A Fibronectin Self-Assembly Site Involved in Fibronectin Matrix Assembly: Reconstruction in a Synthetic Peptide

Alex Morla and Erkki Ruoslahti

Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, California 92037

Abstract. The active form of fibronectin is its extracellular matrix form, which allows for the attachment of cells and influences both the growth and migration of cells. The matrix form is assembled by cells; however, many cells are defective in this regard. Several regions within fibronectin have been shown to play a role in matrix assembly by cells. One such region has been localized into the first type III repeat of fibronectin (Chernousov, M. A., F. J. Fogerty, V. E. Koteliansky, and D. F. Mosher. *J. Biol. Chem.* 266:10851–10858). We have identified this site as a fibronectin–fibronectin binding site and reproduced it as a synthetic peptide. This site is contained in a 14-kD fragment that corresponds to portions of the first two type III repeats. The 14-kD fragment was found to bind to cell monolayers and to inhibit fibronectin matrix assembly. The 14-kD fragment only slightly reduced the binding of fibronectin to cell surfaces but it significantly inhibited the subsequent incorporation of fibronectin into the extracellular matrix. The 14-kD fragment also bound to purified fibronectin and inhibited fibronectin–fibronectin binding. A synthetic 31-amino acid peptide (P1) representing a segment of the 14-kD fragment retained the ability to inhibit fibronectin–fibronectin binding. Peptide P1 specifically bound fibronectin from plasma in affinity chromatography, whereas a column containing another peptide from the 14-kD fragment did not. These results define a fibronectin–fibronectin binding site that appears to promote matrix assembly by allowing the assembly of fibronectin molecules into nascent fibrils. The 14-kD fragment and the P1 peptide that contain this site inhibit matrix assembly by competing for the fibronectin–fibronectin binding.

As a constituent of the extracellular matrix, fibronectin is important for allowing cells to attach to the matrix and influences both the growth and migration of cells (McDonald, 1988; Ruoslahti, 1988; Ruoslahti and Giancotti, 1989; Hynes, 1990; Mosher et al., 1991). Normal fibroblasts in tissue culture secrete fibronectin and assemble it into a matrix that is essential to their adhesion and growth. While many tumorigenic cells continue to produce fibronectin, they do not assemble the fibronectin into a matrix, and this lack of matrix assembly is thought to contribute to the invasive properties of malignant cells (Ruoslahti and Giancotti, 1989; Hynes, 1990). Thus, one important stage in the progression of cancer may be the transition from assembly to nonassembly of the extracellular matrix.

Matrix assembly requires the binding of fibronectin to cell surfaces followed by assembly into fibrils and stabilization of the fibrils by disulfide cross linking. Several regions within fibronectin are needed for the assembly process. One such region is the amino-terminal 29-kD heparin-binding domain. Oh et al. (1981) found that truncated fibronectin molecules that lacked amino- and carboxy-terminal regions were not incorporated into extracellular matrices in vivo. Further evidence for the importance of amino-terminal regions of fibronectin in matrix assembly was obtained by McKeown-Longo and Mosher (1985) who demonstrated that an amino-terminal 70-kD fragment of fibronectin, when added to fibroblast culture medium, inhibited the binding of exogenous fibronectin to cells and its subsequent incorporation into the matrix. They found that the active area within the 70-kD fragment was the amino-terminal 29-kD heparin-binding domain, whereas the 40-kD gelatin-binding domain did not inhibit matrix assembly. The sites on cells to which the 70-kD fragment binds have been localized to the edges of cells (Peters and Mosher, 1987), suggesting the existence of distinct matrix assembly sites. Also, two candidate cell surface receptors for the 70-kD fragment have been detected (Limper et al., 1991; Blystone and Kaplan, 1992). The 70- and 29-kD fragments have also been shown to inhibit matrix assembly of endogenous fibronectin (McDonald et al., 1987). In addition, cells have been shown to organize fibronectin fragments into fibrils only when an RGD-containing cell-binding domain and heparin-binding fragments were coated onto dishes simultaneously (Woods et al., 1988). The importance of the 29-kD heparin-binding domain was further underscored by the finding that recombinant fibronectin molecules that lacked the 29-kD region were not incorporated into the matrix (Schwarzbauer, 1991). Moreover, molecules composed only of the 29-kD region plus the carboxy-terminal half of the protein were efficiently incorporated into the extracellular matrix (Schwarzbauer, 1991). The role of the

29-kD region appears to be to mediate the binding of fibronectin to the cell surface (McKeown-Longo and Mosher, 1985; Quade and McDonald, 1988).

Another region involved in matrix assembly is the RGD-containing cell-binding domain of fibronectin. mAbs directed to the cell-binding domain of fibronectin were found to inhibit matrix assembly (McDonald et al., 1987). Two mAbs have been described that bind close to, but not directly to, the RGD site and block the binding of cells to fibronectin (Nagai et al., 1991). These antibodies also blocked fibronectin matrix assembly. The receptor that binds to the RGD site in fibronectin is, in most cells, the $\alpha_5\beta_1$ integrin (Pierschbacher and Ruoslahti, 1984; Pytela et al., 1985). Accordingly, mAbs directed against the α_5 and β_1 integrin subunits were also found to inhibit fibronectin matrix assembly and the binding of fibronectin to matrix assembly sites (Akiyama et al., 1991; Fogerty et al., 1990). In addition, overexpression of $\alpha_5\beta_1$ in CHO cells resulted in increased fibronectin matrix assembly (Giancotti and Ruoslahti, 1990). These findings establish the importance of the interaction between fibronectin and the $\alpha_5\beta_1$ integrin during matrix assembly.

A third region in fibronectin has recently been shown to be involved in matrix assembly. A 56-kD fragment from fibronectin, containing the 40-kD gelatin-binding domain, plus the first type III repeat, inhibits the incorporation of exogenous fibronectin into the matrix and mAbs localize the active site into the first type III repeat of fibronectin (Chernousov et al., 1991).

In this report we describe a 14-kD fragment from the first two type III repeats of fibronectin. We provide evidence that the region defined by this fragment represents a fibronectin–fibronectin binding site, and show that the 14-kD fragment inhibits fibronectin matrix assembly. In addition, we show that a synthetic peptide derived from the 14-kD region also binds directly to fibronectin and inhibits fibronectin–fibronectin association. In contrast to the 29- and 70-kD fragments, which block an initial event in matrix assembly (fibronectin binding to cells), the 14-kD fragment may inhibit an intermediate step in matrix assembly, fibronectin self-association, before the disulfide cross linking that stabilizes the fibronectin matrix.

Materials and Methods

Materials

Alpha-minimal essential medium (α-MEM) was purchased from Gibco Laboratories (Grand Island, NY), FCS from Tissue Culture Biologicals (Tulare, CA), and Glutamine Pen-Strep from Irvine Scientific (Santa Ana, CA). Immulon 2 Removawell strips were obtained from Dynatech Laboratories (Chantilly, VA). Iodo-Gen was purchased from Pierce Chemical Co. (Rockford, IL). CNBr-activated Sepharose, heparin-Sepharose, and gelatin-Sepharose were obtained from Pharmacia LKB (Piscataway, NJ). Precast SDS-PAGE gels were purchased from BioRad Laboratories (Richmond, CA) and Novex (San Diego, CA). Lab-Tek 8-well Chamber Slides were obtained from Nunc (Naperville, IL). HPLC columns were purchased from Vydac (Hesperia, CA). Collagen type I was obtained from Collaborative Research (Bedford, MA). All other reagents were acquired from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

IMR-90 and HT-1080 cells were cultured in α-MEM supplemented with 10% heat-inactivated FCS and Glutamine Pen-Strep. IMR-90 cells used for experiments were between passages number 11 and 20; cells in later passages were not used.

Fibronectin Fragments

Human fibronectin was obtained from the Blood Transfusion Service of the Finnish Red Cross in Helsinki. To prepare the heparin-binding fragments, fibronectin in PBS was digested with α-chymotrypsin (0.1% by weight, TLCK treated) for 4 h at 25°C. The digestion was stopped by adding PMSF (20 μg/ml final concentration) and the preparation was passed over a gelatin-Sepharose column (Engvall and Ruoslahti, 1977). After washing the gelatin-Sepharose column with PBS, gelatin-bound material was eluted with 8 M urea, 50 mM Tris-HCl, pH 7.5, followed by extensive dialysis against distilled water and lyophilization. The flow-through from the gelatin-Sepharose column was collected and passed over a heparin-Sepharose column preequilibrated with PBS. The heparin-Sepharose column was washed with PBS, then heparin-bound fibronectin fragments were eluted with 1 M NaCl, 50 mM Tris-HCl, pH 7.5, then dialyzed against distilled water and lyophilized.

The amino-terminal 70-kD fragment was produced as previously described by McKeown-Longo and Mosher (1985). The 14-kD fragment was purified from heparin-binding fragments by reverse phase HPLC using a C-4 column. After applying heparin-binding fragments to the HPLC column in 0.06% trifluoroacetic acid, the column was eluted with a linear gradient of 0–60% acetonitrile in 0.06% trifluoroacetic acid. The 14-kD fragment was eluted in the 45% acetonitrile fractions.

Peptides representing various regions of fibronectin were synthesized at the Protein Chemistry Laboratory at the La Jolla Cancer Research Foundation (La Jolla, CA). All peptides used in experiments were purified by reverse phase HPLC. Peptide P1 represents the region from amino acids 600–630 (sequence; NAPQPSHISKYILRWRPKNSVGRWKEATIPG); peptide P2 from 625–656 (sequence; EATIPGHLNSYTIKGLKPGVVYEGQLISIQQ); peptide P3 from 650–680 (sequence; LISIQQYGHQEVTRFDFTTTSTSTPVTSNTV); and peptide P4 from 675–708 (sequence; VTSNTVTGETTPFSPLVATSESVTEITASSFVVS) of the mature protein according to the numbering method of Kornblihtt et al. (1985).

Iodination of Proteins

Proteins (20–100 μg of protein in 0.1 ml 50 mM KPO_4, pH 7.5) were iodinated by using Iodo-Gen as previously described (Fraker and Speck, 1978). Typical values for specific activity were 10^9 μCi/mmol for fibronectin, 5×10^8 μCi/mmol for 70 kD, 5×10^8 μCi/mmol for 14 kD, and 0.5 μCi/μg for heparin-binding fragments.

Matrix Assembly Assays

Matrix assembly assays were performed by using ^{125}I-fibronectin, essentially as described previously (McKeown-Longo and Mosher, 1983, 1985). Before the assay, cells were grown to confluence in 96-well dishes in α-MEM + 10% FCS. Cells were incubated in α-MEM + 10% fibronectin-deficient FCS plus 5 μCi/ml of ^{125}I-fibronectin. Fibronectin-deficient FCS was prepared by passing FCS over a gelatin-Sepharose column to remove fibronectin (Engvall and Ruoslahti, 1977). The concentration of unlabeled fibronectin in α-MEM + 10% fibronectin-deficient FCS was ~0.2 μg/ml as determined by ELISA using antibovine fibronectin antibodies. Where indicated, cells were incubated in the presence of excess nonradioactive competitor proteins such as fibronectin, the 70-kD fragment, or the 14-kD fragment. Cells that were labeled for 1 h were washed four times with ice-cold PBS, then lysed in 1 N NaOH, and cell-bound radioactivity was measured in the NaOH soluble fraction. Cells that were incubated with ^{125}I-heparin-binding fragments were also washed with PBS, then cells were solubilized with SDS-PAGE sample buffer (2% SDS, 67 mM Tris-HCl, pH 6.8, 10% glycerol, 0.03% bromophenol blue), and proteins were separated on BioRad 4-20% Ready Gels, followed by autoradiography. Cells that were incubated with ^{125}I-fibronectin for 24 h were washed as described above and proteins were separated into 1% deoxycholate soluble and insoluble pools (pools I and II) as described by McKeown-Longo and Mosher (1985). Specific binding was defined as that amount of binding which was competed by 2 μM unlabeled fibronectin, and was typically 60–70% of the total ^{125}I-fibronectin binding. Cells that were labeled with ^{125}I-70 kD or ^{125}I-14 kD also received 5 μCi/ml of labeled protein. As with ^{125}I-fibronectin labeling, specific binding was defined by inhibiting the binding of the radiolabel with unlabeled ligand.

Protein–Protein Binding Assays

Protein-protein binding assays were performed on Immulon 2 Removawell strips. Proteins were coated onto wells in 100 mM Na_2CO_3, pH 9.5, in a moist chamber at 4°C overnight. The wells were washed three times with PBS followed by blocking with 0.2% BSA in PBS (0.2% BSA) at 37°C for 1 h. 100 μl of 5 μCi/ml radiolabeled protein solution in 0.2% BSA was added to each well. Proteins were allowed to bind for 2 h at 37°C, then the wells were washed four times with 0.2% BSA, the wells were removed, and the bound ^{125}I was measured.

Affinity Chromatography with Peptide Columns

Peptides were coupled to CNBr-activated Sepharose CL-4B according to the manufacturer's recommendations. The concentration of peptide was typically 8–10 mg peptide/ml of resin. In affinity chromatography, 10-ml aliquots of a 250-μg/ml solution of purified human plasma fibronectin in PBS were applied to freshly made, 1-ml columns of peptide P1, or peptide P2-Sepharose, or gelatin-Sepharose as a positive control. The flow-through fractions were collected and each column was then washed with 20 ml of PBS. The wash fractions were collected, and the fibronectin bound to each column was eluted with 5 ml of 8 M urea in PBS. The amount of fibronectin in the samples was quantitated by measuring the A_{280}.

Alternatively, 1-ml columns of peptide P1, peptide P2, or gelatin-Sepharose received 5-ml aliquots of human plasma which were diluted fivefold with PBS + 5 mM EDTA (PBS/EDTA). The flow-through fractions were collected and the columns were washed with 20 ml of PBS/EDTA. Bound proteins were then eluted with step gradients of urea by using 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 M urea in PBS/EDTA. At each step, 2 ml of urea solution were passed over the columns and the eluates were collected. Equal volumes of each fraction were analyzed by SDS-PAGE under reducing conditions on Novex 4–20% Tris-Glycine gels, and proteins were visualized by staining with Coomassie blue.

Fluorescence Assays for Fibronectin Fibril Formation

IMR-90 cells were seeded onto Lab-Tek 8-well Chamber Slides. Wells were precoated with 50 μg/ml collagen type I to enhance the attachment of cells to the wells. We found that cells not seeded onto collagen-coated wells tended to detach when treated with high concentrations of the 70-kD fragment for prolonged periods of time. Cells were allowed to attach and spread for 1 h at 37°C, followed by washing once with α-MEM + 10% fibronectin-deficient FCS, and incubation in this medium plus either no additions or addition of the 70-kD fibronectin fragment or the 14-kD fragment as described in the legend to Fig. 9. 24 h before fixation for microscopy, fluorescein-labeled fibronectin (prepared as described by McKeown-Longo and Mosher, 1985) was added to the cultures to a final concentration of 10 μg/ml. At the appropriate time, cells were fixed with 3.7% paraformaldehyde, 60 mM sucrose, in PBS, pH 7.4, for 30 min at room temperature. Cell layers were then washed three times with 0.2% BSA in PBS and mounted for microscopy.

Results

Inhibition of Matrix Assembly by a 14-kD Heparin-binding Fibronectin Fragment

To identify new fibronectin fragments that would define sites important for matrix assembly, fibronectin was digested with chymotrypsin and the preparation was separated into heparin-binding and gelatin-binding fragments. The fragment preparations were then tested for their ability to inhibit fibronectin matrix assembly by using ^{125}I-fibronectin and IMR-90 cells. As shown previously, unlabeled fibronectin and an amino-terminal 70-kD cathepsin-D fibronectin fragment inhibited matrix assembly in this assay (McKeown-Longo and Mosher, 1985). Among the chymotryptic fragments, the heparin-binding fragments inhibited matrix assembly, whereas the gelatin-binding fragments had little effect (not shown).

To determine which fragments in the heparin-binding fragment preparation were responsible for inhibiting matrix assembly, ^{125}I-labeled heparin-binding fragments were incubated with IMR-90 cell monolayers. Only two of the many heparin-binding fragments, a 29- and a 14-kD fragment,

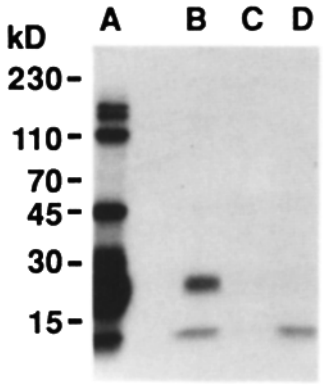

Figure 1. Binding of 29- and 14-kD fragments to IMR-90 cells. IMR-90 cells were incubated for 1 h at 37°C with ^{125}I-labeled heparin-binding fragments (2 μCi/ml) in the presence or absence of unlabeled heparin-binding fragments (250 μg/ml), or 70-kD (1 μM). Cells were then washed with PBS and harvested for analysis by SDS-PAGE as described in Materials and Methods. (Lane *A*) A sample of the ^{125}I-labeled heparin-binding fragment starting material; (lane *B*) fragments that bound in the absence of competition; (lane *C*) fragments bound in the presence of unlabeled heparin-binding fragments; (lane *D*) fragments bound in the presence of unlabeled 70-kD. The positions of molecular mass standards are indicated to the left of the gel.

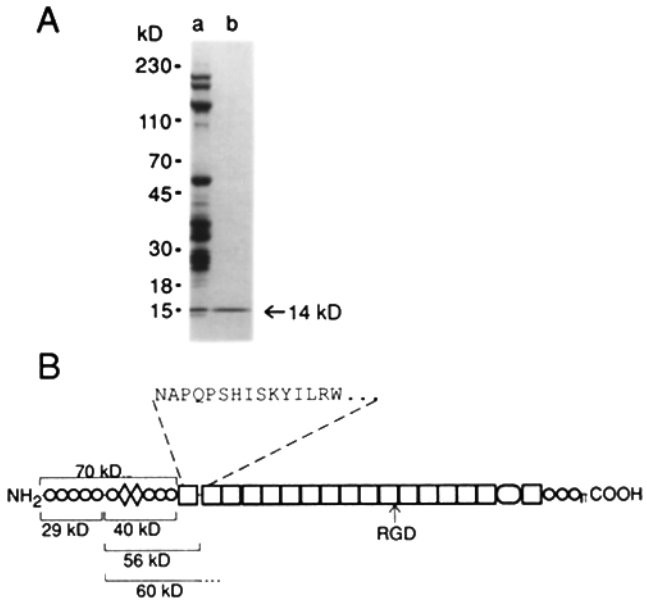

Figure 2. Purification of the 14-kD fibronectin fragment and its location in fibronectin. The 14-kD heparin-binding fragment was purified to homogeneity by reverse phase HPLC. *A* shows SDS-PAGE analysis of the heparin-binding fragment starting material (lane *a*), and the purified 14-kD preparation (lane *b*). The gel was stained with Coomassie blue. The positions of molecular mass standards are indicated to the left of the gel. The position of the 14-kD fragment is indicated to the right of the gel. *B* shows a diagram of fibronectin, outlining the locations of the 14-kD fragment and the various other fragments relevant to this work. The three repeating units of fibronectin are depicted as follows: type I repeats (*circles*); type II repeats (*diamonds*); type III repeats (*squares*). The CS1 region is depicted by a shaded oval. The amino-terminal sequence of the 14-kD fragment is shown with dashed lines extending to the location on the diagram representing the area covered by the 14-kD fragment.

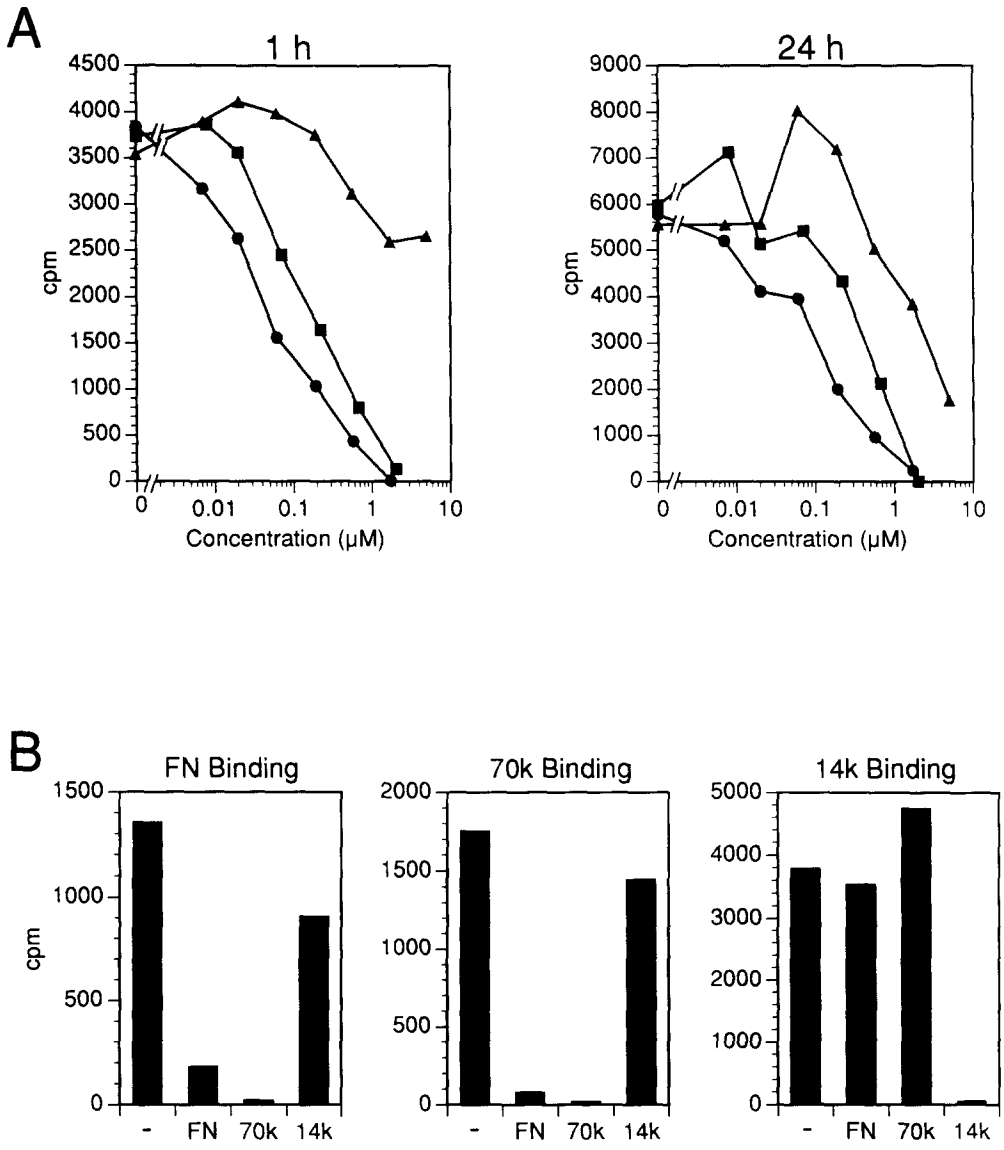

Figure 3. Inhibition of fibronectin binding to cells and matrix assembly by the 14-kD fragment. (*A*) Confluent monolayers of IMR-90 cells were incubated with ^{125}I-fibronectin in the presence of various concentrations of unlabeled fibronectin (■), 70-kD fragment (●), or 14-kD fragment (▲) for either 1 or 24 h, as indicated. Cells that were incubated for 1 h were washed and the total radioactivity bound was measured. Cells that were incubated for 24 h were washed and the amount of ^{125}I-fibronectin in the deoxycholate insoluble pool was measured. Each data point is the average of duplicate determinations. (*B*) Semiconfluent IMR-90 cells layers were incubated for 1 h with either ^{125}I-fibronectin (*left*), ^{125}I-70-kD fragment (*center*), or ^{125}I-14-kD fragment (*right*) in the presence or absence of unlabeled fibronectin (*FN*), 70-kD fragment (*70k*), or 14-kD fragment (*14k*) as indicated. After the 1-h labeling period cells were washed and the total radioactivity bound was measured.

bound to the IMR-90 cells (Fig. 1, lane *B*). The binding of both fragments was shown to be specific by competition with excess unlabeled heparin-binding fragments (Fig. 1, lanes *B* and *C*). Unlabeled 70-kD fragment competed for the binding of the 29-kD heparin-binding fragment, indicating that this fragment represents the amino-terminal heparin-binding domain (Fig. *1*, lane *D*). Interestingly, the 70-kD fragment did not compete for the 14-kD heparin-binding fragment (Fig. 1, lane *D*), suggesting that the 14-kD region is not represented in the 70-kD fragment.

To characterize the 14-kD fragment it was purified to homogeneity by using reverse phase HPLC (Fig. 2 *A*, lane *b*). Amino acid sequencing yielded the following amino-terminal sequence: NAPQPSHISKYILRW. This sequence corresponds to a region just past the beginning of the first fibronectin type III repeat (see Fig. 2 *B*), starting at amino acid residue 600 of the mature protein (according to the numbering of Kornblihtt et al., 1985). Judging from the size of the fragment, it is likely to encompass a sequence that extends partially into the second type III repeat.

Because fibronectin does not bind to monolayers of cells that do not deposit a fibronectin matrix, we next determined the binding of the 14-kD fragment to HT-1080 cells, which produce no matrix. While ~50–60% of the ^{125}I-14-kD fragment bound specifically to IMR-90 cells, which construct an extensive fibronectin matrix, no specific binding to HT-1080 cells was detected (not shown).

Both fibronectin and the 70-kD fragment completely inhibited the binding of ^{125}I-fibronectin to IMR-90 cells in a 1-h binding assay, but the 14-kD fragment was only partially inhibitory (Fig. 3 *A*, *left*). The 14-kD fragment had a much more pronounced effect on the amount of fibronectin incorporated into the extracellular matrix after 24 h of incubation of cells with ^{125}I-fibronectin. As shown in Fig. 3 *A* (*right*), the 14-kD fragment inhibited the fibronectin matrix assembly defined by this assay by ~70% (at 5 μM, the highest concentration tested). The IC_{50} of the 14-kD fragment was between 1 and 2 μM, which was 5–10-fold higher than that of fibronectin or the 70-kD fragment. Thus, the purified 14-kD fragment inhibits fibronectin matrix assembly.

The 14-kD fragment did not significantly reduce ^{125}I-70-kD binding to cells, although, as expected, this was com-

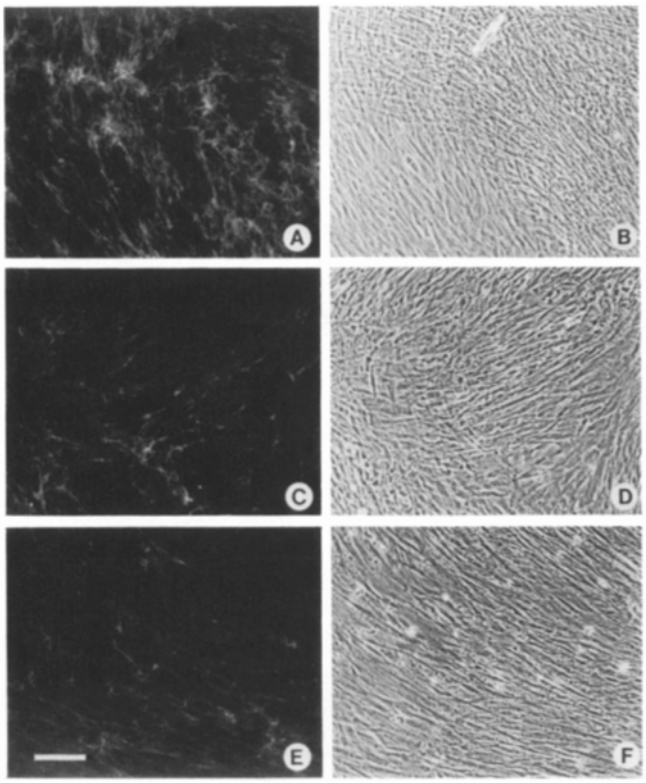

Figure 4. Inhibition of fibronectin fibril formation by the 14-kD fragment. IMR-90 cells were seeded onto slides that had been precoated with collagen type I. After attaching for 1 h at 37°C, cells were cultured for 5 d in 10% fibronectin-deficient medium plus either no additions (*A* and *B*), or 200 μg/ml 70-kD fragment (*C* and *D*), or 100 μg/ml 14-kD fragment (*E* and *F*). On day 5 cells were given 10 μg/ml of fluorescein-labeled fibronectin for 24 h. Cells were then fixed with paraformaldehyde and the fluorescent fibronectin in the matrix was visualized by fluorescence microscopy. The panels show representative fields from each culture. *A*, *C*, and *E* are fluorescent images, and *B*, *D*, and *F* are the corresponding phase-contrast images. Bar, 25 μm.

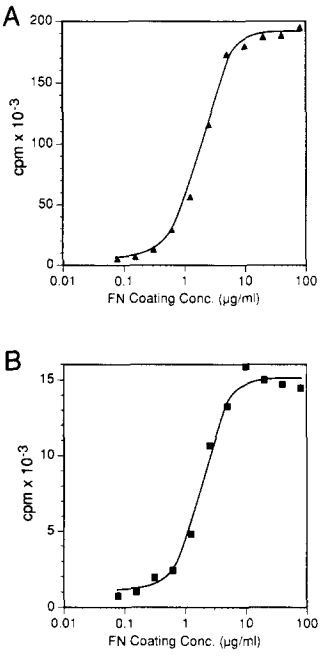

Figure 5. Binding of the 14-kD fragment to fibronectin. Plastic wells were coated with various concentrations of fibronectin, blocked with BSA, then probed with ^{125}I-14-kD (*A*), or ^{125}I-fibronectin (*B*) for 2 h at 37°C. The amount of radioiodinated protein bound was measured after washing extensively with 0.2% BSA in PBS. Each data point is the average of duplicate determinations.

pletely inhibited by fibronectin and the 70-kD fragment (Fig. 3 *B*, *center*). In the reciprocal experiment, the binding of ^{125}I-14-kD fragment to cells was completely blocked by unlabeled 14-kD fragment, whereas fibronectin inhibited only slightly and the 70-kD fragment was not inhibitory (Fig. 3 *B*, *right*). These data indicate that the 14-kD fragment does not compete for the binding of the 70-kD fragment to cells, and thus must inhibit matrix assembly by some mechanism other than the inhibition of the binding of fibronectin to matrix assembly sites.

When matrix assembly was measured by incorporation of fluorescent fibronectin into fibrils in the IMR-90 cell layers, both the 70- and 14-kD fragments significantly reduced the degree of fibril formation, while not significantly affecting cell morphology (Fig. 4). This provided further evidence that the 14-kD fibronectin fragment is able to inhibit fibronectin matrix assembly.

The Purified 14-kD Fragment Binds to Fibronectin and Inhibits Fibronectin–Fibronectin Association

To examine the mechanism by which the 14-kD fragment inhibits matrix assembly, the ability of this fragment to interact with fibronectin was tested. As shown in Fig. 5, ^{125}I-fibronectin and ^{125}I-14-kD fragment both bound to fibronectin coated on the plastic in a dose-dependent manner. The total ^{125}I-14-kD bound was higher than the total amount of ^{125}I-fibronectin bound (compare Fig. 5, *A* to *B*). Taking into account the specific activities, the levels of binding indicated that the maximal binding of the 14-kD fragment to fibronectin was ~10-fold more efficient on a molar basis than fibronectin binding to fibronectin. Typically, 1–2% of the fibronectin added in solution bound to fibronectin-coated dishes, while 15–20% of the 14-kD fragment bound to fibronectin-coated dishes.

Unlabeled 14-kD fragment competed efficiently for the binding of ^{125}I-14-kD fragment to fibronectin, thereby demonstrating the specificity of this binding (Fig. 6 *A*). Yet, unlabeled fibronectin did not compete for the binding of ^{125}I-14-kD fragment to fibronectin. One explanation for this is that the unlabeled fibronectin is binding to the fibronectin coating, and that ^{125}I-14-kD fragment then binds to either the coated or the adsorbed fibronectin.

Besides competing for 14-kD–fibronectin binding, the excess unlabeled 14-kD fragment also competed for fibronectin–fibronectin binding (Fig. 6 *B*). At the highest concentration tested (5 μM), the 14-kD fragment inhibited the binding of fibronectin to fibronectin by >50%. As with 14-kD-fibronectin binding, unlabeled fibronectin did not compete for the fibronectin–fibronectin binding, probably due to the reasons mentioned above. Thus, the 14-kD fragment of fibronectin that inhibited matrix assembly, also binds to fibronectin directly, and inhibits fibronectin–fibronectin association.

In similar assays, radiolabeled 70-kD fragment did not bind to fibronectin-coated plastic (not shown). This indicates that no sites responsible for fibronectin–fibronectin binding are contained within the amino-terminal 70-kD region.

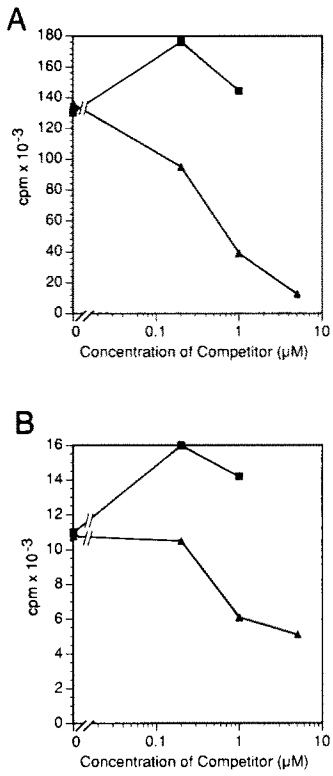

Figure 6. Inhibition of fibronectin–fibronectin binding by the 14-kD fragment. Plastic wells were coated with 5 μg/ml fibronectin, blocked with BSA, then probed with ^{125}I–14 kD (*A*), or ^{125}I-fibronectin (*B*), in the presence of various concentrations of unlabeled 14 kD (▲) or fibronectin (■). The solutions were incubated for 2 h at 37°C, followed by extensive washing with 0.2% BSA in PBS, and measurement of the radioactivity bound to the wells. Each data point is the average of duplicate determinations.

A 31-Amino Acid Peptide from the 14-kD Fragment Inhibits Fibronectin–Fibronectin Binding

Four peptides (of 30–34 amino acids each) were synthesized, representing the region of fibronectin encompassed by the 14-kD fragment (peptides P1–P4). Peptide P1 was the most efficient at inhibiting the binding of the 14-kD fragment to fibronectin, with an IC_{50} of 1 μM; peptides P2 and P3 were ~100-fold less potent (Fig. 7 *A*). Peptide P4 did not inhibit the 14-kD–fibronectin association, rather, at concentrations above 100 μM, it stimulated this association (not shown). The reason for the enhancement of binding by peptide P4 is not clear; it is possible that peptide P4 represents part of a fibronectin-binding domain. However, since peptide P4 did not inhibit the binding of 14-kD to fibronectin, it is not likely to be as important in fibronectin self-association as the peptide P1 region. As shown in Fig. 7, a nonrelated peptide (a peptide representing the cytoplasmic domain of the integrin α_5 subunit) had no effect on 14-kD–fibronectin association.

Peptide P1 also proved to be the most potent among the peptides at inhibiting fibronectin–fibronectin binding, giving an IC_{50} of ~1 μM (Fig. 7 *B*). The only other peptide that inhibited fibronectin–fibronectin binding was peptide P3 with an IC_{50} of 200–300 μM (Fig. 7 *B*).

One unexpected result was found. While peptide P1 inhibited fibronectin–fibronectin association at low concentrations (0.1–50 μM), at high concentrations it actually enhanced the binding of fibronectin to the wells (Fig. 7 *B*). We have found that at high concentrations peptide P1 aggregates and can be pelleted by high speed centrifugation, and that this does not happen with peptide P2 (not shown). It is possible that at high concentrations peptide P1 aggregates into multimers and binds to the coated fibronectin and that the ^{125}I-fibronectin probe becomes incorporated into these peptide P1/fibronectin complexes. This could lead to the observed increase in signal seen with P1 concentrations above 100 μM, because as shown below, fibronectin binds directly to peptide P1. Thus, the ability of the 14-kD fragment to inhibit fibronectin–fibronectin binding was also shared by peptide P1, which was modeled after the amino-terminal 31 residues of the 14-kD fragment.

Peptide P1 Binds to Fibronectin

To study the binding of fibronectin to peptide P1 or the other peptides, the peptides were coupled to Sepharose beads and the resins were tested in affinity chromatography for their ability to bind purified fibronectin. As shown in Fig. 8 all

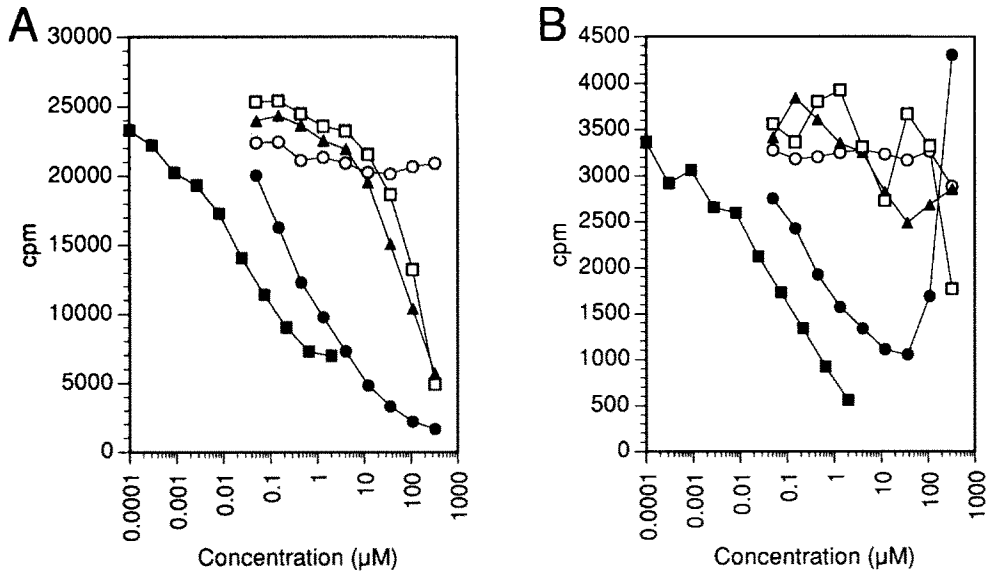

Figure 7. Inhibition of fibronectin–fibronectin association by a peptide from the 14-kD region. Plastic wells were coated with 5 μg/ml fibronectin, blocked with BSA, then probed with ^{125}I–14-kD (*A*), or ^{125}I-fibronectin (*B*), in the presence of various concentrations of unlabeled heparin-binding fragments (■), peptide P1 (●), peptide P2 (▲), peptide P3 (□), or an α_5 cytoplasmic domain peptide as a negative control (○). The solutions were incubated for 2 h at 37°C, followed by extensive washing with 0.2% BSA in PBS, and measurement of the radioactivity bound to the wells. The concentration values shown for heparin-binding fragments refer to the final concentrations of the 14-kD fragment in the solutions.

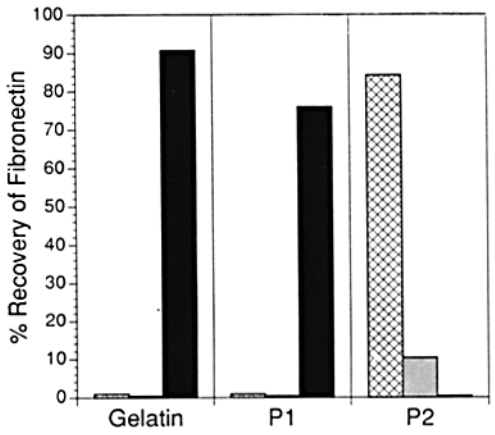

Figure 8. Binding of purified fibronectin to peptide P1 in affinity chromatography. 1-ml Sepharose columns coupled to either gelatin, peptide P1, or peptide P2 received 10 ml of a 250-μg/ml solution of purified human plasma fibronectin in PBS. The unbound fraction from each column was collected and the columns were washed with 20 ml of PBS, followed by elution with 5 ml of 8 M urea in PBS. The total amount of fibronectin recovered in the unbound fractions (*cross-hatched bars*), the wash fraction (*shaded bars*), and the eluted fractions (*solid bars*) was quantitated by measuring the absorbance of each solution at 280 nm.

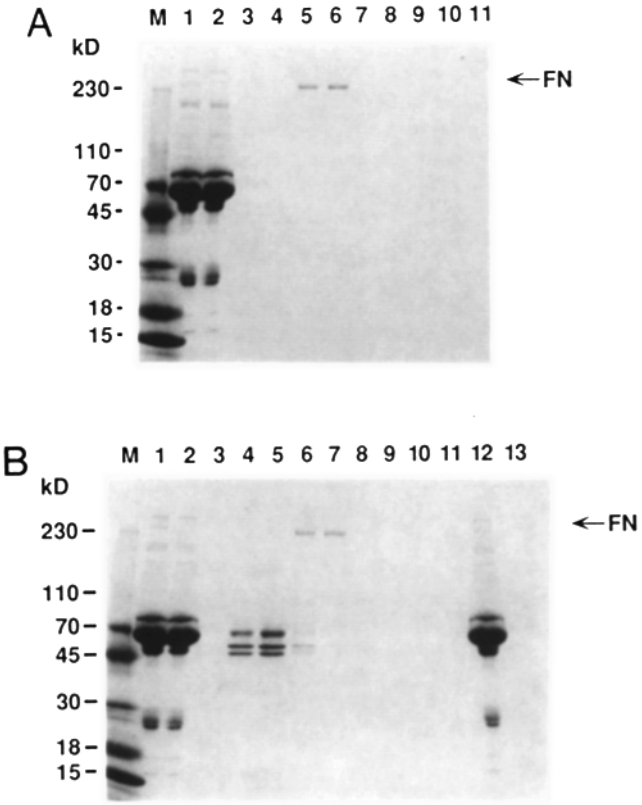

Figure 9. Affinity chromatography of human plasma on peptide columns and gelatin-Sepharose. Human plasma was applied to either gelatin-Sepharose (*A*) or columns made of peptide P1 (*B*, lanes *1–11*) or P2 (*B*, lanes *12* and *13*) coupled to Sepharose. The unbound fraction of proteins was collected, and the columns were washed with PBS + 5 mM EDTA (PBS/EDTA). Proteins remaining bound to the columns were eluted with stepwise urea gradients in the case of gelatin-Sepharose and P1-Sepharose, or 8 M urea in the case of P2-Sepharose. *A*, lane 1 shows the starting material, and lane 2 shows the unbound fraction from the gelatin column. Lanes 3–11 show the fractions eluted from gelatin-Sepharose by 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 M urea, in that order. *B*, lanes 1–11 are analogous to those of *A* except that they represent samples from the peptide P1 column. *B*, lane 12 is the unbound fraction from the peptide P2 column and lane 13 is the 8 M urea elution of the P2 column and represents all of the proteins eluted from the P2 column. The proteins in the gels were stained with Coomassie blue. The lanes marked M depict molecular mass standards whose sizes are indicated to the left of each gel. The position of fibronectin is indicated to the right of each gel.

of the fibronectin in the solution bound to either the gelatin-Sepharose or P1-Sepharose columns. None of the fibronectin bound to the P2-Sepharose column. The binding capacity of the P1-Sepharose column was comparable to that of gelatin-Sepharose; both columns were able to bind at least 2.5 mg of fibronectin per ml of resin.

We next tested the ability of the P1 and P2 columns to bind fibronectin selectively from a complex mixture of proteins by using human plasma as a source of fibronectin. All of the fibronectin detectable by gel electrophoresis was removed from plasma by passage over either a gelatin or a P1 column (Fig. 9 *A*, lane *2*, and 9 *B*, lane *2*), whereas the peptide P2 column did not remove any of the fibronectin (Fig. 9 *B*, lane *12*). The bound fibronectin eluted from the gelatin-Sepharose column in 2–3 M urea (Fig. 9 *A*, lanes *5* and *6*), while 3–4 M urea was required to remove fibronectin from the P1-Sepharose column (Fig. 9 *B*, lanes *6* and *7*). Apart from fibronectin, the preparation eluted from the P1 column contained some other plasma proteins, but the majority of these proteins eluted in the 1–2 M urea fractions (Fig. 9 *B*, lanes *4* and *5*), indicating that they had a lower affinity for P1-Sepharose than did fibronectin. These experiments demonstrated that peptide P1 was able to bind directly and selectively to fibronectin.

Discussion

We have confirmed the previous finding by Chernousov et al. (1991) that the first type III repeat of fibronectin is important for matrix assembly. In addition, we show that this site constitutes a fibronectin–fibronectin binding site and have reproduced some of the essential activities of this site with a synthetic 31-amino acid peptide from that repeat.

The role of the first type III repeat in matrix assembly is likely to be different from the role of the amino-terminal 70- and 29-kD regions. In contrast to the 70-kD fragment, which inhibits the binding of fibronectin to cell surfaces, our 14-kD fragment which represents the first type three repeat region only has a small effect on the initial binding of fibronectin to cells, but has a pronounced effect on the subsequent incorporation of fibronectin into the matrix. This indicates that the site contained in the 14-kD fragment functions during matrix assembly at a stage which is later than the initial binding of fibronectin to cells. Fibronectin binds to cell layers through its amino-terminal 70-kD region (McKeown-Longo and Mosher, 1985), which does not contain the 14-kD region (see Fig. 2). Two proteins have been detected that are able to bind the 70-kD fragment (Limper et al., 1991; Blystone

and Kaplan, 1992); these may serve as the cell surface receptors for the binding of fibronectin to cell layers. In this report we show that the 14-kD fragment also binds to cell layers. The identity of the molecule on the cell surface that binds to the 14-kD region was not directly tested in our study, however, since our results reveal a strong affinity in the 14-kD fragment for fibronectin, it is likely that the 14-kD fragment is binding to cell surface-bound fibronectin.

Several lines of evidence suggest that the 14-kD fragment contains a site that allows fibronectin to bind to itself but that this site is partly cryptic in intact fibronectin. We find that while the binding of radiolabeled 14-kD fragment to cell layers is readily inhibited by excess unlabeled 14-kD fragment, intact fibronectin is a poor inhibitor of this interaction. Similarly, the binding of radiolabeled 14-kD fragment to fibronectin coated onto plastic is also blocked by unlabeled 14-kD fragment but not by unlabeled fibronectin. The reason that fibronectin does not compete effectively for the binding may be that it also associates with cell surface-bound fibronectin (or fibronectin bound to a dish), and that the 14-kD fragment can then bind to the adsorbed fibronectin. The higher binding efficiency of the 14-kD–fibronectin interaction as compared to the fibronectin–fibronectin interaction may be due to the binding site in the 14-kD fragment being partially cryptic. However, since the 14-kD fragment was able to inhibit the self-association of intact fibronectin, the 14-kD region is at least partially active in intact fibronectin molecules. The parallel between the two assays indicates that the mechanism of binding may be the same in both cases. It is therefore likely that the 14-kD fragment is binding to fibronectin on the surface of cells when it binds to cell layers.

Our results indicate that while the role of the 14-kD region during matrix assembly is distinct from that of the 70- and 29-kD regions, it may be more similar to that of the 56-kD region. The 14-kD region and the peptide P1 site are contained in the 56-kD region, and a 40-kD gelatin-binding fragment, which constitutes the majority of the 56-kD region, is known not to inhibit matrix assembly (McDonald et al., 1987; Quade and McDonald, 1988). Therefore, the 14-kD region would appear to be the active site in the 56-kD region. However, Chernousov et al. (1991) did not detect any binding of the 56-kD fragment to fibronectin, whereas we found that both the 14-kD fragment and peptide P1 did bind to it. On the other hand, a 60-kD gelatin and heparin-binding chymotryptic fragment of fibronectin which would appear to be essentially the same as the 56-kD fragment was found to contain a fibronectin-binding site (Ehrismann et al., 1981, 1982). Since the high affinity of the 14-kD region for fibronectin is likely to be cryptic, this may explain the variations in the fibronectin binding of the various larger fragments.

By expressing recombinant forms of rat fibronectin in mouse cells, Schwarzbauer et al. (1987) have identified regions of fibronectin that are required for matrix assembly (Schwarzbauer et al., 1987; Schwarzbauer, 1991). The smallest recombinant fibronectin that was efficiently incorporated into the matrix contained the amino- and carboxy-terminal regions of the molecule with an internal deletion comprising the first seven type III repeats (Schwarzbauer, 1991). This recombinant molecule did not contain the 14-kD region, seemingly indicating that this region may not be required for fibronectin matrix assembly. However, although the recombinant fibronectins were incorporated into a deoxycholate-insoluble matrix, they may not have been disulfide cross linked into high molecular weight multimers (Fig. 5 in Schwarzbauer, 1991), as is always the case with wild type fibronectin in the matrix (Hynes and Destree, 1977; Keski-Oja et al., 1977; Ali and Hynes, 1978). It remains to be seen whether the incorporation of the recombinant fibronectins is identical to that of wild type fibronectin. In addition, it is known that exogenous and endogenous fibronectin molecules can be incorporated into the same fibrils (Peters et al., 1990). It is therefore possible that the incorporation of the recombinant fibronectins into the matrix may have relied partially on the wild type, endogenous fibronectin that was being produced by the cells during the assay.

The results presented in this study, as well as results obtained by other investigators (McDonald, 1988; Akiyama et al., 1991; Chernousov et al., 1991; Mosher et al., 1991; Nagai et al., 1991; Schwarzbauer, 1991), are consistent with the following multi-step model for fibronectin matrix assembly. This model is similar to the models proposed by Mosher et al. (1991) and other investigators (McDonald, 1988; Hynes, 1990). The first step in the model is the capture of fibronectin dimers from the environment. This step is likely to involve the $\alpha_5\beta_1$ integrin and the molecules which bind to the amino-terminal 70-kD region, since antibodies to $\alpha_5\beta_1$ and the 70-kD fragment of fibronectin both inhibit the binding of fibronectin to cells. Since the 14-kD fragment only has a minor effect on the binding of fibronectin to cells, it probably does not inhibit matrix assembly at this step. The second step is translocation of the captured fibronectin to the growing end of a fibronectin fiber. This step may not be necessary if a new fiber can be nucleated at the original location. The third step is fibronectin–fibronectin alignment. This step is likely to involve regions of fibronectin self-association, and we propose that this is probably the step which is inhibited by the 14-kD fragment. The fourth step is disulfide cross linking of the aligned molecules, resulting in covalent stabilization of the fibronectin fibril.

There is evidence to suggest an inverse relationship between the ability of a cell to migrate and the ability to assemble a fibronectin matrix. For example, CHO cells which overexpress a recombinant $\alpha_5\beta_1$ integrin produce more matrix than cells which do not overexpress the integrin (Giancotti and Ruoslahti, 1990). Migration studies with these cells indicate that the cells which produce the most matrix migrate the least (Giancotti and Ruoslahti, 1990). The 14-kD fragment and peptide P1 may provide useful probes of the effects of matrix production on cell attachment and migration. While the P1 peptide clearly contains a fibronectin-binding site, we have not been able to achieve satisfactory inhibition of matrix formation in living cells by using the synthetic peptides (results not shown). The low activity of peptide P1 requires the use of high concentrations of the peptide for an effect on matrix formation and these effective concentrations are too close to the concentrations causing nonspecific effects for the matrix inhibition to be convincing. While the 14-kD fragment at this time remains the only fully effective probe for the fibronectin–fibronectin binding site, our identification of this site in the synthetic peptide should allow for the design of improved probes for matrix assembly.

We thank Dr. George Davis for helpful discussions; John Grzesiak for supplying heparin-binding and gelatin-binding fragments of fibronectin; Dr.

Ralf Butzow for help with reverse phase HPLC; and Khanh Nguyen for technical assistance with protein sequencing and peptide synthesis. We also thank Drs. Eva Engvall, Kathryn Ely, Bingcheng Wang, Lars Rasmussen, Axel Hildebrand, and Bruce Vogel for critical review of the manuscript.

This work was supported by grants CA42507 and Cancer Center Support Grant CA30199 (to E. Ruoslahti) from the National Cancer Institute. A. Morla is supported by a Damon Runyon-Walter Winchell Cancer Research Fund Fellowship, DRG-1098.

Received for publication 24 January 1991 and in revised form 27 April 1992.

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