Altered Rate of Fibronectin Matrix Assembly by Deletion of the First Type III Repeats

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Abstract. The assembly of fibronectin (FN) into a fibrillar matrix is a complex stepwise process that involves binding to integrin receptors as well as interactions between FN molecules. To follow the progression of matrix formation and determine the stages during which specific domains function, we have developed cell lines that lack an endogenous FN matrix but will form fibrils when provided with exogenous FN. Recombinant FNs (recFN) containing deletions of either the RGD cell-binding sequence (RGD^-) or the first type III repeats ($FN\Delta III_{1-7}$) including the III_1 FN binding site were generated with the baculovirus insect cell expression system. After addition to cells, recFN matrix assembly was monitored by indirect immunofluorescence and by insolubility in the detergent deoxycholate (DOC). In the absence of any native FN, $FN\Delta III_{1-7}$ was assembled into fibrils and was converted into DOC-insoluble matrix. This process could be inhibited by the amino-terminal 70 kD fragment of FN, showing that $FN\Delta III_{1-7}$ follows an assembly pathway similar to FN. The progression of $FN\Delta III_{1-7}$ assembly differed from native FN in that the recFN became DOC-insoluble more quickly. In contrast, RGD^- recFNs were not formed into fibrils except when added in combination with native FN. These results show that the RGD sequence is essential for the initiation step but fibrils can form independently of the III_{1-7} modules. The altered rate of $FN\Delta III_{1-7}$ assembly suggests that one function of the missing repeats might be to modulate an early stage of matrix formation.

Fibronectin (FN)[1] is a multifunctional component of the extracellular matrix (for review see Mosher, 1989; Hynes, 1990). From within the matrix, FN interacts with cells to control cell adhesion, cytoskeletal organization, and intracellular signaling. It also forms a structural framework for cell migration, differentiation, cell–cell interactions, and deposition of other matrix proteins. The importance of FN has been underscored by recent results with an FN-null mutation which result in embryonic lethality in mice (George et al., 1993). Moreover, while the loss of an FN matrix is characteristic of many tumorigenic cells, restoration of this matrix can suppress the transformed phenotype (Giancotti and Ruoslahti, 1990).

Models for assembly of FN into a fibrillar matrix propose a stepwise process initiated by binding to cell surface receptors followed by assembly and reorganization into fibrils (for reviews see McDonald, 1988; Mosher et al., 1992; Mosher, 1993). As increasing amounts of FN bind to cells, dimeric FN is converted into a complex network of fibrils that is insoluble in the detergent deoxycholate (DOC) and consists of high molecular weight aggregates (Hynes and Destree, 1977; Keski-Oja et al., 1977; Choi and Hynes, 1979; McKeown-Longo and Mosher, 1983). The $\alpha5\beta1$ integrin appears to be a major receptor for matrix assembly (Ruoslahti, 1991; Wu et al., 1993). Antibodies that interfere with the interaction between $\alpha5\beta1$ and the RGD site block this process (Akiyama et al., 1989; McDonald et al., 1987; Roman et al., 1989; Darribere et al., 1990; Fogerty et al., 1990). Two other RGD-dependent integrins, $\alpha IIb\beta3$ and $\alpha v\beta3$, have also been shown to participate (Wu et al., 1995*c*; Wennerberg et al., 1996) and may account for the observation that cells from $\alpha5$ integrin null mice are able to assemble an FN matrix (Yang et al., 1993). A number of other integrins bind to FN but do not initiate fibril formation (Busk et al., 1992; Zhang et al., 1993; Wu et al., 1995*a*). The absolute necessity of the RGD sequence has been brought into question with the observations that recFNs lacking RGD are incorporated into fibrils (Schwarzbauer, 1991) indicating that interactions with RGD might not be required at all stages of matrix formation.

After binding to the cell surface, interactions occur between FN molecules leading to the formation of fibrils. Several FN domains that contribute to this process have been identified. The amino-terminal domain, particularly

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1. *Abbreviations used in this paper:* DOC, deoxycholate; FN, fibronectin; recFN, recombinant fibronectin.

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modules I_{1-5}, are critical for FN binding and matrix assembly (McKeown-Longo and Mosher, 1985; Quade and McDonald, 1988; Schwarzbauer, 1991; Aguirre et al., 1994). The carboxy-terminal cysteine pair that forms the covalent disulfide-bonded dimer is also required, as only dimeric FN becomes incorporated into a matrix (Schwarzbauer, 1991; Sottile and Wiley, 1994). In addition, a third region encompassing modules I_9 and III_1 is involved. Monoclonal antibodies against this region inhibit matrix assembly (Chernousov et al., 1988, 1991; Darribere et al., 1992). More recently, the III_1 module has been shown to be a major site of FN–FN binding (Morla and Ruoslahti, 1992; Aguirre et al., 1994; Hocking et al., 1994) and is capable of inducing the formation of disulfide cross-linked FN in vitro (Morla et al., 1994). This module is apparently not critical for fibril formation as a recombinant FN lacking repeats III_{1-7} was readily incorporated into a fibrillar matrix by SVT2 fibroblasts (Schwarzbauer, 1991). We have designed an FN matrix assembly system based on cell lines that are capable of assembling exogenous recombinant FNs (recFNs) but lack their own endogenous matrix. In contrast to the SVT2 cell system previously described (Schwarzbauer, 1991), this system is not complicated by the presence of normal endogenous FN. As a result, the type and concentration of FN added to the system can be completely controlled and the progression of fibril formation can be followed at specific time points after addition of FN. We have found that a recFN lacking repeats III_{1-7} (FNΔIII_{1-7}) can be assembled into fibrils in the absence of native FN while deletion of the RGD cell-binding sequence abolishes fibril formation. The ability of RGD^- polypeptides to be incorporated when added along with native FN demonstrates that the RGD sequence is required for initiation of matrix formation but is not needed in all incoming FN molecules incorporated at later steps. Differences in the rate of FNΔIII_{1-7} assembly compared to native FN suggest that the III_{1-7} region plays a regulatory role in the process.

Materials and Methods

Cell Culture

Mouse AtT-20 pituitary cells were transfected with human α5 integrin cDNA in a retroviral vector (Hynes et al., 1992) using Lipofectin reagent (Life Technologies/GIBCO-BRL, Gaithersburg, MD). G418-resistant clones were screened for expression of α5 integrin by adhesion to FN-coated surfaces. Expression was confirmed by immunoprecipitation of metabolically labeled cell lysates (Marcantonio and Hynes, 1988). Clone AtT-20α5 No. 11 was used for all experiments described. AtT-20α5 cells were grown in a 50:50 mixture of Ham's F12 and DMEM supplemented with 20 mM Hepes, pH 7.4, 4 mM L-glutamine, 10% Nu-serum (Collaborative Research, Bedford, MA), 10% FCS (Hyclone Labs, Logan, UT), and 0.25 mg/ml Geneticin (Life Technologies/GIBCO-BRL).

CHO-K1 cells were transfected by electroporation using 8×10^6 cells with 20 μg of a human α5 integrin cDNA in vector pBJ-1 (Lin et al., 1990) and 1 μg of pFneo DNA containing a neomycin-resistance gene. Transfected cells were maintained in DMEM supplemented with 10% FCS at 37°C in 6% CO_2 for 2 d, and then transferred to the same medium containing 100 μg/ml G418 (Life Technologies/GIBCO-BRL) and harvested after 10–14 d of selection. The expression level of α5 integrin was confirmed by flow cytometric analysis in FACScan (Becton-Dickinson, Mountain View, CA) with monoclonal antibody 16 against human α5 (Akiyama et al., 1989).. Clone CHOα5-17, used for all experiments, was obtained by single cell sorting of the G418-resistant cells with monoclonal antibody 16 in FACStar (Becton-Dickinson). Monoclonal antibody PB1 (Brown and Juliano, 1985) was used for detection of endogenous hamster α5. The level of expression of human α5 was threefold higher than hamster α5. Production of endogenous FN was detected by metabolic labeling of cells with [^{35}S]methionine followed by immunoprecipitation with polyclonal antibody R39 or gelatin binding of labeled medium as described (Schwarzbauer et al., 1989). CHOα5 cells were maintained in DMEM, 10% FCS (Hyclone Labs), 2 mM glutamine, 1% nonessential amino acids, and 100 μg/ml Geneticin (Life Technologies/GIBCO-BRL).

BTI-TN-5B1-4 (High Five) insect cell line (Invitrogen Corp., San Diego, CA) was maintained in TNM-FH (Grace's medium supplemented with yeastolate and lactalbumin hydrolysate) supplemented with 10% FCS. For purification of recombinant protein, High Five cells were grown in either Sf-900 II or Express Five serum-free medium (Life Technologies/GIBCO-BRL).

To prepare FN-depleted serum, fetal calf and Nu-serum were passed over a gelatin-agarose column two times as described (Engvall and Ruoslahti, 1977) resulting in 99.7% depletion of FN. The amount of fibronectin remaining in the FCS was calculated to be ~62 ng/ml, translating to 6 ng/ml in complete medium.

FN cDNA Constructions

Baculovirus vector pVL1392 was used for expression of all recombinant FNs (recFNs). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Construction of FNΔIII_{1-7} and 70 kD have been previously described (Schwarzbauer, 1991; Aguirre et al., 1994).

Full-length recFN FNA^-B^- was created by digesting a rat FN cDNA spanning the region coding for I_9 through III_{11} with NheI–RsrII. The resulting 4,071-bp fragment was ligated to NheI–RsrII digested pVL1392-FNΔIII_{1-7}. For FNΔIII_{1-7}(RGD^-), a 2,215-bp RsrII–StuI fragment isolated from pLJ-I_{1-9}/C110-RGDS (Schwarzbauer, 1991) was ligated to pVL1392-FNΔIII_{1-7} which had been digested with RsrII–StuI. From the resulting plasmid, a 1.6-kb RsrII–SacI fragment was isolated and ligated to RsrII–SacI digested pVL1392-FNΔIII_{1-7} in order to create $FNA^-B^-(RGD^-)$.

Recombinant Protein Production and Purification

Recombinant baculoviruses were generated by cotransfection of High Five cells with FN constructions in baculovirus vector pVL1392 and Baculogold DNA (Pharmingen, San Diego, CA). Single viral clones were obtained by limiting dilution cloning of transfection supernatants. High titer stocks were amplified as described by Summers and Smith (1987).

High Five cells grown in serum-free medium were infected with high titer recombinant virus stock at an m.o.i. of 10. Culture medium was collected 3–4 d postinfection. PMSF (0.5 mM) and EDTA (10 mM) were added to inhibit proteolysis. Recombinant protein and rat plasma FN (pFN) were purified by gelatin-agarose chromatography essentially as described by Engvall and Ruoslahti (1977). Recombinant protein preparations were free of contaminating intact FN since High Five cells do not produce FN. Purity of preparations was confirmed by silver stain analysis (Merril et al., 1984) and immunoblot after SDS-PAGE.

Immunofluorescence

Polyclonal rabbit anti-FN antiserum R39 (Schwarzbauer et al., 1989) which recognizes rat, hamster, bovine, and human FNs was used at a dilution of 1:50. Culture supernatant from hybridoma cells producing rat-specific monoclonal antibody IC3 was diluted 1:10.

AtT-20α5 and CHOα5 cells were seeded on glass coverslips in 24-well dish or Lab Tek Chamber Slides (Nunc Inc., Naperville, IL) at concentrations of 4×10^5 and 1.5×10^5 cells/cm^2, respectively. Cells were seeded in medium containing FN-depleted serum and incubated for ~24 h until cells were almost confluent. AtT-20α5 cells were seeded into medium containing FN-depleted 10% Nu serum/ 20% FCS. Fresh medium, serum-free for short incubations or with FN-depleted serum for longer incubations, was then added along with pFN or recFNs and 70 kD as indicated. At the end of the incubation period, cells were washed with PBS and fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. Coverslips were then washed with PBS and incubated with primary antisera diluted in 2% ovalbumin in PBS in a moist chamber at 37°C for 30 min.

Coverslips incubated with polyclonal antibody were washed with PBS and incubated with goat anti–rabbit biotinylated IgG (Life Technologies/GIBCO-BRL) at a 1:100 dilution followed by rhodamine-avidin (ICN

Biochemicals, Costa Mesa, CA) at 1:300. After several washes with PBS, coverslips incubated with monoclonal antibodies were incubated with fluorescein-conjugated goat anti-mouse IgG (Molecular Probes, Inc., Eugene, OR) at a dilution of 1:320. All coverslips were then washed for a final time and mounted with FITC-Guard (Testog, Inc., Chicago, IL). Fibrils were visualized with a Nikon Optiphot microscope with epifluorescence using a 40× phase/fluorescence or 60× plan-apochromatic objective. Photography was performed as described in Schwarzbauer (1991).

Isolation and Detection of DOC-soluble and -insoluble Material

AtT-20α5 and CHOα5 cells were cultured in wells of a 24-well dish, in the absence of glass coverslips essentially as described above. For inhibition experiments with the 70-kD fragment, cells were cultured in a 96-well tissue culture plate. RecFNs or pFN were incubated with the cultured cells for defined periods of time. At the end of the incubation period, the cell layers were washed with serum-free DMEM, and then lysed in 200 μl deoxycholate (DOC) lysis buffer (2% deoxycholate, 0.02 M Tris-HCl, pH 8.8, 2 mM PMSF, 2 mM EDTA, 2 mM iodoacetic acid, and 2 mM *N*-ethylmaleimide) per well. DOC-insoluble material was isolated by centrifugation and then solubilized in 1% SDS, 25 mM Tris-HCl, pH 8.0, 2 mM PMSF, 2 mM EDTA, 2 mM iodoacetic acid, and 2 mM *N*-ethylmaleimide. Aliquots of DOC-soluble and insoluble material were electrophoresed on a 5% SDS polyacrylamide gel nonreduced and reduced with DTT.

Proteins were transferred to nitrocellulose (Sartorius Corp., Bohemia, NY) for immunodetection by using a Mini-Protean II transfer apparatus (BioRad Labs, Hercules, CA) according to manufacturer's instructions. Filters were blocked overnight in buffer A (25 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) at room temperature. IC3 hybridoma cell culture supernatant was diluted 1:100 in buffer A and incubated with the filter for 1 h at room temperature followed by three washes in buffer A. Biotinylated goat anti-mouse IgG (Life Technologies/GIBCO-BRL) was added at a 1:10,000 dilution in buffer A, incubated 1 h at room temperature, and washed with buffer A. A 1:10,000 dilution of streptavadin-horseradish peroxidase (Life Technologies/GIBCO-BRL) was added, incubated 30 min at room temperature, and washed. Immunoblots were then developed with chemiluminescent reagents (New England Nuclear Dupont, Boston, MA) according to instructions of the manufacturer and exposed to film (X-omat; Eastman Kodak Co., Rochester, NY).

Quantitation of DOC-soluble and -insoluble Material

DOC-soluble and -insoluble material was extracted from CHOα5 cells incubated with pFN or recFNs as described above. Total protein in the DOC-soluble and -insoluble fractions was measured by a BCA protein assay (Pierce, Rockford, IL) in order to standardize each of the collected samples. Equal amounts of total protein from either DOC-insoluble or DOC-soluble fractions from various time points were reduced with DTT and electrophoresed on a 5% SDS polyacrylamide gel. Protein was transferred to nitrocellulose as described above. Filters were blocked overnight at room temperature with 5% BSA in TBS (50 mM Tris-HCl, 200 mM NaCl). IC3 hybridoma culture supernatant was diluted 1:100 and incubated with the filter for 1 h at room temperature. After three washes in TBS, the filter was incubated with rabbit anti-mouse IgG (Pierce) diluted to 1 μg/ml in 5% BSA in TBS for 1 h at room temperature, and then washed three times with TBS. Approximately 6 μCi ^{125}I-Protein A (New England Nuclear Dupont) was then added to the filter in 10 ml 5% BSA in TBS. The ^{125}I-Protein A was incubated with the blot for 1 h at room temperature and then washed four times with buffer A until background signal was minimal. Washed blots were exposed to a phosphor storage screen and analyzed using a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Total cell-associated FN for each of the six time points was calculated as DOC-insoluble counts plus DOC-soluble counts. DOC-insoluble and -soluble values were expressed in terms of percent of total cell-associated FN.

Results

Fibronectin Matrix Assembly System

Cell lines, lacking an endogenous FN matrix but capable of assembling a matrix when supplied with exogenous FN, were used to develop a matrix assembly system dependent on recombinant FNs (recFNs). The mouse pituitary cell line AtT-20 does not produce either the α5 integrin subunit or endogenous FN. mRNAs were not detectable by Northern blot analysis (α5) and reverse transcriptase PCR (FN). The absence of protein was confirmed by immunoprecipitations with anti-α5 (data not shown) and anti-FN antibodies (Castle et al., 1995). Transfection of these cells with human α5 cDNA in a retroviral vector resulted in expression of the α5 subunit and presentation at the cell surface as a complex with the endogenous β1 subunit. As they produce no endogenous FN, the AtT-20α5 cells expressing the chimeric α5β1 FN receptor do not form a FN matrix (Fig. 1 *A*). These cells are, however, capable of assembling exogenous rat plasma FN (pFN) into a fibrillar matrix. When added to cell culture medium at 25 μg/ml, pFN accumulates in the form of fibrils at the cell surface as detected by immunofluorescence (Fig. 1 *C*). At concentrations below 25 μg/ml, a sparser pattern of FN fibrils was observed (Fig. 1 *B*), with no apparent fibril formation below 5 μg/ml. At concentrations above 25 μg/ml, AtT-20α5 cells became less adherent and detached from the substrate.

The AtT-20α5 cell line provides a unique system for determining the activity of various FN domains during initiation of matrix assembly in the absence of any endogenous FN. The fact that they become less adherent to substrate upon prolonged incubation with FN makes them less suitable for examining the later steps of matrix formation. For these types of studies a CHOα5 cell line generated by transfection of human α5 cDNA into CHO-K1 cells was used. This particular CHOα5 cell clone expresses a very low level of endogenous FN, which is insufficient to produce an FN matrix as determined by immunofluorescence (Fig. 1 *D*) or by analysis of DOC-insoluble matrix even in the presence of recFNs (data not shown). Like AtT-20α5 cells, CHOα5 cells are capable of assembling exogenous FN into a fibrillar network (Fig. 1, *E* and *F*). Incubation with 1 μg/ml or 5 μg/ml of pFN over a 15-h time period resulted in no detectable FN fibrils. At 10 μg/ml sparse fibrils mostly limited to short connections between cells were formed (Fig. 1 *E*). A network of fibrils became evident with the addition of 25 μg/ml and increased in density with 50 μg/ml (Fig. 1 *F*). CHOα5 cells remained adherent to substrate even with the addition of higher concentrations of FN and at prolonged incubation times.

Use of the AtT-20α5 cells lacking endogenous FN production and the CHOα5 cells severely deficient in FN production provides a model system for investigating FN matrix assembly. As neither cell line produces a preformed matrix, the type and concentrations of FN added to the system can be controlled. Therefore, the roles of specific FN domains during fibril formation as well as the progression of matrix assembly can be dissected with this system.

Production of Recombinant FNs in a Baculovirus Expression System

The large quantities of recFNs needed for these studies were expressed and purified using the baculovirus insect cell system. Expression in insect cells allows for proper disulfide bond formation which is required for the formation of FN dimers as well as folding of the type I and II re-

AtT-20α5

CHOα5

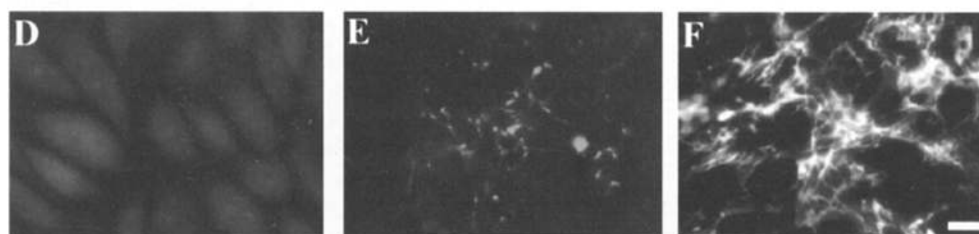


Figure 1. Assembly of FN by AtT-20α5 (*top*) and CHOα5 cells (*bottom*). Cells were cultured in medium with FN-depleted serum, fixed, and stained with a polyclonal anti-FN antibody (*A* and *D*). No FN fibrils were observed for either AtT-20α5 (*A*) or CHOα5 (*D*). Background staining of cells by rhodamine-avidin is visible in *D*. Occasional fibrils were observed upon addition of 10 μg/ml rat pFN to culture medium followed by staining with a rat-specific monoclonal antibody IC3 (*B* and *E*). A more extensive fibrillar matrix was observed upon the addition of 25 μg/ml rat pFN to AtT-20α5 cells (*C*) and 50 μg/ml rat pFN to CHOα5 cells (*F*). Bar equals 10 μm.

peats. Fig. 2 illustrates the recFNs expressed and purified from infected insect cell culture medium. FNA$^-$B$^-$ is a full-length rat FN, lacking only the alternatively spliced EIIIA and EIIIB regions. FNΔIII_{1-7} has an 80-kD internal deletion of repeats III_{1-7} (referred to as I_{1-9}/C110 in Schwarzbauer, 1991). In FNA$^-$B$^-$(RGD$^-$) and FNΔIII_{1-7} (RGD$^-$), the RGDS cell-binding sequence in repeat III_{10} has been deleted. All of the recFNs contain the two carboxy-terminal cysteines required for dimer formation and the alternatively spliced V120 region, which is required for efficient dimer secretion (Schwarzbauer et al., 1989). All four recFNs were efficiently secreted from insect cells primarily as disulfide bonded dimers.

A RecFN Lacking Repeats III_{1-7} Is Capable of Independent Fibril Formation

Recombinant FNΔIII_{1-7} is assembled into a fibrillar matrix when expressed in SVT2 fibroblasts (Schwarzbauer, 1991). However, since SVT2 cells produce low levels of endogenous FN, it was not possible to ascertain whether or not this recFN was capable of independent fibril formation. FNΔIII_{1-7} was tested for assembly by AtT-20α5 and CHOα5 cells in order to address whether repeats III_{1-7} are required for this process. Both AtT-20α5 cells (Fig. 3, *A* and *B*) and CHOα5 cells (Fig. 3, *C* and *D*) assembled FNΔIII_{1-7} into fibrils. These results demonstrate that repeats III_{1-7}, including the FN-binding site in repeat III_1, are not required for FN fibril formation in this system.

As an FN matrix forms, dimeric FN at the cell surface is converted from a DOC-soluble form to one which is insoluble. A proportion of this DOC-insoluble material exists as high molecular weight multimers that remain either at the top of the stacking gel or penetrate the separating gel but migrate more slowly than dimeric FN under nonreduced conditions. Exogenous pFN also becomes incorporated into these high molecular weight aggregates (McKeown-Longo and Mosher, 1983). To determine whether FNΔIII_{1-7} can be incorporated into a DOC-insoluble matrix, DOC-soluble and -insoluble material was isolated from AtT-20α5 and CHOα5 cells ~15 h after addition to the culture medium. As shown in Fig. 4, both cell lines assembled FNΔIII_{1-7} into matrix-associated, DOC-insoluble material. High molecular weight multimers of FNΔIII_{1-7} were present at the top of the stacking gel and at the interface of the stacking and separating gel. These aggregates were not present upon reduction of insoluble material (data not shown) or in nonreduced soluble fractions (Fig. 4). High molecular weight DOC-insoluble material was not

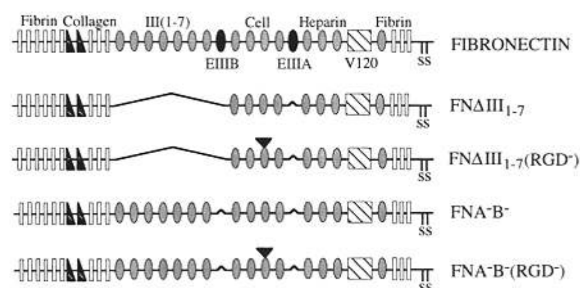


Figure 2. Schematic representation of FN and recFNs expressed in the baculovirus insect cell system. The structural organization of FN is shown at the top. The three types of repeats which comprise the FN molecule are indicated as: type I (*open rectangles*), type II (*triangles*), and type III (*stippled ovals*). Darkened ovals represent the alternatively spliced EIIIA and EIIIB repeats, neither of which was included in any of the recombinants. The V120 variant of the alternatively spliced V region (*cross hatch box*) was used for all recombinants. Location of the carboxy-terminal cytesine pair required for FN dimer formation is indicated (SS). Each of the four recombinants was constructed from rat FN cDNAs. A solid triangle is used to indicate deletion of the RGDS sequence in repeat III_{10}. RecFNs FNA$^-$B$^-$ and FNA$^-$B$^-$(RGD$^-$) are full-length FNs. RecFNs FNΔIII_{1-7} and FNΔIII_{1-7}(RGD$^-$) contain and internal 80-kD deletion of repeats III_{1-7}.

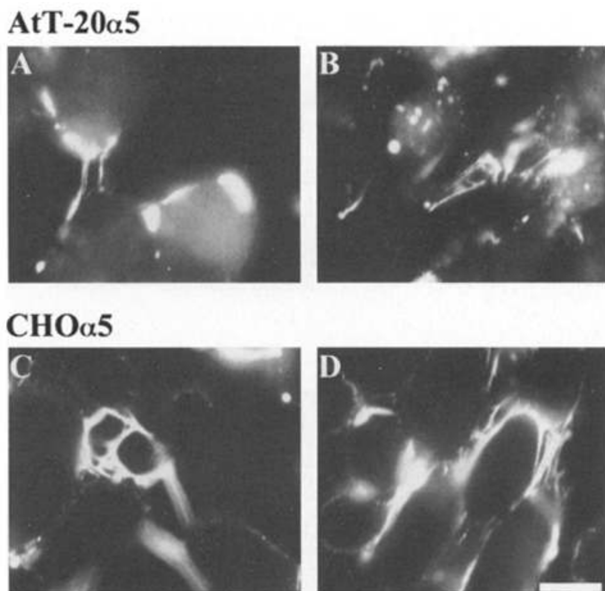


Figure 3. Immunofluorescence staining of FNΔIII_{1-7} fibrils. AtT-20α5 (*top panel*) and CHOα5 (*bottom panel*) cells were incubated for 15 h in serum-free medium containing 25 μg/ml FNΔIII_{1-7} for AtT-20α5 cells (*A* and *B*) or 50 μg/ml FNΔIII_{1-7} for CHOα5 cells (*C* and *D*). Fibrils were detected by indirect immunofluorescence using rat-specific monoclonal antibody IC3 and fluorescein-conjugated goat anti–mouse IgG. Bar equals 10 μm.

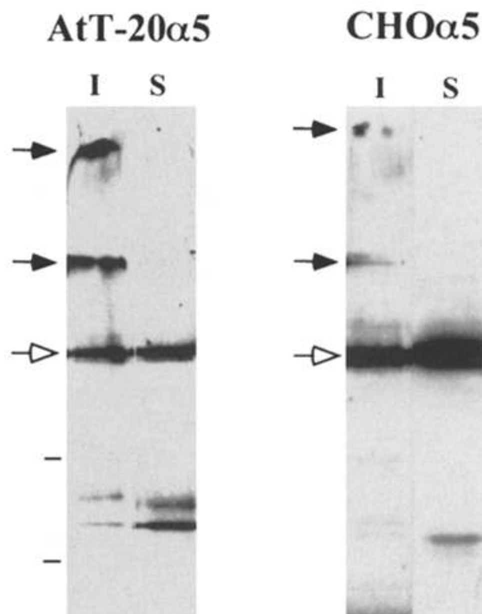


Figure 4. Analysis of DOC-soluble and -insoluble material. DOC-soluble and -insoluble material was isolated from AtT-20α5 and CHOα5 cells incubated for 15 h with 25 μg/ml or 50 μg/ml FNΔIII_{1-7}, respectively. DOC-soluble (*S*) and -insoluble (*I*) fractions were separated by 5% SDS-PAGE without reduction and transferred to nitrocellulose. FN was detected with a rat-specific monoclonal antibody IC3 and chemiluminescence reagents. 360-kD dimeric FNΔIII_{1-7} is present in all fractions (*open arrows*). High molecular weight multimers of FNΔIII_{1-7} are present at the top of the stacking gel and at the interface of stacking and separating gels from insoluble fractions only (*solid arrows*). Molecular mass standards of 180 and 116 kD are marked by dashes on the left.

present in the purified protein or after overnight incubation in the absence of cells (not shown). These data show the formation of fibrils or the incorporation of these fibrils into DOC-insoluble high molecular weight multimers can occur independent of repeats III_{1-7}.

Progression of Fibril Formation

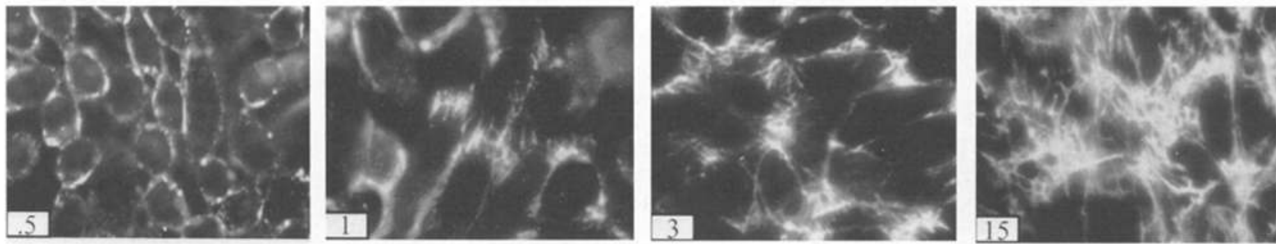
A major advantage of this system is that it allows one to follow the de novo formation of an FN matrix at specific time points after the addition of FN to cells. Fig. 5 illustrates the time course of fibril formation by CHOα5 cells for rat pFN as well as the two recFNs, FNA^-B^- and FNΔIII_{1-7}. At the 30-min time point, binding of FN to the periphery of the cells was observed for all three FNs. Fibril formation by FNA^-B^- was virtually identical to that of rat pFN. Within 1 h of incubation, FN accumulated at the cell surface and resulted in the formation of short fibrils between cells. A fibrillar network formed by 3 h of incubation and increased in density over time.

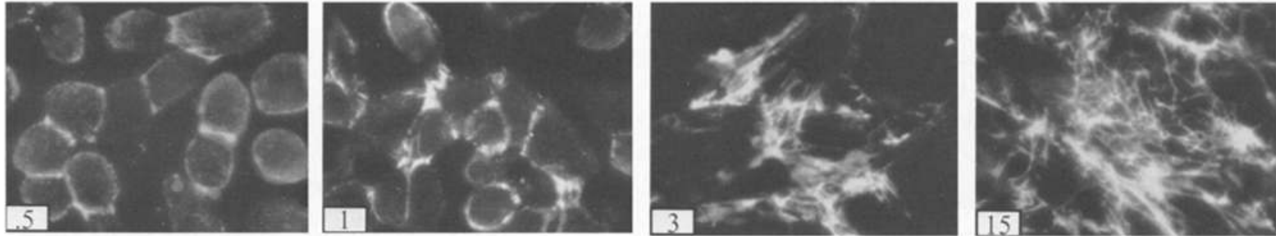
While the organization of FNΔIII_{1-7} at the cell surface was very similar to the other FNs at 30 min, as more protein accumulated a different pattern of FNΔIII_{1-7} matrix was observed (Fig. 5). Rather than the formation of distinct fibrils, as was characteristic of pFN and FNA^-B^-, fluorescent aggregates appeared on the cell surface. By 24 h many of the aggregates had been remodeled into fibrils that extended from one cell to another and appeared somewhat thicker than native FN fibrils. By 48 h, a complex network of fibrils had formed. Therefore, FNΔIII_{1-7} fibrils developed over a significantly longer time frame, taking greater than 12 h longer to form a fibrillar network than full-length FN.

FNΔIII_{1-7} Is Incorporated into DOC-insoluble Material More Rapidly than pFN

FNΔIII_{1-7} associates rapidly with cells followed by gradual assembly into fibrils. To determine the stage at which FNΔIII_{1-7} is incorporated into DOC-insoluble matrix, DOC-soluble and -insoluble fractions were isolated from CHOα5 cells incubated with either pFN, FNA^-B^-, or FNΔIII_{1-7}. All three FNs accumulated as cell-associated DOC-soluble material that was subsequently converted into DOC-insoluble matrix (Fig. 6). DOC-soluble FNs were associated with cells at all time points (*right panels*). High molecular weight, disulfide-stabilized aggregates characteristic of an insoluble matrix increased with time in non-reduced samples from all three matrices (*middle panels*). These high molecular weight multimers were not present in DOC-soluble material. Compared to pFN, significantly more FNA^-B^- and FNΔIII_{1-7} were insoluble by 4 h after addition. Furthermore, analysis of DOC-insoluble protein under reducing conditions suggests that FNΔIII_{1-7} accumulates more rapidly in this fraction than either full-length FN (*left panels*).

Quantitation of DOC-soluble and -insoluble FN revealed a marked difference in the rate of matrix formation between FNΔIII_{1-7} and full-length FN (Fig. 7). First, DOC-insoluble material appeared much faster with FNΔIII_{1-7}. Second, the amount of insoluble FNΔIII_{1-7} reached





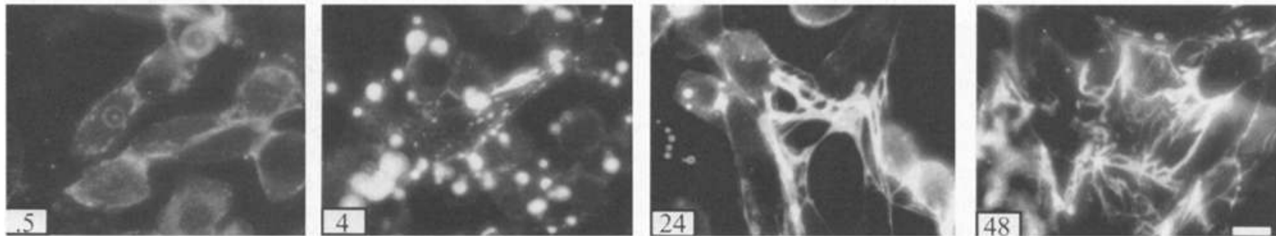


Figure 5. Immunofluorescence analysis of fibril formation at increasing times. Rat pFN (*top*), FN(A^-B^-) (*middle*), and FNΔIII_{1-7} (*bottom*) were added to CHOα5 cells at a concentration of 50 μg/ml. Cells were fixed, stained with rat-specific monoclonal antibody IC3, and visualized by immunofluorescence. Rat pFN and FNA^-B^- were incubated with cells for 0.5, 1, 3, and 15 h (*boxed numbers*). FNΔIII_{1-7} incubations were 0.5, 4, 24, and 48 h as indicated.

maximal amounts (70% of total cell-associated FN) between 4 and 7 h of incubation. In contrast, DOC-insoluble material from rat pFN and FNA^-B^- reached maximal amounts between 7 and 15 h and represented at least 90% of the total cell-associated FN. These results demonstrate an alteration in the timing of the FN matrix assembly process indicating that the region lacking in FNΔIII_{1-7} may play a role in the temporal regulation of fibril formation.

Matrix formation with FNA^-B^- also differed slightly from rat pFN (Fig. 7). The appearance of DOC-insoluble material occurred more quickly for FNA^-B^- than rat pFN, although not nearly to the extent of FNΔIII_{1-7}. The most notable structural difference between FNA^-B^- and pFN is in the alternatively spliced V region. FNA^-B^- consists of V120-V120 homodimers while pFN consists primarily of V^+-V0 heterodimers.

pFN and both recFNs formed disulfide-bonded high molecular weight multimers. However, at the 15-h time point, the percentage of FNΔIII_{1-7} in the multimeric form appeared somewhat less than either pFN or FNA^-B^- (Fig. 6). Reduced accumulation of insoluble multimers might parallel the slower rate of fibril formation by FNΔIII_{1-7}. To test this possibility, FNΔIII_{1-7} and pFN were incubated with CHOα5 cells for 48 h followed by analysis of equal amounts of DOC-insoluble material. As shown in Fig. 8 *A*, the proportion of FNΔIII_{1-7} in the form of high molecular weight multimers was equivalent to that of pFN.

FNΔIII_{1-7} Assembly Is Inhibited by Excess 70 kD

Fibronectin interactions involving the amino-terminal domain are essential for matrix assembly. Inhibition of these interactions by inclusion of excess 70 kD fragment or antibodies against this region disrupts the assembly process (McKeown-Longo and Mosher, 1985; McDonald et al., 1987; Quade and McDonald, 1988). To determine whether FNΔIII_{1-7} is assembled by the same mechanism as FN, we tested whether the addition of 70 kD fragment could inhibit fibril formation. Increasing concentrations of 70 kD fragment caused similar reductions in the amount of DOC-insoluble material formed by both pFN and FNΔIII_{1-7} (Fig. 8 *B*). In addition, fewer fibrils were detected in cultures incubated with pFN or FNΔIII_{1-7} plus 70 kD (Fig. 9). Therefore, similar types of interactions involving the amino-terminal region are used in the assembly of both FNΔIII_{1-7} and pFN. These results also show that the ag-

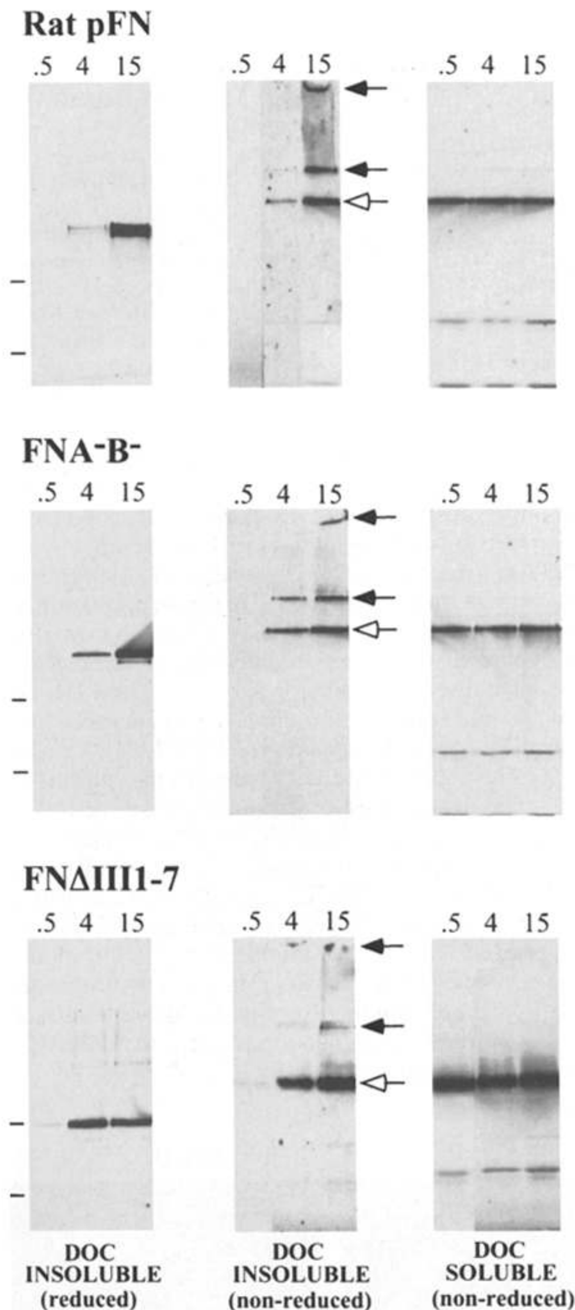


Figure 6. Time course of incorporation of FN and recFNs into DOC-insoluble matrix. DOC-soluble and -insoluble material was isolated from CHOα5 cells after incubation with rat pFN (*top*), FNA^-B^- (*middle*), or $FN\Delta III_{1-7}$ (*bottom*) for 0.5, 4, and 15 h. DOC-insoluble (reduced and nonreduced) and soluble (nonreduced) fractions were separated by 5% SDS-PAGE and FN was detected as in Fig. 4. Dimeric FN and recFN (*open arrows*) accumulate in DOC-insoluble fractions with time. High molecular weight multimers are present at the top of the stacking gel and interface of stacking and separating gels of nonreduced DOC-insoluble material from all three FNs (*solid arrows*). Positions of molecular mass standards of 180 and 116 kD are marked by dashes on the left.

gregation of $FN\Delta III_{1-7}$ observed early in the assembly process involves specific interactions between FN molecules.

RecFNs Lacking RGD Cannot Support Matrix Formation

Antibody blocking experiments have demonstrated the importance of α5β1/RGD interaction for matrix formation (McDonald et al., 1987; Akiyama et al., 1989; Roman et al., 1989; Darribere et al., 1990; Fogerty et al., 1990). However, a recFN lacking RGD (I_{1-9}/C110-RGDS) was fully capable of fibril formation when expressed in SVT2 fibroblasts (Schwarzbauer, 1991). Two recFNs lacking RGD, $FN\Delta III_{1-7}(RGD^-)$, and $FNA^-B^-(RGD^-)$ were tested for their ability to independently support matrix formation. Addition of each of these recFNs to CHOα5 cells resulted only in the binding of FN to the periphery of the cells, principally at regions of cell–cell contact (Fig. 10, *A* and *C*). A fibrillar network was never formed from either of the two proteins, even when added at high concentrations (up to 75 μg/ml). Additions of as much as 50 μg/ml of $FNA^-B^-(RGD^-)$ to AtT-20α5 cells resulted in no detectable fibril formation and no localization to areas of cell–cell contact as observed with CHOα5 cells (Fig. 10 *E*). In addition, neither DOC-soluble nor -insoluble FN was detected from cell lysates with either $recFN(RGD^-)$ (data not shown). Deletion of the RGD from $FN\Delta III_{1-7}$ abolished the ability of this recFN to become incorporated into either DOC-insoluble aggregates or a fibrillar form demonstrating that $FN\Delta III_{1-7}$ matrix assembly requires specific interactions with the cell surface.

To determine whether the RGD sequence is required at all stages of assembly, native FN was included in the incubation. In the presence of human pFN, both $FN\Delta III_{1-7}$ (RGD^-) and $FNA^-B^-(RGD^-)$ were incorporated into DOC-insoluble material (data not shown) and into fibrils as detected by indirect immunofluorescence using a monoclonal antibody specific for rat FN (Fig. 10, *B*, *D*, and *F*). Thus RGD^- recFNs are capable of fibril formation in the presence of native FN. Human pFN was only required to initiate the process of assembly. CHOα5 cells incubated with human pFN for 3 h, washed, and incubated for an additional 14 h with fresh medium containing only $FNA^-B^-(RGD^-)$ formed a recFN matrix similar to that in Fig. 10 *D*. Therefore, the RGD sequence is required for the initiation of matrix formation but is not essential in all incoming FN molecules added at later steps during fibril elongation.

Discussion

In this report, we have described an FN matrix assembly system based on cell lines that are capable of forming a fibrillar matrix solely from exogenous FN. A major advantage of this system is the ability to dissect the independent assembly of recFNs from the earliest stages of binding to cells. In addition, the progression of fibril formation can be analyzed at specific time points after addition of FN, making it possible to determine the stages at which certain FN domains function. These cell lines assemble a recFN lacking repeats III_{1-7} into a matrix, and, like native FN, assembly is dependent on both the 70-kD region and RGD

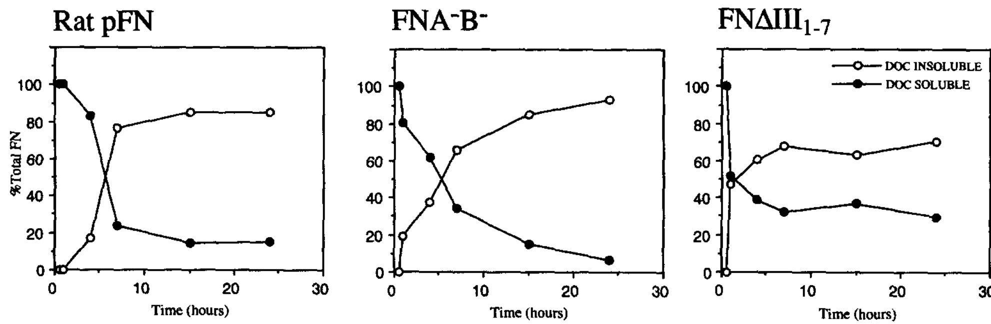


Figure 7. Rate of incorporation of pFN, FNA⁻B⁻, and FNΔIII_{1-7} into DOC-insoluble matrix. Quantitative immunoblot analysis was performed on DOC-soluble and -insoluble cell extracts isolated from CHOα5 cells incubated with each of the three FNs over the indicated periods of time as described under Materials and Methods. Values are expressed in terms of percentage of total cell-associated FN (DOC-soluble plus insoluble) at each time point. In each case, DOC-soluble material (*closed circles*) is converted into DOC-insoluble matrix (*open circles*).

sequence. Differences in the rate of its assembly suggest that the deleted region plays a regulatory role during fibril formation.

Within the first seven type III repeats reside a major FN-binding site in the III_1 module and two low affinity heparin-binding regions in repeat III_1 and repeats III_{4-6} (Hynes, 1990). The FN-binding site appears to be cryptic (Morla and Ruoslahti, 1992; Hocking et al., 1994) but, when exposed, it is able to interact with the first five type I repeats (Aguirre et al., 1994; Hocking et al., 1994). FN–FN interactions involving III_1 or other as yet unidentified sites within repeats III_{1-7} could play a role in regulating matrix assembly. We have postulated that intramolecular interactions between FN-binding sites in I_{1-5} and III_1 domains could hold soluble FN dimers in a compact form that is inactive for assembly (Aguirre et al., 1994). Conformational transitions in FN upon binding to the cell surface could disrupt the intramolecular interactions, activating FN by making the binding sites in I_{1-5} and III_1 available for association with adjacent FN dimers. Removal of one of the binding sites, as in FNΔIII_{1-7}, might yield a constitutively active molecule that behaves differently during the first stages of assembly. Our data fit well with this model. FNΔIII_{1-7} becomes DOC-insoluble significantly faster than full-length FN after binding to α5β1 on the cell surface. The increased rate of accumulation suggests that FNΔIII_{1-7} is already in an activated state.

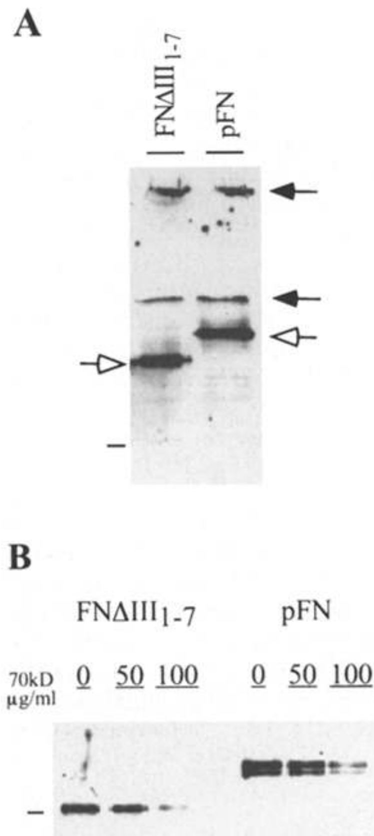


Figure 8. Characterization of DOC-insoluble matrix. (*A*) CHOα5 cells were incubated with medium containing 50 μg/ml FNΔIII_{1-7} or rat pFN for 48 h. DOC-insoluble material was isolated and equal amounts of total protein were analyzed by SDS-PAGE under nonreduced conditions. FN was detected with IC3 antibody and chemiluminescence reagents. High molecular weight multimers (*solid arrows*) and dimeric FN (*open arrows*) are indicated. (*B*) CHOα5 cells were incubated with medium containing 25 μg/ml FNΔIII_{1-7} or rat pFN and 0, 50, or 100 μg/ml 70 kD fragment for 16 h. DOC-insoluble material was isolated and FN detected under reduced conditions as described above. Position of 180 kD molecular mass standard is indicated by dash.

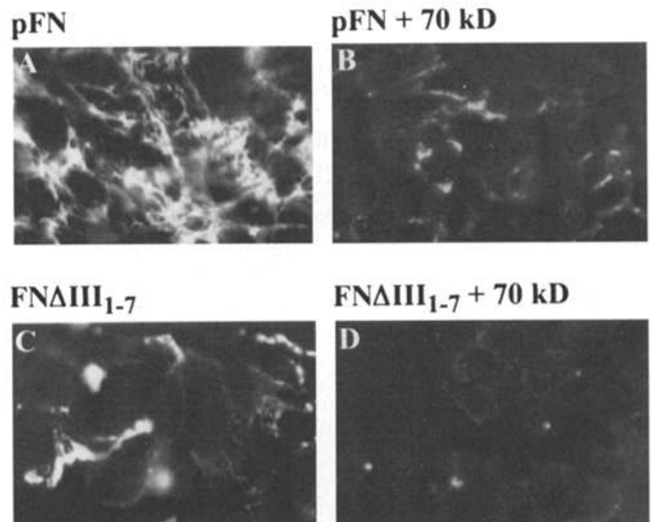
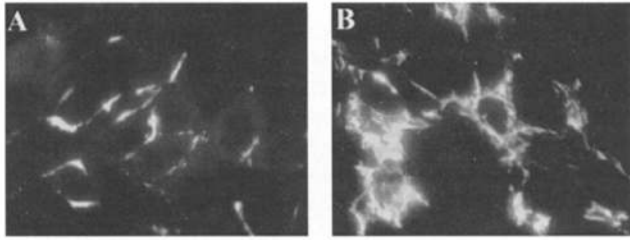
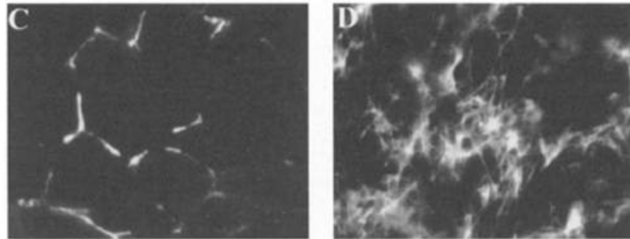


Figure 9. Inhibition of fibril formation with the 70-kD amino-terminal fragment. CHOα5 cells were incubated with 50 μg/ml rat pFN (*A* and *B*) or FNΔIII_{1-7} (*C* and *D*) for 16 h followed by staining for immunofluorescence. In *B* and *D*, ~1 mg/ml 70 kD fragment was included in the incubation.

$CHO\alpha5 + FN\Delta III_{1\text{-}7}(RGD^-)$



$CHO\alpha5 + FNA^-B^-(RGD^-)$



$AtT\text{-}20\alpha5 + FNA^-B^-(RGD^-)$

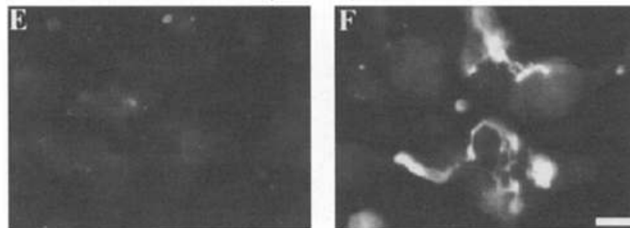


Figure 10. Immunofluorescence staining of recFN fibrils lacking the RGD sequence. CHOα5 or AtT-20α5 cells were incubated with medium containing 50 μg/ml $FN\Delta III_{1\text{-}7}(RGD^-)$ or $FNA^-B^-(RGD^-)$ for 16 h followed by staining for immunofluorescence (*A*, *C*, and *E*). In *B*, *D*, and *F*, cells were incubated with a mixture of 25 μg/ml human pFN plus 25 μg/ml $recFN(RGD^-)$ and stained with monoclonal antibody IC3 which detects only the rat recFNs. Bar equals 10 μm.

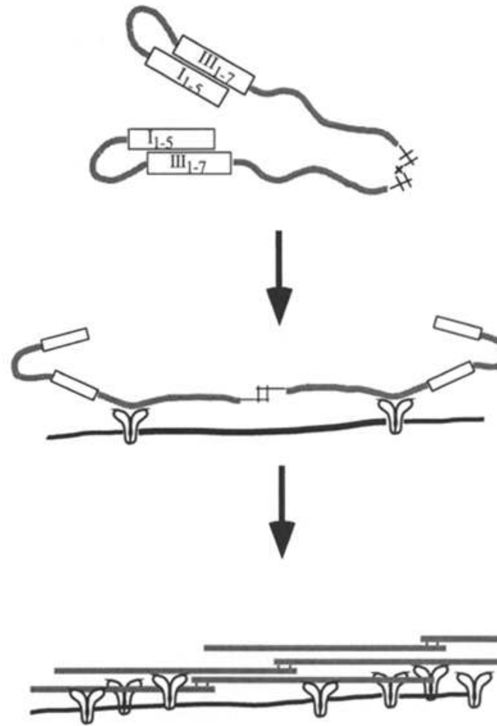


Figure 11. Model for fibronectin matrix assembly. Dimeric fibronectin is secreted from the cell in an inactive form. Intramolecular interactions between FN-binding sites such as the first type III repeats and $I_{1\text{-}5}$ prevent interactions with other FN molecules (*top*). The binding of compact soluble FN to an activated integrin receptor triggers a conformational change and subsequent release of amino-terminal binding sites which are then available for intermolecular interactions and initiation of fibril formation (*middle*). As integrins cluster and the local concentration of cell-associated FN molecules increases, disulfide cross-linking and fibril elongation occur (*bottom*).

A requirement for activation of FN in order for assembly to proceed adds another step to the complex process of matrix assembly. Our model for the major steps is illustrated in Fig. 11. Compact soluble FN binds to α5β1 or other integrin receptors. This activates the molecule by dissociating the intramolecular interactions near the amino terminus and exposing the FN-binding sites. These sites can then participate in FN–FN interactions thus initiating fibril formation. As more FN dimers bind and integrins cluster, the local accumulation of FN results in fibril formation and DOC-insolubility. The matrix then becomes stabilized into high molecular weight disulfide-bonded multimers. There appear to be at least two mechanisms for assembly into fibrils, one involving integrins at the cell surface and another involving interactions with existing FN fibrils. The latter pathway would be the primary route of RGD^-recFN assembly. Fragment inhibition experiments also support the existence of two mechanisms. Fragments containing either the amino-terminal 70 kD or the central cell-binding domain can inhibit FN binding to cell surfaces and thus prevent matrix assembly (McDonald et al., 1987; this report). In contrast, fragments containing III_1 cannot inhibit the initial interactions of FN at the cell surface and do not block de novo assembly (Morla and Ruoslahti, 1992). However, III_1 fragments can inhibit FN incorporation into an established matrix (Chernousov et al., 1991; Morla and Ruoslahti, 1992). Inhibition of distinct steps illustrates the sequential nature of the assembly process. Perhaps different domains and interactions can dominate depending on the site and stage of assembly.

Binding of an activated $FN\Delta III_{1\text{-}7}$ allows the FNs to skip the activation step and proceed more quickly to the next step. Without proper tethering of both ends of the fibrils, they could collapse into aggregates that must be subsequently remodeled and stretched into fibrils. The in vivo formation of $FN\Delta III_{1\text{-}7}$ fibrils might be similar to the in vitro process reported by Morla et al. (1994). In their experiments, FN incubated with a fragment of repeat III_1 formed DOC-insoluble aggregates that contained fibrillar structures visible only after stretching. The DOC-insoluble $FN\Delta III_{1\text{-}7}$ is not completely fibrillar during the early stages of assembly but it can be subsequently remodeled into fibrils. This remodeling takes longer than native FN fibril formation indicating that the $III_{1\text{-}7}$ region plays a role

in coordinating fibrillogenesis with conversion into DOC-insoluble matrix possibly by regulating the rate of activation of FN at the cell surface. It seems unlikely that the rate of assembly is affected by the length of this recFN because inclusion of low levels of native FN were able to modulate the progression of assembly. When FNΔIII_{1-7} (RGD^-) was added to cells along with human pFN, recFN fibril assembly proceeded normally and no cell-associated aggregates were detected by immunofluorescence even at early times (Sechler, J.L., and J.E. Schwarzbauer, unpublished observations). In addition, no aggregates were observed when FNΔIII_{1-7} was expressed in SVT2 fibroblasts (Schwarzbauer, 1991).

Changes in activation state have been clearly demonstrated for integrin receptors (for review see Ginsberg et al., 1992) and represent an important regulatory mechanism. Cells expressing low affinity receptors do not adhere to ligand while those in the high affinity or activated state are able to bind ligand effectively. Faull et al. (1993) have postulated that the activation state of α5β1 integrin might play an important role in FN fibrillogenesis. This is supported by the observation that osteosarcoma cells stimulated with lysophosphatidic acid exhibit enhanced binding of FN (Zhang et al., 1994). More recently it has been shown that the αIIbβ3 integrin supports matrix assembly only when in an activated state (Wu et al., 1995*c*). We propose that the activation of the FN ligand also plays a regulatory role in fibril formation. When assembly is required, inactive FN may bind to an activated receptor which in turn leads to activation of the FN molecule. The dual requirement for activated integrin and activated FN may provide two check points to assure that a matrix is formed only at the appropriate time and place.

While a recFN lacking III_{1-7} can initiate fibrillogenesis independent of full-length FN, recFNs lacking an RGD sequence cannot. Neither AtT-20α5 nor CHOα5 cells were capable of assembling RGD^-FNs into DOC-insoluble material. Indirect immunofluorescence of AtT-20α5 cells incubated with $FNA^-B^-(RGD^-)$ showed no detectable fibrils. However, CHOα5 cells localized a small amount of this recFN to regions of cell–cell contact. The different uses of $FNA^-B^-(RGD^-)$ between the two cell types could be due to interactions with the very low level of FN produced by CHOα5 cells. However, the CHO FN was not detected in DOC-insoluble matrix formed by FNΔIII_{1-7} and low levels of pFN) (< 5 μg/ml) were unable to cause either RGD^-recFN to be detected at the cell surface or in DOC-insoluble material when added to AtT-20α5 cells (Sechler, J.L., and J.E. Schwarzbauer, unpublished observations). It seems more likely that RGD^-recFNs may be interacting with receptors or other proteins present on CHOα5 cells but not on AtT-20α5 cells. For example, Wu et al. (1995*b*) have implicated α3β1 and entactin in the deposition of FN into a matrix by CHO cells in a mechanism which is independent of RGD. Therefore, while α5β1 binding to the RGD sequence appears to be the major mechanism for assembly, other routes are possible.

Although essential, the presence of the RGD sequence is only required during the initial stage of assembly. Preincubation of cells with native human pFN provided sufficient nucleation sites for subsequent incorporation of RGD^-recFNs. These results demonstrate that FN assembly involves RGD-dependent and -independent phases. It is also likely that other regions of FN influence matrix formation. For example, FNA^-B^- which consists of V120-V120 homodimers was able to assemble into DOC-insoluble material at a slightly faster rate than V^+-V0 pFN. The V region apparently has weak FN-binding activity (Aguirre et al., 1994) which could give V120-V120 homodimers a slight advantage in fibril formation or alignment. It is therefore possible that alternative splicing of FN may provide a mechanism to modulate matrix assembly.

In summary, our results demonstrate differential requirements for the RGD sequence and the first type III repeats during FN matrix assembly. RGD-dependent interactions with α5β1 are required to initiate assembly in this system. In contrast, the first type III repeats apparently play a role in regulating the conversion to DOC-insolubility and the rate of fibril formation.

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