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 (FNAIIII_7) 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, FNAIIII_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 FNAIIII_7 follows an assembly pathway similar to FN. The progression of FNAIIII_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_7 modules. The altered rate of FNAIIII_7 assembly suggests that one function of the missing repeats might be to modulate an early stage of matrix formation.

Fibronectin (FN) 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 α5β1 integrin appears to be a major receptor for matrix assembly (Ruoslahti, 1991; Wu et al., 1993). Antibodies that interfere with the interaction between α5β1 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, αIββ3 and αvβ3, have also been shown to participate (Wu et al., 1995c; Wennerberg et al., 1996) and may account for the observation that cells from α5 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., 1995a). 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...
modules I-1-I-7 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; Sottille and Wiley, 1994). In addition, a third region encompassing modules I-3 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-I-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-I-7 (FNΔIII-I-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-I-7 assembly compared to native FN suggest that the III-I-7 region plays a regulatory role in the process.

Materials and Methods

Cell Culture

Mouse A1T-20 pituitary cells were transfected with human a5 integrin cDNA in a retroviral vector (Hyynes et al., 1992) using Lipofectin reagent (Life Technologies/GIBCO-BRL, Gaithersburg, MD). G418 resistant clones were screened for expression of a5 integrin by adhesion to FN-coated surfaces. Expression was confirmed by immunoprecipitation of metabolically labeled cell lysates (Marcantonio and Hyynes, 1988). Clone A1T-20a5 No. 11 was used for all experiments described. A1T-20a5 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 (HyClone Labs, Logan, UT), and 0.25 mg/ml Geneticin (Life Technologies/GIBCO-BRL).

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-I-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 I3 through III-I with NheI-RsrlI. The resulting 4,071 bp fragment was ligated to NheI-RsrlI digested pVL1392-FNΔIII-I-7. For FNΔIII-I-7(RGD)-, a 2,215 bp RsrI-Stul fragment isolated from pLF-1,aCl10-RGDS (Schwarzbauer, 1991) was ligated to pVL1392-FNΔIII-I-7 which had been digested with RsrI-Stul. From the resulting plasmid, a 1.6 kb RsrI-Sall fragment was isolated and ligated to RsrI-Sall digested pVL1392-FNΔIII-I-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 (Merrill et al., 1984) and immunoblot after SDS-PAGE.

Immunofluorescence

Polyclonal rabbit anti-FN antisera 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-20a5 and CHOa5 cells were seeded on glass coverslips in 24-well dishes or Lab Tek Chamber Slides (Nunc Inc., Naperville, IL) at concentrations of 4 x 103 and 1.5 x 103 cells/cm2, respectively. Cells were seeded in medium containing FN-depleted serum and incubated for ~24 h until cells were almost confluent. AtT-20a5 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 inoculated 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 were 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 40X phase/fluorescence or 60X plan-achromatic objective. Photography was performed as described in Schwarzbauer (1991).

**Isolation and Detection of DOC-soluble and -insoluble Material**

AT-20x5 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 iodoacetate 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 iodoacetate 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-Protein 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 B. 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 streptavidin-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, 0.15 M 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 125I-Protein A (New England Nuclear Dupont) was then added to the filter in 10 ml 5% BSA in TBS. The 125I-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 AT-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 AT-20x5 cells express 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, AT-20x5 cells became less adherent and detached from the substrate.

The AT-20x5 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 AT-20x5 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 AT-20x5 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-
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.

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 cysteine 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 III10. RecFNs FNA-B- and FNA-B-(RGD-) are full-length FNs. RecFNs FNAIII1-7 and FNAIII1-7(RGD-) contain and internal 80-kD deletion of repeats III1-7.

A RecFN Lacking Repeats III1-7 Is Capable of Independent Fibril Formation

Recombinant FNAIII1-7 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. FNAIII1-7 was tested for assembly by AtT-20α5 and CHOα5 cells in order to address whether repeats III1-7 are required for this process. Both AtT-20α5 cells (Fig. 3, A and B) and CHOα5 cells (Fig. 3, C and D) assembled FNAIII1-7 into fibrils. These results demonstrate that repeats III1-7, including the FN-binding site in repeat III1, 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 FNAlIII1-7 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 FNAIII1-7 into matrix-associated, DOC-insoluble material. High molecular weight multimers of FNAIII1-7 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...
Immunofluorescence staining of FN\TIl\1 fibrils. AtT-20α5 (top panel) and CHOα5 (bottom panel) cells were incubated for 15 h in serum-free medium containing 25 μg/ml FN\TIl\1 for AtT-20α5 cells (A and B) or 50 μg/ml FN\TIl\1 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.

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 FN\TIl\1. 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 FN\TIl\1 at the cell surface was very similar to the other FNs at 30 min, as more protein accumulated a different pattern of FN\TIl\1 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, FN\TIl\1 fibrils developed over a significantly longer time frame, taking greater than 12 h longer to form a fibrillar network than full-length FN.

FNA\TIl\1 Incorporates into DOC-insoluble Material More Rapidly than pFN

FNA\TIl\1 associates rapidly with cells followed by gradual assembly into fibrils. To determine the stage at which FNA\TIl\1 is incorporated into DOC-insoluble matrix, DOC-soluble and -insoluble fractions were isolated from CHOα5 cells incubated with either pFN, FNA-B-, or FNA\TIl\1. 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 FNA\TIl\1 were insoluble by 4 h after addition. Furthermore, analysis of DOC-insoluble protein under reducing conditions suggests that FNA\TIl\1 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 FNA\TIl\1 and full-length FN (Fig. 7). First, DOC-insoluble material appeared much faster with FNA\TIl\1. Second, the amount of insoluble FNA\TIl\1 reached
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 FNA1111.7 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 FNA1111.7. 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 V7-V0 heterodimers.

pFN and both recFNs formed disulfide-bonded high molecular weight multimers. However, at the 15-h time point, the percentage of FNA1111.7 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 FNA1111.7. To test this possibility, FNA1111.7 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 FNA1111.7 in the form of high molecular weight multimers was equivalent to that of pFN.

**FNA1111.7 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 FNA1111.7 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 FNA1111.7 (Fig. 8 B). In addition, fewer fibrils were detected in cultures incubated with pFN or FNA1111.7 plus 70 kD (Fig. 9). Therefore, similar types of interactions involving the amino-terminal region are used in the assembly of both FNA1111.7 and pFN. These results also show that the ag-

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**Figure 5.** Immunofluorescence analysis of fibril formation at increasing times. Rat pFN (top), FN(A-B-) (middle), and FNA1111.7 (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). FNA1111.7 incubations were 0.5, 4, 24, and 48 h as indicated.
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 FNΔIII1-7 (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.

Ggregation of FNΔIII1-7 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 (I₇₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋_-₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋วิทยา(307,151),(471,183)

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 III1-7 into a matrix, and, like native FN, assembly is dependent on both the 70-kD region and RGD...
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 III1 module and two low affinity heparin-binding regions in repeat III1 and repeats III4-6 (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 III1 or other as yet unidentified sites within repeats III1-7 could play a role in regulating matrix assembly. We have postulated that intramolecular interactions between FN-binding sites in I1-5 and III1 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 I1-5 and III1 available for association with adjacent FN dimers. Removal of one of the binding sites, as in FNAIII1-7, might yield a constitutively active molecule that behaves differently during the first stages of assembly. Our data fit well with this model. FNAIII1-7 becomes DOC-insoluble significantly faster than full-length FN after binding to α5β1 on the cell surface. The increased rate of accumulation suggests that FNAIII1-7 is already in an activated state.

Figure 7. Rate of incorporation of pFN, FNA-B`, and FNAIII1-7 into DOC-insoluble matrix. Quantitative immunoblot analysis was performed on DOC-soluble and -insoluble cell extracts isolated from CHoO5 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).

Figure 8. Characterization of DOC-insoluble matrix. (A) CHoO5 cells were incubated with medium containing 50 μg/ml FNAIII1-7 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) CHoO5 cells were incubated with medium containing 25 μg/ml FNAIII1-7 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. CHoO5 cells were incubated with 50 μg/ml rat pFN (A and B) or FNAIII1-7 (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.
Figure 10. Immunofluorescence staining of recFN fibrils lacking the RGD sequence. CHOα5 or AtT-20α5 cells were incubated with medium containing 50 μg/ml FNΔIII_{1,7}(RGD^{-}) or FNα-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 II_{1,5} 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 αβ1 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 II_{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 FNΔIII_{1,7} allows the FNα 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 FNΔIII_{1,7} 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 FNΔIII_{1,7} 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_{1,7} 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 FNAIII<sub>7</sub> (RGD<sup>-</sup>) 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. Schwarzbrauer, unpublished observations). In addition, no aggregates were observed when FNAIII<sub>7</sub> was expressed in SVT2 fibroblasts (Schwarzbrauer, 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., 1995c). 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<sub>7</sub> can initiate fibrillogenesis independent of full-length FN, recFNs lacking an RGD sequence cannot. Neither α<sub>IT</sub>-<sub>20</sub>α5 nor CHOα5 cells were capable of assembling RGD-containing FN into DOC-insoluble material. Indirect immunofluorescence of α<sub>IT</sub>-<sub>20</sub>α5 cells incubated with FNA<sub>B</sub>(RGD<sup>-</sup>) 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<sub>B</sub>(RGD<sup>-</sup>) 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 FNAIII<sub>7</sub> and low levels of pFN (<5 μg/ml) were unable to cause either RGD<sup>-</sup>recFN to be detected at the cell surface or in DOC-insoluble material when added to α<sub>IT</sub>-<sub>20</sub>α5 cells (Sechler, J.L., and J.E. Schwarzbrauer, unpublished observations). It seems more likely that RGD<sup>-</sup>recFNs may be interacting with receptors or other proteins present on CHOα5 cells but not on μ<sub>IT</sub>-<sub>20</sub>α5 cells. For example, Wu et al. (1995b) 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<sup>-</sup>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<sub>B</sub> which consists of V120-V120 homodimers was able to assemble into DOC-insoluble material at a slightly faster rate than V<sup>+</sup>-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.

We thank Jun-Lin Guan and Richard Hynes for the gift of the human α5 construct. We are also grateful to Marty Fonseca for preparation of the IC5 antibody, Jennifer Luzak and Wilma Fuzon-McLaughlin for technical assistance, and Siobhan Garbett, Kerry Brenner, and Mercedes Doshi-Castro for helpful discussions.

This work was supported by National Institutes of Health grants CA-44627 (to J.E. Schwarzbrauer) and GM-47157 (to Y. Takada), an American Cancer Society grant No. CB-79 (to J.E. Schwarzbrauer), and a grant-in-aid from the New Jersey Commission on Cancer Research (to J. E. Schwarzbrauer). J.L. Sechler is supported by postdoctoral fellowship No.795-052 from the New Jersey Commission on Cancer Research.

Received for publication 18 September 1995 and in revised form 1 May 1996.

References

Aguirre, K.M., R.J. McCormick, and J.E. Schwarzbrauer. 1994. Fibronectin self-association is mediated by complementary sites within the amino-terminal one-third of the molecule. J. Biol. Chem. 269:27863-27868.

Akiyama, S.K., S.S. Yamada, W.-T. Chen, and K.M. Yamada. 1989. Analysis of fibronectin receptor function with monoclonal antibodies: roles in cell adhesion, migration, matrix assembly, and cytoskeletal organization. J. Cell Biol. 109:863-875.

Brockway, P.J. and R.L. Juliano. 1985. Selective inhibition of fibronectin-mediated cell adhesion by monoclonal antibodies to a cell-surface glycoprotein. Science (Wash, DC). 228:1448-1451.

Bunk, M., R. Pytelu, and D. Sheppard. 1992. Characterization of the integrin αβ<sub>1</sub> as a fibronectin-binding protein. J. Biol. Chem. 267:5790-5796.

Castle, A.M., J.E. Schwarzbrauer, R.L. Wright, and J.D. Castle. 1995. Differen- tial targeting of recombinant fibronectin in αIT-20 cells based on their effi- ciency of aggregation. J. Cell Sci. 108:3837-3837.

Chernousov, M.A., A.I. Faerman, M.G. Frid, O. Yu. Prinsenva, and V.E. Kote- liansky. 1988. Monoclonal antibody to fibronectin which inhibits extracellu- lar matrix assembly. FEBS Lett. 217:124-128.

Chernousov, M.A., F.J. Fogerty, V.E. Kotliansky, and D.F. Mosher. 1991. Role of the I-9 and III-1 modules of fibronectin in the formation of an extra- cellular fibronectin matrix. J. Biol. Chem. 266:10851-10858.

Choi, M.G., and R.O. Hynes. 1979. Biosynthesis and processing of fibronectin in NIL.8 hamster cells. J. Biol. Chem. 254:12090-12095.

Darribere, T., K. Guida, H. Larijava, K.E. Johnson, K.M. Yamada, J.-P. Thiery, and J.-C. Boucault. 1990. In vivo analysis of integrin β1 subunit function in fibro- nectin matrix assembly. J. Cell Biol. 110:1813-1822.

Darrabere, T., V.E. Kotliansky, M.A. Chernousov, S.K. Akiyama, K.M. Ya- mada, J.P. Thiery, and J.-C. Boucault. 1992. Distinct regions of human fi- bronectin are essential for fibral assembly in an in vitro developing system. Dev. Dyn. 194:63-70.

Engvall, E., and R. Rouslahti. 1977. Binding of soluble form of fibroblast sur- face protein, fibronectin, to collagen. Int. J. Cancer. 20:1-5.

Faull, R.J., N.L. Kovach, J.M. Harlan, and M.H. Ginsberg. 1993. Affinity modu- lation of integrin αβ1: regulation of the functional response by soluble fi- bronectin. J. Cell Biol. 121:155-162.

Fogerty, F.J., S.K. Akiyama, K.M. Yamada, and D.F. Mosher. 1990. Inhibition of binding of fibronectin to matrix assembly sites by anti-integrin (αβ1) an- tibodies. J. Cell Biol. 111:699-708.

George, E.L., A.N. Georges-Labouesse, R.S. Patel-King, H. Rayburn, and R.O. Hynes. 1993. Defects in mesoderm, neural tube and vascular development in

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mouse embryos lacking fibronectin. Development. 119:1079–1091.

Giancotti, F.G., and E. Ruoslahti. 1990. Elevated levels of the α5β1 fibronectin receptor suppress the transformed phenotype of the Chinese hamster ovary cells. Cell. 60:849–859.

Ginsberg, M.H., X. Du, and E.F. Plow. 1992. Inside-out integrin signalling. Curr. Opin. Cell Biol. 4:766–771.

Hocking, D.C., J. Sottile, and P. McKeown-Longo. 1994. Fibronectin's III-1 module contains a conformation-dependent binding site for the amino-terminal region of fibronectin. J. Biol. Chem. 269:19183–19191.

Hynes, R.O. 1990. Fibronectins. Springer-Verlag, New York. 544 pp.

Hynes, R.O., and A.T. Destree. 1977. Extensive disulfide bonding at the mammalian cell surface. Proc. Natl. Acad. Sci. USA. 74:2855–2859.

Hynes, R.O., E.L. George, E.N. Georges, J.L. Guan, H. Rayburn, and J.T. Yang. 1992. Toward a genetic analysis of cell-matrix adhesion. Cold Spring Harbor Symp. Quans. Biol. 58:249–258.

Keski-Oja, J., D.F. Mosher, and A. Vaheri. 1977. Dimeric character of fibronectin: a major cell surface-associated glycoprotein. Biochem. Biophys. Res. Commun. 74:699–706.

Lin, A.Y., B. Devaux, A. Green, C. Sagerstrom, J.F. Elliott, and M.M. Davis. 1990. Expression of T cell antigen receptor heterodimers in a lipid-linked form. Science (Wash. DC). 249:677–679.

Marcantonio, E.E., and R.O. Hynes. 1988. Antibodies to the conserved cytoplasmic domain of the integrin β1 subunit react with proteins in vertebrates, invertebrates, and fungi. J. Cell Biol. 106:1765–1772.

McDonald, J.A. 1988. Extracellular matrix assembly. Annu. Rev. Cell Biol. 4:183–207.

McDonald, J.A., B.J. Quade, T.J. Broekelman, R. LaChance, K. Forsman, E. Hasegawa, and S. Akilyama. 1987. Fibronectin’s cell-adhesive domain and an amino-terminal matrix assembly domain participate in its assembly into fibroblast pericellular matrix. J. Biol. Chem. 262:2957–2967.

McKeown-Longo, P.J., and D.F. Mosher. 1983. Binding of plasma fibronectin to cells layers of human skin fibroblasts. J. Cell Biol. 97:466–472.

McKeown-Longo, P.J., and D.F. Mosher. 1985. Interaction of the 70,000-mol wt amino terminal fragment of fibronectin with matrix-assembly receptor of fibroblasts. J. Cell Biol. 100:364–374.

Merril, C.R., D. Goldman, and M.L. VanKeuren. 1984. Gel protein stains: silver stain. Methods Enzymol. 104:441–447.

Morla, A., and E. Ruoslahti. 1992. A fibronectin self-assembly site involved in fibronectin matrix assembly: reconstruction in a synthetic peptide. J. Cell Biol. 118:421–429.

Morla, A., Z. Zhang, and E. Ruoslahti. 1994. Superfibronectin is a functionally distinct form of fibronectin. Nature (Lond.). 367:193–196.

Mosher, D.F., editor. 1989. Fibronectin. Academic Press, New York. 474 pp.

Mosher, D.F. 1993. Assembly of fibronectin into extracellular matrix. Curr. Opin. Struct. Biol. 5:214–222.

Mosher, D.F., J. Sottile, C. Wu, and J.A. McDonald. 1992. Assembly of extracellular matrix. Curr. Opin. Cell Biol. 4:810–818.

Quade, B.J., and J.A. McDonald. 1988. Fibronectin's amino-terminal matrix assembly site is located within the 29-KDa amino-terminal domain containing five type I repeats. J. Biol. Chem. 263:19002–19009.

Roman, J., R.M. LaChance, T.J. Broekelmann, C.J.R. Kennedy, E.A. Wagner, W.G. Carter, and J.A. McDonald. 1989. The fibronectin receptor is organized by extracellular matrix fibronectin: implications for oncogenic transformation and for cell recognition of fibronectin matrices. J. Cell Biol. 108:2529–2543.

Ruoslahti, E. 1991. Integrins. J. Clin. Invest. 87:1–5.

Schwarzbauer, J.E. 1991. Identification of the fibronectin sequences required for assembly of a fibrillar matrix. J. Cell Biol. 113:1463–1473.

Schwarzbauer, J.E., C.S. Spencer, and C.L. Wilson. 1989. Selective secretion of alternatively spliced fibronectin variants. J. Cell Biol. 109:3445–3545.

Sottile, J., and S. Wiley. 1984. Assembly of amino-terminal fibronectin dimers into the extracellular matrix. J. Biol. Chem. 269:17192–17198.

Sumners, M.D., and G.E. Smith. 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experimental Station Bulletin 1555. 55 pp.

Wenneberg, K., L. Lehikangas, D. Gullberg, M. Pfaff, S. Johansson, and R. Fassler. 1996. β1 integrin-dependent and -independent polymerization of fibronectin. J. Cell Biol. 132:227–238.

Wu, C., J.S. Bauer, R.L. Juliano, and J.A. McDonald. 1993. The α5 β1 integrin fibronectin receptor, but not the α5 cytoplasmic domain, functions in an early and essential step in fibronectin matrix assembly. J. Biol. Chem. 268:21883–21888.

Wu, C., A.J. Fields, B.A.E. Kapteijin, and J.A., McDonald. 1995a. The role of the α5 β1 integrin fibronectin receptor in cell motility and fibronectin matrix assembly. J. Cell Sci. 108:821–829.

Wu, C., A.E. Chung, and J.A. McDonald. 1995b. A novel role for α3 β1 integrins in extracellular matrix assembly. J. Cell Sci. 108:2511–2523.

Wu, C., V. Kevins, T.E. O'Toole, J.A. McDonald, and M.H. Ginsberg. 1995c. Integrin activation and cytoskeletal interaction are critical steps in the assembly of a fibronectin matrix. Cell. 83:715–724.

Yang, J.T., H. Rayburn, and R.O. Hynes. 1993. Embryonic mesodermal defects in α5 integrin-deficient mice. Development. 119:1093–1105.

Zhang, Q., W.J. Checovich, D.M. Peters, R.M. Albrecht, and D.F. Mosher. 1994. Modulation of cell surface fibronectin assembly sites by lysosphosphatidic acid. J. Cell Biol. 127:1447–1459.

Zhang, Z., A.O. Morla, K. Vuori, J.S. Bauer, R.L. Juliano, and E. Ruoslahti. 1993. The α v β 1 integrin functions as a fibronectin receptor but does not support fibronectin matrix assembly and cell migration on fibronectin. J. Cell Biol. 122:235–242.