 37°C for 1 h and 4°C for 3 h with sheep anti-rabbit immunoglobulin as the second antibody.

The immune precipitate was washed three times with cold PBS, and the amount of ^{125}I-fibronectin in the precipitate was measured in a gamma counter.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous gel electrophoresis with a 3% stacking gel and a 5% resolving gel was performed in a Tris-buffered system according to Laemmli (20). For unlabeled proteins, the gels were stained with Coomassie Blue to visualize the polypeptide bands. For radiolabeled proteins, the gels were first dried on filter pads (Whatman, Inc., Clifton, N. J.) and then exposed to Kodak x-ray films for autoradiography. Reduced human plasma fibronectin (220,000 M_r), β-galactosidase (116,000 M_r), bovine serum albumin (68,000 M_r), and bromphenol blue were used as calibration markers.

RESULTS

Affinity Chromatography of Human Plasma on Actin-Sepharose

To test the binding of fibronectin to actin, total human plasma was chromatographed on actin-Sepharose and gelatin-Sepharose conjugates. Under standard conditions, <10% of the total protein was retained by either column. The columns were then rinsed with the loading buffer supplemented with 0.3 M KCl to remove loosely bound proteins. The proteins retained on the column were finally eluted with 1 M KBr. A complete quantitative dissociation of fibronectin from gelatin-Sepharose conjugates was achieved by using 1 M KBr (6; our unpublished observation). The elution pattern of proteins as monitored by the A_{280} of the effluent fractions is shown in Fig. 1. The net amount of plasma proteins bound to gelatin-Sepharose (Fig. 1 A) is lower than that bound to actin-Sepharose (Fig. 1 B). Virtually all of the bound protein is recovered from gelatin-Sepharose in the 1 M KBr wash; a very small amount (<5%) of the protein elutes with 0.3 M KCl (Fig. 1 A) and contains no detectable fibronectin (see below). A considerable amount of actin-bound protein (~30%) elutes in the 0.3 M KCl wash, and the remainder is released with 1 M KBr (Fig. 1 B).

The proteins of the column eluates were analyzed on a reducing SDS-PAGE (Fig. 2). For both columns, the unbound material contained little or no fibronectin detectable by Coomassie Blue staining. The 0.3 M KCl eluate of the actin-Sepharose column contained only low levels of fibronectin and several low molecular weight proteins (<65,000) that have not been resolved. The small amount of protein in the 0.3 M KCl eluate of the
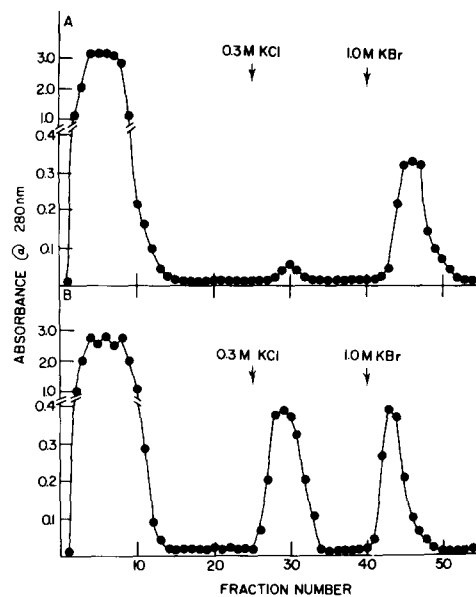

FIGURE 1 Elution of human plasma proteins from gelatin-Sepharose and actin-Sepharose conjugates. Total human citrate plasma, dialyzed against 20 mM Tris (pH 8.0)–5 mM MgCl₂–50 mM KCl, was chromatographed in parallel through (A) gelatin-Sepharose and (B) actin-Sepharose conjugates. Fractions (1.5 ml each) were collected and monitored for protein content by measuring the absorbance at 280 nm. Arrows indicate the beginnings of the elutions with 0.3 M KCl and 1 M KBr.

gelatin-Sepharose column showed no fibronectin or other Coomassie Blue-stained bands in the SDS-PAGE analysis. The 1 M KBr eluate from the gelatin-Sepharose column was >90% homogeneous fibronectin. Approximately 60% of the protein recovered in the 1 M KBr eluate of the actin-Sepharose column comigrated with the subunits of control fibronectin (~220,000 M_r) as a closely spaced doublet (Fig. 2, lane 3). Minor polypeptide bands migrating at >75,000 were also detected; these polypeptides have not been further characterized.

To further analyze the 1 M KBr eluates of actin-Sepharose and gelatin-Sepharose conjugates, the fractions were tested in a competitive radioimmunoassay for fibronectin (Fig. 3). The slopes of the competition curves with the 1 M KBr eluates from actin-Sepharose are indistinguishable from the slope obtained with fibronectin purified by other means. The approximate titers of fibronectin in the 1 M KBr eluates, as calculated from the competition curves, suggest a higher purity (>90% fibronectin) for the gelatin-bound material than
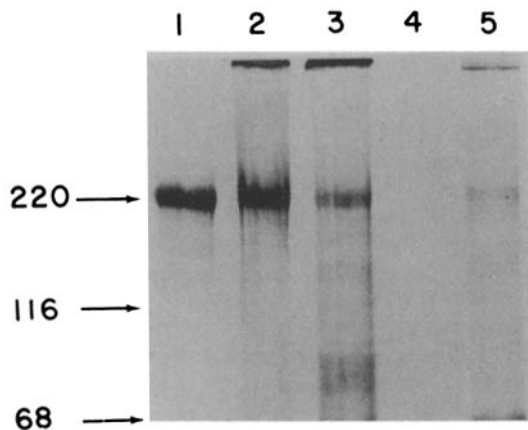
FIGURE 2 SDS polyacrylamide gel electrophoresis of human plasma proteins bound to actin-Sepharose and gelatin-Sepharose conjugates as shown in Fig. 1. Proteins in column effluents were concentrated by precipitation with 15% trichloroacetic acid at 4°C followed by centrifugation and then analyzed on a 5% polyacrylamide gel. (1) Purified human plasma fibronectin marker. (2) 1.0 M KBr eluate from gelatin-Sepharose. (3) 1.0 M KBr eluate from actin-Sepharose. (4) 0.3 M KCl eluate from gelatin-Sepharose. (5) 0.3 M KCl eluate from actin-Sepharose. Arrows indicate the positions of fibronectin (220,000), β-galactosidase (116,000), and bovine serum albumin (68,000) protein markers. (Insoluble protein aggregates [mol wt $>1.6 \times 10^6$], probably of fibronectin, were found in samples from both columns [lanes 2 and 3] after precipitation with trichloracetic acid.)

coupled with Sepharose, and ovalbumin coupled to Sepharose) bound only ~5% of the input fibronectin.

Binding of Fibroblast-derived Fibronectin to Actin

We examined the direct binding of cell-derived fibronectin, which had been radiolabeled in vivo (see Materials and Methods), from a human fibroblast culture (CCL 137). Serum-free culture supernate from the labeled cells was tested by affinity chromatography using actin-Sepharose and gelatin-Sepharose conjugates. Proteins in the starting material, in the unbound fraction, and in the 1 M KBr eluate of each column were precipitated with trichloracetic acid and then analyzed on SDS polyacrylamide gels (Fig. 4). Both column matrices effectively retained fibronectin from the culture supernate (Fig. 4), and it was missing from the unretained fractions in both cases. The eluted cell-derived proteins were also able to compete with radiolabeled plasma fibronectin in radioimmunoassays. All of the bound, radioactive fibronectin was efficiently eluted with 1 M KBr. Again, in the case of actin-bound proteins, additional minor polypeptide bands that have not been further characterized were seen. The 1 M KBr eluate of gelatin-bound material contained, at much reduced levels, minor protein bands.

for the actin-bound proteins. About 0.8 mg of fibronectin was recovered from the eluates of both actin (0.3 M KCl and 1 M KBr eluates) and gelatin (1 M KBr eluate) columns in this experiment. In these studies, actin was immobilized by coupling to Sepharose particles. Therefore, the affinity or stoichiometry of the binidng between the components might not reflect the true in vivo interaction, and it was considered futile to calculate binding constants or binding stoichiometry from the column data. In any case, there is a relatively high affinity between these proteins.

Normal human plasma was subjected to similar affinity chromatography on Sepharose conjugates containing denatured or native calf thymus DNA. Whereas native DNA-Sepharose failed to bind detectable amounts of immunoreactive fibronectin in our assay, denatured DNA-Sepharose efficiently retained fibronectin (see below). The specificity control columns (Sepharose beads washed with bovine serum albumin [BSA, 2 mg/ml], BSA
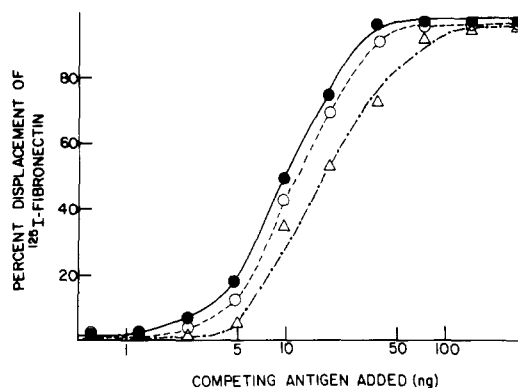

FIGURE 3 Radioimmunoassay of human plasma proteins bound to actin-Sepharose and gelatin-Sepharose conjugates. The proteins recovered in the 1 M KBr eluate of actin-Sepharose ($\triangle$) and gelatin-Sepharose ($\bigcirc$) columns were analyzed in a radioimmunoassay specific for human plasma fibronectin, as described in Materials and Methods. Purified human fibronectin was used as a control ($\bullet$).
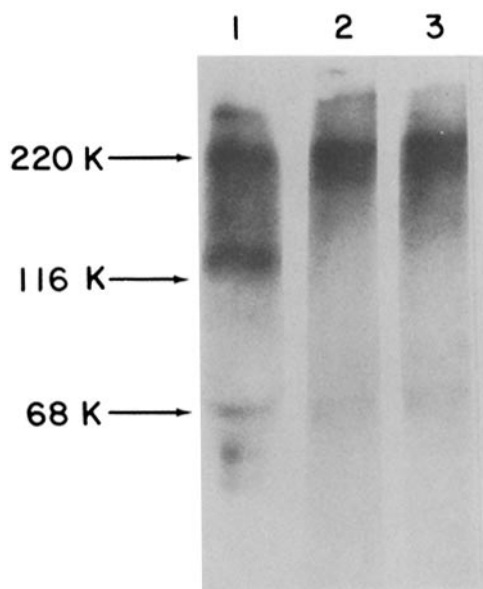
I 2 3

220 K——→

116 K——→

68 K——→

FIGURE 4 SDS polyacrylamide gel electrophoresis of in vitro radiolabeled human fibroblast-derived proteins bound to actin-Sepharose and collagen-Sepharose. A human fibroblast culture (CCL 137) was labeled with [³⁵S]methionine as described, and serum-free culture supernates from these cells were analyzed by chromatography on actin and collagen. (*1*) Total serum-free supernatant. (*2*) Proteins bound to actin-Sepharose. (*3*) Proteins bound to gelatin-Sepharose. Arrows indicate the positions of fibronectin (220,000), β-galactosidase (116,000), and bovine serum albumin (68,000) protein markers.

Affinity Chromatography of Collagen Type I on Actin-Sepharose

Purified rat skin collagen type I was dissolved in the column buffer (100 μg/ml) and slowly passed three times through a 1-ml actin-Sepharose column. The column was washed with five column volumes of the buffer and then eluted with 1 M KBr. The eluate was analyzed for protein by the method of Lowry et al. (21). The eluate contained only trace amounts of protein, suggesting that collagen type I does not bind to actin.

The Affinity of Binding of Purified Fibronectin to Actin and Collagen

To exclude the possibility that some protein other than fibronectin might cross-link actin and fibronectin molecules, we used a radioimmunoassay for human fibronectin to compare the relative

affinities of binding of the fibronectin in plasma and of purified, electrophoretically homogeneous fibronectin to gelatin, actin, BSA, and DNA molecules.

Equal volumes of fresh human plasma were applied to actin-Sepharose, gelatin-Sepharose, BSA-Sepharose, native DNA-Sepharose, and denatured DNA-Sepharose columns in parallel. In each case, the loading material was cycled twice to ensure complete binding. The columns were then washed with the loading buffer. Each column was then eluted stepwise with increasing concentrations of KBr (at 0.1 M intervals). The amount of eluted fibronectin in each fraction were determined by radioimmunoassay (Fig. 5). Both the actin and the gelatin columns almost quantita-
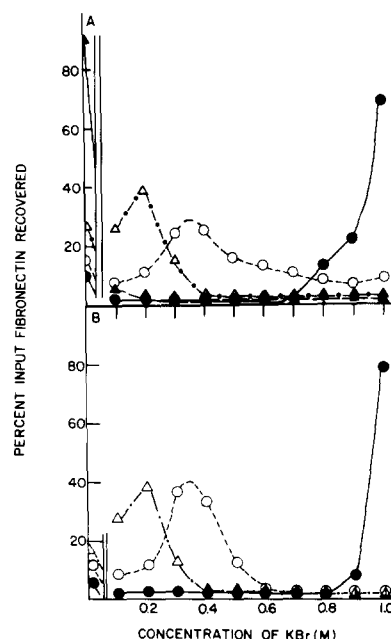

FIGURE 5 The elution of fibronectin from human plasma and purified human fibronectin from various conjugates as a function of increasing concentrations of KBr. Total plasma (1.5 ml) or purified fibronectin (100 μg) was applied to gelatin-Sepharose (●), actin-Sepharose (○), denatured DNA-Sepharose (△), or native DNA-Sepharose (▲) columns. Unbound proteins were removed by washing with loading buffer. Bound proteins were eluted with increasing concentrations of KBr added to the loading buffer, and the amount of fibronectin in each fraction was measured by radioimmunoassays. (*A*) Total human plasma. (*B*) Purified human plasma fibronectin.

tively retained the input fibronectin (Fig. 5 A). Whereas double-stranded DNA-Sepharose or BSA-Sepharose did not bind fibronectin, denatured DNA-Sepharose conjugate retained 60–70% of the input fibronectin. During the elution with increasing concentrations of KBr of bound material from the collagen-Sepharose column, 80–85% of the immunoreactive fibronectin was recovered at 0.9–1.0 M KBr. The actin-bound material eluted heterogeneously at 0.3–0.8 M KBr. The fibronectin bound to single-stranded DNA was eluted at 0.1–0.3 M KBr.

In the parallel experiment, purified plasma fibronectin (100 μg per column) was used (Fig. 5 B). Gelatin-Sepharose again retained >80% of the bound fibronectin up to 0.9 M KBr concentration. The elution from actin-Sepharose was relatively homogeneous compared with the elution profile obtained with total plasma, as is shown by the 80% recovery at 0.3–0.5 M KBr. Denatured DNA-Sepharose retained fibronectin up to 0.1–0.3 M KBr. Cellular fibronectin from cultured fibroblasts could also be eluted from actin at 0.3–0.5 M KBr (data not shown).

DISCUSSION

We demonstrate in this study that fibronectins from human plasma and from the serum-free medium of cultured human fibroblasts can efficiently bind directly to actin. This observation is based on the following data: (a) affinity chromatography of the soluble forms of fibronectin on actin conjugated to Sepharose showed effective, if not quantitative, binding of a 220,000 M_r protein to actin, whereas the specificity control columns retained <10% of input fibronectin; (b) the eluted molecules comigrated in SDS polyacrylamide gels with the subunits of purified fibronectin molecules; and (c) the actin-bound proteins competed in a radioimmunoassay specific for human fibronectin.

Fibronectins are high molecular weight glycoproteins present in mammalian cells and plasma in immunologically similar forms (30), and they have a number of known biological interactions (reviewed in references 11, 35, and 37). Collagen, a major component of the extracellular matrix, can bind to fibronectin (7) and codistributes with it in cultured cells (34). Fibronectin can also interact with heparin, fibrinogen, and fibrinogen-fibrin complexes in the cold (22). Fibronectin is susceptible to the action of transglutaminases (23) and has a tendency to form disulfide-bonded complexes on the cell surface (13).

In this report, we present biochemical evidence from experiments with purified components that the direct binding of fibronectin to actin is possible. Using total plasma, purified plasma fibronectin, or fibroblast-derived fibronectin, we demonstrated specific binding between actin and fibronectin. Both molecules, like collagens, are conserved proteins, and an in vitro interaction between them might have biological significance. It has previously been shown that neither albumin nor the fibrous proteins tropoelastin and keratin bind fibronectin (8). We were unable to show any binding of collagen type I to actin in this study. The tightness of the association of fibronectin with actin as determined by stepwise elution with increasing salt is less than that found with gelatin, but it apparently is comparable to the affinity of fibronectin for native collagens (8). We have recently shown binding of a murine sarcoma virus-associated protein kinase to immobilized actin molecules (32).

A transmembrane connection between cell surface fibronectin and the internal cytoskeleton has been suggested from several lines of experiments (10, 12, 33). On the other hand, it has been shown that certain gangliosides can block the attachment of cells to collagen-fibronectin complexes (16), suggesting that specific gangliosides or related molecules on the cell surface may act as the receptors for fibronectin. Immunological purification of fibronectin from cell extracts was accompanied by copurification of an actinlike protein (3, 19). Some actin was also found in the cell-free fibronectin matrices of mouse fibroblasts (4).

However, the role of the affinity of fibronectin for actin in vivo and in culture remains to be elucidated in further studies. Plasma fibronectin might play a role in wound healing by binding to exposed actin molecules and serving as a temporary matrix for fibroblasts (23). Fibronectin has also been referred to as a major nonspecific opsonin of blood (31), and one of the molecular mechanisms might be the binding of fibronectin to actin-containing targets.

If the interaction between actin and fibronectin molecules plays a role in the normal anchorage, morphology, and growth of cells, a modification of either actin or fibronectin might regulate their mutual interaction. Our studies on the direct binding between actin and fibronectin in vitro thus suggest an in vivo molecular mechanism for the observed association. The studies also offer the background for the direct examination of the na-

ture of intermolecular interaction between cyto-skeleton and cell-surface proteins in normal cells during different stages in cell cycle and during cell transformation.

ADDENDUM

We found that fibronectin can bind to some extent to denatured DNA. After this paper was submitted Zardi et al. showed in an assay system not directly comparable to ours that fibronectin can bind also to native DNA in affinity chromatography (39). Recently, Parsons et al. (25) isolated, from plasma of cancer patients, a DNA-binding protein that cross-reacts with antifibronectin antibodies in immunodiffusion.

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