A Novel Role for the Integrin-binding III-10 Module in Fibronectin Matrix Assembly

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Abstract. Fibronectin matrix assembly is a cell-dependent process which is upregulated in tissues at various times during development and wound repair to support the functions of cell adhesion, migration, and differentiation. Previous studies have demonstrated that the $\alpha_5\beta_1$ integrin and fibronectin's amino terminus and III-1 module are important in fibronectin polymerization. We have recently shown that fibronectin's III-1 module contains a conformationally sensitive binding site for fibronectin's amino terminus (Hocking, D.C., J. Sottile, and P.J. McKeown-Longo. 1994. *J. Biol. Chem.* 269: 19183–19191). The present study was undertaken to define the relationship between the $\alpha_5\beta_1$ integrin and fibronectin polymerization. Solid phase binding assays using recombinant III-10 and III-1 modules of human plasma fibronectin indicated that the III-10 module contains a conformation-dependent binding site for the III-1 module of fibronectin. Unfolded III-10 could support the formation of a ternary complex containing both III-1 and the amino-terminal 70-kD fragment, suggesting that the III-1 module can support the simultaneous binding of III-10 and 70 kD. Both unfolded III-10 and unfolded III-1 could support fibronectin binding, but only III-10 could promote the formation of disulfide-bonded multimers of fibronectin in the absence of cells. III-10–dependent multimer formation was inhibited by both the anti–III-1 monoclonal antibody, 9D2, and amino-terminal fragments of fibronectin. A fragment of III-10, termed III-10/A, was able to block matrix assembly in fibroblast monolayers. Similar results were obtained using the III-10A/RGE fragment, in which the RGD site had been mutated to RGE, indicating that III-10/A was blocking matrix assembly by a mechanism distinct from disruption of integrin binding. Texas red–conjugated recombinant III-1,2 localized to β_1-containing sites of focal adhesions on cells plated on fibronectin or the III-9,10 modules of fibronectin. Monoclonal antibodies against the III-1 or the III-9,10 modules of fibronectin blocked binding of III-1,2 to cells without disrupting focal adhesions. These data suggest that a role of the $\alpha_5\beta_1$ integrin in matrix assembly is to regulate a series of sequential self-interactions which result in the polymerization of fibronectin.

FIBRONECTINS are a family of high molecular weight, multidomain glycoproteins composed of two structurally similar subunits which are joined at the carboxyl terminus by disulfide bonds (17). Fibronectin, like many other proteins of the extracellular matrix, is a mosaic protein composed of modular units (17). The primary structure of fibronectin is organized into three types of repeating homologous units, termed types I, II, and III (17, 30). These modules in turn are organized into functional domains which have been shown to contain multiple binding sites, including those for sulfated glycosaminoglycans, gelatin, fibrin, and cell surface integrin receptors (26, 30, 51).

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Fibronectin is secreted as a dimer into the blood and other body fluids, where it circulates at micromolar concentrations as a soluble, protomeric molecule. In addition, an insoluble, multimeric form of fibronectin is found within the extracellular spaces of connective tissue, basement membranes, and cultured cells (17). This multimeric form of fibronectin is its primary functional form, mediating a variety of adhesive and migratory events during embryogenesis, angiogenesis, thrombosis and hemostasis, inflammation, and wound repair (17).

Polymerization of soluble fibronectin into insoluble fibrils is a dynamic, multistep, cell-dependent process which occurs at the surface of substrate-attached cells (28). The mechanism by which a fibronectin matrix is assembled, however, is only partially understood. In one of the initial steps of matrix assembly, cell surfaces bind the amino-terminal region of fibronectin in a reversible and saturable manner (22, 35). Subsequent homophilic-binding interac-

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tions are thought to lead to the formation of disulfide-stabilized high molecular mass multimers (5, 24, 25). The molecule(s) which mediates the initial binding of the amino terminus of fibronectin to cell surfaces has not been identified. These amino-terminal binding sites, termed matrix assembly sites (22), have been defined operationally by ligand binding assays using ^{125}I-labeled amino-terminal fragments of fibronectin (22, 35). The expression of these binding sites on cell surfaces has been shown to be regulated by the $\alpha_5\beta_1$ integrin receptor for fibronectin (9).

Cellular adhesion to fibronectin is mediated primarily by the $\alpha_5\beta_1$ integrin, which binds to fibronectin via the Arg-Gly-Asp sequence contained within the III-10 module (16, 32). The importance of the $\alpha_5\beta_1$ integrin receptor for fibronectin in the assembly of a fibronectin matrix has been demonstrated in several studies. Overexpression of the $\alpha_5\beta_1$ integrin in Chinese hamster ovary (CHO) cells results in an increase in the level of fibronectin deposition by these cells (11). Transfection of the α_5 subunit into mutant CHO cells deficient in α_5 expression restores the ability of these cells to assemble a fibronectin matrix (48). Antibodies directed against either $\alpha_5\beta_1$ (2) or the integrin-binding site in the III-10 module of fibronectin (20) inhibit fibronectin fibril formation. In addition, anti-β_1 integrin antibodies have been shown to inhibit binding of the amino-terminal 70-kD fragment to the cell surface, suggesting that the $\alpha_5\beta_1$ integrin can regulate the expression of matrix assembly sites (9).

We recently identified a conformation-dependent site within the III-1 module of fibronectin which binds to the amino-terminal region of fibronectin with high affinity (15). Based on these and other studies, we proposed a model in which the fibronectin molecule, bound to the integrin, undergoes conformational changes resulting in the exposure of the 70-kD binding site in III-1. These series of interactions form a "nucleation site" which serves as a template for the subsequent binding and incorporation of incoming fibronectin molecules into the extracellular matrix. The present study was undertaken to identify a mechanism by which the cryptic 70-kD binding site in III-1 may be exposed in the intact fibronectin molecule. We postulated that regulation of the conformation of the 70-kD binding site in III-1 by the $\alpha_5\beta_1$ integrin may be mediated by an interaction between fibronectin's integrin-binding III-10 module and the 70-kD-binding III-1 module. In this study, we have used recombinant fibronectin modules to characterize a conformation-dependent binding site in the III-10 module of fibronectin which binds to III-1. The interaction of III-1 in intact fibronectin with III-10 promotes the formation of disulfide-stabilized high molecular mass multimers in the absence of cells, via an interaction involving the amino-terminal region of fibronectin. A fragment of III-10 which blocks the formation of in vitro fibronectin multimers also inhibits fibronectin polymerization by cells. In addition, we examined the expression of III-1 binding sites on cell surfaces and found that exogenously added III-1,2 localizes at regions of focal adhesions and that localization to focal adhesions is dependent on the III-10 module. Taken together, these data suggest that $\alpha_5\beta_1$ integrin regulation of fibronectin matrix assembly may be mediated, in part, by an interaction between the III-1 and III-10 modules of fibronectin.

Materials and Methods

Reagents

Materials for autoradiography were from Kodak (Rochester, NY). Gel electrophoresis supplies were from BioRad Labs (Richmond, VA). Unless otherwise indicated, chemical reagents were obtained from Sigma Chem. Co. (St. Louis, MO).

Cell Culture

Human foreskin fibroblasts, A1-F, were a gift from Dr. Lynn Allen-Hoffmann (University of Wisconsin, Madison, WI). A1-Fs were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% FBS (Sterile Systems, Logan, UT). 9D2 hybridoma cells (5) were a gift from Dr. Deane Mosher (University of Wisconsin, Madison, WI). Hybridoma cells were cultured in DMEM containing 20% heat-inactivated FBS.

Gel Electrophoresis

SDS-PAGE was performed according to Laemmli (19) using a discontinuous buffer system (BioRad Labs). Samples were diluted 1:1 in gel buffer containing 4% SDS and 20% glycerin in 0.05 M Tris, pH 6.8. Some samples were reduced with 2% β-mercaptoethanol before electrophoresis. Proteins were visualized with 0.1% Coomassie blue (47). Iodinated proteins were visualized by autoradiography. III-10 preparations were characterized on 16.5% polyacrylamide gels containing 13% glycerol, using a discontinuous tricine-SDS-PAGE system, according to the method of Shägger and von Jagow (39). Proteins were visualized with 0.025% Serva blue G (Serva, Paramus, NJ).

Purification of Fibronectin and Fibronectin Fragments

Human plasma fibronectin was purified from a fibronectin- and fibrinogen-rich by-product of Factor VIII production by ion exhange chromatography on DEAE-cellulose (Pharmacia LKB Biotechnology, Piscataway, NJ), as previously described (22). Fig. 1 shows a schematic of the various fragments used in this study. The 70-kD amino-terminal fragment of fibronectin was generated by limited digestion of intact fibronectin with cathepsin D, followed by gelatin affinity chromatography, as previously described (22). The 70-kD fragment was further cleaved into a 27-kD amino-terminal fragment and a 40-kD gelatin-binding fragment by limited digestion with trypsin (4). The 27- and 40-kD fragments were then separated by gelatin affinity chromatography. A preparation containing the 160/180-kD cell- and gelatin-binding fragments was generated by limited digestion of fibronectin with trypsin, and then isolated by retention on gelatin-Sepharose, as previously described (22). Purity of all fragments was assessed by SDS-PAGE and proteins were frozen at −80°C until use.

Preparation of Recombinant Fibronectin Modules

Recombinant III-1 and III-2 were expressed in BL21(DE3) bacteria as fusion proteins composed of glutathione-*S*-transferase (GST)[1] and either III-1 or III-2, as previously described (15). To separate III-1 or III-2 from GST, fusion proteins were incubated with TPCK-trypsin-agarose beads (1 U/mg) for 30 min at 20°C. After incubation, the TPCK-trypsin-agarose beads were removed by centrifugation. III-1 was further purified by passing the digested material over a fast protein liquid chromatography (FPLC) SP-5PW column (Waters, Boston, MA), as previously described (15). The III-2 digest was purified by passing the digest over an FPLC Protein-Pak Q 8HR column (Waters). III-2 was eluted with a salt gradient from 0.015 M NaCl, 20 mM phosphate, pH 7.0 to 0.25 M NaCl, 20 mM phosphate, pH 7.0. III-2 eluted from the column in one sharp peak and was the only protein detected by SDS-PAGE.

Polymerase chain reaction (PCR) was used to amplify human fibronectin cDNA encoding the first and second type III modules (III-1,2) of fibronectin (bases 1745 through 2347). This DNA encodes amino acids from Ser-578 through Thr-778. Bases are numbered from the A in the codon for the first amino acid of the mature protein (EMBL accession number X02761), and amino acids are numbered from the amino-terminal

1. *Abbreviations used in this paper*: FPLC, fast protein liquid chromatography; GST, glutathione-*S*-transferase.

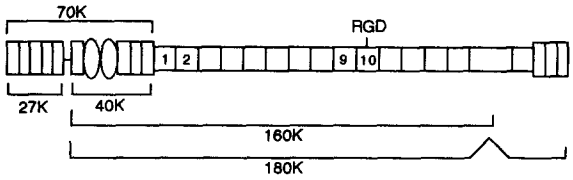

Figure 1. Fibronectin fragments. Schematic representation of fibronectin molecule. Shown are the 27- (cathepsin-trypsin: amino-terminal), 40- (cathepsin-trypsin: gelatin-binding), 70- (cathepsin: amino-terminal), and 160/180-kD (trypsin; gelatin and cell binding) fragments. Type I modules, rectangles; type II modules, ovals; type III modules, squares.

pyroglutamic acid (30). PCR was also used to amplify human fibronectin cDNA encoding the fifth type III module (III-5) (bases 2906 through 3175; Lys 965 through Leu 1054), the tenth type III module (III-10) (bases 4259 through 4518; Val-1415 through Thr-1508), and the ninth and tenth type III modules (III-9,10) of fibronectin (bases 3989 through 4518; Gly-1325 through Thr-1508). The sense primers for the III-1,2 construct (5′-CCC**GGATCC**AGTGGTCCTGTCGAAGTATT), the III-5 construct (5′-CCC**GGATCC**AAACTGGATGCTCCCACTAACC), and the III-10 construct (5′-CCC**GGATCC**GTTTCTGATGTTCCGAGGGAC) were synthesized with a BamHI site (shown in bold) at the 5′ end. The sense primer for the III-9,10 construct (5′-CCC**AGATCT**GGTCTTGATTCCCCAACTGG) was synthesized with a BglII site (shown in bold) at the 5′ end. The antisense primers for the III-1,2 construct (5′-CCC**GAATTC**CTATGTTGTTTGTGAAGTAGACAGG), the III-5 construct (5′-CCC**GAATTC**CTACAGTGTGGTAAAGACTCCAGTG), and the III-10 construct (5′-CCC**GAATTC**CTATGTTCGGTAATTAATGGAAATTG) were synthesized with an EcoRI site (shown in bold) at the 5′ end. The antisense primer for the III-9,10 construct (5′-CCC**CCCGGG**CTATGTTCGGTAATTAATGGAAATTG) was synthesized with a SmaI site (shown in bold) at the 5′ end. Underlined bases introduce a stop codon after the last base in the sequence to be amplified. The III-10/RGE mutant was produced using recombinant PCR (14). The two mutant primers were 5′-CCGTGGAGA**A**AGCCCCGCAAGC (sense primer) and 5′-GCTTGCGGGGC**T**TCTCCACGG (antisense primer). The mutated base is shown in bold. The two outer primers used were the same as those used to amplify nonmutant III-10. PCR was performed according to established procedures (37), using human full-length fibronectin cDNA, pFH100 (a gift of Dr. Jean Thiery, Paris, France) as a template. After restriction enzyme digestion, the PCR amplified DNA was cloned into the bacterial expression vector pGEX-2T (Pharmacia). PCR amplified DNA was sequenced to confirm that no base changes had been introduced during amplification of the DNA and to establish the presence of the mutated base in III-10/RGE (38). DNA encoding either III-1,2, III-10, III-10/RGE, or III-9,10 was cloned in frame with the bacterial glutathione-*S*-transferase DNA.

DNA was transfected into BL21(DE3) bacteria using standard procedures (37). Fusion proteins were isolated by passing bacterial lysates over glutathione agarose, as previously described (15). Fusion proteins were then digested with 1 U/mg TPCK-trypsin-agarose for 30 min at 20°C. This cleavage gives rise to an additional glycine and serine residue at the amino terminus of the isolated modules. The III-5 and III-1,2 constructs were isolated by passing the digested materal over an FPLC Protein-Pak Q 8HR column (Waters) and eluting with a salt gradient from 0.015 M NaCl, 20 mM phosphate, pH 7.0 to 0.25 M NaCl, 20 mM phosphate, pH 7.0. Amino acid sequencing of III-1,2 yielded the following amino terminal sequence: GSSGPVEVFI, corresponding to the region beginning at the amino-terminus of III-1, starting at Ser^{578}. III-9,10 was isolated by passing the digested material over a glutathione agarose column. To isolate III-10, the TPCK-trypsin–digested material was passed twice over a high performance liquid chromatography Protein-Pak 125 gel filtration column (Waters). For some studies, GST/III-10 and GST/III-10/RGE were digested with 0.5 U/mg trypsin-acrylic beads (non-TPCK-treated) for 30 min at 20°C (III-10A and III-10A/RGE) and purified as described for III-10. The apparent molecular masses of the III-10 preparations were determined by tricine-SDS-PAGE and are as follows: III-10 = 17 kD, III-10A and III-10A/RGE = 14.5 kD. Amino acid sequencing of III-10, III-10A, and III-10A/RGE yielded identical amino-terminal sequences: GSVSDVPRDL. This sequence corresponds to the region beginning at the amino terminus of III-10, starting at Val-1415.

Preparation of 9D2 IgG and Fab′ Fragments

Ascites of 9D2 were prepared as described (13). Immunoglobulins (IgGs) were isolated by passing the ascites over a protein A–Sepharose CL-4B column (Pharmacia) equilibrated with 100 mM sodium phosphate (pH 7.0). Bound IgG was eluted with 1 M acetic acid, 100 mM glycine (pH 3.0). The eluate was neutralized to pH 7.4 with 1 M Tris (pH 10.0) and dialyzed against PBS. 9D2 IgG, diluted in 0.1 M sodium citrate buffer, pH 4, was digested with pepsin (1:200 wt/wt) for 15 h at 37°C to produce $F(ab')_2$ fragments (13). Monovalent Fab′ fragments were obtained after reduction of $F(ab')_2$ with 10 mM β-mercaptoethanol for 2 h at 37°C and alkylation with 10 mM iodoacetamide for 4 h at 20°C (13). Fab′ fragments were dialyzed against PBS, and then passed over a protein A–Sepharose CL-4B column (Pharmacia) to remove intact IgG. Purity was assessed by SDS-PAGE and aliquots were stored at −80°C before use. Nonimmune mouse $F(ab')_2$ fragments (Cappel, Durham, NC) were similarly reduced, alkylated, and dialyzed against PBS to produce Fab′ fragments.

Iodination of Fibronectin and Fibronectin Fragments

For solid phase and cell binding assays, 27 and 70 kD, fibronectin, and III-1 and III-2 were iodinated with 1.0 mCi $Na^{125}I$ (New England Nuclear, Boston, MA) using chloramine T, as previously described (22). The iodination reaction was stopped with sodium metabisulfite (Fisher Scientific, Fair Lawn, NJ) and free iodine was removed by chromatography on G-25M Sephadex (Pharmacia). Iodinated proteins were dialyzed against PBS and frozen at −80°C until use. Integrity of the labeled proteins was assessed by gel electrophoresis and autoradiography. Specific activities of labeled proteins ranged from 2.1–11.9 × 10^4 Ci/mol.

Solid Phase Binding Assay

Solid phase binding assays were performed essentially as described (15). III-1, III-2, III-5, or III-10 was diluted to 10 μg/ml in PBS containing 2 mM PMSF, incubated for 10 min at 90°C, and added to wells of 24-well cluster plates (Falcon, Bedford, MA). Plates were then incubated for 90 min at 80°C. For studies involving nondenatured proteins, proteins were diluted to 10 μg/ml in PBS containing 2 mM PMSF, added to wells, and plates were incubated at 20°C for 90 min. After this incubation, unbound protein was removed and unreacted sites were blocked with 1% protease-free BSA (Intergen Co., Purchase, NY) in PBS. In studies (not shown) using radiolabeled proteins, ~50% less protein bound to wells coated at 80°C than at 20°C. For some studies, wells were coated with 10 mg/ml BSA in PBS for 2 h at 37°C. Protein-coated wells were washed three times with PBS containing 0.2% BSA and incubated at 37°C with 10^6 cpm/ml ^{125}I-labeled protein diluted in PBS with 0.2% BSA. Nonspecific binding was determined in separate wells by the addition of excess unlabeled protein to the binding medium. Specific binding was calculated by subtracting nonspecific binding from total binding.

Cell Binding Assays

^{125}I-fibronectin binding assays were conducted as previously described (21). Briefly, A1-F fibroblasts were seeded at 5 × 10^4 cells/well into 24-well cluster dishes (Falcon) in DMEM containing 10% FBS and allowed to grow to confluence for 3–4 d. Cells were washed three times with serum-free DMEM and incubated for 24 h with 0.5 × 10^6 cpm/ml ^{125}I-fibronectin in DMEM containing 0.2% BSA in the absence or presence of either III-10, III-10A, or III-10A/RGE. After this incubation, unbound protein was removed, cells were washed with PBS, and bound protein was solubilized with 1 N NaOH. Nonspecific binding was determined by incubating the cells in the presence of excess unlabeled fibronectin (400 μg/ml).

Immunofluorescence Microscopy

III-1,2 was diluted to 0.5 mg/ml in 0.05 M sodium borate buffer, pH 9.0, and directly conjugated to Texas red sulfonyl chloride (Molecular Probes, Eugene, OR) using N,N-dimethyl formamide, according to the manufacturer's protocol. The protein conjugate was separated from unreacted Texas red by gel filtration chromatography on G-25M Sephadex (Pharmacia). Protein concentration was determined from the absorbance at 280 nM.

18-mm glass coverslips were coated for 2 h at 37°C with 10 μg/ml of either fibronectin, the 160/180-kD fragment of fibronectin, the III-9,10 modules of fibronectin, vitronectin, or laminin in PBS. Unbound protein was removed and A1-F fibroblasts were seeded onto coverslips in 12-well cluster dishes (Falcon) at 4 × 10^4 cells/well in DMEM containing 0.2% BSA.

To inhibit endogenous synthesis of fibronectin, cycloheximide was added at a concentration of 20 μg/ml (23). Cells were then incubated overnight at 37°C. After this incubation, 10 μg/ml Texas red–labeled-III-1,2 was added directly to the culture media and cells were incubated an additional 90 min. In some studies, before the incubation with Texas red III-1,2, 50 μg/ml of either 9D2, the monoclonal antibody clone 3E3 (31) (GIBCO BRL), or the monoclonal antibody LAB (a gift of Deane Mosher, University of Wisconsin, Madison, WI) were added directly to the culture medium. After a 1-h incubation, Texas red III-1,2 was added to the culture media and cells were incubated an additional 90 min. The epitope recognized by the Lab monoclonal antibody is contained within the region III-4 to III-8 (5). The epitope recognized by the 3E3 monoclonal antibody is contained within the 15-kD cell-binding fragment of fibronectin (31). Media was then removed and cells were fixed with 3.7% paraformaldehyde in Small's cytoskeletal buffer (41) and permeabilized with 0.5% Triton X-100. Cells were stained for β_1 integrins using the monoclonal anti-β_1 antibody, AIIB2 (a gift of Caroline Damsky, University of California, San Francisco, CA), followed by incubation with a fluorescein-conjugated goat anti–rat antibody (Cappel) for 60 min. Cells were washed, mounted, and then examined using an Olympus microscope.

Results

Identification of a Binding Site within the Integrin-binding III-10 Module of Fibronectin for III-1

Cellular adhesion to fibronectin is mediated primarily by the $\alpha_5\beta_1$ integrin, which binds to fibronectin via the Arg-Gly-Asp sequence contained within the III-10 module (16, 32, 34). A role for the integrin in fibronectin matrix assembly has previously been demonstrated in studies in which addition of antibodies directed against the $\alpha_5\beta_1$ integrin was shown to inhibit fibronectin polymerization and decrease 70 kD binding to cell surfaces (9). Our previous study indicated that the III-1 module of fibronectin contains a conformation-dependent site which binds to the amino-terminal region of fibronectin with high affinity (15). Based on these studies, we postulated that expression of 70 kD binding sites within the III-1 module may be regulated by an interaction between the III-1 and III-10 modules. To determine whether III-1 binds to III-10, solid phase binding studies were conducted using recombinant III-1 and III-10 modules. As shown in Fig. 2 *A*, ^{125}I-III-1 bound to wells coated with heat-denatured III-10, whereas ^{125}I-III-2 did not. In addition, ^{125}I-III-1 did not bind to III-10 which was not heat-denatured before its adsorption onto tissue culture plastic (Fig. 2 *A*), suggesting that the binding site in the III-10 module for III-1 is conformation dependent. To determine whether III-1 bound selectively to heat-denatured III-10, the binding of ^{125}I-III-1 to other heat-denatured modules was assessed. As shown in Fig. 2 *B*, both heat-denatured III-10 and heat-denatured III-5 supported higher levels of ^{125}I-III-1 binding than did heat-denatured III-1 or heat-denatured III-2. These results indicate that III-1 can bind to heat-denatured III-10 and may also bind to other heat-denatured type III modules in solid phase binding assays. However, in additional experiments described in this study, we were unable to detect any functional significance for the interaction between III-1 and III-5.

Inhibition of Fibronectin Binding to Heat-denatured III-10 by III-1

To determine whether heat-denatured III-10 is able to recognize III-1 within the context of the intact fibronectin molecule, binding of radiolabeled fibronectin to heat-denatured III-10 was assessed in the presence of increasing concentrations of unlabeled III-1. Binding of ^{125}I-labeled fibronectin to heat-denatured III-10 was inhibited in a dose-dependent manner by III-1 (Fig. 3). In contrast, III-2 had no effect on fibronectin binding to heat-denatured III-10 (Fig. 3). In addition, ^{125}I-fibronectin binding to heat-denatured III-10 was inhibited by the addition of the anti–III-1 monoclonal antibody 9D2 (data not shown). These data indicate that intact fibronectin can bind to conformationally altered III-10 and that this binding is dependent on the III-1 module.

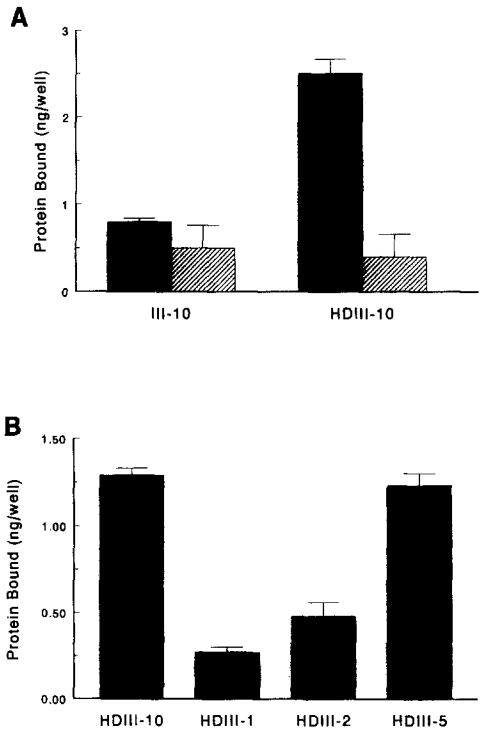

Figure 2. ^{125}I-III-1 binding to heat-denatured III-10. (*A*) 24-well plates were coated with 10 μg/ml nonheat-denatured III-10 (III-10) or heat-denatured III-10 (HDIII-10), as indicated under Materials and Methods, and incubated for 60 min at 37°C with 0.5 × 10^6 cpm/ml of either ^{125}I-III-1 (*closed bars*) or ^{125}I-III-2 (*hatched bar*). In *B*, 24-well plates were coated with either 10 μg/ml of the following heat-denatured proteins: III-1 (HDIII-1), III-2 (HDIII-2), III-5 (HDIII-5), or III-10 (HDIII-10), and incubated for 60 min at 37°C with 0.5 × 10^6 cpm/ml of ^{125}I-III-1. Plates were then rinsed, bound protein was solubilized in 1 N NaOH, and radioactivity was determined. Nonspecific binding was determined in separate wells by performing the assay in the presence of 25 μg/ml unlabeled III-1 or III-2. Nonspecific binding, which was ~30% of total binding, was subtracted from total binding to determine specific binding. Data are presented as the amount of protein bound per well and represent one of three experiments performed in duplicate.

Inhibition of III-1 Binding to Heat-denatured III-10 by 9D2

Previous studies (5, 24, 25) have demonstrated a role for III-1 in self-interactive events thought to be involved in the polymerization of fibronectin on cell surfaces. In matrix assembly assays done on fibroblast monolayers, the anti–III-1 monoclonal antibody, 9D2, blocked the polymerization of fibronectin by fibroblasts at a step subse-

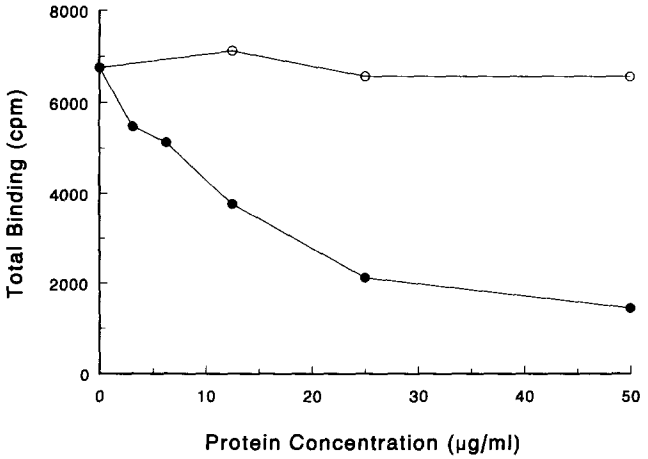

Figure 3. ^{125}I-fibronectin binding to heat-denatured III-10. Plates were coated with 10 μg/ml heat-denatured III-10, as indicated under Materials and Methods, and incubated for 60 min at 37°C with 0.5 × 10^6 cpm/ml of ^{125}I-fibronectin in the absence or presence of increasing concentrations of unlabeled III-1 (●) or III-2 (○). Plates were then rinsed, bound protein was solubilized in 1 N NaOH, and radioactivity was determined. Data represent one of three experiments performed in duplicate.

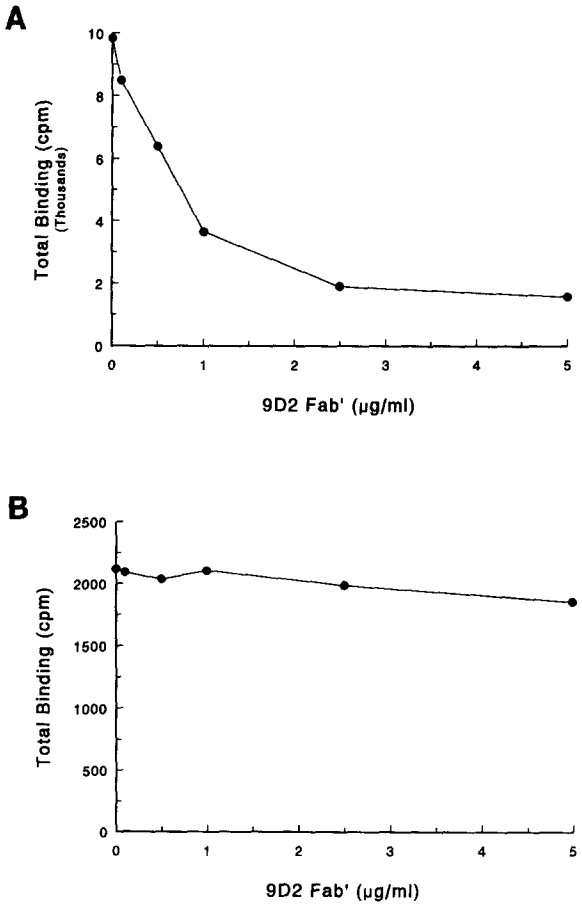

Figure 4. Inhibition of III-1 binding to heat-denatured III-10 by 9D2. Plates were coated with 10 μg/ml of either heat-denatured III-10 (*A*) or heat-denatured III-1 (*B*), as indicated under Materials and Methods. Plates coated with heat-denatured III-10 (*A*) were incubated for 60 min at 37°C with 0.5 × 10^6 cpm/ml of ^{125}I-III-1 (~50 ng/ml) in the presence of increasing concentrations of 9D2 Fab' fragments. Plates coated with heat-denatured III-1 (*B*) were incubated for 60 min at 37°C with 0.5 × 10^6 cpm/ml of ^{125}I-70 kD (~100 ng/ml), in the presence of increasing concentrations of 9D2 Fab' fragments. Plates were then rinsed, bound protein was solubilized in 1 N NaOH, and radioactivity was determined. Data represent one of three experiments performed in duplicate.

quent to the initial amino-terminal binding of fibronectin to the cells (5). The complementary site for III-1 binding was not identified. To determine whether 9D2 recognizes the III-10 binding site within the III-1 module, binding of ^{125}I-III-1 to heat-denatured III-10 was assessed in the presence of increasing concentrations of either 9D2 or nonimmune mouse Fab' fragments. As shown in Fig. 4 *A*, 9D2 Fab' fragments inhibited ^{125}I-III-1 binding to heat-denatured III-10 in a dose-dependent manner. A 50% inhibition of ^{125}I-III-1 binding to heat-denatured III-10 was obtained at a concentration of 1 μg/ml 9D2 Fab' (Fig. 4 *A*). This concentration of 9D2 is similar to that previously shown to cause a 50% inhibition of ^{125}I-fibronectin binding to cell surfaces (5). In contrast, nonimmune Fab' fragments had no effect on ^{125}I-III-1 binding to heat-denatured III-10 (not shown).

We previously reported that the III-1 module contains a conformation-dependent binding site for the amino-terminal 70-kD fibronectin fragment (15). To test whether 9D2 inhibits the interaction of 70 kD with heat-denatured III-1, binding of ^{125}I-70 kD to heat-denatured III-1 was assessed in the presence of increasing concentrations of 9D2 Fab' fragments. In contrast to the results described above (Fig. 4 *A*), 9D2 Fab' fragments had no effect on the binding of ^{125}I-70 kD to heat-denatured III-1 (Fig. 4 *B*). The inability of 9D2 to block ^{125}I-70 kD binding to heat-denatured III-1 was not due to an inability of 9D2 to recognize the conformationally altered form of III-1. In separate studies using an enzyme-linked immunoassay (8), 9D2 recognized III-1 and heat-denatured III-1 equally well (not shown).

Binding of Amino-Terminal Fragments to Heat-denatured III-10 in the Presence of III-1,2

Solid phase binding assays were conducted to determine if a complex could be formed between heat-denatured III-10, III-1, and the amino terminus of fibronectin. Tissue culture wells coated with heat-denatured III-10 were incubated with ^{125}I-labeled 70 kD amino-terminal fragment of fibronectin in the absence or presence of either III-1, III-2, or III- 1,2. Whereas ^{125}I-70 kD itself did not bind to heat-denatured III-10 (Fig. 5 *A*), preincubation of heat-denatured III-10 with increasing concentrations of III-1,2 resulted in a dose-dependent increase in ^{125}I-70 kD binding (Fig. 5 *A*). Similar results were obtained using the ^{125}I-27-kD fragment (data not shown). In contrast, addition of III-1,2 to plates coated with heat-denatured III-5 did not promote ^{125}I-70 kD binding (Fig. 5 *A*). Neither III-1 nor III-2 alone could support the formation of this ternary complex on heat-denatured III-10 (Fig. 5 *A*). In parallel studies (not shown), the binding of ^{125}I-III-1 to heat-denatured III-10 was greater than the binding of ^{125}I-III-1,2 to heat-denatured III-10, indicating that the inability of III-1 to

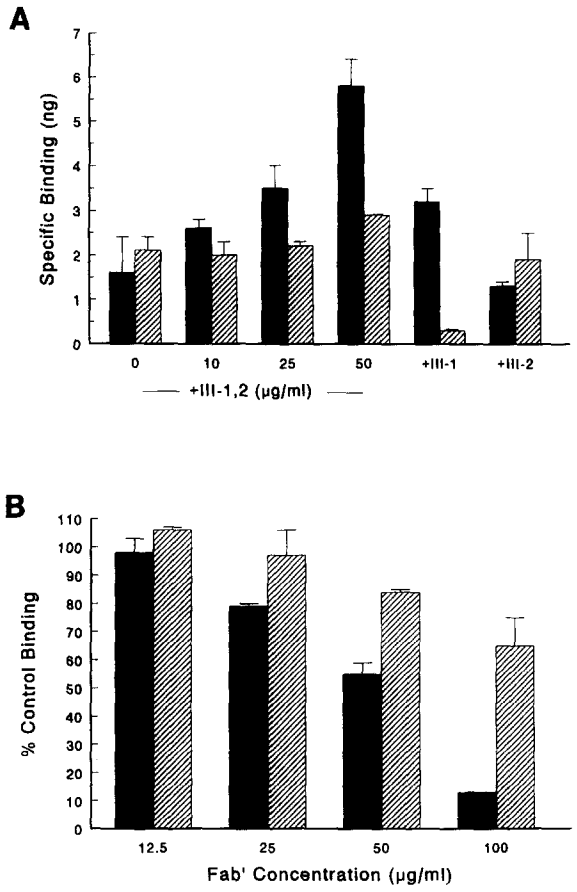

Figure 5. ^{125}I-70 kD binding to heat-denatured III-10 in the presence of III-1,2. (*A*) Plates were coated with 10 μg/ml heat-denatured III-10 (*solid bars*) or heat-denatured III-5 (*hatched bars*), as indicated under Materials and Methods. Wells were then incubated with 10^6 cpm/ml ^{125}I-70 kD for 2 h at 37°C in the absence or presence of increasing concentrations of III-1,2, III-1 (50 μg/ml), or III-2 (50 μg/ml). Plates were rinsed, bound protein was solubilized in 1 N NaOH, and radioactivity was determined. Nonspecific binding was determined in separate wells by performing the assay in the presence of 50 μg/ml unlabeled 70 kD. Nonspecific binding, which was ~40% of total binding, was subtracted from total binding to determine specific binding. The amount of 70 kD bound was normalized to the amount of heat-denatured protein adsorbed to the plate. In *B*, wells coated with heat-denatured III-10 were incubated with 50 μg/ml III-1,2 and 2×10^6 cpm/ml of ^{125}I-27 kD in the absence or presence of increasing concentrations of either 9D2 (*solid bars*) or nonimmune (*hatched bars*) Fab' fragments. Data are presented as percent of specific binding measured in the absence of antibody and represent one of three experiments performed in duplicate.

promote ^{125}I-70 kD binding was not the result of decreased binding to heat-denatured III-10. These data suggest that the presence of III-2 in the III-1,2 construct may stabilize III-1 in the appropriate conformation to support the ternary complex formation. As shown in Fig. 5 *B*, ^{125}I-27 kD binding to the III-1,2/III-10 complex was dependent on the binding of III-1 to heat-denatured III-10, since addition of 9D2 Fab' fragments resulted in an inhibition of ^{125}I-27 kD binding. Similar results were obtained using the ^{125}I-70 kD fragment (data not shown). Taken together, these results indicate that a ternary complex can be formed in which III-1 simultaneously binds to both III-10 and the amino terminus of fibronectin.

Formation of Fibronectin Multimers by Heat-denatured III-10

Upon binding to cells, fibronectin accumulates as high molecular mass aggregates which do not penetrate the stacking gel when analyzed by SDS-PAGE. These aggregates, which are thought to represent fibronectin in the extracellular matrix, can be dissociated to monomeric fibronectin with reducing agents (21). Morla et al. (25) have recently shown that in the absence of cells, a fibronectin construct containing two thirds of the III-1 module can induce the formation of fibronectin multimers. The authors speculate that the activity of their III-1 construct may be masked in the intact fibronectin molecule but may be exposed on the cell surface as a result of fibronectin binding to the $\alpha_5\beta_1$ integrin. To determine if the interaction of conformationally altered III-10 with III-1 within the intact fibronectin molecule could promote the formation of disulfide-stabilized high molecular mass fibronectin multimers, solid phase binding assays were conducted using assay wells coated with either heat-denatured III-10, heat-denatured III-1, nonheat-denatured III-10, or BSA. Incubation of ^{125}I-fibronectin in wells coated with either heat-denatured III-10 or heat-denatured III-1 resulted in specific fibronectin binding (Fig. 6 *A*). Samples containing equal cpm were then analyzed by SDS-PAGE followed by autoradiography. Incubation of ^{125}I-fibronectin with heat-denatured III-10 resulted in the formation of high molecular mass fibronectin aggregates which did not penetrate the stacking gel (Fig. 6 *B*, *arrow*). To quantitate these aggregates, gel slices were removed and counted (Fig. 6 *C*). In the presence of a reducing agent, no high molecular mass aggregates were detected (Fig. 6 *C*); only monomeric subunits were present (Fig. 6 *B*). In addition, the III-10 module must be conformationally altered in order to induce fibronectin multimer formation, since incubation of ^{125}I-fibronectin with wells coated with III-10 which was not heat-denatured did not result in either specific fibronectin binding (Fig. 6 *A*) or multimer formation (Fig. 6 *C*). Whereas ^{125}I-fibronectin bound avidly to wells coated with heat-denatured III-1 (Fig. 6 *A*), when analyzed by SDS-PAGE, no high molecular mass multimers were detected (Fig. 6 *B*, *arrow* and Fig. 6 *C*). The binding of fibronectin to heat-denatured III-1 was completely inhibited by the addition of excess amino-terminal 70-kD fragment (not shown), indicating that fibronectin binding to heat-denatured III-1 was dependent upon the amino-terminal region. Heat-denatured III-5, although capable of binding III-1 in solid phase assays (Fig. 2), was not active in promoting disulfide-stabilized fibronectin multimers (data not shown).

Inhibition of III-10–induced Fibronectin Multimers with 9D2 or 27 kD

To test whether the III-1 module in intact fibronectin interacts with conformationally altered III-10 during the formation of fibronectin multimers, ^{125}I-fibronectin was incubated in wells precoated with heat-denatured III-10, in the presence of increasing concentrations of 9D2 Fab' frag-

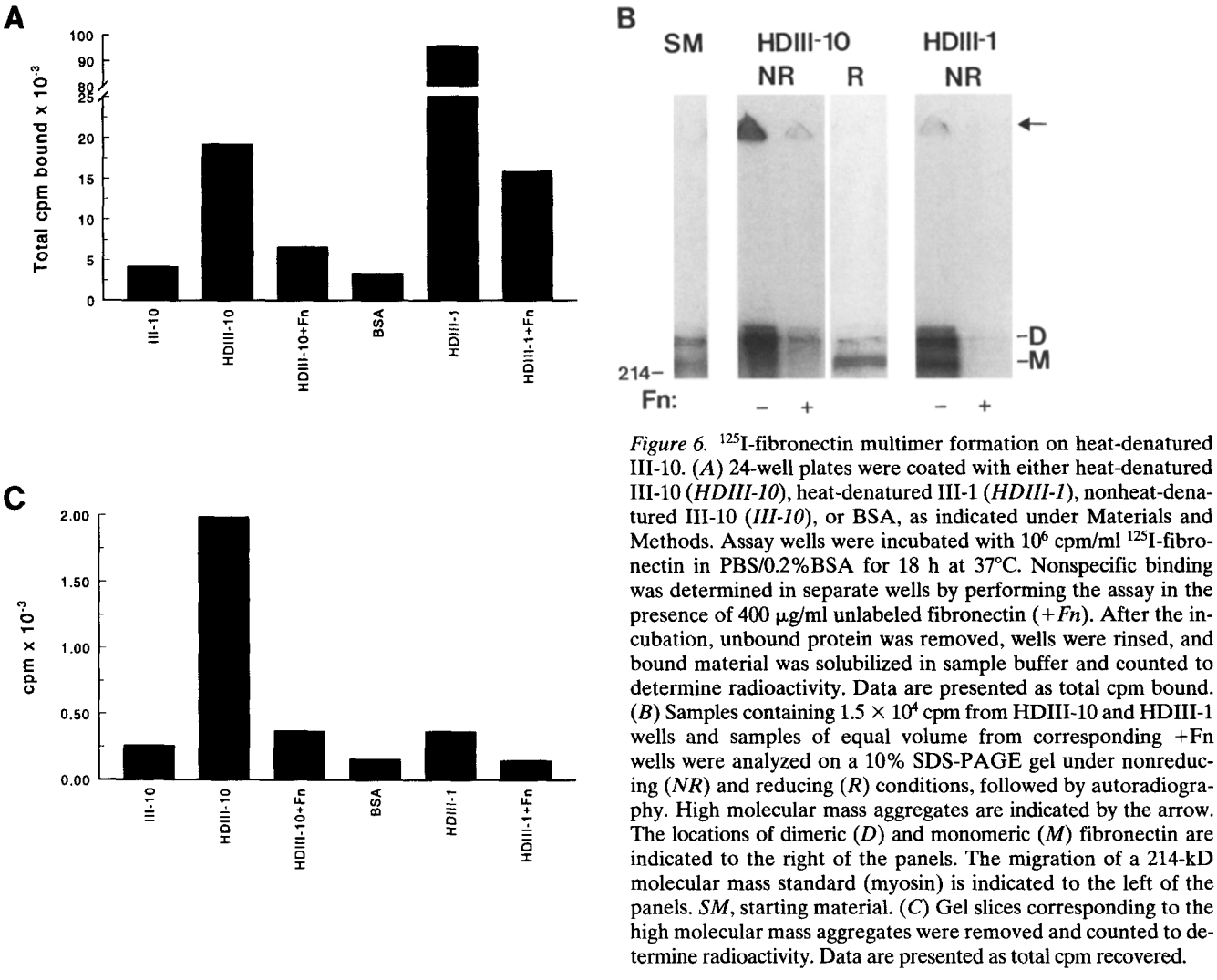

Figure 6. ^{125}I-fibronectin multimer formation on heat-denatured III-10. (*A*) 24-well plates were coated with either heat-denatured III-10 (*HDIII-10*), heat-denatured III-1 (*HDIII-1*), nonheat-denatured III-10 (*III-10*), or BSA, as indicated under Materials and Methods. Assay wells were incubated with 10^6 cpm/ml ^{125}I-fibronectin in PBS/0.2%BSA for 18 h at 37°C. Nonspecific binding was determined in separate wells by performing the assay in the presence of 400 μg/ml unlabeled fibronectin (+*Fn*). After the incubation, unbound protein was removed, wells were rinsed, and bound material was solubilized in sample buffer and counted to determine radioactivity. Data are presented as total cpm bound. (*B*) Samples containing 1.5×10^4 cpm from HDIII-10 and HDIII-1 wells and samples of equal volume from corresponding +Fn wells were analyzed on a 10% SDS-PAGE gel under nonreducing (*NR*) and reducing (*R*) conditions, followed by autoradiography. High molecular mass aggregates are indicated by the arrow. The locations of dimeric (*D*) and monomeric (*M*) fibronectin are indicated to the right of the panels. The migration of a 214-kD molecular mass standard (myosin) is indicated to the left of the panels. *SM*, starting material. (*C*) Gel slices corresponding to the high molecular mass aggregates were removed and counted to determine radioactivity. Data are presented as total cpm recovered.

ments. Addition of 9D2 Fab′ fragments resulted in a dose-dependent decrease in fibronectin binding (Fig. 7 *A*) and a parallel decrease in multimer formation (not shown). In contrast, nonimmune Fab′ fragments had no effect on ^{125}I-fibronectin binding to heat-denatured III-10 (Fig. 7 *A*).

Addition of either the 27-kD or 70-kD amino-terminal fibronectin fragment to cells blocks the polymerization of fibronectin on cell surfaces (22, 35). To determine whether the formation of fibronectin multimers on conformationally altered III-10 also involves the amino-terminal region of fibronectin, ^{125}I-fibronectin was incubated with wells precoated with heat-denatured III-10 in the presence of increasing concentrations of the 27-kD fibronectin fragment. Addition of 27 kD resulted in a dose-dependent decrease in fibronectin binding (Fig. 7 *B*) and a parallel decrease in multimer formation (not shown). In contrast, addition of the 40-kD gelatin-binding fibronectin fragment to the reaction had no effect (Fig. 7 *B*). These data indicate that both III-1 and the amino terminus are involved in the formation of III-10–induced disulfide-stabilized fibronectin multimers. In addition, these data suggest that the intramolecular interactions involved in the polymerization of fibronectin on cell surfaces and in the formation of III-10–induced fibronectin multimers are similar.

A Fragment of III-10 Inhibits In Vitro Multimer Formation

An amino-terminal fragment of III-10 (III-10A), with an apparent molecular mass of 14.5 kD, was generated by digestion of the GST/III-10 fusion protein with trypsin. Amino acid sequencing of III-10 and III-10A yielded identical results, indicating that III-10A is generated by cleavage of carboxy-terminal residues from III-10. Whereas intact III-10 had no effect on the binding of ^{125}I-III-1 to heat-denatured III-10, addition of III-10A to assay wells blocked the binding of ^{125}I-III-1 to heat-denatured III-10 (not shown), suggesting that the conformation-dependent binding site for III-1 is exposed in the III-10A fragment. Results from our studies with the 9D2 antibody indicate that the interaction of III-1 with the conformation-dependent site in III-10 is involved in the formation of fibronectin multimers. To test whether III-10A could similarly inhibit the formation of III-10–induced fibronectin multimers, ^{125}I-fibronectin was incubated in wells coated with heat-denatured III-10 in the presence of either intact III-10 or the III-10A fragment. Addition of III-10A resulted in a dose-dependent inhibition of binding of ^{125}I-fibronectin to heat-denatured III-10 (Fig. 7 *C*) and subsequent

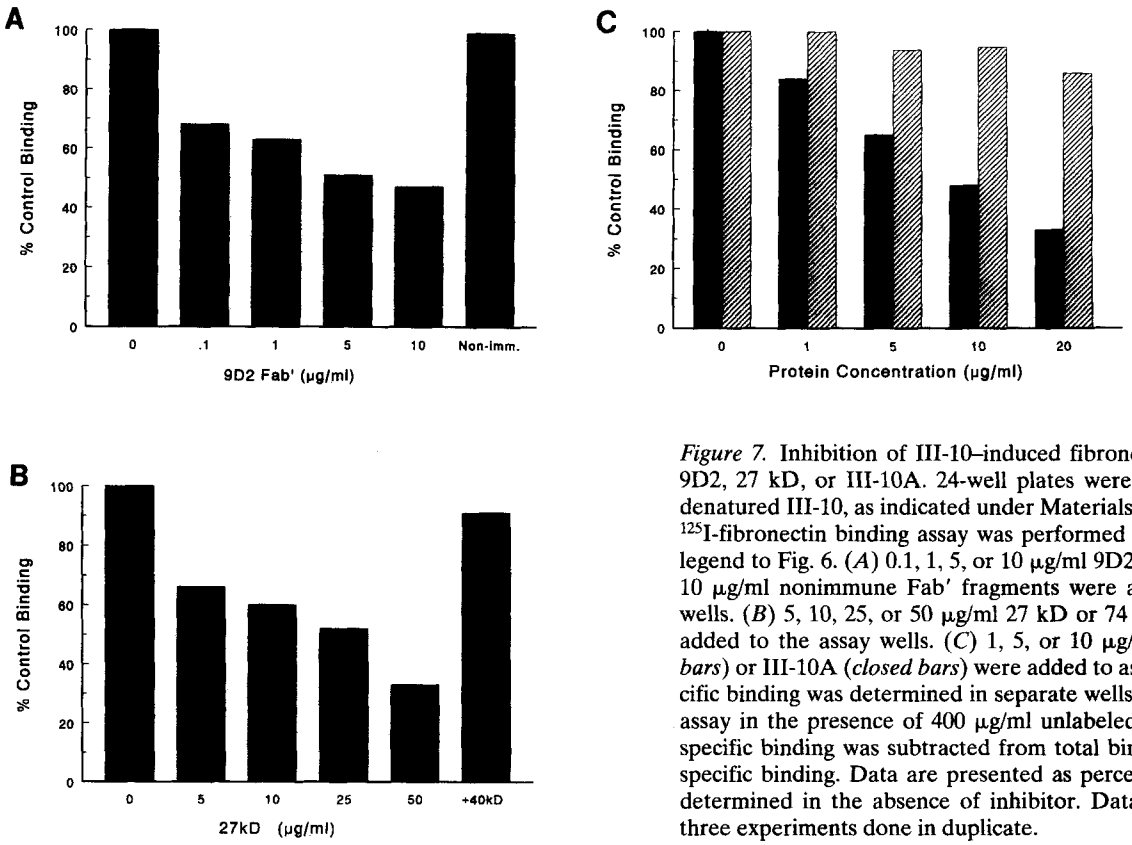

Figure 7. Inhibition of III-10–induced fibronectin multimers by 9D2, 27 kD, or III-10A. 24-well plates were coated with heat-denatured III-10, as indicated under Materials and Methods. The ^{125}I-fibronectin binding assay was performed as indicated in the legend to Fig. 6. (*A*) 0.1, 1, 5, or 10 μg/ml 9D2 Fab' fragments or 10 μg/ml nonimmune Fab' fragments were added to the assay wells. (*B*) 5, 10, 25, or 50 μg/ml 27 kD or 74 μg/ml 40 kD were added to the assay wells. (*C*) 1, 5, or 10 μg/ml III-10 (*hatched bars*) or III-10A (*closed bars*) were added to assay wells. Nonspecific binding was determined in separate wells by performing the assay in the presence of 400 μg/ml unlabeled fibronectin. Nonspecific binding was subtracted from total binding to determine specific binding. Data are presented as percent specific binding determined in the absence of inhibitor. Data represent one of three experiments done in duplicate.

multimer formation (not shown). In contrast, intact III-10 had no effect on ^{125}I-fibronectin binding to heat-denatured III-10 (Fig. 7 *C*). These results suggest that the III-1 binding site in III-10 is exposed by proteolytic removal of carboxy-terminal amino acids.

Inhibition of Fibronectin Matrix Assembly by III-10A

Addition of the anti–III-1 monoclonal antibody, 9D2, to cell monolayers inhibits the polymerization of fibronectin by fibroblasts (5). Our data indicate that the 9D2 antibody can inhibit the interaction of III-1 with conformationally altered III-10, and that the interaction of III-1 with conformationally altered III-10 is involved in the formation of in vitro fibronectin multimers. It is therefore possible that 9D2 blocks fibronectin matrix assembly by binding to the III-1 module of fibronectin molecules and inhibiting the subsequent interaction of III-1 with III-10 modules on the cell surface. If so, addition of III-10A to cell monolayers should similarly inhibit fibronectin polymerization by cells. To test this hypothesis, ^{125}I-fibronectin binding to cell monolayers was determined in the presence of exogenously added III-10A. As shown in Fig. 8, addition of III-10A to confluent A1-F fibroblast monolayers resulted in a dose-dependent decrease in ^{125}I-labeled fibronectin binding to cells in a 24-h fibronectin incorporation assay. No apparent change in cell morphology was observed following incubation of cells with III-10A (data not shown). Complete inhibition of ^{125}I-fibronectin binding to cell surfaces by III-10A was obtained at a concentration of 20 μg/ml (Fig. 8). This concentration of III-10A is approximately 100-fold less than the concentration of the 11-kD cell-binding fragment needed to disrupt adhesion of cells to fibronectin (31). No change in ^{125}I-fibronectin binding was observed with the addition of either III-2 or intact III-10 to confluent cell monolayers (not shown).

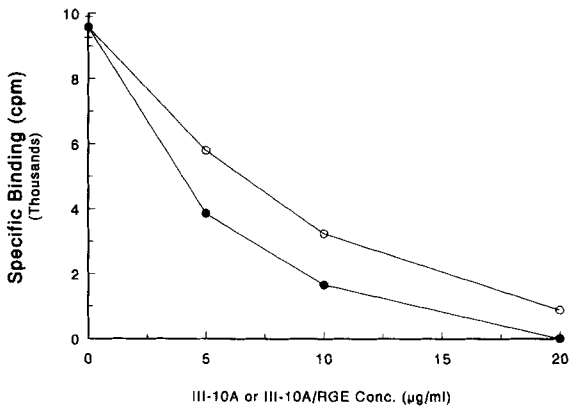

Figure 8. ^{125}I-fibronectin binding to fibroblasts in the presence of III-10A or III-10/RGE. A1-F fibroblasts were seeded at 5×10^4 cell/ml in 24-well tissue culture plates and grown to confluence. Media was removed and cells were washed with DMEM. Cells were then incubated with 0.25 ml DMEM/0.2%BSA containing 0.5×10^6 cpm/ml ^{125}I-fibronectin, in the absence or presence of 5, 10, or 20 μg/ml III-10A (○) or III-10/RGE (●) for 24 h at 37°C. Cell layers were then washed three times with PBS and bound protein was solubilized with 1 N NaOH. Data are expressed as mean ± standard error and represent one of two experiments performed in triplicate.

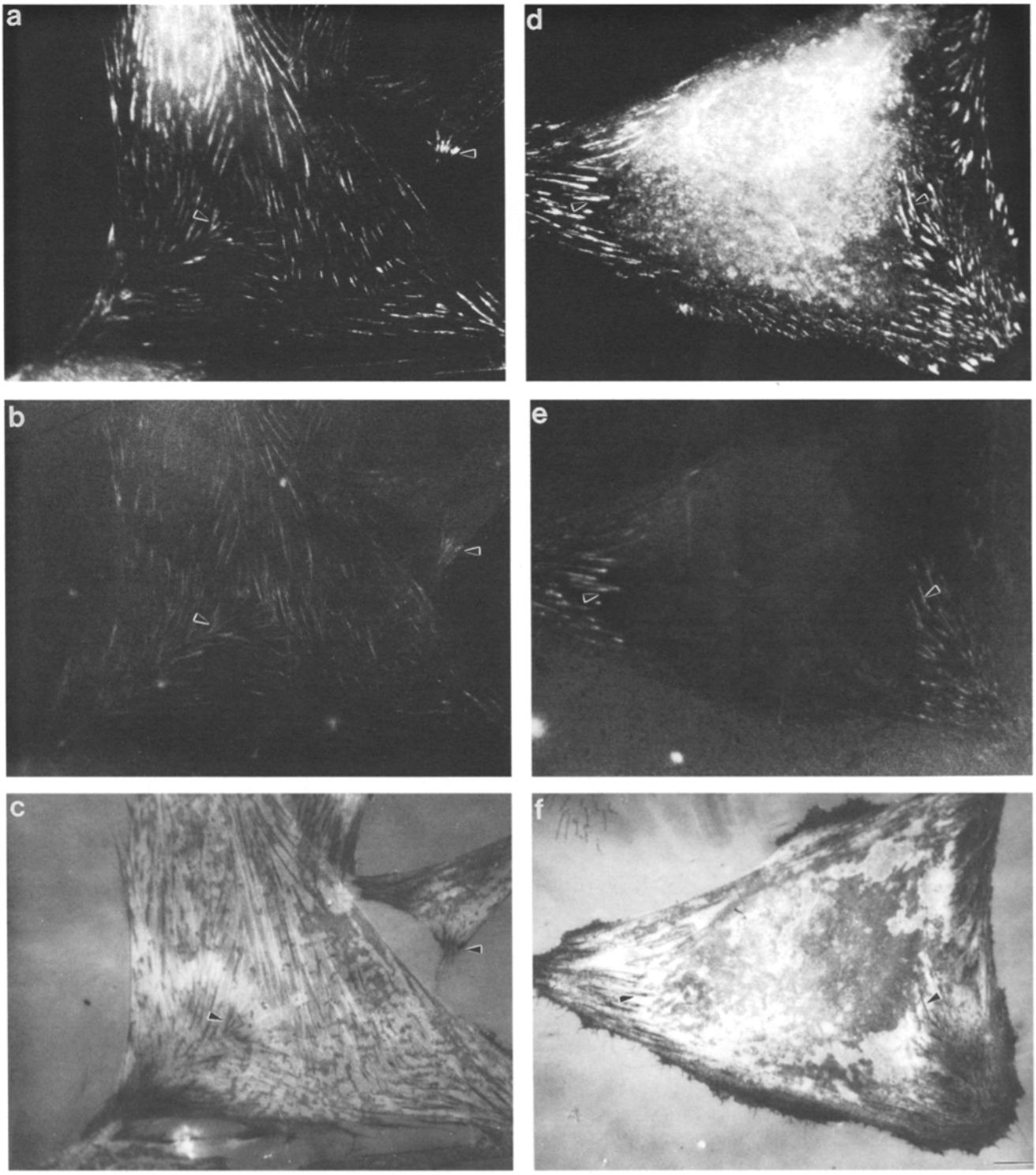

Figure 9. Immunofluorescent colocalization of III-1,2 and β_1 integrins on fibroblasts. A1-Fs (4×10^4 in DMEM/0.2%BSA with 20 μg/ml cycloheximide) were incubated for 18 h on coverslips coated with 10 μg/ml of either fibronectin (*a–c*) or 160/180-kD fibronectin fragment (*d–f*). Cells were then stained with 10 μg/ml Texas red–conjugated-III-1,2 (*b* and *e*) for 90 min at 37°C. Cells were fixed, permeabilized, and stained for β_1 integrins using a monoclonal anti-β_1 antibody followed by an FITC-conjugated goat anti–rat Fab_2 (*a* and *d*). The IRM image is shown in panels *c* and *f*. Arrowheads indicate colocalization of III-1,2 and β_1 integrin staining at sites of focal contacts.

Previous studies have demonstrated that the addition of Arg-Gly-Asp peptides to cell monolayers disrupts the interaction of fibronectin with the $\alpha_5\beta_1$ integrin (32) and inhibits fibronectin matrix assembly (6). To determine whether the effects of the III-10A fragment on matrix assembly were due to the disruption of fibronectin-integrin interactions, a recombinant III-10 fragment was produced in which the Arg-Gly-Asp sequence was mutated to Arg-

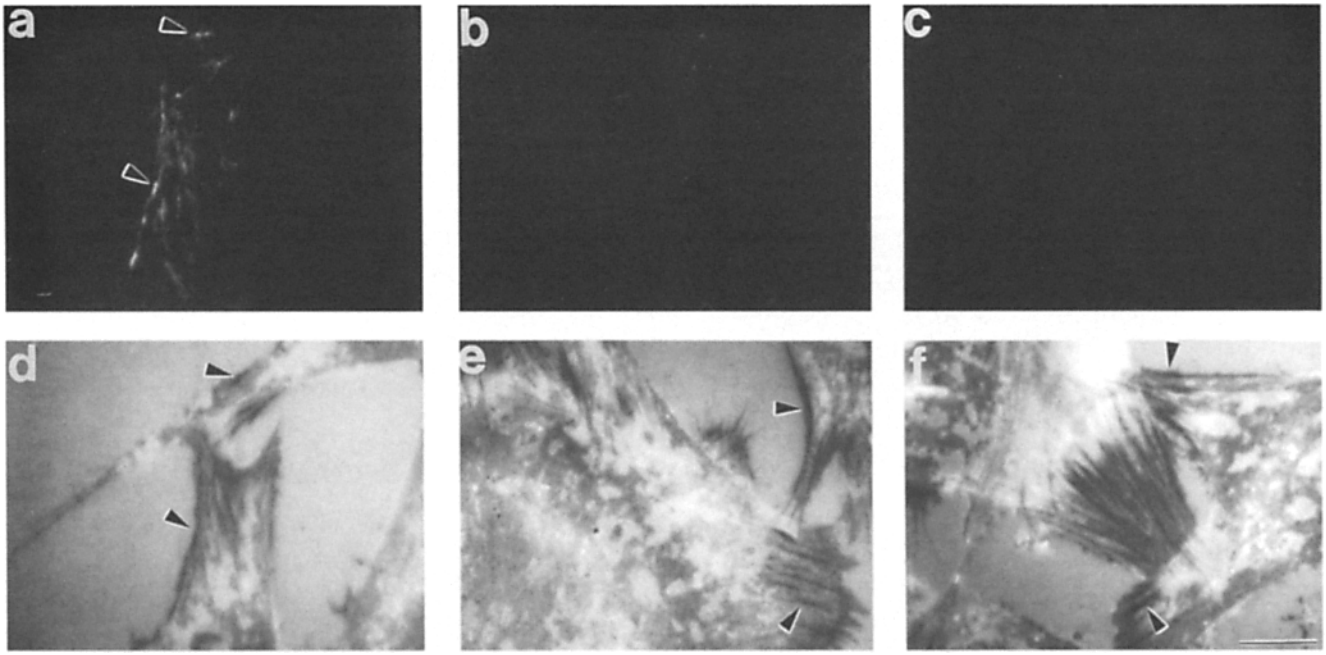

Figure 10. Localization of III-1,2 binding sites. A1-Fs (4×10^5 in DMEM/0.2%BSA with 20 μg/ml cycloheximide) were incubated for 18 h on coverslips coated with 10 μg/ml of III-9,10. Cells were then incubated with either media alone (panels *a* and *d*), 9D2 (panels *b* and *e*), or 3E3 (panels *c* and *f*). Cells were stained with 10 μg/ml Texas red–conjugated-III-1,2 (*a–c*) for 90 min at 37°C. Arrowheads indicate localization of III-1,2 at sites of focal contacts. The IRM image is shown in *d*, *e*, and *f*.

Gly-Glu (III-10A/RGE). The III-10A/RGE module was as effective as the nonmutant III-10A in inhibiting fibronectin binding to fibroblast monolayers (Fig. 8), indicating that the inhibition of fibronectin matrix assembly by III-10A was not due to the disruption of fibronectin-$\alpha_5\beta_1$ integrin interactions.

Colocalization of III-1,2 with β_1 Integrins

We postulated that upon binding of fibronectin to the $\alpha_5\beta_1$ integrin, a conformational change may occur in the III-10 module which serves to expose the III-1 binding site. If so, cell surface binding sites for exogenously added III-1 should colocalize with β_1 integrins. To test this hypothesis, A1-F fibroblasts were seeded onto fibronectin- or 160/180 kD fragment-coated coverslips in the presence of cycloheximide and allowed to adhere and spread for 18 h. Cells were then incubated with Texas red–conjugated III-1,2 for 90 min. The III-1,2 construct was chosen for use in cell assays based on its ability to promote the ternary complex with III-10 and the amino terminus of fibronectin (Fig. 5 *A*). In addition, a 14-kD chymotryptic fragment, containing portions of both the first and second type III modules, has previously been shown to bind to cell surfaces and inhibit fibronectin assembly (24). Cells were fixed, permeabilized, and stained for β_1 integrins using a monoclonal anti-β_1 antibody followed by an FITC-conjugated secondary antibody. III-1,2 and β_1 integrins were colocalized on the same cell using epifluorescence microscopy. As shown in Fig. 9, Texas red–conjugated III-1,2 staining (panels *b* and *e*) was detected on cells seeded onto either fibronectin (panels *a–c*) or the 160/180-kD fibronectin fragment (panels *d–f*). In contrast, III-1,2 staining was not detected on cells which had been seeded onto either laminin or vitronectin (not shown). Moreover, Texas red–conjugated III-1,2 staining was localized to regions of β_1-integrin staining (Fig. 9, *a* and *b*), which occurred at areas of focal adhesions, as observed under IRM optics (Fig. 9, *c* and *f*). Similar results were observed with cells seeded onto the 160/180-kD fragment of fibronectin (Fig. 9, *d–f*), which does not contain the amino-terminal region, indicating that the III-1,2 was not binding directly to the amino terminus.

Localization of Cell Surface III-1,2 Binding Sites to III-10

To further localize cell surface III-1,2 binding sites, Texas red III-1,2 was incubated with A1-F fibroblasts which had been seeded overnight onto coverslips coated with a fibronectin construct containing the III-9 and III-10 modules (III-9,10). This construct contains the synergy site within the III-9 module that is required for cell spreading (3, 29). Binding sites for Texas red III-1,2 were observed on the surface of cells adherent to III-9,10 (Fig. 10 *a*, *arrowheads*). These sites also localized to focal adhesions, as observed under IRM optics (Fig. 10 *d*, *arrowheads*). ^{125}I-III-1,2, in the absence of cells, did not bind to coverslips coated with III-9,10 (not shown), indicating that the interaction of the cell with III-9,10 was required for the expression of III-1,2 binding sites. To localize III-1,2 binding to the III-10 module, cells adherent to III-9,10 were incubated with 50 μg/ml of either the anti–III-1 monoclonal antibody, 9D2, or the monoclonal antibody 3E3. Clone 3E3 has previously been shown to recognize the 15-kD cell-binding fragment of fibronectin (33) and recognizes the III-9,10 construct in Western blots (data not shown). After 1-h incubation, Texas red III-1,2 was added directly to the culture media and cells were incubated an addi-

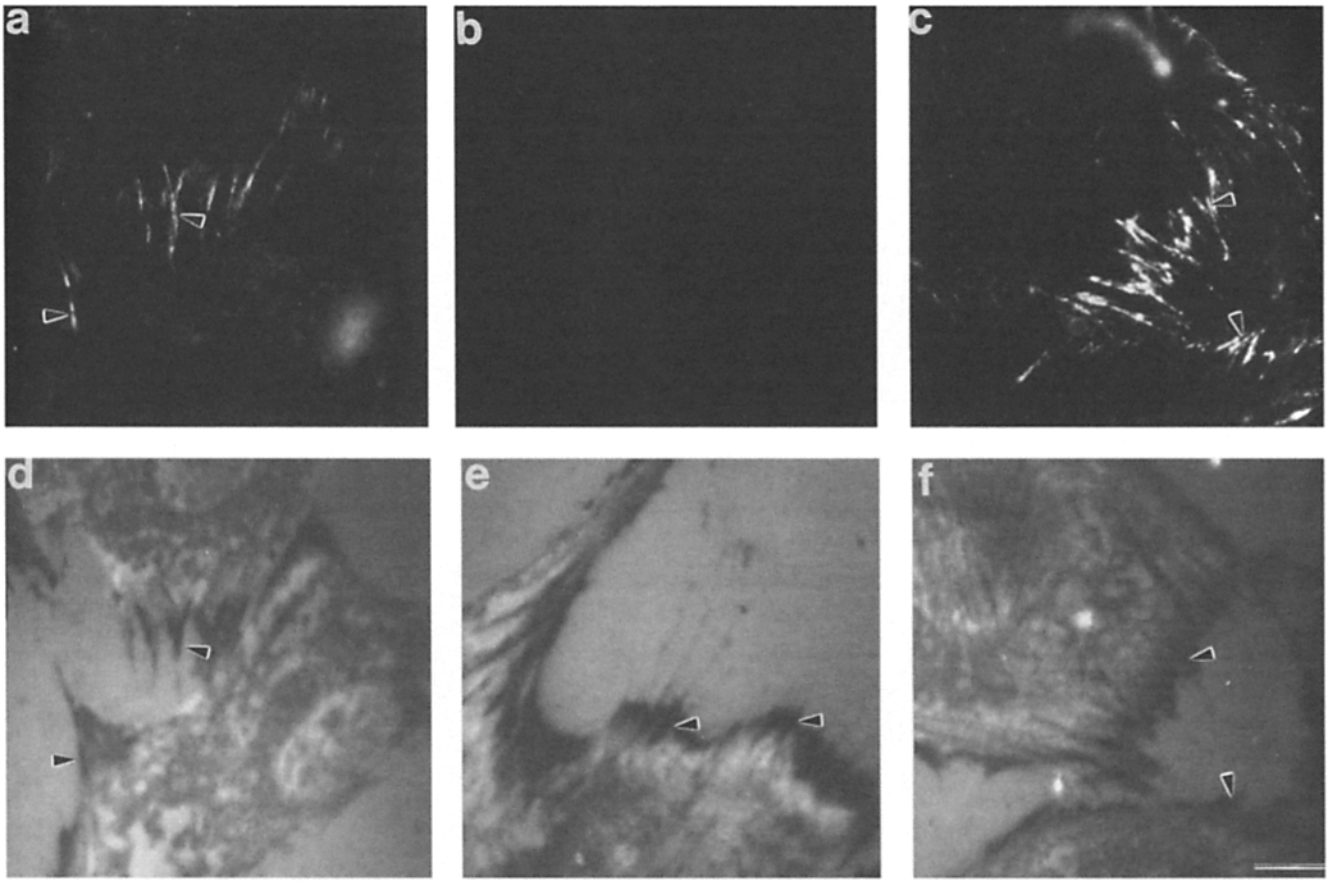

Figure 11. Inhibition of III-1,2 binding site expression by monoclonal antibody 3E3. A1-Fs (4×10^5 in DMEM/0.2%BSA with 20 μg/ml cycloheximide) were incubated for 18 h on coverslips coated with 10 μg/ml of the 160/180-kD fragments of fibronectin. Cells were then incubated with either media alone (*a* and *d*), 3E3 (*b* and *e*), or LAB (*c* and *f*). Cells were stained with 10 μg/ml Texas red–conjugated-III-1,2 (*a–c*) for 90 min at 37°C. Arrowheads indicate localization of III-1,2 at sites of focal contacts. The IRM image is shown in *d*, *e*, and *f*.

tional 90 min. Addition of either 9D2 (Fig. 10 *b*) or 3E3 (Fig. 10 *c*) completely inhibited the binding of III-1,2 to cell surfaces. Inhibition of III-1,2 binding by the monoclonal antibodies was not due to disruption of cell adhesion, since sites of focal adhesions were observed in the presence of either 9D2 or 3E3 (Fig. 10, *e* and *f*, *arrowheads*). These data suggest that the binding of III-1 to III-10 is required for the localization of III-1,2 to the integrin/III-9,10 complex.

To determine if type III modules of fibronectin other than III-10 could serve as III-1,2 binding sites on cell surfaces during the initiation of fibronectin polymerization, A1-F fibroblasts were plated onto coverslips coated with the 160/180-kD fragments of fibronectin and allowed to adhere and spread for 18 h. As previously shown (Fig. 9 *b*), cells adherent to the 160/180-kD fragments of fibronectin expressed III-1,2 binding sites on their surfaces (Fig. 11 *a*, *arrowheads*). Addition of either 3E3 (Fig. 11 *b*) or 9D2 (not shown) inhibited the expression of cell surface III-1,2 binding sites. In contrast, addition of the monoclonal antibody Lab, which recognizes an epitope within the region III-4 to III-8 (5), had no efffect on III-1,2 binding site expression (Fig. 11 *c*). Similar results were obtained with cells seeded onto intact fibronectin (not shown). Taken together, these data suggest that on cells newly adherent to protomeric fibronectin, the III-10 module serves as a cell surface binding site for III-1.

Discussion

We have recently reported that the amino terminus of fibronectin binds to a conformation-dependent site within the III-1 module of fibronectin (15). In addition, the amino-terminal region of fibronectin was shown to bind to trypsinized cell surface fibronectin fragments which contained the III-1 module (15) and to a recombinant fusion protein containing the first two type III repeats (1), suggesting that the interaction of the amino terminus with III-1 may be one of the homophilic interactions which occurs during the process of fibronectin matrix assembly. In the present study, we have defined a second homophilic interaction which may also function during the process of fibronectin polymerization. Our data indicate that the III-1 module also binds to a conformation-dependent site in the integrin-binding III-10 module. In addition, our data indicate that in the absence of cells, the interaction of III-1 in intact fibronectin with the conformation-dependent site in III-10 leads to the formation of high molecular mass fibronectin multimers through an interaction which involves

the amino terminus. Taken together, our results suggest a mechanism whereby the interaction of III-10 with III-1 promotes a conformational change within the III-1 module which unmasks the amino-terminal binding site and triggers the self-polymerization of fibronectin.

A role for the cell-binding domain of fibronectin in matrix assembly was previously demonstrated in a study by McDonald et al. (20), in which addition of either a 105-kD cell-binding fibronectin fragment or antibodies directed against this fragment inhibited matrix assembly. In addition, studies have demonstrated a role for III-1 in fibronectin matrix assembly (5, 24). The anti–III-1 monoclonal antibody, 9D2, has been shown to block the incorporation of exogenous fibronectin into the extracellular matrix (5). In addition, a 14-kD fibronectin fragment, beginning 20–amino acid residues into the III-1 module, was shown to bind to both intact fibronectin and cell layers, and to block matrix assembly (24). Neither the 9D2 antibody nor the 14-kD fragment inhibited the binding of the 70-kD fragment to cells (5, 24). Similarly, in the present study, 9D2 had no effect on the ability of the 70-kD fragment to bind to III-1. On the other hand, 9D2 was able to completely inhibit the interaction of III-1 with III-10, at concentrations similar to those previously shown to block fibronectin polymerization on cell surfaces (5). It is possible, therefore, that one mechanism by which both 9D2 and the 14-kD fragment inhibit fibronectin polymerization on cell surfaces is by inhibiting the interaction of III-1 with III-10. In support of this is our observation that addition of the III-10 fragment (III-10A) to fibroblast monolayers also resulted in the inhibition of fibronectin polymerization. The ability of III-10A to inhibit fibronectin polymerization was not due to a disruption of cell adhesion, since the III-10A/RGE construct, in which the integrin-binding RGD sequence was mutated to RGE (32), was also able to inhibit fibronectin polymerization. These results suggest that the III-10 module in fibronectin participates in a step in matrix assembly which is distinct from its integrin-binding activity.

Upon binding of the amino-terminal region to the cell surface, fibronectin accumulates in the extracellular matrix in the form of high molecular mass aggregates (21). Cell-mediated fibronectin polymerization can be blocked by the addition of either amino-terminal fragments of fibronectin (22, 35) or the 9D2 antibody (5). In the present study, the interaction of fibronectin with conformationally altered III-10, in the absence of cells, resulted in the formation of disulfide-stabilized high molecular mass fibronectin multimers, via an interaction involving the amino-terminal region of fibronectin. Although earlier studies have demonstrated in vitro fibronectin multimer formation through either the oxidation of free sulfhydryls (27), the use of reducing agents (46), or the addition of metal ions (43), the ability of both 9D2 and the 27-kD fibronectin fragment to inhibit III-10–induced fibronectin multimer formation suggests that the mechanisms involved in the formation of III-10–induced multimers may be similar to those which mediate polymerization of fibronectin on cell surfaces. Morla et al. (25) have demonstrated that a fibronectin construct containing a portion of the III-1 module was able to induce fibronectin multimer formation by a mechanism which also involved the amino-terminal region of fibronectin. The authors suggested that the site in the III-1 construct which promotes multimer formation is cryptic within the III-1 module of intact fibronectin (25). In the present study, the ability of conformationally altered III-10 to trigger fibronectin multimer formation involved both the III-1 module and the amino terminus, suggesting that the cryptic site in III-1 may be unmasked upon its interaction with III-10. In addition, whereas binding of fibronectin to heat-denatured III-1 could be completely inhibited by the addition of excess amino-terminal 70-kD fragment, this interaction failed to trigger fibronectin multimer formation, suggesting that both the III-10 and amino-terminal binding sites in III-1 must be accessible during multimer formation.

On cell surfaces, fibronectin fibrils (36) and amino-terminal 70-kD fibronectin fragment binding sites (7) have been shown to colocalize with $\alpha_5\beta_1$ integrins in focal adhesions. In addition, anti-β_1 integrin antibodies inhibit binding of the amino-terminal 70-kD fragment to the cell surface, suggesting that the $\alpha_5\beta_1$ integrin can regulate the expression of amino-terminal binding sites (9). One of the functions of integrins in matrix assembly, therefore, may be to provide adhesion sites in which fibronectin, bound to the integrin, undergoes a series of conformational changes resulting in the exposure of the amino-terminal binding site in III-1. In one of the initial steps of matrix assembly, binding of the III-10 module to the $\alpha_5\beta_1$ integrin may directly alter the conformation of III-10 to expose the III-1 binding site. To explore the role of $\alpha_5\beta_1$ integrins in the regulation of this conformation-dependent binding site within the III-10 module, we examined the expression of III-1 binding sites on cell surfaces. In the present study, cell surface binding sites for exogenously added III-1,2 colocalized with β_1 integrins at sites of focal adhesions on cells which had been plated onto fibronectin. In addition, III-1,2 binding sites were expressed on cell surfaces following adhesion to the III-9,10 modules. Moreover, monoclonal antibodies directed against either III-1 or the 15-kD cell-binding fragment blocked binding of III-1,2 to cells plated onto either the III-9,10 modules or the 160/180-kD fragments of fibronectin. Since the 160/180-kD fragments contain all of the fibronectin type III repeats, these data suggest that on cells adherent to protomeric fibronectin, the III-10 module serves as an initial cell surface binding site for III-1. The ability of HDIII-5 to support III-1 binding (Fig. 2 *B*) suggests that following the nucleation of fibronectin matrix assembly and the deposition of fibronectin into the extracellular matrix, other fibronectin type III modules may serve as additional matrix-derived binding sites for III-1, particularly during the lateral growth of a fiber. In the present study, which defines the initial steps involved in the initiation of fibronectin polymerization from protomeric fibronectin, the 3E3 monoclonal antibody was found to inhibit completely the cell surface expression of III-1,2 sites on cells plated onto the III-9,10 construct, the 160/180-kD fragments of fibronectin, and protomeric fibronectin. Taken together, these data are consistent with the hypothesis that binding of fibronectin to the $\alpha_5\beta_1$ integrin regulates the unfolding of the III-10 module to expose the III-1 binding site during the initial nucleation of fibronectin matrix assembly from cell surfaces.

Although several studies (2, 9, 11, 48) have implicated $\alpha_5\beta_1$ integrins in the formation of fibronectin matrices, fi-

broblastic cells derived from α_5-null embryos retain the ability to form focal contacts and assemble a fibronectin matrix, suggesting that other fibronectin receptors may substitute for the $\alpha_5\beta_1$ integrin during fibronectin polymerization (52). The ability of other α subunits to substitute for α_5 in fibronectin matrix assembly has been examined using α_5-deficient CHO cells. Expression of $\alpha_4\beta_1$, which binds to the CS1 site located in the alternatively spliced IIICS region of fibronectin (10, 12, 45), restored adhesion and spreading of α_5-deficient CHO cells on fibronectin, but did not restore the ability of these cells to assemble a fibronectin matrix, suggesting that direct binding of the integrin to the III-10 module may be necessary for the initiation of matrix assembly (49). Expression of $\alpha_v\beta_1$, which binds to the RGD site in the III-10 module of fibronectin (44), restored the ability of α_5-deficient CHO cells to adhere to fibronectin, but did not restore their ability to assemble a fibronectin matrix (53). In contrast to $\alpha_5\beta_1$-expressing cells, $\alpha_v\beta_1$-expressing cells demonstrated a lower affinity for fibronectin and did not localize $\alpha_v\beta_1$ to focal adhesions (53). More recently, the activated form of $\alpha_{IIb}\beta_3$ has been shown to promote fibronectin matrix assembly (50). These data suggest that several components may be important in the initiation of matrix assembly, including the direct interaction of the integrin with the III-10 module, the affinity of the integrin–fibronectin interaction, and the ability of the integrin–fibronectin complex to localize to focal adhesions.

In the present study, we have demonstrated that the III-10 module of fibronectin contains a conformation-dependent binding site for the III-1 module and that the interaction of the III-1 module in intact fibronectin with the unfolded III-10 module promotes the generation of high molecular mass multimers through an interaction involving the amino-terminal region of fibronectin. Our data are consistent with a model in which de novo nucleation of fibronectin matrix assembly is initiated by sequential interactions involving the integrin and the III-10 and III-1 modules of fibronectin. The interaction between III-10 and III-1 is blocked by the 9D2 monoclonal antibody. The inability of the 9D2 monoclonal antibody to inhibit either 70-kD binding to cell monolayers (5) or to heat-denatured III-1 (Fig. 4 *B*) suggests that the initial binding of the amino terminus of fibronectin to the cell surface is mediated by another molecule. Our model, depicted in Fig. 12, predicts that once the III-10/III-1 complex is formed, the amino terminus is transferred from the binding site on the cell to the III-1 module. This model addresses the de novo nucleation of matrix asembly from cells adherent to protomeric fibronectin, and does not preclude the possibility that fibronectin polymerization may be initiated or propagated from other sites when cells are adherent to other matrix molecules.

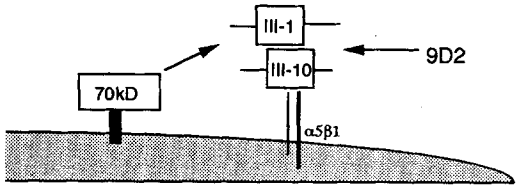

Figure 12. Model of III-1/III-10/integrin nucleation site. Schematic representation of fibronectin nucleation site in which the binding of the $\alpha_5\beta_1$ integrin to the III-10 module in protomeric fibronectin exposes the III-1 binding site in III-10. Upon binding of a III-1 module to the unfolded III-10, the 70-kD binding site within the III-1 module is exposed, allowing the amino terminus of an incoming fibronectin molecule to be transferred to the III-1 module of the growing fibril. Addition of either 9D2 or III-10A inhibits the interaction of III-1 with III-10 and prevents the movement of 70 kD onto the nucleation complex.

The presence of an established fibronectin matrix may support the incorporation of dimeric fibronectin constructs lacking either the RGD sequence or the III-1 module (18, 40). These studies did not distinguish between the ability of the fibronectin construct to bind to a preexisting fibronectin matrix and to actually undergo polymerization into fibrils. The studies presented here would suggest that such constructs would not undergo polymerization in the absence of a preexisting fibronectin matrix. In agreement with this, a dimeric construct containing only the amino-terminal 70-kD region and the carboxy-terminal bridge region of fibronectin has been shown to be incorporated into the extracellular matrix (42). However, the dimeric 70-kD construct accumulated in the matrix of cycloheximide-treated cells only in the presence of intact fibronectin (42). These data suggest that once the nucleation site is established, by either endogenous fibronectin or exogenously added fibronectin, subsequent interactions involving the unfolded III-1 module in the nucleation site, and the amino terminus of an incoming fibronectin molecule leads to the incorporation of the fibronectin molecule into the extracellular matrix. We propose that following initial adhesion of cells to protomeric fibronectin, nucleation of fibronectin matrix assembly occurs by an integrin-dependent unfolding of the III-10 domain which regulates the subsequent interaction between the unfolded III-10 module and the III-1 module of another fibronectin molecule. By controlling the interaction of III-10 with III-1, the β_1 integrin thereby regulates the conformation of III-1, and, consequently, the polymerization of incoming fibronectin molecules into fibrils. Taken together, these data suggest that the nucleation of fibronectin polymerization proceeds by a series of sequential self-interactions which are regulated by the integrin-dependent unfolding of the III-10 module.

We thank Drs. Jane Sottile and Susan LaFlamme for helpful advice and critically reviewing this manuscript.

This work was supported by grants P01-GM-40761 and T32-GM-07033 of the National Institutes of Health and 950210 of the American Heart Association, New York State Affiliate.

Received for publication 22 May 1995 and in revised form 16 September 1995.

References

1. Aguirre, K.M., R.J. McCormick, and J.E. Schwarzbauer. 1994. Fibronectin self-association is mediated by complementary sites within the amino-terminal one-third of the molecule. *J. Biol. Chem.* 269:27863–27868.
2. Akiyama, S.K., S.S. Yamada, W.T. Chen, and K.M. Yamada. 1989. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. *J. Cell Biol.* 109:863–875.
3. Aota, S., T. Nagai, and K.M. Yamada. 1991. Characterization of regions of fibronectin besides the arginine-glycine-aspartic acid sequence required for adhesive function of the cell-binding domain using site-directed mu-

tagenesis. *J. Biol. Chem.* 266:15938–15943.
4. Balian, G., E.M. Click, and P. Bornstein. 1980. Localization of a collagen-binding domain in fibronectin. *J. Biol. Chem.* 255:3234–3236.
5. Chernousov, M.A., F.J. Fogerty, V.E. Koteliansky, and D.F. Mosher. 1991. Role of the I-9 and III-1 modules of fibronectin in formation of an extracellular matrix. *J. Biol. Chem.* 266:10851–10858.
6. Darribere, T., K. Guida, H. Larjava, K.E. Johnson, K.M. Yamada, J. Thiery, and J. Boucat. 1990. In vivo analysis of integrin β-1 subunit function in fibronectin matrix assembly. *J. Cell Biol.* 110:1813–1823.
7. Dzamba, B.J., H. Bultmann, S.K. Akiyama, and D.M. Peters. 1994. Substrate-specific binding of the amino terminus of fibronectin to an integrin complex in focal adhesions. *J. Biol. Chem.* 269:19646–19652.
8. Engvall, E. 1981. Enzyme immunoassay ELISA and EMIT. *Methods Enzymol.* 70:419–439.
9. Fogerty, F.J., S.K. Akiyama, K.M. Yamada, and D.F. Mosher. 1990. Inhibition of binding of fibronectin to matrix assembly sites by anti-integrin ($\alpha_5\beta_1$) antibodies. *J. Cell Biol.* 111:699–708.
10. Garcia-Pardo, A., E.A. Wayner, W.G. Carter, and O. Ferreira, Jr. 1990. Human B lymphocytes define an alternative mechanism of adhesion to fibronectin. The interaction of the α4β1 integrin with the LHGPEILDVPST sequence of the type III connecting segment is sufficient to promote cell attachment. *J. Immunol.* 144:3361–3366.
11. Giancotti, F.G., and E. Ruoslahti. 1990. Elevated levels of the α5β1 fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell.* 60:849–859.
12. Guan, J.-L., and R.O. Hynes. 1990. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor α4β1. *Cell.* 60:53–61.
13. Harlow, E., and D. Lane. 1988. Antibodies: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp. 121–123, 630.
14. Higuchi, R. 1992. PCR Technology. W.H. Freeman and Co., New York. 61 pp.
15. Hocking, D.C., J. Sottile, and P.J. McKeown-Longo. 1994. Fibronectin's III-1 module contains a conformation-dependent binding site for the amino-terminal region of fibronectin. *J. Biol. Chem.* 269:19183–19191.
16. Hynes, R.O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.
17. Hynes, R.O. 1990. Fibronectins. Springer-Verlag, New York. pp. 24–364.
18. Ichihara-Tanaka, K., T. Maeda, K. Titani, and K. Sekiguchi. 1992. Matrix assembly of recombinant fibronectin polypeptide consisting of amino-terminal 70 kDa and carboxyl-terminal 37 kDa regions. *FEBS Lett.* 299: 155–158.
19. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
20. McDonald, J.A., B.J. Quade, T.J. Broekelmann, R. LaChance, K. Forsman, E. Hasegawa, and S. Akiyama. 1987. Fibronectin's cell-adhesive domain and an amino-terminal matrix assembly domain participate in its assembly into fibroblast pericellular matrix. *J. Biol. Chem.* 262:2957–2967.
21. McKeown-Longo, P.J., and D.F. Mosher. 1983. Binding of plasma fibronectin to cell layers of human skin fibroblasts. *J. Cell Biol.* 97:466–472.
22. McKeown-Longo, P.J., and D.F. Mosher. 1985. Interaction of the 70,000-mol-wt amino-terminal fragment of fibronectin with the matrix-assembly receptor of fibroblasts. *J. Cell Biol.* 100:364–374.
23. McKeown-Longo, P.J., and C.A. Etzler. 1987. Induction of fibronectin matrix assembly in human fibrosarcoma cells by dexamethasone. *J. Cell Biol.* 104:601–610.
24. Morla, A., and E. Ruoslahti. 1992. A fibronectin self-assembly site involved in fibronectin matrix assembly: reconstruction in a synthetic peptide. *J. Cell Biol.* 118:421–429.
25. Morla, A., Z. Zhang, and E. Ruoslahti. 1994. Superfibronectin is a functionally distinct form of fibronectin. *Nature (Lond.).* 367:193–196.
26. Mosher, D.F. 1984. Physiology of fibronectin. *Annu. Rev. Med.* 35:561–575.
27. Mosher, D.F., and R.B. Johnson. 1983. *In vitro* formation of disulfide-bonded fibronectin multimers. *Ann. NY Acad. Sci.* 408:583–594.
28. Mosher, D.F., J. Sottile, C. Wu, and J.A. McDonald. 1992. Assembly of extracellular matrix. *Curr. Opin. Cell Biol.* 4:810–818.
29. Obara, M., M.S. Kang, and K.M. Yamada. 1988. Site-directed mutagenesis of the cell-binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. *Cell.* 53:649–657.
30. Petersen, T.E., K. Skorstengaard, and K. Vibe-Pedersen. 1989. Primary structure of fibronectin. *In* Fibronectin. D.F. Mosher, editor. Academic Press, New York. pp. 1–24.
31. Pierschbacher, M.D., and E. Ruoslahti. 1984. Variants of the cell recognition site of fibronectin that retain attachment-promoting activity. *Proc. Natl. Acad. Sci. USA.* 81:5985–5988.
32. Pierschbacher, M.D., and E. Ruoslahti. 1984. The cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature (Lond.).* 309:30–33.
33. Pierschbacher, M.D., E.G. Hayman, and E. Ruoslahti. 1981. Location of the cell-attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. *Cell.* 26:259–267.
34. Pytela, R., M.D. Pierschbacher, and E. Ruoslahti. 1985. Identification and isolation of a 140 kD cell surface glycoprotein with properties of a fibronectin receptor. *Cell.* 40:191–198.
35. Quade, B.J., and J.A. McDonald. 1988. Fibronectin's amino-terminal matrix assembly site is located within the 29-kDa amino terminal domain containing five type I repeats. *J. Biol. Chem.* 263:19602–19609.
36. Roman, J., R.M. LaChance, T.J. Broekelmann, C.J.R. Kennedy, E.A. Wayner, W.G. Carter, and J.A. McDonald. 1989. The fibronectin receptor is organized by extracellular matrix fibronectin: implications for oncogenic transformation and for cell recognition of fibronectin matrices. *J. Cell Biol.* 108:2529–2543.
37. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. pp. 1.74–1.84.
38. Sanger, F., S. Niklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.
39. Schagger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368–379.
40. Schwarzbauer, J.E. 1991. Identification of the fibronectin sequences required for assembly of a fibrillar matrix. *J. Cell Biol.* 113:1463–1473.
41. Small, J.V., and J.E. Celis. 1978. Filament arrangements in negatively stained cultured cells: the organization of actin. *Eur. J. Cell Biol.* 16:308–325.
42. Sottile, J., and S. Wiley. 1994. Assembly of amino-terminal fibronectin dimers into the extracellular matrix. *J. Biol. Chem.* 269:17192–17198.
43. Vartio, T. 1986. Disulfide-bonded polymerization of plasma fibronectin in the presence of metal ions. *J. Biol. Chem.* 261:9433–9437.
44. Vogel, B.E., G. Tarone, F.G. Giancotti, J. Gailit, and E. Ruoslahti. 1990. A novel fibronectin receptor with an unexpected subunit composition (αvβ1). *J. Biol. Chem.* 265:5934–5937.
45. Wayner, E.A., A. Garcia-Pardo, M.J. Humphries, J.A. McDonald, and W.G. Carter. 1989. Identification and characterization of the lymphocyte adhesion receptor for an alternative cell attachment domain in plasma fibronectin. *J. Cell Biol.* 109:1321–1330.
46. Williams, E.C., P.A. Janmey, R.B. Johnson, and D.F. Mosher. 1983. Fibronectin: effect of disulfide bond reduction on its physical and functional properties. *J. Biol. Chem.* 258:5911–5914.
47. Wray, W., R. Boulikas, V.P. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* 118:197–203.
48. Wu, C., J.S. Bauer, R.L. Juliano, and J.A. McDonald. 1993. The $\alpha_5\beta_1$ integrin fibronectin receptor, but not the α_5 cytoplasmic domain, functions in an early and essential step in fibronectin matrix assembly. *J. Biol. Chem.* 268:21833–21888.
49. Wu, C., A.J. Fields, B.A.E. Kapteijn, and J.A. McDonald. 1995. The role of α4β1 integrin in cell motility and fibronectin matrix assembly. *J. Cell Sci.* 108:821–829.
50. Wu, C., V.M. Keivens, T.E. O'Toole, J.A. McDonald, and M.H. Ginsberg. 1995. Integrin activation and cytoskeletal interaction are essential for the assembly of a fibronectin matrix. *Cell.* 83:715–724.
51. Yamada, K. 1989. Fibronectin domains and receptors. *In* Fibronectin. D.F. Mosher, editor. Academic Press, New York. pp. 47–121.
52. Yang, J.T., H. Rayburn, and R.O. Hynes. 1993. Embryonic mesodermal defects in α5 integrin-deficient mice. *Development.* 119:1093–1105.
53. Zhang, Z., A.E. Morla, K. Vuori, J.S. Bauer, R.L. Juliano, and E. Ruoslahti. 1993. The $\alpha_v\beta_1$ integrin functions as a fibronectin receptor but does not support fibronectin matrix assembly and cell migration on fibronectin. *J. Cell Biol.* 122:235–242.