Interaction of the 70,000-mol-wt Amino-terminal Fragment of Fibronectin with the Matrix-assembly Receptor of Fibroblasts

PAULA J. McKEOWN-LONGO and DEANE F. MOSHER
Department of Medicine, University of Wisconsin, Madison, Wisconsin 53706. Dr. McKeown-Longo's present address is Department of Biological Sciences, State University of New York at Albany, Albany, New York 12222.

ABSTRACT Plasma fibronectin binds saturably and reversibly to substrate-attached fibroblasts and is subsequently incorporated into the extracellular matrix (McKeown-Longo, P. J., and D. F. Mosher, 1983, *J. Cell Biol.*, 97:466–472). We examined whether fragments of fibronectin are processed in a similar way. The amino-terminal 70,000-mol-wt catheptic D fragment of fibronectin bound reversibly to cell surfaces with the same affinity as intact fibronectin but did not become incorporated into extracellular matrix. The 70,000-mol-wt fragment blocked binding of intact fibronectin to cell surfaces and incorporation of intact fibronectin into extracellular matrix. Binding of the 70,000-mol-wt fragment to cells was partially abolished by cleavage into 27,000-mol-wt heparin-binding and 40,000-mol-wt gelatin-binding fragments and more completely abolished by reduction and alkylation of disulfide bonds. Binding of the 70,000-mol-wt fragment to cells was not blocked by gelatin or heparin. When coated onto plastic, the 70,000-mol-wt fragment did not mediate attachment and spreading of suspended fibroblasts. Conversely, fibronectin fragments that had attachment and spreading activity did not block binding of exogenous fibronectin to substrate-attached cells. These results indicate that there is a cell binding site in the 70,000-mol-wt fragment that is distinct from the previously described cell attachment site and is required for assembly of exogenous fibronectin into extracellular matrix.

In vivo, fibronectin is a large, dimeric glycoprotein of plasma and most body fluids, and an insoluble constituent of loose connective tissue and basement membranes. It is synthesized by a wide variety of cell types in culture and is found in culture medium and in the insoluble extracellular matrix around cultured cells (22, 34, 47). The fibronectin in the matrix of cultured fibroblasts is found in fibrillar structures that are 5–10 nm in diameter (7, 12, 19, 23, 51). Similar structures have been identified in granulation tissue of skin wounds (52). Although there are structural differences between fibronectin purified from plasma and fibronectin synthesized by cultured cells (2, 16), fibronectin of the extracellular matrix is derived from both plasma (or serum) and local cells (18, 32, 38). Furthermore, both plasma (32) and cellular (5, 8, 21, 25, 31, 57) fibronectin are present in the matrix as high molecular weight, disulfide-bonded multimers. Multimerization of plasma fibronectin probably occurs by disulfide exchange in the 70,000-mol-wt amino-terminal region of the molecule (33).

Fibronectin is believed to mediate cell-matrix adhesion. When coated onto plastic or glass substrata, fibronectin promotes the attachment and spreading of cells. The cell adhesive activity of fibronectin has been localized, first to increasingly smaller regions within the fibronectin subunit (15, 43, 44), and then to a specific tetrapeptide sequence (42). It has been postulated that cells in suspension interact with this region of the fibronectin molecule by means of a specific receptor present on the cell surface. Cell surface gangliosides (27, 60), proteins (1, 40, 55), and wheat germ agglutinin receptors (39) have all been suggested as cell surface molecules important in the attachment and adhesion of cells to fibronectin-coated substrata.

We recently described a presumptive receptor for soluble fibronectin on substrate-attached fibroblasts. This receptor

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appeared to be different from the presumptive receptor on suspended fibroblasts that allows adhesion of cells to substrate-bound fibronectin (32). We proposed that the receptor on substrate-attached fibroblasts mediates the assembly of soluble fibronectin into the insoluble fibrils of the extracellular matrix. To learn more about the interaction of soluble fibronectin with this receptor, we have tested the binding and blocking activities of various proteolytic fragments of fibronectin. In this paper, we describe several fragments that bind to substrate-attached cells. One of these fragments, derived from the amino-terminal third of the fibronectin subunit, was studied in greater detail. It blocked assembly of intact plasma fibronectin into extracellular matrix, although it itself was not assembled into the matrix.

MATERIALS AND METHODS

Materials: Medium for cell culture was from Gibco Laboratories (Grand Island, NY). Fetal bovine serum was from Sterile Systems (Logan, UT). Human embryonic skin cells (Detroit 551) and human embryonic lung cells (IMR-90) were from the American Type Culture Collection (Rockville, MD). Cathepsin D, heparin, and 1,4-diazabicyclo[2.2.2]octane were from Sigma Chemical Co. (St. Louis, MO). L-1-tosylamido-2-phenylethyl choromethyl ketone-treated trypsin was from Worthington Diagnostics Div. (Freehold, NJ). Na ^{125}I was from New England Nuclear (Boston, MA). Type I collagen was purified from fetal bovine skin (4). Cyanogen bromide fragment CB-7 of the α-1(I) collagen chain (26) was a gift from Dr. Hynda Kleinman, National Institute of Dental Research. Heparan sulfate (bovine kidney, super special grade) was from Miles Laboratories (Elkhart, IN). Fluorescein isothiocyanate was from Cappel Laboratories (Cochranville, PA). Materials for autoradiography were from Kodak (Rochester, NY). Gel electrophoresis supplies were from Bio-Rad Laboratories (Richmond, CA).

Cell Culture: Human embryonic skin cells (S132) were of locally established strains (Dr. Michael Gould, University of Wisconsin) and cultured in Ham's F-12 nutrient medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 50 μg/ml streptomycin. Detroit 551 cells were cultured in Eagle's minimal essential medium containing 1.0 mM sodium pyruvate supplemented with 10% fetal calf serum. IMR-90 cells were cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum. Cells for passage were customarily split 1:5 every 7 d. All experiments were performed on confluent cell monolayers. Fibronectin and 70,000-mol-wt fragment binding studies were similar for all three cell strains.

Purification of Human Plasma Fibronectin and Fibronectin Fragments: Human plasma fibronectin was purified from a fibronectin- and fibrinogen-rich by-product of Factor VIII production (36). The plasma fraction was dissolved in 0.01 M Tris and 0.4 M sodium chloride (pH 7.4). Fibrinogen was precipitated by heating at 56°C for 3 min. The solution was clarified by centrifugation and chromatographed on DEAE-cellulose. The fibronectin peak was pooled, and the protein was precipitated with ammonium sulfate, dialyzed against Tris-buffered saline (TBS),[1] and frozen at −70°C until used.

The 70,000-mol-wt, amino-terminal, gelatin-binding fragment of fibronectin was purified from cathepsin D digests by a modification of Balian's procedure (3). 310 μg/ml cathepsin D was preincubated with 1 μg/ml soybean trypsin inhibitor, and 14 mM phenylmethylsulfonyl fluoride in 0.1 M Tris (pH 7.4) to inhibit contaminating serine proteases. 2 mg/ml fibronectin was digested by 1–8 μg/ml cathepsin D for 12 h at 37°C in 50 mM sodium acetate (pH 3.5). The digestion was terminated by addition of pepstatin to a final concentration of 0.3 μM. The mixture was dialyzed against three changes of TBS and applied to a column of gelatin-agarose, 0.8 ml bed volume/mg of starting fibronectin, equilibrated, and washed with TBS. The 70,000-mol-wt fragment was eluted with 3 M guanidine hydrochloride in TBS, dialyzed against TBS, and clarified by centrifugation at 8,800 *g* for 20 min. At this point, the preparation was ~90% pure as judged by SDS PAGE, containing a small amount of 40,000-mol-wt gelatin-binding fragment. This fragment was presumably generated by trace amounts of a contaminating protease still present in the cathepsin preparation. In some preparations, the 40,000-mol-wt fragment was separated from the 70,000-mol-wt fragment by chromatography on DEAE-cellulose. The presence of contaminating 40,000-mol-wt in the 70,000-mol-wt preparations had no detectable effect on the cell binding activity of the 70,000-mol-wt fragment. The yield of 70,000-mol-wt fragment in several preparations was 16–22%, approximately two-thirds of the expected yield of 33%.

The tryptic amino-terminal 27,000-mol-wt fragment of fibronectin was purified as described previously (35). The gelatin-binding 160,000- to 180,000-mol-wt tryptic fragments were purified as described previously (37). The final 160,000- to 180,000-mol-wt preparation consisted of both fragments in approximately equal amounts. The tryptic carboxyl-terminal 31,000-mol-wt fragment was purified as described by Smith et al. (54).

Modification of disulfides in types I and II homology regions of fibronectin greatly increases the susceptibility of the regions to trypsinization (59). Using this strategy, large "fingerless" fragments of fibronectin (i.e., fragments lacking the type I homology "fingers") were produced by disulfide modification followed by mild trypsinization. Fibronectin was reduced with 10 mM dithiothreitol in 0.15 M sodium chloride, 0.15 M Tris (pH 7.4), at 22°C for 3 h and alkylated with 60 mM iodoacetic acid for 1 h at 22°C in the dark. Excess reagents were removed by extensive dialysis against TBS. Modified fibronectin (3.6 mg/ml) was then digested with trypsin (1 μg/ml) for 15 min at 37°C. The digestion was terminated by adding soybean trypsin inhibitor to a final concentration of 5 μg/ml, and the digestion mixture, 10 ml, was applied to a 3- × 70-cm column of Sepharose 6B equilibrated and eluted with TBS. The initial peak was concentrated by ultrafiltration. Approximately 13 mg of a mixture of fragments with molecular weights of ~170,000, 160,000, and 150,000 were obtained from 35 mg of the starting material. The fragments bound to heparin-agarose but did not bind to gelatin-agarose under conditions in which an equal quantity of intact fibronectin bound completely to both affinity supports (59).

Trypsinization and Carboxyamidomethylation of the 70,000-mol-wt Fragment: To cleave the 70,000-mol-wt fragment into 27,000-mol-wt heparin-binding and 40,000-mol-wt gelatin-binding domains, we incubated 500 μg/ml of 70,000-mol-wt fragment with 4 μg/ml trypsin for 2 min at 37°C in TBS. The reaction was stopped with 20 μg/ml soybean trypsin inhibitor. SDS PAGE demonstrated complete conversion of the 70,000-mol-wt fragment into the two smaller fragments. More extensive trypsinization was accomplished by incubation with 20 μg/ml trypsin for 10 min at 37°C. SDS PAGE with and without reduction indicated that the 27,000- and 40,000-mol-wt fragments had been cleaved within disulfide-bonded loops. To form carboxyamidomethylated (Cam)-70,000-mol-wt fragment, we alkylated the fragment (450 μg/ml) with iodoacetamide in the presence of 10 mM dithiothreitol as described above for intact fibronectin. Cam-70,000-mol-wt fragment was dialyzed against TBS. SDS PAGE demonstrated that Cam–70,000-mol-wt fragment migrated the same with and without reduction, whereas unmodified 70,000-mol-wt fragment migrated further in the absence than in the presence of reducing agent because of extensive disulfide bonding.

Iodination of Fibronectin or Fibronectin Fragments: 400 μg of purified plasma fibronectin, 100 μg of 70,000-mol-wt fragment and 400 μg of 160,000–180,000-mol-wt fragments were iodinated with 1 μCi Na ^{125}I using 50 μg of chloramine T in 400 μl of 0.04 M phosphate buffer (pH 7.4). After 60 s, 5 mg of bovine albumin was added to the mixture, and labeled proteins were purified on gelatin-agarose. ^{125}I-Fibronectin and the ^{125}I-160,000–180,000-mol-wt fragments were eluted from the column with 1 M sodium bromide in 50 mM sodium acetate (pH 5.0). ^{125}I-70,000-mol-wt fragment was eluted with 3 M guanidine hydrochloride in TBS. Labeled proteins were then dialyzed against TBS and frozen in portions at −70°C until used. The specific activities of ^{125}I-fibronectin, ^{125}I-70,000-mol-wt fragment, and ^{125}I-160,000–180,000-mol-wt fragments were 300 μCi/mg, 140 μCi/mg, and 40 μCi/mg, respectively. Purities on the labeled proteins were assessed by SDS PAGE with and without reduction followed by autoradiography.

The 31,000-mol-wt carboxyl-terminal tryptic fragment was iodinated by a similar method except that albumin was not added, and the labeled protein was separated from free isotope by gel filtration on Sephadex G-25.

Binding of ^{125}I-labeled Fibronectin or Fibronectin Fragments to Cultured Cells: Binding of iodinated proteins to cell layers was done in F-12 binding medium supplemented with 10% fibronectin-depleted human serum (32) or in F-12 supplemented with 0.2% bovine albumin. Cultures were rinsed twice with Hank's balanced salt sodium before addition of labeled medium. After incubation with labeled medium at 37°C, cultures were rinsed four times in Hank's balanced salt sodium, and cell layers were either sequentially extracted in 1% deoxycholate followed by 4% SDS or scraped directly into 1 N sodium hydroxide. The sequential extraction procedure was used to distinguish cell surface–associated radioactivity (deoxycholate-soluble, Pool I) from radioactivity incorporated into the extracellular matrix (deoxycholate-insoluble, Pool II) (32). Degradation of labeled protein was assessed by SDS PAGE of medium containing labeled protein and by quantification of the percentage of labeled protein soluble in 10% trichloroacetic acid. Electrophoretic analysis of proteins in the absence of reducing agent was used to assess formation of disulfide-bonded multimer of the labeled protein.

[1] *Abbreviations used in this paper:* Cam, carboxyamidomethyl; TBS, Tris-buffered saline (0.14 M sodium chloride, 10 mM Tris, pH 7.4).

To determine specific binding of iodinated molecules, we incubated cell layers with ^{125}I-fibronectin or ^{125}I-70,000-mol-wt fragment for 30 min in the absence (total binding) or presence (nonspecific binding) of unlabeled fibronectin or fragment (200 μg/ml). Specific binding of 70,000-mol-wt fragment in several experiments was 70–80%. Specific binding of intact fibronectin was 60–70%.

Cell Attachment Assay: For quantification of cell attachment and spreading on substrata coated with fibronectin, 70,000-mol-wt fragment, or fingerless fragment, plastic tissue culture plates were incubated for 1 h at room temperature in TBS containing 5, 10, or 25 μg/ml of these three molecules or of bovine albumin. This was followed by a second 1-h incubation at room temperature in 0.2% bovine albumin in TBS. The plates were then rinsed three times with TBS and incubated with a 1-ml suspension of human fibroblasts (Detroit 551, 1.5 × 10^5 cells/ml) in Dulbecco's modified Eagle's medium for 1 h at 37°C. Plates were then shaken on a rotary shaker (180 strokes/min) for 1 min. The medium was removed and the plates were rinsed three times with TBS. For visualization and counting, the attached cells were fixed with 3% paraformalydehyde for 10 min and stained with Coomassie Brilliant Blue. This method was based on the assay described by Grinnell et al. (14).

Incorporation of Fluoresceinated Fibronectin or 70,000-mol-wt Fragment into Cell Cultures: 1 mg/ml fibronectin or 1 mg/ml 70,000-mol-wt fragment was dialyzed against 50 mM carbonate buffer (pH 9.5) containing 0.15 M sodium chloride. 500 μg fluorescein isothiocyanate in 1 ml of carbonate buffer was added to a final concentration of 10 μg/ml. The protein-fluorescein solution was left for 1 h at room temperature with constant stirring. Fluoresceinated fibronectin or 70,000-mol-wt fragment was separated from free fluorescein by chromatography on Sephadex G-25 in phosphate-buffered saline (pH 7.4).

Binding of fluoresceinated proteins was done on cultures of human skin fibroblasts (Detroit 551) grown on glass coverslips. Equimolar amounts of fluoresceinated fibronectin or 70,000-mol-wt fragment (1 × 10^{-7} M) were incubated with cells in F-12 supplemented with 0.2% bovine albumin. After 15 min or 24 h, medium was removed. Cell layers were rinsed three times, fixed with 3.5% paraformaldehyde for 30 min, rinsed, mounted on glass slides in 50% glycerine-phosphate-buffered saline containing 1 mM 1,4-diazabicyclo[2.2.2]octane, and photographed on a Nikon microscope equipped with epifluorescence and phase-contrast.

PAGE: SDS PAGE was performed on slabs of either 6% or 10% polyacrylamide and 3.3% polyacrylamide stacking gels using a discontinuous buffer system (28). Marker proteins were visualized by staining with Coomassie Brilliant Blue. For visualizing ^{125}I-labeled proteins, slabs were dried and autoradiographed with Kodak X-Omat R x-ray film.

RESULTS

Binding of Fibronectin Fragments to Cell Layers

Treatment of fibronectin with various proteases results in fragments that retain specific binding activities. These fragments, therefore, contain one or more of the "binding domains" of fibronectin. Recent studies have described three different types of amino acid sequence homologies, I, II, and III, that make up more than 90% of the amino acid sequence of fibronectin (41, 48, 53). The type I and type II homology units all contain a pair of disulfide bonds. The loops of polypeptide chain defined by these bonds have been called "fingers." The type III homology region of the molecule does not contain disulfide bonds, but does contain the two free sulfhydryls per subunit that are found in plasma fibronectin (48, 54, 56). Cell adhesive activity is located in the type III homology region. The model in Fig. 1 illustrates some of the more well-characterized "binding domains" of fibronectin, identifies the type(s) of amino acid homologies found in each, and localizes the catheptic D and tryptic fragments that are important in the present study.

Fragments of fibronectin prepared by limited tryptic or catheptic D digestion of human plasma fibronectin were tested for their ability to bind to cell monolayers of cultured human fibroblasts and, once bound, to be transferred to the deoxycholate-insoluble extracellular matrix. Digestion of ^{125}I-fibronectin with cathepsin D resulted in eight radiolabeled

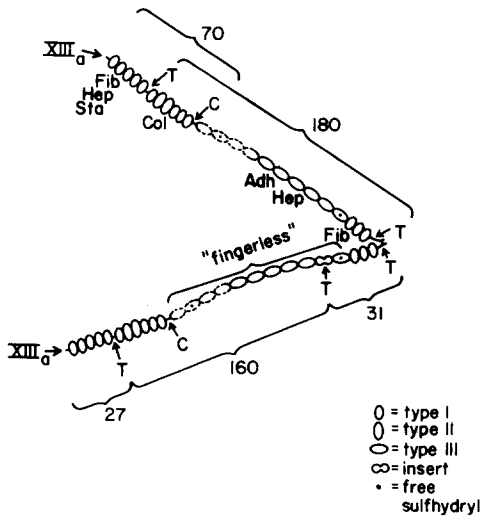

FIGURE 1 Diagram of plasma fibronectin dimer. The two subunits are depicted as two arms. Amino-termini are to the left; carboxyl-termini are to the right. The two arms are joined through disulfide bonds at their extreme carboxyl-termini. All except the region shown as dashed lines have been analyzed by protein sequencing (41, 53) or cDNA sequencing (48). The 45-residue type I homology units are shown as small beads, the 50-residue type II homology units are shown as slightly larger beads, and the 90-residue type III homology units are shown as large beads. Because of their distinctive pattern of disulfide loops, the type I homology units have been called "fingers" (41). A protease-sensitive insertion in the type III homology region is shown as a peanut. Free sulfhydryls are indicated by dots; the exact location of the free sulfhydryl in the dashed region is not known. The following binding activities are localized: for fibrin (*Fib*), for heparin (*Hep*), for *Staphylococcus aureus* cell surface (*Sta*), for collagen and gelatin (*Col*), and for cell adhesion (*Adh*). The plasma transglutaminase ($XIII_a$) cross-linking site is also depicted. Sites of important cleavages by trypsin (*T*) or cathepsin D (*C*) are indicated by arrows, and the resulting fragments are indicated by brackets. The preparation of a "fingerless" fragment containing the type III homology units is described in the text.

fragments with molecular weights ranging from 20,000 to 140,000. Only one fragment, the 70,000-mol-wt gelatin-binding fragment, bound when cell layers were incubated for 30–60 min with these fragments (data not shown). Of the fragments generated by mild trypsin digestion, fragments with molecular weights of 180,000 and larger bound preferentially to cells (data not shown). As illustrated in Fig. 1, the 70,000-mol-wt fragment contains the 27,000-mol-wt amino-terminal heparin- and fibrin-binding domain as well as the adjacent 40,000-mol-wt gelatin-binding domain (3, 46). The 180,000-mol-wt fragment contains all of the binding domains except those of the 27,000-mol-wt amino-terminal region (17, 49, 54). It also does not contain the extreme carboxyl-terminal region of the subunit and is monomeric rather than dimeric, because it lacks the interchain disulfide bonds.

To characterize the interaction of these fragments with cells over a longer time course, we purified the 70,000-mol-wt catheptic D fragment and a mixture of 180,000- and 160,000-mol-wt tryptic fragments from digestion mixtures by chromatography on gelatin-agarose, radiolabeled them with ^{125}I, and incubated them with fibroblasts for up to 24 h. Fig. 2 shows the time courses, performed in parallel, of binding of ^{125}I-labeled fibronectin, 70,000-mol-wt fragment, and mixture of 160,000- and 180-000-mol-wt fragments to cell layers. Amounts of labeled protein bound to the deoxycholate-solu-

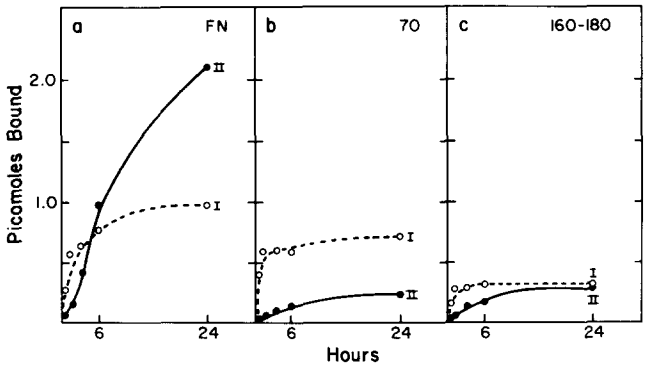

FIGURE 2 Time course of binding ^{125}I-fibronectin (*FN*), ^{125}I-70,000-mol-wt fragment (*70*), and ^{125}I-160,000–180,000-mol-wt fragments (*160–180*) to cell layers. Confluent monolayers of human embryonic skin cells (S132) in 35-mm diameter dishes incubated with 1 ml of binding medium containing 0.2% bovine albumin and equimolar concentrations (5×10^{-8} M; specific activity 10 μCi/nmol) of ^{125}I-fibronectin, ^{125}I-70,000-mol-wt fragment, or ^{125}I-160,000–180,000-mol-wt fragments. At the designated time points, binding in Pool I (deoxycholate soluble, ○) and Pool II (deoxycholate-insoluble, ●) was determined. Dimeric fibronectin was assumed to contain two binding sites per molecule and therefore was assigned a molecular weight of 200,000. The mixture of 160,000–180,000-mol-wt fragments was assigned a molecular weight of 170,000. Determinations represent the average from duplicate cultures.

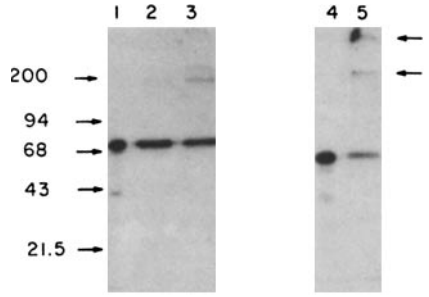

FIGURE 3 Autoradiograph of ^{125}I-70,000-mol-wt fragment bound to cell layers after 24 h. ^{125}I-70,000-mol-wt fragment extracted from cells at the 24-h time point shown in Fig. 2 was analyzed by SDS PAGE. Material from Pool I (lane *2*) or Pool II (lanes *3* and *5*) was electrophoresed into 10% acrylamide gels in the presence (lanes *2* and *3*) or absence (lane *5*) of reducing agent. Lanes *1* and *4* contained the preparation of ^{125}I-70,000-mol-wt fragment used in the experiment, analyzed with (lane *1*) or without (lane *4*) reduction. The arrows on the right mark the tops of the stacking and separating gels. Molecular weights of reduced protein standards are shown on the left. The 200,000-mol-wt standard was human plasma fibronectin. Equal amounts of radioactivity were electrophoresed in each lane.

ble, cell-surface pool (Pool I) and the deoxycholate-insoluble, extracellular matrix (Pool II) were quantified. The behavior of ^{125}I-fibronectin was as described previously (32). Initial binding was to Pool I (Fig. 2*a*). This reached a steady state after 6 h. After a 30-min lag period, fibronectin began to bind to Pool II. After 24 h, 2.1 pmol of fibronectin per plate had accumulated in the extracellular matrix. Binding of the 70,000-mol-wt fragment in Pool I reached a steady state by 1 h and was ~70% of the level of intact fibronectin when compared on a mole basis at 24 h (Fig. 2*b*). There was very little binding of the 70,000-mol-wt fragment to Pool II. Binding of labeled 160,000–180,000-mol-wt fragments to Pool I also reached a steady state at 1 h, and binding at 24 h was ~25% of the binding of intact fibronectin (Fig. 2*c*). More labeled 160,000–180,000-mol-wt fragments than labeled 70,000-mol-wt fragment accumulated in Pool II, but the 160,000–180,000-mol-wt fragments did not accumulate to the extent that intact fibronectin did. Binding of all three labeled proteins was inhibited by excess unlabeled ligand. There was little degradation of the labeled proteins by the cells; after 24 h of incubation with the cell layer, <2.0% of the added radioactivity was soluble in 10% trichloroacetic acid.

To investigate the possibility that trace contaminants in the preparations of ^{125}I-labeled fragments accounted for the Pool II binding found after 24 h, we extracted cell layers and analyzed them by SDS PAGE (Figs. 3 and 4).

Fig. 3 shows the results with the 70,000-mol-wt fragment. The labeled protein in Pool I (lane *2*) consisted mostly of labeled 70,000-mol-wt fragment whereas Pool II (lane *3*) also contained labeled intact fibronectin. Labeled intact fibronectin could not be detected in the starting material (lanes *1* and *4*), and therefore its presence in Pool II represented a substantial enrichment. The small amount of labeled 40,000-mol-wt gelatin-binding fragment that was present in the original preparation (lanes *1* and *4*) was not present in the cell layer at 24 h (lanes *2*, *3*, and *5*). In the absence of reduction (lane *5*), a considerable proportion of the radiolabeled protein in Pool II did not penetrate the stacking gel. This material represents disulfide-bonded multimers (32, 33). It is not known how much of the multimeric material was derived from intact labeled fibronectin and how much was derived from labeled 70,000-mol-wt fragment.

Fig. 4 shows the results with the labeled 160,000–180,000-mol-wt preparation (lanes *3*, *4*, *7*, and *8*) and, for comparison, labeled fibronectin (lanes *1*, *2*, *5*, and *6*). The 160,000–180,000-mol-wt preparation (lanes *3* and *7*) contained small amounts of contaminants that migrated close to the position of the subunit of intact fibronectin when analyzed with reduction (lane *3*), but were both monomeric and dimeric when analyzed without reduction (lane *7*). The monomeric polypeptide probably represents fibronectin that has been cleaved only at the extreme carboxyl terminus (see Fig. 1). Analysis of labeled 160,000–180,000-mol-wt material extracted from Pool I (not shown) demonstrated the 180,000-mol-wt fragment, none of the 160,000-mol-wt fragment, and only traces of polypeptides with molecular weights larger than 180,000. In contrast, approximately equal amounts of the larger proteins and the 180,000-mol-wt fragment were extracted from Pool II (lane *4*). In the absence of reducing agent, some of the labeled proteins in Pool II migrated as disulfide-bonded multimers (lane *8*). Thus, these results indicate that the cells selectively bind and accumulate molecules with weights of 180,000 and greater from the mixture in the labeled 160,000–180,000-mol-wt fragment preparation.

In a single experiment, specific binding of ^{125}I-labeled 31,000-mol-wt fragment to Pool I was demonstrated (data not shown). The 31,000-mol-wt fragment represents the difference between the 180,000- and 160,000-mol-wt fragments (17, 49, 54; see Fig. 1).

Binding of the 70,000-mol-wt Fragment to Pool I

Reversibility of binding of the 70,000-mol-wt fragment and fibronectin to cells was tested by incubation of cell layers for 20 min at 37°C with ^{125}I-70,000-mol-wt fragment or ^{125}I-

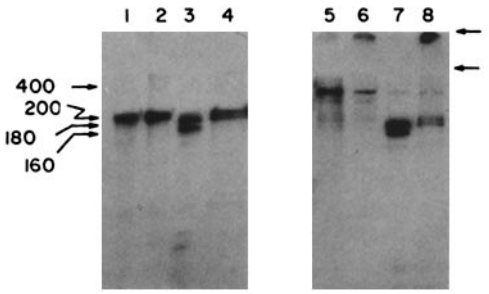

FIGURE 4 Autoradiograph of ^{125}I-fibronectin and ^{125}I-160,000-180,000-mol-wt fragments bound to Pool II after 24 h. The preparations of iodinated proteins used in the experiment shown in Fig. 2 and labeled proteins extracted from Pool II at the 24-h time point were analyzed by SDS PAGE on 6% gels in the presence (lanes *1-4*) and absence (lanes *5-8*) of reducing agent. Lanes *1* and *5*, ^{125}I-fibronectin; lanes *2* and *6*, Pool II-bound ^{125}I-fibronectin; lanes *3* and *7*, ^{125}I-160,000-180,000-mol-wt preparation; and lanes *4* and *8*, Pool II-bound ^{125}I-160,000-180,000-mol-wt fragments. The arrows on the right indicate the tops of the stacking and the separating gels. The arrows on the left indicate bands corresponding to intact fibronectin and 180,000- and 160,000-mol-wt fragments. Equal amounts of radioactivity were electrophoresed into each lane.

fibronectin, washing away of unbound protein, and further incubation of cell layers in medium without ligand (Fig. 5). After 3 h, >70% of the bound 70,000-mol-wt fragment was chased from the cell layer. In the same experiment, only 50% of the bound fibronectin was chased from the cell layer. Some of the fibronectin that remained associated with the cell layer after the 3-h chase probably represented bound fibronectin that had transferred into Pool II. Pool II binding is irreversible (32).

Inhibition of Binding of ^{125}I-Fibronectin to Pool I by Fibronectin Fragments

Binding of ^{125}I-fibronectin to cells was inhibited by the presence of increasing concentrations of unlabeled 70,000-mol-wt fragment or fibronectin (Fig. 6). Half maximal inhibiton (K_i) of ^{125}I-fibronectin binding was obtained at concentrations of 2.5×10^{-8} M for both fibronectin and the 70,000-mol-wt fragment. This indicates that the 70,000-mol-wt fragment is as effective as fibronectin in preventing the binding of ^{125}I-fibronectin to Pool I.

In other experiments, the mixture of 160,000- and 180,000-mol-wt fragments and the 31,000-mol-wt fragment generated from the carboxyl-terminal region by tryptic cleavage (Fig. 1) were also found to be inhibitory. The K_i of these fragments were not determined. However, the fragments were less inhibitory than the same molar concentrations of 70,000-mol-wt fragment (data not shown).

Cell Attachment Assay Using the 70,000-mol-wt Fragment

As described in the beginning of this paper, the cell adhesive site of fibronectin is the region that mediates the attachment of suspended cells to fibronectin-coated substrata and has been localized to a tetrapeptide in the type III homology region (Fig. 1). Our data, however, indicated that efficient binding of fibronectin to substrate-attached cells required a number of type I homology finger sequences. To separate more definitively the two types of binding phenomena, we

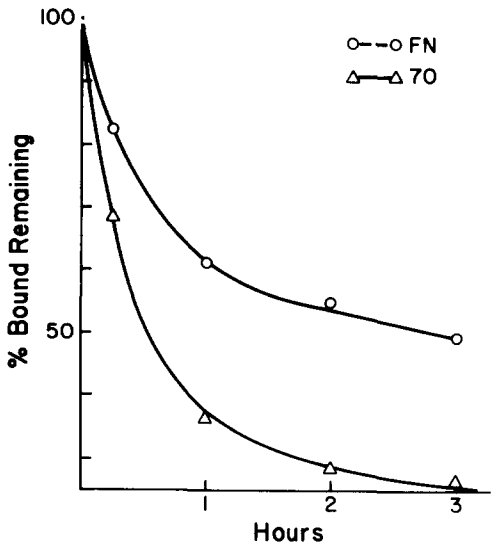

FIGURE 5 Reversibility of binding of ^{125}I-fibronectin and ^{125}I-70,000-mol-wt fragment to Pool I. Confluent monolayers of human embryonic skin fibroblasts (Detroit 551) in 60-mm diameter dishes were incubated with 2 ml of binding medium containing 0.2% bovine albumin and equimolar (1×10^{-8} M; specific activity 10 μCi/nmol) concentrations of ^{125}I-fibronectin (*FN*) or ^{125}I-70,000-mol-wt fragment (*70*). Incubations were at 37°C for 20 min. After 20 min, the plates were rinsed, and the medium was replaced with F-12 containing 0.2% bovine albumin. At the designated time points, cell layers were scraped into 1 ml of 1 N sodium hydroxide, and the amount of ^{125}I-fibronectin (○) or ^{125}I-70,000-mol-wt fragment (△) remaining in the cell layer was determined. Determinations represent the average from duplicate cultures.

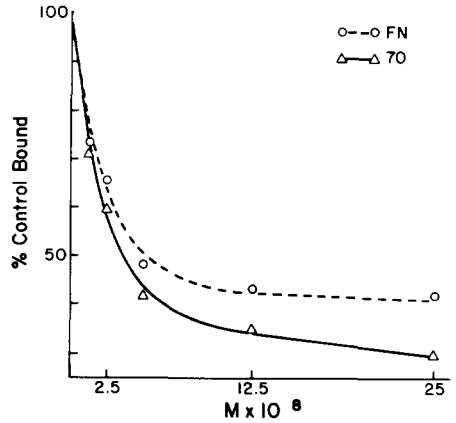

FIGURE 6 Inhibition of ^{125}I-fibronectin binding to Pool I by 70,000-mol-wt fragment. Confluent monolayers of human embryonic skin fibroblasts (Detroit 551) in 35-mm diameter dishes were incubated with ^{125}I-fibronectin (450 ng, 10^6 cpm) in 0.9 ml of binding medium containing 0.2% bovine albumin and increasing concentrations of unlabeled fibronectin (○) or 70,000-mol-wt fragment (△). Medium of control plates contained only albumin. Cultures were incubated for 30 min at 37°C, rinsed, and scraped into 1 ml of 1 M sodium hydroxide. Determinations represent the average from duplicate plates. So that binding affinities could be compared with previous data (32), fibronectin has been assigned a molecular weight of 400,000, rather than 200,000 as in Fig. 2.

compared fibronectin, the 70,000-mol-wt fragment that does not contain the cell adhesion site, and fingerless fragments that contain the cell adhesion site but not intact disulfide-looped fingers (59, see Fig. 1), for their ability to promote cell attachment and spreading (see Fig. 7 and Table I). The results

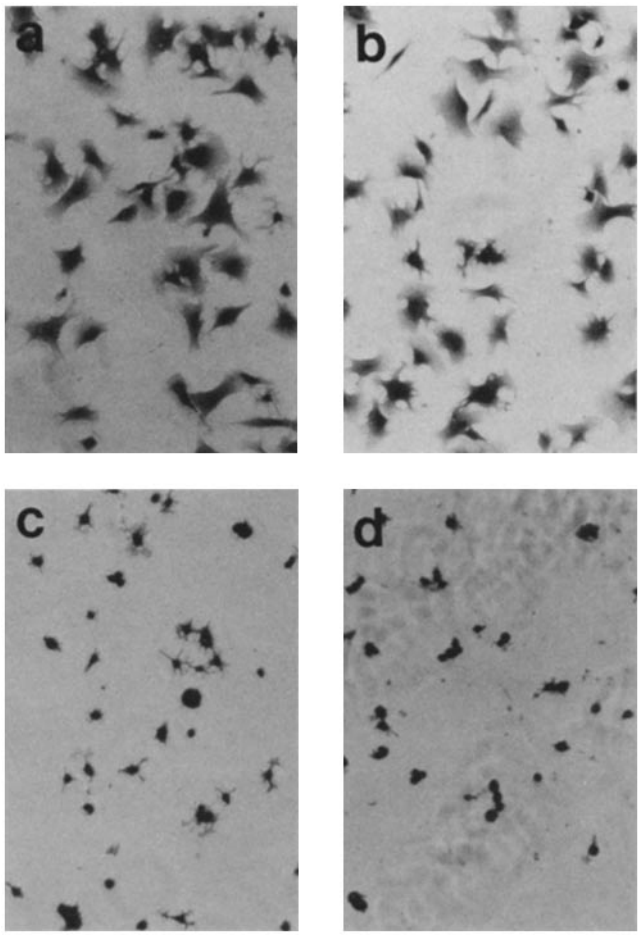

FIGURE 7 Cell attachment to fibronectin, fingerless fragments and 70,000-mol-wt fragment. Human embryonic skin cells (Detroit 551) in suspension (2×10^5 cells/ml) were allowed to attach to plastic tissue culture dishes coated with (a) fibronectin (10 μg/ml), (*b*) fingerless fragments (10 μg/ml), (c) 70,000-mol-wt fragment (25 μg/ml), or (*d*) bovine albumin (25 μg/ml). Attachment was for 1 h at 37°C. Cells were fixed and stained with Coomassie Brilliant Blue before photography.

TABLE I

Effect of Preadsorption of Fibronectin or Fibronectin Fragments on Cell Attachment and Spreading on Tissue Culture Plastic

Protein coating	Concentration of coating (μg/ml)	Cells per sq mm (X ± SEM, n = 10)* Attached only	Attached and spread
Fibronectin	5	<1	59 ± 10
	10	<1	86 ± 15
	25	<1	100 ± 10
Fingerless fragments	5	<1	50 ± 3
	10	<1	105 ± 15
	25	<1	71 ± 8
70,000-mol-wt fragment	5	9 ± 2	<1
	10	15 ± 4	<1
	25	23 ± 4	<1
Bovine albumin	5	14 ± 5	<1
	10	5 ± 2	<1
	25	5 ± 1	<1

* The numbers of trypsinized human skin fibroblasts attached or attached and spread after a 1-h incubation period were counted in 10 different areas chosen at random on two different plates (five areas per plate).

presented in Table I demonstrate that both fibronectin and the fingerless fragment promote cell attachment and spreading, although cells were slightly less spread on fingerless fragments as compared with fibronectin (Fig. 7). Attachment of cells to 70,000-mol-wt fragment was similar to the control levels of attachment obtained with albumin (Table I). At the highest coating concentration, cells attached to 70,000-mol-wt fragment in some regions of the plate (Fig. 7*c*). However, these cells were not spread, and the same attachment was seen with albumin coating (Fig. 7*d*).

Inhibition of Incorporation of Fibronectin into the Extracellular Matrix by the 70,000-mol-wt Fragments

Previous studies have suggested that the assembly of fibronectin into the extracellular matrix (Pool II) requires the participation of the presumptive cell surface receptors that mediate binding to Pool I (32). To verify this hypothesis, we tested the 70,000-mol-wt fragment that bound to cells primarily in Pool I (Fig. 2) and competed for binding of intact fibronectin to Pool I (Fig. 6) for its ability to block the binding of fibronectin in Pool II. Confluent cultures were incubated for 4 h with ^{125}I-fibronectin in the presence of increasing concentrations of unlabeled 70,000-mol-wt fragment or, as a control, fingerless fragments. Inhibition of ^{125}I-fibronectin binding to Pool I was accompanied by a corresponding inhibition of binding to Pool II (Fig. 8). This result indicates that Pool I binding of fibronectin is required for its subsequent incorporation into the matrix. The fingerless fragment of fibronectin had no effect on ^{125}I-fibronectin binding to either pool. This is further evidence that the ability of fibronectin to bind to cell surfaces of substrate-attached cells is independent of its cell adhesive activity for cells in suspension.

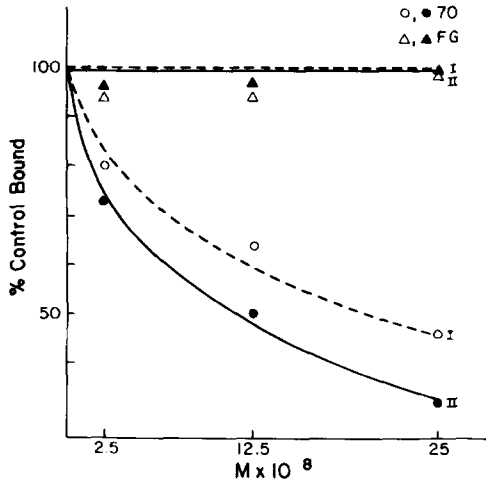

FIGURE 8 Inhibition of incorporation of fibronectin into extracellular matrix by 70,000-mol-wt fragment. Confluent monolayers of human embryonic skin fibroblasts (Detroit 551) in 35-mm diameter dishes were incubated with 1.0 ml of binding medium containing 0.2% bovine albumin, ^{125}I-fibronectin (400 ng, 5×10^5 cpm) and increasing concentrations of unlabeled fingerless fragment (*FG*, △ and ▲) or 70,000-mol-wt fragment (*70*, ○ and ●). Medium in control plates contained only albumin and ^{125}I-fibronectin. Cultures were incubated for 4 h at 37°C, rinsed, and binding to Pool I (open symbols) and Pool II (closed symbols) was determined as described in Materials and Methods. Determinations represent the average from duplicate cultures.

Effects of Collagen and Heparin on Binding of 70,000-mol-wt Fragment and Fibronectin to Pool I

Because the 70,000-mol-wt fragment of fibronectin contains both collagen- and heparin-binding activities, it was of interest to learn whether these molecules had any effect on the binding of fibronectin or its 70,000-mol-wt fragment to cells. When ^{125}I-fibronectin was preincubated with type I collagen, gelatin, or cyanogen bromide fragment 7 (CB-7) of the α-1(I) chain of type I collagen (this is the collagen fragment which has greatest affinity for fibronectin [26]), binding of ^{125}I-fibronectin and ^{125}I-70,000-mol-wt fragment to cells was enhanced (Table II). Collagen and gelatin enhanced binding of ^{125}I-fibronectin 1.5- to 2-fold. Lesser enhancement was noted with the CB-7 fragment. Binding of ^{125}I-70,000-mol-wt fragment to cells was only slightly enhanced by the collagenous molecules. Heparin enhanced binding to ^{125}I-fibronectin by 30%, but had no effect on binding of the ^{125}I-70,000-mol-wt fragment. Heparan sulfate had little effect on binding of either ^{125}I-fibronectin or ^{125}I-70,000-mol-wt fragment.

Effects of Modification of the 70,000-mol-wt Fragment on Its Binding to Cells

To determine which region of the 70,000-mol-wt fragment was binding to the cell surface, we tested purified 27,000- and 40,000-mol-wt tryptic fragments of fibronectin for their ability to inhibit the binding to ^{125}I-70,000-mol-wt fragment to cells. As shown in Table III, the 27,000-mol-wt fragment was sixfold more effective than the 40,000-mol-wt fragment in preventing the binding of the ^{125}I-70,000-mol-wt fragment to the cell surface but fourfold less effective than the 70,000-mol-wt fragment in the inhibition assay. Mild trypsinization of the 70,000-mol-wt fragment into the 27,000- and 40,000-mol-wt fragments resulted in a fourfold decrease in inhibitory activity (Table III). Thus, the inhibitory activity of the mixture of fragments was similar to that of the 27,000-mol-wt fragment.

TABLE II

Effect of Fetal Calf Skin Collagen, Gelatin, Cyanogen Bromide Fragment 7 of Alpha-1(I) Collagen Chains (CB-7), Heparin, or Heparan on Binding of ^{125}I-Fibronectin or ^{125}I-70,000-mol-wt Fibronectin Fragment

Molecule	μg/ml	% Control bound	
		^{125}I-Fibronectin	^{125}I-70,000-mol-wt
Collagen	5	150	117
	25	216	129
Gelatin	5	192	129
	25	159	136
CB-7	5	126	119
	25	129	127
Heparin	5	133	102
	25	135	97
Heparan	5	106	105
	25	105	96

Confluent monolayers of human embryonic skin cells (Detroit 551) (collagen experiments) or human embryonic lung cells (IMR-90) (heparin and heparan experiments) were incubated with ^{125}I-fibronectin (900 ng, 3×10^5 cpm) or ^{125}I-70,000-mol-wt fragment (200 ng, 8×10^5 cpm) in 1 ml of binding medium containing 20 mM HEPES (pH 7.4), 0.2% bovine albumin without (control) or with the designated concentration of protein or polysaccharide. Determinations represent the average from duplicate plates.

TABLE III

Inhibition by Trypsinized 70,000-mol-wt Fragment, Cam-70,000-mol-wt Fragment, and 27,000- or 40,000-mol-wt Fragment of Binding of ^{125}I-70,000-mol-wt Fibronectin Fragment to Cells

Fragment	Concentration required for half maximal inhibition
	(M × 10^8)
70,000-mol-wt	2.5
Trypsinized 70,000-mol-wt	11
Cam-70,000-mol-wt	>60
27,000-mol-wt	10
40,000-mol-wt	65

Confluent monolayers of human embryonic skin cells (Detroit 551) were incubated with 1 ml of binding medium containing ^{125}I-70,000-mol-wt (200 ng, 7×10^5 cpm), 0.2% bovine albumin, and increasing concentrations of unlabeled 70,000-mol-wt fragment, trypsinized 70,000-mol-wt fragment, Cam-70,000-mol-wt fragment, or 27,000- or 40,000-mol-wt fragment. Incubations were for 30 min at 37°C. Cell layers were rinsed and scraped into 1 N sodium hydroxide for radioactivity determinations. Maximal inhibition was defined as the inhibition obtained with 70,000-mol-wt fragment, 50 μg/ml (7×10^{-7} M).

The 27,000-, 40,000-, and 31,000-mol-wt regions all contain several units of Type I amino acid sequence homology. The secondary structure of the units is undoubtedly maintained by disulfide bonds. To test whether the integrity of these disulfides is needed for binding to cells, we reduced the 70,000-mol-wt fragment and alkylated it with iodoacetamide (to form the carboxyamidomethyl- or Cam- derivative). Cam-70,000-mol-wt fragment did not compete for binding of ^{125}I-70,000-mol-wt fragment to cells (Table III) at concentrations 30-fold greater than the K_I of unmodified 70,000-mol-wt fragment. As further evidence that intact finger regions are important for effective binding of the 70,000-mol-wt fragment to cells, vigorous trypsinization of the 70,000-mol-wt fragment, resulting in cleavages within fingers, produced greater decreases in inhibitory activity than that observed when the 70,000-mol-wt fragment was simply cleaved into 27,000- and 40,000-mol-wt fragments (data not shown).

Localization of Fibronectin and the 70,000-mol-wt Fragment in Cell Layers

Fluorescein-labeled fibronectin or 70,000-mol-wt fragment was incubated with cell layers for 15 min or 24 h and localized by fluorescence microscopy. After 15 min of binding, both molecules were detected in fine linear patterns that appeared to be between cells (Fig. 9, *a* and *c*). When viewed at higher magnification (not shown), linear fluorescence was localized to the extreme edges of the cells. After 24 h (Fig. 9*b*), there was extensive accumulation of fluorescein-labeled fibronectin by the cells (note the fivefold shorter exposure time). Fluorescence was in fibrils that were coarser and thicker than those observed at the earlier time and formed an extensive extracellular meshwork that completely surrounded the cells. This was the same pattern seen when the cell layers were stained for endogenous fibronectin matrix by indirect immunofluorescence using antifibronectin antibodies (not shown). In contrast, there was no accumulation of fluorescein-labeled 70,000-mol-wt fragment by the cell layers at 24 h (Figure 9*d*). The fluorescence was distributed in a linear pattern. When viewed at a higher magnification (Fig. 10), the fluorescence was localized to the edges of cells. When one focused up and down, there was no evidence of fluorescence underneath or on top of the cells.

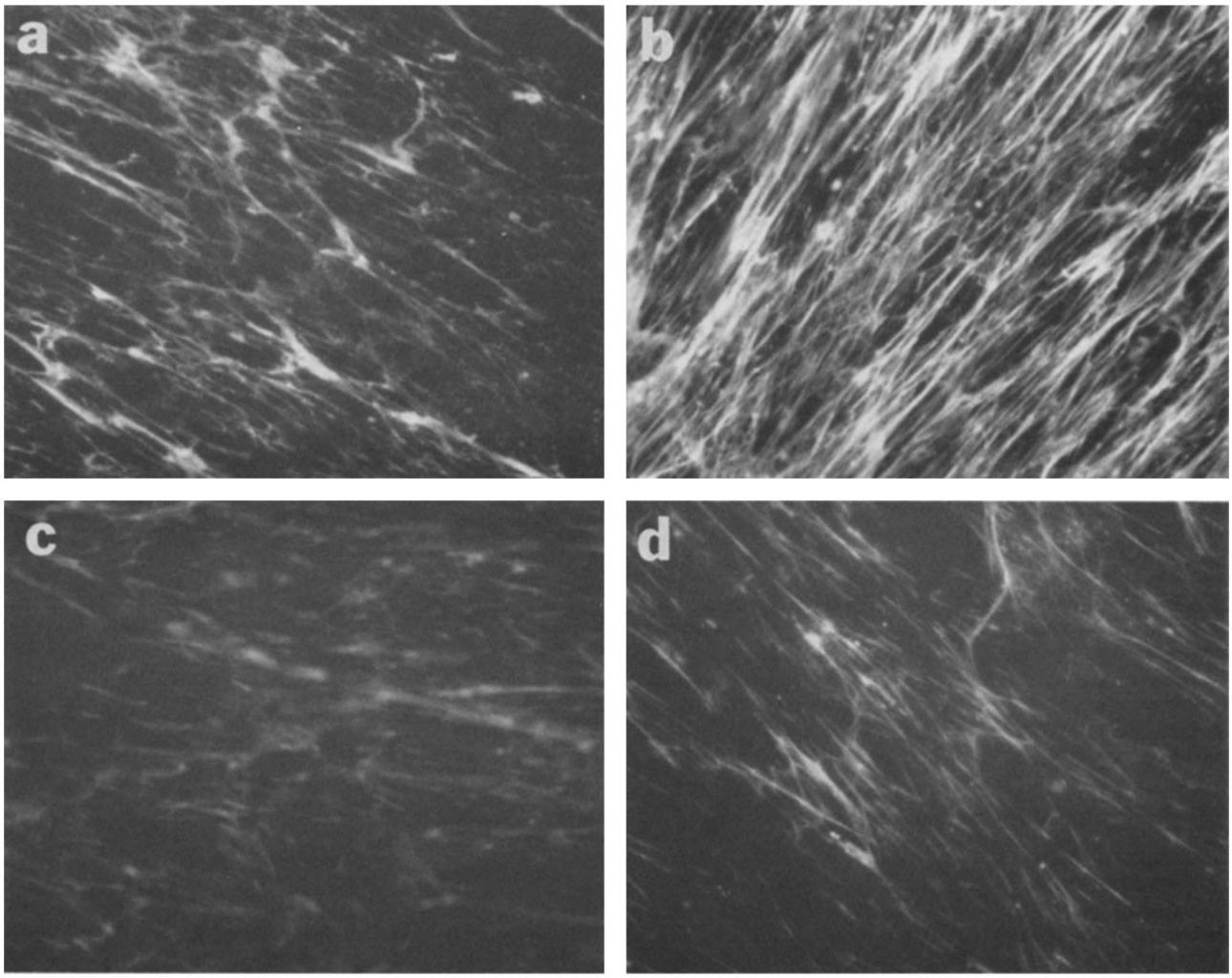

FIGURE 9 Localization of cell layer-bound fibronectin and 70,000-mol-wt fragment by fluorescence microscopy. Fluorescein-labeled derivatives of fibronectin and 70,000-mol-wt fragment (1×10^{-7} M) were incubated with confluent cell layers of cultured human fibroblasts for either 15 min or 24 h. Cell layers were rinsed, fixed, and viewed under the fluorescent microscope. (*a*) Fibronectin, 15-min incubation; (*b*) fibronectin, 24-h incubation; (*c*) 70,000-mol-wt fragment, 15-min incubation; and (*d*) 70,000-mol-wt fragment, 24-h incubation. Exposure times were as follows: (*a*) 147 s; (*b*) 30 s; (*c*) 180 s; and (*d*) 185 s. × 300.

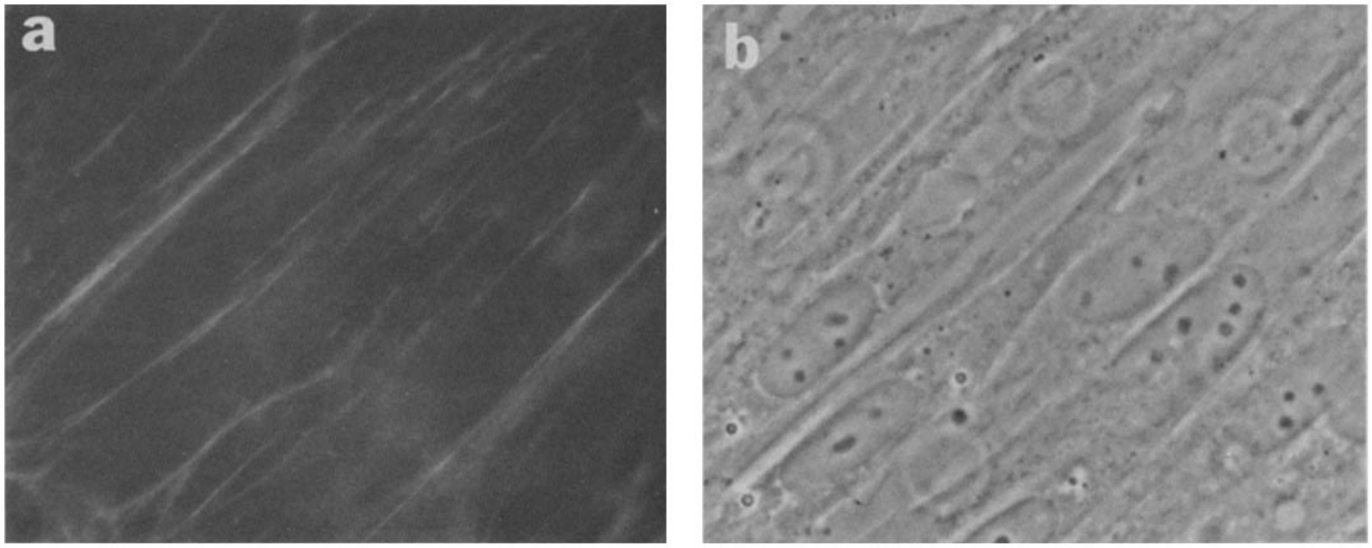

FIGURE 10 Localization of 70,000-mol-wt fragment bound to the cell layer after 24 h. Fluorescein-labeled 70,000-mol-wt fragment (1×10^{-7} M) was incubated with cell layers for 24 h. Cell layers were rinsed, fixed, and 70,000-mol-wt fragment was visualized by (*a*) fluorescent and (*b*) phase-constrast microscopy. × 750.

DISCUSSION

In a previous communication, we reported that soluble plasma fibronectin binds to monolayers of cultured human fibroblasts (32). Two pools of bound fibronectin in cell layers were defined on the basis of their differential solubility in 1% deoxycholate. The deoxycholate soluble pool, Pool I, was proposed to represent fibronectin binding to receptors on the cell surface, and the deoxycholate insoluble pool, Pool II, was proposed to represent fibronectin incorporated into the detergent-insoluble extracellular matrix. It seemed likely that Pool I binding of fibronectin represented binding to a receptor present on the cell surface because binding to Pool I was specific and saturable, and fluorescence microscopic localization indicated that Pool I binding occurred at the edges of the cells and not in the fibrils of the extracellular matrix (see also Figs. 9 and 10). Furthermore, Pool I binding seemed to be required for transfer of fibronectin to Pool II because fibronectin did not accumulate into the matrix in the absence of cells (32).

Binding studies presented here using the 70,000-mol-wt fragment indicate that a principal cell binding site on fibronectin is in the amino-terminal one-third of the molecule. In these studies, the binding affinities of fibronectin and the 70,000-mol-wt fragment appeared similar, as judged by concentrations needed for half maximal inhibition of binding, $K_I = 2.5 \times 10^{-8}$ M. This is in good agreement with previous binding studies, analyzed by the method of Scatchard, that gave a dissociation constant for fibronectin and its receptor of 3.8×10^{-8} M (32).

Preincubation of ^{125}I-fibronectin or ^{125}I-70,000-mol-wt fragment with collagen or heparin did not inhibit binding to the cells. Thus, the cell binding site present on the 70,000-mol-wt fragment appears to be independent of the heparin and collagen binding sites. Both heparin and collagen enhanced the binding of fibronectin to cells over 30 min. This is in agreement with a previous report in which collagen was shown to enhance the accumulation of soluble fibronectin in cell layers over a longer period (45). The binding of CB-7 to fibronectin in solution has been reported to cause the amino-terminal part of fibronectin to be more mobile (58). Thus, the increased binding seen in the presence of collagen may be due to increased accessibility of the 27,000-mol-wt region of fibronectin to cell receptors. Heparin enhanced the binding of fibronectin, but not the 70,000-mol-wt fragment, to the cells. Binding of heparin to the more carboxyl-terminal of the heparin binding sites (see Fig. 1) may also mobilize the amino-terminal end of the molecule. Alternatively, the heparin may bind several fibronectin molecules that subsequently attach to the cell surface. Both collagen and heparin conceivably could enhance binding of fibronectin to cell layers by first complexing to fibronectin and then binding to cell surface receptors for collagen (13) or heparin. Heparan sulfate had no effect on fibronectin binding. This is consistent with the observation by Sekiguchi et al. (50) that heparan sulfate does not bind to intact fibronectin under physiological conditions.

Although the 70,000-mol-wt fragment was bound into Pool I, very little was incorporated into the extracellular matrix over a 24-h period (Fig. 2). The 180,000-mol-wt fragment did accumulate in the matrix but to a lesser extent than intact fibronectin (Fig. 2). Previously, it was shown that only intact fibronectin becomes associated with connective tissue matrices when injected in vivo (38). These results suggest that, although fragments of fibronectin bind to cell surface receptors, only intact molecules are effectively assembled into the matrix. That binding of fibronectin to these receptors is required for matrix assembly was shown by the finding that the 70,000-mol-wt fragment that bound to Pool I and was transferred poorly to the matrix blocked the incorporation of fibronectin into the extracellular matrix (Fig. 7). Fluorescence microscopy (Figs. 9 and 10) indicated that binding sites are not uniformly distributed over the cell surface, but are restricted to the edges of the cell. Consistent with this observation, indirect immunofluorescence using antifibronectin antibodies has shown that the formation of fibronectin fibrils by newly seeded cells begins at the outer edges of the cell (6).

The "cell binding site" present in the 70,000-mol-wt fragment is distinct from the previously characterized "cell attachment" or "cell adhesion site" (42–44). The cell adhesion site of fibronectin has been characterized using cell attachment assays in which cells in suspension attach and spread on fibronectin adsorbed onto plastic or glass substrata. Inhibition of cell adhesion with soluble fibronectin has resulted in the characterization of low affinity receptors ($K_D = 10^{-7}$ to 10^{-5} M) for fibronectin (24, 30, 61). Cell binding studies using fibronectin adsorbed onto latex beads (30) probably also represent a variation of the cell attachment assay. Our studies indicate that there is a "matrix assembly site" on fibronectin for a "matrix assembly receptor" on substrate-attached cells and a cell adhesion site on fibronectin for a cell adhesion receptor on suspended cells. The cell adhesion receptor and the matrix assembly receptor can be distinguished by several criteria. The matrix assembly receptor recognizes preferentially the part of the fibronectin molecule present in the 70,000-mol-wt fragment. The 70,000-mol-wt cell binding fragment did not exhibit cell adhesion activity (Fig. 7 and Table I). Fragments that contained the cell adhesion site and mediated cell adhesion did not bind to the matrix assembly receptor (Fig. 8). HT-1080 fibrosarcoma cells attached and spread on substrate-adsorbed fibronectin but did not bind fibronectin in either Pool I or Pool II (32). It is likely that the cell adhesion receptor is functional in these transformed cells but the matrix assembly receptor is not. The matrix assembly receptor exhibited relatively high affinity for soluble fibronectin (Fig. 6) when compared to the cell adhesion receptor (24, 30, 61).

We were unable to localize the matrix assembly site unequivocally to a site within the 70,000-mol-wt fragment. Because all three regions of fibronectin that contain type I homology units seem to contribute to binding (i.e., the 70,000-mol-wt fragment inhibited better than the 27,000- or 40,000-mol-wt fragment, and the 180,000-mol-wt fragment that contained the 40,000- and 31,000-mol-wt regions bound better than the 160,000-mol-wt fragment that contained only the 40,000-mol-wt region), binding to the matrix assembly receptor may be a general property of the type I sequence. Inhibition studies using tryptic fragments derived from the 70,000-mol-wt fragments, however, suggest that the principal binding site is within the 27,000-mol-wt amino-terminal region. Although the 27,000-mol-wt fragment was significantly less active than the 70,000-mol-wt fragment in blocking the binding of labeled 70,000-mol-wt fragment to cells (Table III), in high enough concentration it did block completely. In addition, the 27,000-mol-wt fragment was considerably more effective than the 40,000-mol-wt fragment in blocking the binding of labeled 70,000-mol-wt fragment. If the matrix

assembly site is contained wholly in the 27,000-mol-wt region, the isolated 27,000-mol-wt fragment may not bind to the receptor with the same affinity as the 70,000-mol-wt fragment because of conformational changes mediated by the adjacent 40,000-mol-wt region. Alternatively, the binding site may span both the 27,000- and 40,000-mol-wt regions of the molecule, with sites in both regions requried for optimal binding. Finally, there may be another site in the 31,000-mol-wt region that also interacts with cells and allows efficient transfer of intact bound molecules into extracellular matrix.

Previous studies have suggested that a 60,000-mol-wt chymotryptic fragment from horse serum fibronectin contains a binding site for intact fibronectin (10, 11). The 60,000-mol-wt fragment appears to be quite similar to the 70,000-mol-wt catheptic fragment. This suggests the possibility that the matrix assembly receptor for fibronectin is fibronectin itself. Such a possibility seems unlikely for several reasons: (*a*) Very little plasma fibronectin binds to isolated extracellular matrix (32). (*b*) Early binding of fibronectin or the 70,000-mol-wt fragment was to restricted areas on or near the cell surface and not to the entire fibronectin-containing extracellular matrix. (*c*) Pretreatment of cells with trypsin, under conditions in which 90% of the extracellular fibronectin was removed, has no effect on binding of fibronectin to Pool I (unpublished observations). (*d*) Pretreatment of cells with cycloheximide for several hours to clear the cells of newly synthesized fibronectin has no inhibitory effect on fibronectin binding (unpublished observation). Nevertheless, fibronectin-fibronectin interactions probably occur during assembly of disulfide-bonded multimers (33), and further studies are needed to describe more fully the molecular interactions at the cell surface.

Proteolytic fragments of fibronectin have effects on cellular metabolism that are not shared by the intact molecule. Cathepsin D digests of bovine plasma fibronectin have been shown to stimulate the synthesis of DNA in quiescent fibroblast cultures (20). Gelatin-binding fragments of plasmin- or cathepsin G-treated human plasma fibronectin enhance morphological cell transformation of Rous sarcoma virus-infected chick embryo fibroblasts (9). Trypsin and plasmin digests of fibronectin accelerate the formation of tubular structures in endothelial cell cultures (29). Our experiments indicate that fibronectin fragments can interact with cells in a manner that is different from intact fibronectin, i.e., the fragments can bind to the presumptive matrix assembly receptor but are not assembled into extracellular matrix. It will be of interest to learn whether the effects of fibronectin fragments described above are modulated by interactions of the fragments with the presumptive matrix-assembly receptor.

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