

Fibronectin Binding Site in Type I Collagen Regulates Fibronectin Fibril Formation

Bette J. Dzamba,** Hong Wu,§ Rudolf Jaenisch,|| and Donna M. Peters*

*Department of Laboratory Medicine and Pathology; †Program in Cell and Molecular Biology, University of Wisconsin, Madison, Wisconsin 53706; §Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02139; ||Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract. Mov13 fibroblasts, which do not express endogenous $\alpha 1(I)$ collagen chains due to a retroviral insertion, were used to study the role of type I collagen in the process of fibronectin fibrillogenesis. While Mov13 cells produced a sparse matrix containing short fibronectin fibrils, transfection with a wild type pro- $\alpha 1(I)$ collagen gene resulted in the production of an extensive matrix containing fibronectin fibrils of normal length. To study the amino acids involved in the fibronectin-collagen interaction, mutations were introduced into the known fibronectin binding region of the pro $\alpha 1(I)$ collagen gene. Substitution of Gln and Ala at positions 774 and 777 of the $\alpha 1(I)$ chain for Pro re-

sulted in the formation of short fibronectin fibrils similar to what was observed in untransfected Mov13 cells. Type I collagen carrying these substitutions bound weakly to fibronectin-sepharose and could be eluted off with 1 M urea. The effect of this mutation on fibronectin fibrillogenesis could be rescued by adding either type I collagen or a peptide fragment (CB.7) which contained the wild type fibronectin binding region of the $\alpha 1(I)$ chain to the cell culture. These results suggest that fibronectin fibrillogenesis in tissue culture is dependent on type I collagen synthesis, and define an important role for the fibronectin binding site in this process.

FIBRONECTIN is an extracellular matrix protein containing two nonidentical peptide chains disulfide bonded together at the carboxyl terminal (for a recent review see Hynes, 1989). It is a modular protein composed of three types of repeating sequences, called type I, II, and III homology repeats, and several domains which are known to bind to a variety of matrix components such as heparan sulfate proteoglycans, fibrin, and collagens. The binding site for collagen has been localized to a 14-amino acid sequence that links the second type II homology repeat with the adjacent type I homology repeat (I_7 ; Owens and Baralle, 1986). Recent results from affinity chromatography of fibronectin fragments released after pepsin digestion suggest that a second collagen binding site may also be localized in the 8th and 9th type I homology repeats (Ingham et al., 1989).

Type I collagen is a triple helical molecule composed of two identical $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain. The $\alpha 1(I)$ chain contains a binding region for fibronectin between amino acid residues 757-791 (Kleinman et al., 1978). This region also coincides with the vertebrate collagenase cleavage site which is located between amino acids 775-776 (Gross, 1982).

It is not known what, if any, influence these respective fibronectin and collagen binding sites have on the assembly of fibronectin or collagen into fibrils. Immunofluorescent and immunoelectron microscopy studies of human skin and embryonic chick heart fibroblast cultures (Vaheri et al., 1978; Furcht et al., 1980a,b; Irish and Hasty, 1983; Little

and Chen, 1982) and in situ studies on human skin (Fleischmajer and Timpl, 1984) show that fibronectin co-distributes with type I and type III collagens. In these fibrils, fibronectin shows an axial periodicity of approximately 70 nm which is close to the periodicity of type I collagen fibrils. These observations suggest that fibronectin may become incorporated into fibrils by binding to the fibronectin binding domain in collagen (Dessau et al., 1978; Kleinman et al., 1978).

There is, however, evidence that fibronectin fibrils form independently from type I collagen fibrils. In vivo and in vitro studies show that fibronectin fibrils exist in the absence of type I collagen (Chen et al., 1978; Löhler et al., 1984; Thiery et al., 1989). Collagenase digestion of matrix fibrils does not disrupt fibronectin fibrils, whereas the digestion of matrix with trypsin or thrombin removes both fibronectin and collagen (Vaheri, 1978; Keski-Oja et al., 1981). In cycloheximide-treated human fibroblast cultures, exogenously added plasma fibronectin is assembled into fibrils devoid of type I collagen (Peters et al., 1990). Immunoelectron microscopy studies of subconfluent fibroblast cultures show that fibronectin is mainly found in fibrils devoid of type I collagen and that fibronectin in these fibrils has an axial periodicity of 84 or 42 nm depending on the diameter of the fibril (Dzamba and Peters, 1991).

In the present study, we used Mov13 mouse fibroblasts, which do not produce $\alpha 1(I)$ collagen (Schnieke et al., 1983) and Mov13 fibroblasts transfected with either wild type $\alpha 1(I)$ collagen or $\alpha 1(I)$ collagen genes containing mutations in the

fibronectin binding region (Wu et al., 1990). This approach allowed us to study the effect of growing collagen fibrils containing defined mutations on the formation of fibronectin fibrils *in vivo*. The mutations consisted of amino acid substitutions in the fibronectin binding region of the $\alpha 1(I)$ chain which previously have been shown to affect the sensitivity of the triple helix to digestion with collagenase (Wu et al., 1990). Untransfected Mov13 cells or cells transfected with $\alpha 1(I)$ collagen gene containing Pro substitutions at positions 774 and 777 but not cells transfected with the wild type gene produced a sparse matrix containing short fibronectin fibrils. The effect of these mutations could be rescued by adding type I collagen or a collagen peptide fragment (CB.7) which contains the normal fibronectin binding region. These studies show that although short fibronectin fibrils can form in the absence of type I collagen chains, elongation of fibronectin fibrils is dependent on the presence of type I collagen.

Materials and Methods

Cell Culture

Mov13 fibroblasts transfected with the wild type murine genomic clone for the pro $\alpha 1(I)$ collagen chain (CollA1) and mutant derivatives of the murine Colla1 clone were prepared as described by Schnieke et al. (1987) and Wu et al. (1990). The fibroblasts were grown at 37°C in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 100 U/ml penicillin G and 5 μ M streptomycin sulfate (GIBCO BRL).

Purification of Collagen

Triple-helical collagens were prepared using a modification of the procedure described by Bateman et al. (1984). Cells were grown to confluency in 100 mm dishes and then incubated for 16 h with 5 ml of MEM containing 5% FBS, 50 μ g/ml L-ascorbic acid, 50 μ g/ml β -aminopropionitrile, 100 U/ml penicillin G, 5 μ M streptomycin sulfate, 20 μ Ci/ml L-[2,3- 3 H]-proline (Dupont-New England Nuclear, Boston, MA). After incubation the media was saved and the cell layer was washed with 20 mM sodium phosphate, pH 7.4, containing 150 mM sodium chloride (PBS). The wash was added to the media and collagen was precipitated with 25% ammonium sulfate (ICN Radiochemicals, Costa Mesa, CA) overnight at 4°C, and then centrifuged at 36,000 g at 4°C. The pellets were resuspended in 50 mM Tris HCl, pH 7.4, 150 mM NaCl, and the collagens were immediately reprecipitated with 75% ethanol (final concentration), and centrifuged at 41,000 g at 4°C. The pellets were resuspended in 0.5 N acetic acid, pH 2.0. The collagens were then digested with 0.1 mg/ml pepsin (Worthington, Freehold, NJ) for 3 h at 4°C. The digestion was stopped by adding 15 μ g/ml pepstatin and neutralized with 10 N NaOH. The collagens were precipitated overnight with 75% ethanol and centrifuged as described above. The cell layer was scraped into 0.5 N acetic acid, pH 2.0. Protease inhibitors were added to both the cell layer and media to a final concentration of 25 mM EDTA, 1 mM *p*-aminobenzoic acid, 1 mM *N*-ethylmaleimide, and 0.1 mM PMSF. The cell layer was sonicated for 20 s on ice. Collagen in the cell layer was digested with pepsin and precipitated with 75% ETOH as described above. The pellets from both the cell layer and media were then resuspended in a 0.125 M Tris-HCl, pH 6.8, buffer containing 2% SDS, 10% glycerol, and 0.01% bromophenol blue, and analyzed by SDS-PAGE (Laemmli, 1970) using a 6% separating gel and a 3% stacking gel. Gels were treated with En³Hance (New England Nuclear, Boston, MA), dried, and exposed for fluorography.

Collagen used in the binding assays was isolated from the culture media as described above except that the cultures were incubated with both 20 μ Ci/ml L-[2,3- 3 H]-proline and 20 μ Ci/ml L-[3 H]-glycine (Dupont-New England Nuclear, Boston, MA). The purified collagen was then resuspended in 0.1 M Tris-HCl, pH 7.4, containing 0.1 M NaCl, 5 mM CaCl₂, and 0.5% BSA.

Immunofluorescence Microscopy

Fibroblast cultures were washed three times in HBSS containing 20 mM Hepes, pH 7.0, fixed with either 3.5% formaldehyde in 0.1 M sodium phos-

phate, pH 7.4, for 20 min or permeabilized with acetone for 5 min at -20°C. Cells were then washed with 0.1 M sodium phosphate, pH 7.4, and labeled for 30 min with either a 1:100 dilution of rabbit anti-human fibronectin sera or 5 μ g/ml goat anti-human type I collagen IgG (Southern Biotechnology, Birmingham, AL). The anti-human fibronectin sera was generously provided by Dr. Deane F. Mosher (University of Wisconsin, Madison, WI). All of the labeling was done in 20 mM Tris-HCl, pH 8.3, containing 150 mM sodium chloride, 1% BSA, and 20 mM sodium azide (BSA buffer). Labeled cells were washed and then labeled for 1 h in BSA buffer containing a 1:100 dilution of either donkey anti-goat IgG or mouse anti-rabbit IgG (Jackson Immunolaboratory, West Grove, PA) conjugated to fluorescein or rhodamine. Cells were washed and mounted in TBS containing 1 mg/ml *p*-phenylenediamine (Sigma Immunochemicals, St. Louis, MO).

To label cells for actin, cells were permeabilized with acetone as described above, and then labeled for 20 min with 1 U/ml rhodamine conjugated phalloidin (Molecular Probes, Eugene, OR). Cells were mounted as described above. Labeled fibroblasts were viewed with a Microphot epifluorescent microscope (Nikon Inc., Melville, NY). Areas photographed represented areas of similar cell density and morphology.

Preparation of Fibronectin Sepharose Affinity Column

Plasma fibronectin (10 mg/ml) was generously provided by Dr. Deane F. Mosher (University of Wisconsin, Madison). Plasma fibronectin was dialyzed overnight at 4°C against 0.1 M NaHCO₃ buffer, pH 8.3, containing 0.5 M NaCl (coupling buffer). Fibronectin was then coupled to cyanogen bromide activated sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's instructions. Briefly, plasma fibronectin was incubated overnight at 4°C and 0.3 g cyanogen bromide-activated sepharose which had been washed with 1 mM HCL. Active groups were blocked with 0.2 M glycine for 16 h at 4°C. Excess ligand was removed by alternatively washing the resin with the coupling buffer and 0.1 M sodium acetate buffer, pH 4.0, containing 0.5 M NaCl.

Fibronectin-Collagen Binding Assay

Collagen labeled with [3 H]proline and [3 H]glycine was incubated with 200 μ l fibronectin-sepharose in 0.1 M Tris-HCl, pH 7.4, containing 0.1 M NaCl, 5 mM CaCl₂, and 0.5% BSA by end-over-end rotation in a microcentrifuge tube for 3 h at 37°C using a modification of the procedure described by Jilek and Hörmann (1978). The fibronectin-sepharose was centrifuged at 5,000 rpm in a microfuge (Beckman Instruments, Fullerton, CA) for 3 min. The supernatant was removed and the fibronectin-sepharose was washed three times with 0.1 M Tris-HCl, pH 7.4, containing 0.1 M NaCl, and 5 mM CaCl₂ (column buffer). The fibronectin-sepharose was then washed successively with column buffer containing 1 M urea, 2 M urea, 0.4 M NaCl, and 4 M urea. The supernatants from each wash were precipitated for 30 min on ice with 10% TCA. The precipitates were centrifuged at 10,000 rpm for 2 min in an Eppendorf microcentrifuge (Brinkman Instruments Inc., Westbury, NY), resuspended in a 0.125 M Tris-HCl, pH 6.8, buffer containing 2% SDS, 10% glycerol and 0.01% bromophenol blue, and analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) using a 6% separating gel and a 3% stacking gel. Gels were treated with En³Hance, dried, and exposed for fluorography. In some experiments collagens were denatured for 1 h at 56°C and then loaded onto the fibronectin sepharose column at 37°C. As a control for nonspecific binding, labeled collagens were incubated with cyanogen bromide-activated sepharose as described above. The active sites in the cyanogen bromide-activated sepharose used in the control experiments were blocked with 0.2 M glycine.

To calculate the amounts of type I collagen that eluted off the fibronectin-sepharose, fluorograms were scanned with a Biomed ID/2D laser densitometer. Areas representing $\alpha 1(I)$ and $\alpha 2(I)$ collagen chains were added together and taken to represent the total amount of type I collagen bound to the fibronectin-sepharose. The amount of type I collagen eluted at each step was then expressed as the percent of type I collagen bound.

Fibronectin ELISAs

To determine levels of fibronectin in the cell layers of these cultures, Mov13 cells and Mov13 cells transfected with wild type Colla1 gene and mutant Colla1 genes were grown to confluency in microtiter plates. The cultures were washed with HBSS, fixed, and permeabilized with 100% methanol for 20 min at -20°C. A direct ELISA using a 1:100 dilution of rabbit anti-human fibronectin serum and a 1:1000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Immunochemicals) was done. Anti-

bodies were diluted into 20 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl (TBS) and 0.1% BSA, and incubated with the cell layers for 2 h. The enzyme substrate used was 1 mg/ml *p*-nitrophenyl phosphate hexahydrate and the absorbance was read at 405 nm. A standard curve using varying concentrations of fibronectin (5–500 ng/ml) was used to determine the amount of fibronectin in the samples. All levels were normalized to cell number.

An indirect ELISA as described by Allen-Hoffmann and Mosher (1987) was used to determine the levels of fibronectin in the media. Briefly, two confluent 100 mm tissue culture dishes were incubated overnight with media containing 0.2% BSA. The media was removed and the cell number in each dish determined. The fibronectin in the media was precipitated with 40% ammonium sulfate, pelleted by centrifugation at 28,000 *g* for 40 min at 4°C, and resuspended in 1 ml TBS. Fibronectin was reprecipitated for 1 h with 70% ethanol (final concentration) at 4°C and then centrifuged as described above. The pellet containing fibronectin was resuspended into 1 ml TBS containing 0.1% BSA and incubated overnight with a 1:1,000 dilution of rabbit anti-human fibronectin sera. All levels were normalized to cell number.

Incubation of Mov13 and MII Cells with the Collagen Peptide CB.7, Type I, III, and Type V Collagens

Cultures of Mov13 and MII cells were plated at a density of 1×10^5 in MEM containing 10% FCS. At the time of plating, 20 µg/ml of either CB.7, type I, III, or V collagens were added to the cultures. Cultures were grown to confluency and then prepared for immunofluorescent microscopy as described above. CB.7 which was isolated from type I rat tail collagen was generously provided by Dr. Hynda Kleinman (National Institutes of Health, Bethesda, MD). Human type III and V collagens were purchased from Southern Biotechnology. Rat type I collagen was purchased from Sigma Immunochemicals.

Results

Recently, Wu et al. (1990) generated several constructs that contain mutations in the collagenase cleavage site of the $\alpha 1(I)$ collagen chain. The mutations and their effect on the susceptibility of type I collagen to collagenase cleavage are summarized in Table I. Since the binding region for fibronectin in the $\alpha 1(I)$ chain coincides with the collagenase cleavage site, we examined the effect of these mutations on the assembly of fibronectin fibrils in Mov13 cells. Immunofluorescent studies showed that in fibroblasts transfected with the wild type Colla1 gene (pWTC1), fibronectin fibrils formed an extensive network that could be observed between the cells as well as across the cell surface (Fig. 1 A). In contrast, fibronectin fibrils in confluent Mov13 cells, which do not produce type I collagen, were shorter and were concentrated along the edges of the cells (Fig. 1 B).

The effects of the mutations (MI, MII, MIII, MIV, and

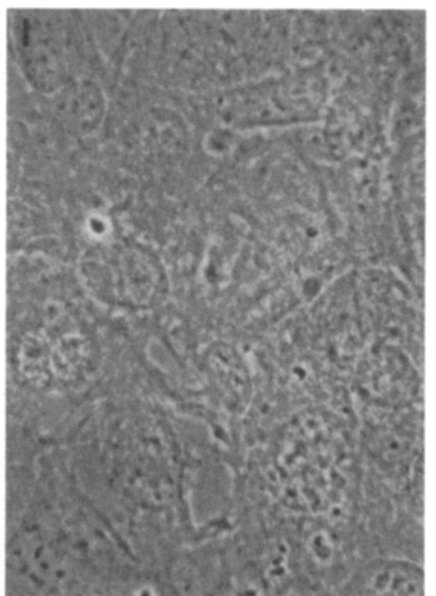
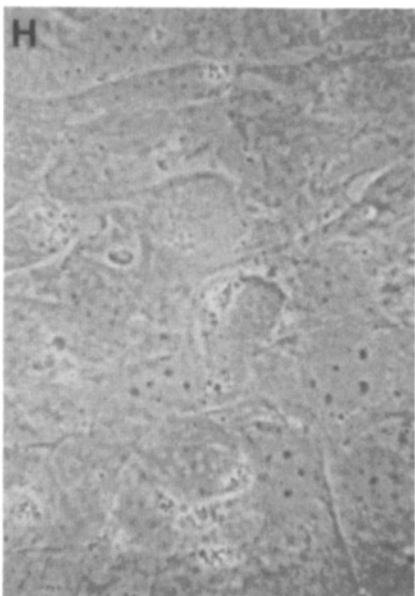
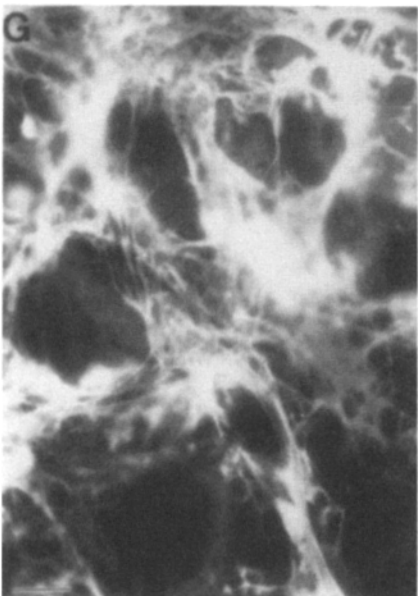
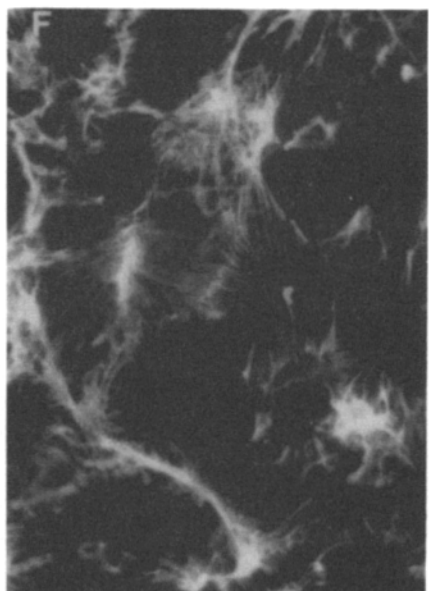
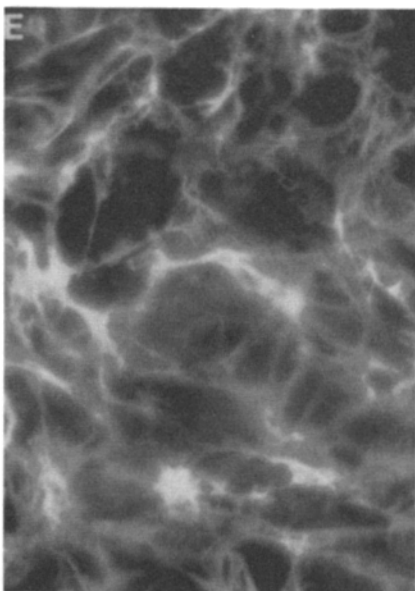
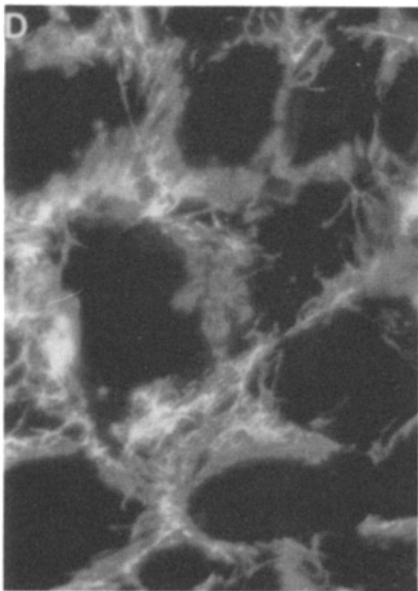
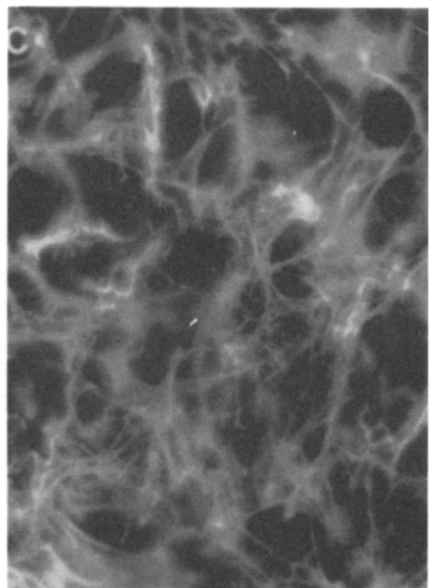
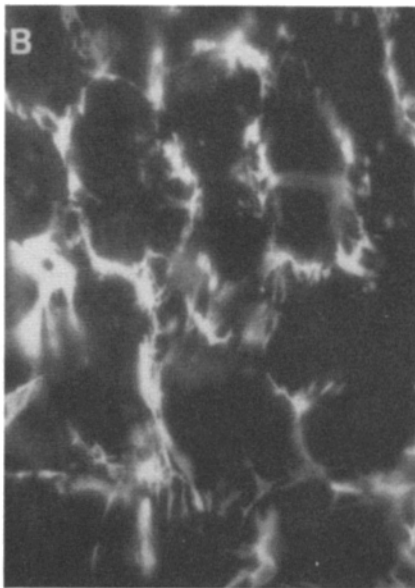
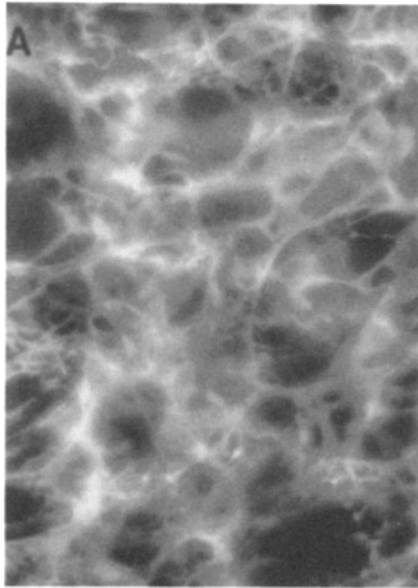
MV) on fibronectin fibrillogenesis are shown in Fig. 1 (C–G). Mutants MI, MIII, and MV produced an extensive fibronectin matrix similar to that produced by wild type pWTC1 cells (compare Fig. 1, C, E, and G to Fig. 1 A). Mutant MII and MIV, on the other hand, produced a matrix similar to that observed in Mov13 cells in which the fibronectin fibrils were shorter and confined to the edges of the cells (Fig. 1, D and F). Fig. 2 shows fibronectin fibrils in subconfluent cultures of pWTC1 and MII. Even in the less well-established matrix of the subconfluent cultures, fibronectin in pWTC1 cells is concentrated on the cell surface whereas cell surface labeling for fibronectin is sparse in MII cultures and is seen mostly on the edges of the cells (Fig. 2, arrow). The morphology of all the cell lines was similar indicating that differences in cell morphology were not responsible for the formation of short fibronectin fibrils in MII and MIV cells (Fig. 1, H and I, and Fig. 2, B and D). Since fluorescent microscopy studies indicated that MII cells formed stress fibers of actin, differences in the organization of the cytoskeleton would not explain the appearance of short fibronectin fibrils (data not shown). Finally, immunofluorescent microscopy studies showed that MII and MIV produced a matrix of very thin type I collagen fibrils similar to that observed in the pWTC1 cultures (data not shown). Thus, the production of short fibronectin fibrils was not due to any problem assembling type I collagen into fibrils.

To determine if reduced levels of fibronectin synthesis were responsible for the production of short and sparse fibronectin fibrils observed in Mov13, MII, and MIV cell lines, fibronectin levels in the cell layer and media were analyzed by direct and indirect ELISAs, respectively. The indirect ELISA (Fig. 3 B) showed that the media of pWTC1, Mov13, MI, MII, MIII, and MV cultures had similar levels of fibronectin and only the level of fibronectin in the media from MIV cultures was significantly different ($P < 0.05$). The direct ELISAs on the levels of fibronectin in the cell layers indicated that levels of fibronectin varied between all the cell lines (Fig. 3 A). These differences, however, do not correlate with the appearance of short fibronectin fibrils in the cell layer, since cell layers of MIV cultures which produced short fibronectin fibrils had levels of fibronectin that were comparable to those observed in pWTC1, MI, MIII, and MV cultures.

Since differences in the levels of fibronectin do not appear to be responsible for the reduction in the fibronectin fibrils

Table I. Sequences Surrounding the Fibronectin Binding Site in Wild Type and Mutant $\alpha 1(I)$ Collagen Chains

| | 774 | 775 | 776 | 777 | | 782 | 783 | Collagenase cleavage | FN matrix | FN binding | | | | | | | | | | |
|------|-----|-----|-----|-----|-----|-----|-----|----------------------|-----------|------------|-----|-----|-----|-----|-----|-----|-----|------|-----------|--------|
| WT | GLY | THR | PRO | GLY | PRO | GLN | GLY | ↓ | ILE | ALA | GLY | GLN | ARG | GLY | VAL | VAL | GLY | + | normal | normal |
| MI | | | | | PRO | | | | | | | | | | ALA | ALA | | 0 | normal | normal |
| MII | | | | | PRO | | | | | | | | | | ALA | ALA | | 0 | decreased | weaker |
| MIII | | | | | | | | | | | | | | | ALA | ALA | | slow | normal | normal |
| MIV | | | | | PRO | | | | MET | | | | | | ALA | ALA | | 0 | decreased | weaker |
| MV | | | | | | | | | MET | | | | | | ALA | ALA | | slow | normal | normal |



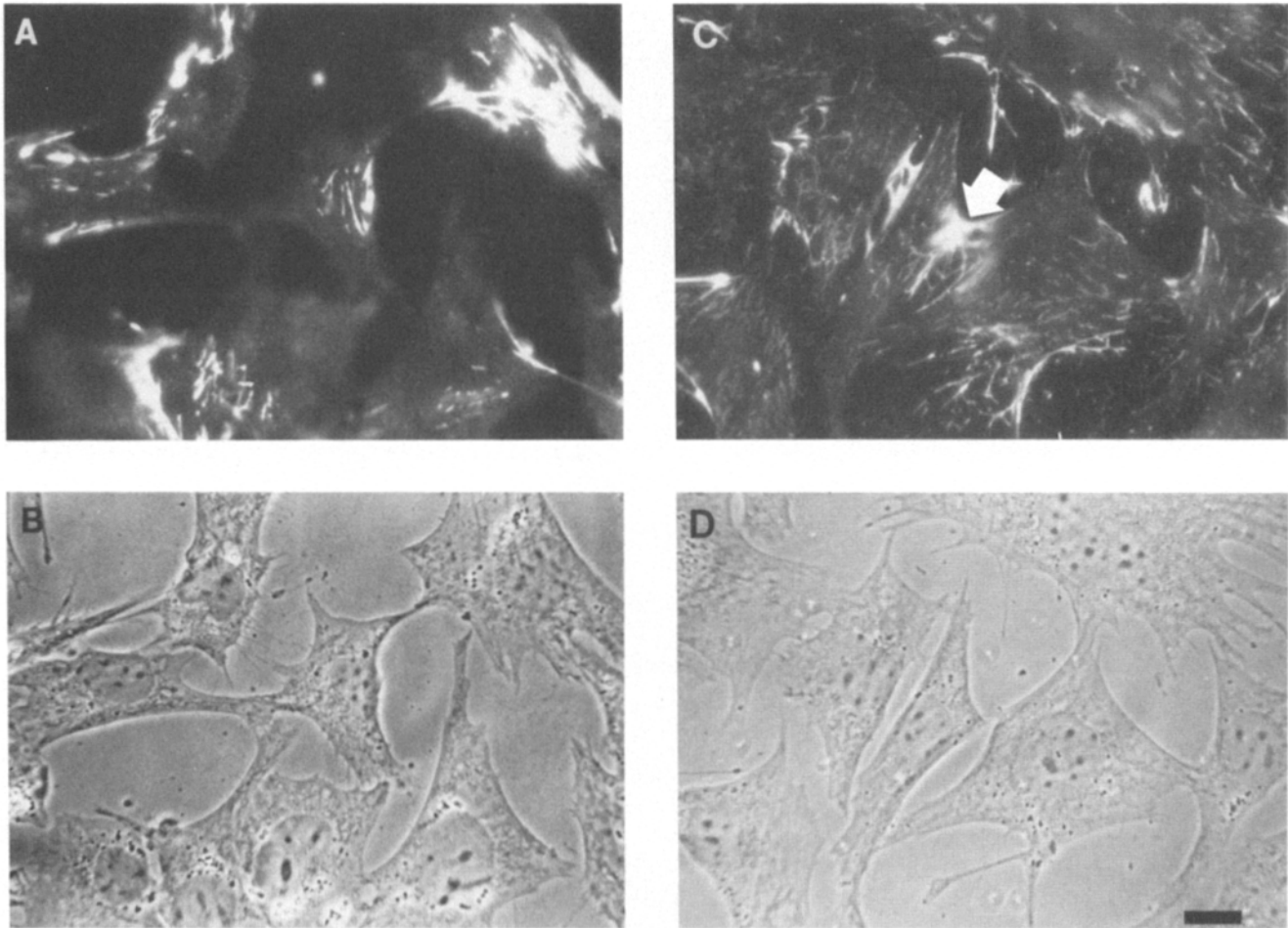


Figure 2. Immunofluorescent localization of fibronectin in subconfluent pWTC1 and MII cells. Subconfluent cultures were fixed with 3.5% paraformaldehyde, labeled with rabbit anti-human fibronectin sera, and then labeled with fluorescein mouse anti-rabbit IgG as described in Materials and Methods. In MII cells (*c* and *d*) the majority of fibronectin appears to be deposited between cells or off the cell surface (*arrow*). In contrast, the majority of fibronectin in pWTC1 cells (*a* and *b*) is located on the cell surface. Bar, 20 μm .

in Mov13 and MII cultures, we determined whether the binding interaction between the mutant $\alpha 1(\text{I})$ collagen chain and fibronectin was altered. To do this, ^3H -labeled collagens were isolated from the media of the Mov13, pWTC1, MI, MII, MIII, MIV, and MV cultures, and then incubated with fibronectin sepharose. Bound ^3H -labeled collagens were sequentially eluted with buffer containing 1 M urea, 2 M urea/0.4 M NaCl, and 4 M urea, and analyzed by SDS-PAGE. Fig. 4 A shows representative gel patterns obtained from collagens isolated from pWTC1, MII, MIII, and MIV. $\alpha 1$ type I collagen chains isolated from all the cultures, except the MII and MIV cultures, were eluted with 2 M urea/0.4 M NaCl. $\alpha 1$ type I collagen chains from the MII and MIV cultures bound less tightly to the fibronectin-sepharose and $\sim 40\%$ of the type I collagen could be eluted with 1 M

urea (Fig. 4 B). When collagen chains in MII cultures were denatured by heating and then applied to the fibronectin sepharose column, the $\alpha 1(\text{I})$ chains eluted with 2 M urea, 0.5 M NaCl (data not shown). Denaturation did not affect $\alpha 1(\text{I})$ chains from pWTC1 cultures which still eluted with 2 M urea, 0.4 M NaCl. Thus denatured $\alpha 1(\text{I})$ chains from MII cell lines behaved like wild type $\alpha 1(\text{I})$ chains. This suggests that the fibronectin binding site in undenatured MII type I collagen is not as readily accessible as that in wild type, type I collagen because the helicity of the region may have been changed by introducing the proline residues at positions 774 and 777 (Gross, 1982). Denatured $\alpha 1(\text{V})$, $\alpha 2(\text{V})$ and $\alpha 2(\text{I})$ collagens chains also bound more tightly and were eluted with 2 M urea, 0.4 M NaCl.

In all cell lines, a small percentage of $\alpha 1(\text{I})$ collagen chains

Figure 1. Immunofluorescent localization of fibronectin in Mov13 cells. Confluent cultures of Mov 13, pWTC1, MI, MII, MIII, MIV, and MV cells were fixed with 3.5% paraformaldehyde as described in Materials and Methods and labeled with rabbit anti-human fibronectin antibodies. Cultures were then labeled with fluorescein mouse anti-rabbit IgG. Immunofluorescent micrographs of pWTC1 cells transfected with wild type CollA1 gene (*A*), Mov 13 cells (*B*), MI cells (*C*), MII cells (*D*), MIII cells (*E*), MIV cells (*F*), and MV cells (*G*). Phase micrographs of pWTC1 cells (*H*) and MII cells (*I*). Bar, 20 μm .

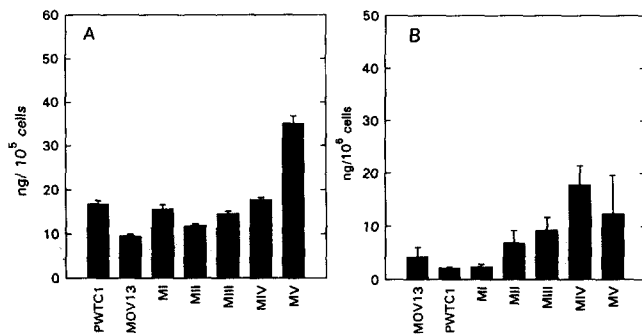


Figure 3. Histogram of fibronectin protein levels. Levels of fibronectin in the cell layer (A) and media (B) were determined using an ELISA as described in Materials and Methods. Fibronectin levels were normalized to cell number.

eluted off with 4 M urea. The amount of $\alpha 1(I)$ collagen chains which eluted with 4 M urea varied from collagen preparation to preparation and was usually between 2–20% of the total type I collagen. It is unclear why some $\alpha 1(I)$ chains eluted with 4 M urea. This elution profile did not seem to reflect the mutation in the $\alpha 1(I)$ chain since $\alpha 1(I)$ collagen chains from pWTC1 and MIV, and MII and MIII, cell lines had similar levels of $\alpha 1(I)$ chains eluting with 4 M urea. The $\alpha 2(I)$ chains in all the cell lines eluted with 1 M urea. None of the collagens bound to the sepharose in the absence of fibronectin (data not shown). This suggests that the substitution of Pro for Glu and Ala in residues 774 and 777 altered the binding interaction between $\alpha 1(I)$ collagen chains and fibronectin.

It is interesting to note that type V collagen did not bind to the fibronectin–sepharose in the presence of mutated $\alpha 1(I)$ chains from either MII or MIV cultures. This suggests that the interaction between type V collagen and fibronectin may be mediated by the presence of “normal” type I collagen chains. The $\alpha 1(I)$ chain in both the MII and MIV mutants also appeared to be a broader band. The reason for this is unclear, but it may reflect variation in the posttranslational modification of $\alpha 1(I)$ chains.

The weaker binding exhibited between fibronectin and $\alpha 1(I)$ collagen chains from either MII or MIV suggests that the fibronectin binding site in type I collagen may be important for fibronectin fibrillogenesis. To test the idea, Mov13 cell cultures which do not produce any type I collagen fibrils and MII cell cultures which produce mutated collagen fibrils were incubated with either type I collagen or a peptide fragment containing the fibronectin binding region of the $\alpha 1(I)$ chains. The extent of fibronectin fibrils formed was then examined by immunofluorescence. These studies showed that addition of type I collagen (data not shown) or CB.7 to the Mov13 or MII (data not shown) cell cultures rescued fibronectin fibrillogenesis. Fibrils formed in the presence of CB.7 (Fig. 5 B) were longer than the fibronectin fibrils produced by Mov13 cultures without CB.7 (Fig. 5 A). The addition of exogenous type III or type V collagen to the cultures did not rescue fibronectin fibril formation, indicating that the elongation of fibronectin fibrils was specific for $\alpha 1(I)$ chains (data not shown).

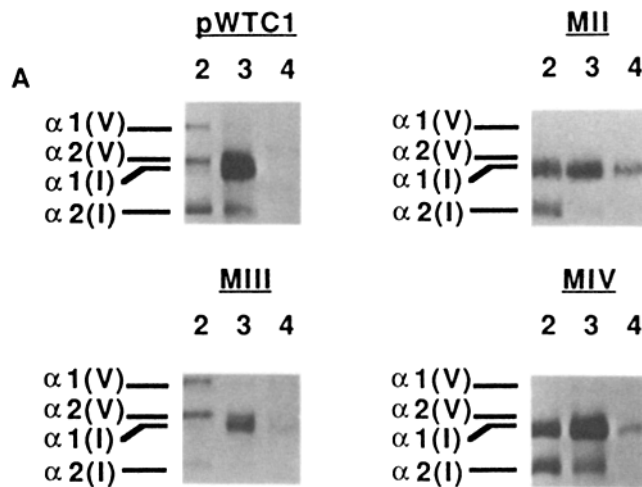


Figure 4. Elution profiles of type I collagen chains from fibronectin–sepharose. (A) Fluorograms of typical elution profiles. Collagens from cultures of pWTC1, MII, MIII, and MIV were labeled with [3 H]proline and [3 H]glycine and incubated with fibronectin–sepharose as described in Materials and Methods. Columns

were then washed with 0.1 M Tris-HCl, pH 7.4, 0.1 M NaCl/5 mM CaCl₂ (step 1) and eluted in a stepwise fashion with 1 M urea (step 2), 2 M urea/0.4 M NaCl (step 3), and 4 M urea (step 4), and then analyzed on nonreduced SDS–polyacrylamide gels. The amount of material loaded on the SDS–polyacrylamide gels was not equivalent and differences in the intensity of the bands between cultures do not reflect the ability of the collagen chains to bind to fibronectin. Three clones of MII, two clones of MIV, two clones of pWTC1 and one clone of MIII were analyzed. One clone of MI and MV were also analyzed (data not shown). (B) Levels of type I collagen eluted off the fibronectin–sepharose column. Gels were scanned with a Biomed Instrument 1D/2D laser densitometer (1D/2D; Biomed. Res. Instrs., Rockville, MD) and the percent of $\alpha 1(I)$ and $\alpha 2(I)$ collagen chains, eluted at each step, was plotted. Solid bars represent the level of type I collagen eluted with 1 M urea. Hatched bars represent the levels of type I collagen eluted with 2 M urea, 0.4 M NaCl.

Discussion

In this study we have used Mov13 cells transfected with $\alpha 1(I)$ collagen genes containing mutations in the fibronectin binding site to examine the role of fibronectin–type I collagen interactions on fibronectin fibril formation. The studies show that only the substitution of prolines for glutamine and alanine at positions 774 and 777 (MII) was sufficient to alter the interaction between $\alpha 1(I)$ collagen chains and fibronectin. The substitution of proline or methionine for isoleucine at position 776 did not effect binding of collagen to fibronectin although substitution of a prolyl residue for Ile at position 776 did inhibit cleavage of type I collagen by vertebrate collagenase (Wu et al., 1990).

While substitutions of proline are conservative in terms of

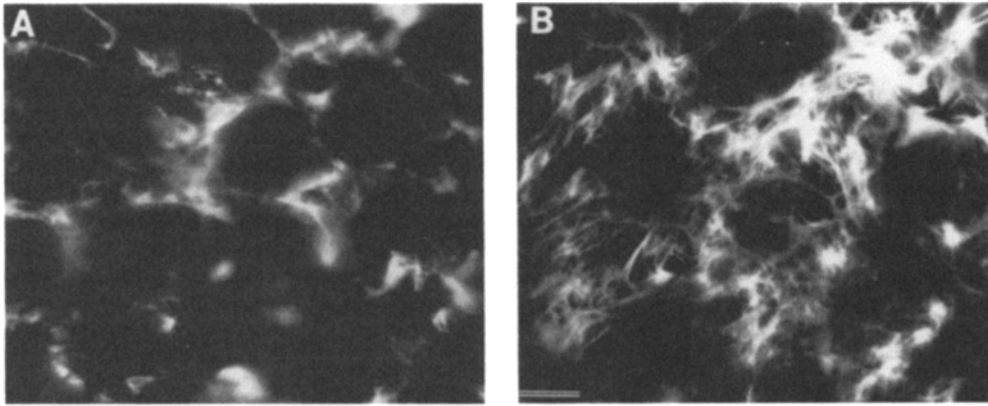


Figure 5. Immunofluorescent localization of fibronectin in Mov13 cells treated with the collagen peptide CB.7. Cultures of Mov13 cells were grown to confluency in the absence (A) or presence (B) of the collagen peptide CB.7. Cultures were fixed with 3.5% paraformaldehyde and labeled as described in Fig. 1. Bar, 20 μ m.

charge, proline is thought to be important for stabilizing the triple helix in collagen (Ramachandran and Ramakrishnan, 1976). A notable feature of the collagenase cleavage/fibronectin binding region is that proline is absent from the y position of Gly-x-y triplet in the stretch of amino acids from 772 to 785. This region is also unusually hydrophobic for collagen sequences. It has been suggested that the triple helix in this region may be less tightly coiled with local denaturation which would allow vertebrate collagenase access to its cleavage site and allow fibronectin access to its binding site. Thus, one reason fibronectin may bind to denatured collagen more strongly than to native collagen (Engvall et al., 1978) is that changes in helicity due to denaturation may expose the fibronectin binding site in α 1(I) chains. Binding studies with triple helical type I collagen and denatured α 1(I) collagens which have proline substitutions in residues 774 and 777 would support this idea and suggest that local changes in the helicity of this region could be responsible for the changes in fibronectin binding and collagenase cleavage observed in the mutants. The importance of the conformation of this region in promoting fibronectin binding has also been demonstrated using a synthetic 20-mer peptide spanning the collagenase cleavage site. In these studies, the synthetic peptide failed to bind fibronectin or inhibit type I collagen binding to fibronectin in a fluid phase binding assay (Ingham et al., 1988). It seems likely, therefore, that the affinity of fibronectin for collagen is dependent on the conformation of collagen.

The weaker binding of the α 1(I) collagen produced by the MII cultures, appears to effect fibronectin fibril formation. In these cultures, fibronectin fibrils are short and confined to the periphery of the cells and appear to be very similar to fibrils produced by Mov13 cultures which do not produce type I collagen. This suggests that although Mov13 and MII cultures can assemble fibronectin fibrils, elongation of fibronectin fibrils into a true fibrillar network requires the interaction of fibronectin with the fibronectin binding site in type I collagen.

It is interesting to note that in MIII cultures which appeared to produce homotrimers of α 1(I) chains, the elution profile of collagen chains was similar to that observed in pWTC1 cells which consisted of heterotrimers of α 1(I) and α 2(I) collagen chains in a 2:1 ratio. In addition, MIII cultures produce an extensive matrix of fibronectin fibrils. This suggests that α 1(I) homotrimers interact with fibronectin in the same manner as heterotrimers and the primary interac-

tion between fibronectin and type I collagen during fibril elongation occurs between the α 1(I) collagen chains and fibronectin. The earlier elution of α 2(I) chains from the fibronectin sepharose column would support this idea. A similar observation was made by Kleinman et al. (1976). Studies by Dessau et al. (1978), however, have suggested that there isn't any difference in the binding activity of α 1(I) and α 2(I) collagen to fibronectin. Clearly, additional studies are needed to determine the role of α 2(I) chains in fibronectin fibril formation.

The ability of normal α 1(I) collagen chains and the CB.7 peptide to rescue fibronectin fibril formation could be the result of the fibronectin binding site in α 1(I) chains inducing a conformational change in fibronectin. Fibronectin in solution is thought to have a folded conformation (Koteliansky et al., 1981; Williams et al., 1982; Benecky et al., 1990). The binding of fibronectin to collagen or CB.7 appears to alter this folded conformation of fibronectin and allow it to unfold (Williams et al., 1982; Ingham et al., 1988). Unfolding may be a necessary step in fibronectin fibrillogenesis, especially during elongation when it could expose fibronectin-fibronectin binding sites for self association. This would provide a mechanism that would enable fibronectin dimers off the cell surface to add onto the tip of a growing fibril (McDonald, 1988). Interestingly, studies by Ingham et al. (1988) suggest that the conformational change induced by type I collagen would cause fibronectin to lose its affinity for collagen. In this respect the interactions between fibronectin and collagen during matrix formation would be transient. This would explain why others have observed that fibronectin fibrils can exist as a separate entity from collagen fibrils (Vaheri et al., 1978; Furcht et al., 1980a; Keski-Oja et al., 1981; Peters et al., 1990; Dzamba and Peters, 1991).

In conclusion, fibronectin fibril formation appears to be a multi-step process where at the early stages of fibrillogenesis, a cell surface molecule such as the α ₅ β ₁ integrin receptor, a proteoglycan or the matrix assembly receptor initiates fibril formation (McDonald, 1988; Fogerty and Mosher, 1990). During later stages of fibrillogenesis, as fibrils extend off the cell surface, collagen interactions might be necessary to hold fibronectin in the unfolded conformation and thus promote fibril elongation. Collagen interactions do not appear to be involved in the initiation of fibronectin fibril formation since Mov13 cells which do not produce type I collagen were able to assemble short fibronectin fibrils. This

agrees with earlier studies by McDonald et al. (1987) which showed that antibodies to the collagen binding domain in fibronectin and collagen binding fragments of fibronectin did not inhibit fibronectin fibrillogenesis. Finally, our studies explain the observation of McKeown-Longo and Mosher, (1985) which showed that collagen enhanced the binding of the amino terminal of fibronectin to cell layers even though the site in fibronectin that interacted with the cell surface was located in the first type I repeats in the amino terminal of fibronectin and not in the collagen binding domain (McKeown-Longo and Mosher, 1985; Quade and McDonald, 1988; Sottile et al., 1991; Schwarzbauer, 1991).

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