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# Modulatory Roles for Integrin Activation and the Synergy Site of Fibronectin during Matrix Assembly

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Initiation of fibronectin (FN) matrix assembly is dependent on specific interactions between FN and cell surface integrin receptors. Here, we show that de novo FN matrix assembly exhibits a slow phase during initiation of fibrillogenesis followed by a more rapid growth phase. $Mn^{2+}$, which acts by enhancing integrin function, increased the rate of FN fibril growth, but only after the initial lag phase. The RGD cell-binding sequence in type III repeat 10 is an absolute requirement for initiation by $\alpha5\beta1$ integrin. To investigate the role of the cell-binding synergy site in the adjacent repeat $III_9$, a full-length recombinant FN containing a synergy mutation, FN(syn$^-$), was tested for its ability to form fibrils. Mutation of this site drastically reduced FN assembly by CHO$\alpha$5 cells. Only sparse short fibrils were formed even after prolonged incubation, indicating that FN(syn$^-$) is defective in progression of the assembly process. These results show that the synergy site is essential for $\alpha5\beta1$-mediated accumulation of a FN matrix. However, the incorporation of FN(syn$^-$) into fibrils and the deoxycholate-insoluble matrix could be stimulated by $Mn^{2+}$. Therefore, exogenous activation of integrin receptors can overcome the requirement for FN's synergy site as well as modulate the rate of FN matrix formation.

## INTRODUCTION

The interaction of cell surface integrin receptors with the extracellular matrix plays a critical role in the regulation of a number of cellular functions including adhesion and migration, cytoskeletal organization, and cell cycle progression (reviewed in Hynes, 1990; 1992; Yamada and Miyamoto, 1995; Assoian, 1997). Fibronectin (FN)[1] is an essential extracellular matrix component as demonstrated by the fact that null mutations in either FN or its integrin receptors result in embryonic lethality (George *et al.*, 1993; reviewed in Fassler *et al.*, 1996). Oncogenically transformed cells generally show reduced FN expression, further supporting a role for the FN matrix in regulating cell morphology and growth (Yamada *et al.*, 1976; Ali *et al.*, 1977; Giancotti and Ruoslahti, 1990).

The majority of integrin-mediated interactions with FN occur through the arg-gly-asp (RGD) cell-binding sequence in repeat $III_{10}$ (Ruoslahti, 1991; Hynes, 1992). For some integrins, such as $\alpha v\beta3$, the RGD sequence alone is sufficient to support adhesion to FN (Bowditch *et al.*, 1994; Danen *et al.*, 1995). Other integrins, including $\alpha5\beta1$ and $\alpha IIb\beta3$, require the presence of additional domains (Aota *et al.*, 1991; Bowditch *et al.*, 1991). For example, adhesion of cells to FN via $\alpha5\beta1$ integrin requires not only the RGD site but a second synergy site corresponding to the sequence pro-his-ser-arg-asp (PHSRN) in repeat $III_9$ (Aota *et al.*, 1994). Both sites are also required for *Xenopus* gastrulation, providing additional functional evidence for the importance of the synergy site (Ramos and DeSimone, 1996). RGD-mediated adhesion can also be modulated by exogenous activators. For example, binding of certain antibodies to integrin extracellular domains can activate the receptors from a low- to high-affinity state for ligand binding (Frelinger *et al.*, 1991; Arroyo *et al.*, 1992; Kovach *et al.*, 1992). Divalent cations such as $Mn^{2+}$ can also stimulate integrin activity and interactions

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[1] Abbreviations used: DOC, deoxycholate; FN, fibronectin; FN(RGD$^-$), recombinant FN lacking the RGD cell-binding sequence; FN(syn$^-$), recombinant FN with mutation of the synergy site; pFN, plasma fibronectin; recFN, recombinant FN; PMA, phorbol 12-myristate 13-acetate.

with ligand (Gailit and Ruoslahti, 1988; Bazzoni *et al.*, 1995; Mould *et al.*, 1995; reviewed in Humphries, 1996; Mould, 1996).

In addition to its role in cell adhesion, the RGD sequence is also essential for assembly of FN into a complex fibrillar matrix. Recombinant FNs (recFNs) containing a deletion of the RGD site are unable to initiate matrix assembly (Sechler *et al.*, 1996). Furthermore, antibodies to $\alpha5\beta1$ integrin or the cell-binding domain of FN can inhibit assembly (Akiyama *et al.*, 1989; Roman *et al.*, 1989; Fogerty *et al.*, 1990; Nagai *et al.*, 1991). Following integrin ligation, this multistep assembly process proceeds through specific interactions between individual FN molecules culminating in the formation of a detergent-insoluble matrix (McDonald, 1988; Schwarzbauer, 1991; Morla and Ruoslahti, 1992; Mosher, 1993; Aguirre *et al.*, 1994; Hocking *et al.*, 1994). Some of the interactions during these later stages are RGD independent, suggesting that integrins might not be involved in all stages of FN assembly.

Although $\alpha5\beta1$ is the major receptor on cells that assemble FN fibrils, other integrins are also able to participate in this process (Wu *et al.*, 1995, 1996; Wennerberg *et al.*, 1996; Yang and Hynes, 1996). For $\alpha$IIb$\beta3$ and $\alpha$v$\beta3$ integrins, efficient assembly is dependent on antibody activation of the receptors to a high-affinity state for ligand binding. As $\alpha$v integrins bind FN via the RGD sequence, this raises the question whether the synergy site plays a role in any of the stages of FN assembly.

In this report, we show that the dual interaction of the RGD and synergy sites with $\alpha5\beta1$ integrin is required to fully support matrix assembly. De novo assembly of FN progressed through a slow phase of fibril initiation which was followed by a more rapid accumulation of a deoxycholate (DOC)-insoluble matrix. In contrast, assembly of FN(syn$^-$), a recFN containing a mutation in the synergy site, was stalled during the initiation phase. Exogenous stimulation of $\alpha5\beta1$ function with $Mn^{2+}$ overcame the block in assembly of FN(syn$^-$). $Mn^{2+}$ activation of $\alpha5\beta1$ also affected assembly of FN, resulting in a dramatic increase in the rate of conversion of fibrils into a DOC-insoluble matrix. With both native and mutant FNs, the stimulatory effect occurred only after the assembly of fibrils had been initiated. These results show that the required levels of integrin activity differ for $\alpha5\beta1$-mediated initiation and for accumulation of dense matrix. In the absence of synergy site binding, the latter step can be induced by exogenous integrin activators. It is also possible that the assembly of a FN matrix may itself activate $\alpha5\beta1$ in an RGD and synergy site-dependent process.

## MATERIALS AND METHODS

### *Antibodies and Reagents*

Ascites fluid was isolated from the previously described hybridoma cells producing rat-specific monoclonal antibody IC3 (Sechler *et al.*, 1996; Sechler and Schwarzbauer, 1997). The function blocking antihamster $\alpha5$ antibody PB1 (Brown and Juliano, 1985, 1988) and function blocking antihuman $\alpha5$ antibody m16 (Akiyama *et al.*, 1989) were kindly provided by R.L. Juliano (University of North Carolina-Chapel Hill, Chapel Hill, NC) and K. Yamada (National Institutes of Health, Bethesda, MD), respectively. The activating anti-$\beta3$ antibody LIBS6 (Frelinger *et al.*, 1991) was provided by M.H. Ginsberg (Scripps Research Institute, La Jolla, CA). Fluorescein-conjugated goat anti-mouse IgG was purchased from Molecular Probes (Eugene, OR). Cycloheximide was purchased from Sigma Chemical Co. (St. Louis, MO).

### *Cell Culture*

CHO$\alpha5$ cells, clone 17, transfected with a cDNA to the human $\alpha5$ integrin subunit have been described previously (Sechler *et al.*, 1996; Sechler and Schwarzbauer, 1997). For all experiments CHO$\alpha5$ cells were cultured in DMEM, 2 mM glutamine, 1% nonessential amino acids, 100 $\mu$g/ml Geneticin (Life Technologies, Grand Island, NY), and 10% fetal calf serum (Hyclone Labs, Logan, UT) depleted of FN by gelatin-agarose affinity chromatography. The CHO K1 $\alpha$v$\beta3$ cell line (described in Wu *et al.*, 1996) was kindly provided by M.H. Ginsberg (Scripps Research Institute) and maintained in DMEM supplemented with 10% fetal calf serum (Hyclone Labs), 2 mM glutamine, and 1% nonessential amino acids. For immunofluorescence experiments, FN-depleted fetal calf serum was used.

### *FN cDNA Construction and Recombinant Protein Purification*

Mutation of the synergy site was generated using polymerase chain reaction (PCR) amplification with a mutant oligonucleotide. The mutant primer, FNPSR/GSE (5′-TAGAATTCTCTGAGCCCG-GCACTCGG-3′), was prepared by the Synthesis and Sequencing Facility (Princeton University, Princeton, NJ) and spans nucleotides 4507 to 4482 of the rat FN cDNA. Base pair changes are underlined and change the codons from proline to glycine and from arginine to glutamate at amino acid positions 1498 and 1500, respectively. PCR amplification was performed using FNPSR/GSE and an upstream primer for 30 cycles under the following conditions: 95°C, 30 s; 37°C, 60 s; and 72°C, 60 s. The 690-bp product was digested with *Rsr*II and *Eco*RI (within the primer) and the resulting 395-bp fragment was then used to insert the synergy site mutation into the FN cDNA in pVL1392. The mutation was confirmed following construction by restriction digests and sequencing.

Creation of recombinant baculovirus and purification of recombinant protein were performed as described (Aguirre *et al.*, 1994; Sechler *et al.*, 1996). For purification of recombinant FN protein, baculovirus-infected BTI-TN-5B-4 (High Five) insect cells (Invitrogen Corp., San Diego, CA) were grown in Express Five serum-free medium (Life Technologies). Rat plasma FN (pFN) and recombinant FN (recFN) were purified by gelatin agarose chromatography. Approximately 1 mg of recFN was purified from $5 \times 10^7$ infected cells. Since insect cells do not synthesize endogenous FN, recFN preparations were free of contaminating FN. RecFNs were stored in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11) and 150 mM NaCl at −80°C.

### *Immunofluorescence*

CHO$\alpha5$ cells were seeded in medium containing FN-depleted serum onto glass coverslips in 24-well dishes at a concentration of $2.0 \times 10^5$ cells/cm$^2$. A nearly confluent monolayer resulted after an overnight incubation. pFN or FN(syn$^-$) was added to cells along with fresh medium and incubated for the specified period of time.

Cells were then washed with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde for 15 min at room temperature. Coverslips were washed with PBS and incubated for 30 min at 37°C with IC3 ascites diluted 1:1000 in PBS with 2% ovalbumin. Following incubation with IC3, coverslips were washed with PBS and incubated with fluorescein-conjugated goat anti-mouse secondary antibody at a concentration of 1:400. After a final wash with PBS, coverslips were mounted onto microscope slides with FITC-guard (Testog, Inc., Chicago, IL).

CHO K1 $\alpha v\beta 3$ cells were seeded at a concentration of $5 \times 10^5$ cells/cm$^2$ onto glass coverslips in a 24-well dish with medium containing FN-depleted fetal calf serum. Cells were allowed to attach for 1 h, washed with medium containing 20 $\mu$g/ml cycloheximide, and incubated with fresh cycloheximide-containing medium for an additional hour. After another wash, medium containing 20 $\mu$g/ml cycloheximide, 40 $\mu$g/ml PB1 antihamster $\alpha 5$ antibody, 300 $\mu$g/ml LIBS6 anti-$\beta 3$-activating antibody, and either 75 $\mu$g/ml pFN or FN(syn$^-$) was added to the cells and incubated for an additional 6 or 22 h. Cycloheximide treatment decreased the production of endogenous FN to undetectable levels as determined by labeling with [$^{35}$S]methionine and gelatin binding. Immunofluorescence staining was then performed as described above. Staining was visualized with a Nikon Optiphot-2 microscope using a 40× planapochromatic objective, and photography was performed as described in the study by Schwarzbauer (1991).

### *Isolation, Detection, and Quantitation of DOC-soluble and -insoluble Material*

CHO$\alpha 5$ cells were cultured in a 24-well dish as described above except in the absence of glass coverslips. pFN and FN(syn$^-$) were incubated with the cells for defined time periods. After the incubation period, cells were washed with serum-free DMEM and lysed in 200 $\mu$l of DOC lysis buffer (2% DOC, 0.02 M Tris-HCl, pH 8.8, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM iodoacetic acid, and 2 mM *N*-ethylmaleimide) per well. DOC-insoluble material was isolated and aliquots of DOC-soluble and -insoluble material were separated by SDS-PAGE. Immunodetection and quantitation were performed as described (Sechler *et al.*, 1996) with the exception that IC3 ascites was used at a dilution of 1:10,000. In initial experiments, immunoblots were developed with chemiluminescent reagents (Pierce, Rockford, IL) and repeated at least twice. To quantify results, experiments were repeated in at least two separate trials using $^{125}$I-labeled protein A as described (Sechler *et al.*, 1996).

### *$Mn^{2+}$ Stimulation and Integrin Function Blocking*

CHO$\alpha 5$ cells were seeded in a 24-well dish with glass coverslips (for immunofluorescence experiments) or without coverslips (for biochemical analysis) and incubated overnight. Fresh medium containing $MnCl_2$ and either pFN or FN(syn$^-$) was then added to the CHO$\alpha 5$ cells and incubated for defined time periods. Incubations in the presence of 0.1 and 0.2 mM $MnCl_2$ were performed for up to 16 h, while exposure to 1 mM $MnCl_2$ was limited to a maximum of 4 h. Anti-integrin function blocking experiments were performed by adding FN(syn$^-$) to CHO$\alpha 5$ cells and incubating the cells for 16 h. One millimolar $MnCl_2$ was then added to culture medium either alone or with 50 $\mu$g/ml m16 and 20 $\mu$g/ml PB1 anti-$\alpha 5$ antibodies. Immunofluorescence and isolation of DOC-insoluble and -soluble material was then performed as described above.

## RESULTS

### *The Synergy Site Is Required for FN Fibril Assembly*

A full-length recFN was constructed to contain a PPSRN to PGSEN mutation in repeat III$_9$ of rat FN in a region corresponding to the human FN PHSRN synergy site (Aota *et al.*, 1994). Recombinant protein [FN(syn$^-$)] was generated with the baculovirus expression system and purified by gelatin-agarose chromatography. FN(syn$^-$) and native pFN were added to the culture medium of CHO$\alpha 5$ cells at a concentration of 25 $\mu$g/ml and incubated for the times specified (Figure 1). These cells lack endogenous FN but will assemble a matrix when provided with exogenous FN (Sechler *et al.*, 1996). FN fibrils were then visualized by indirect immunofluorescence. pFN was assembled into short fibrils by 1 h of incubation (Figure 1A), and an increase in the density and length of these fibrils was observed after longer periods of incubation (Figure 1, B and C). In contrast, at least 6 h of incubation were required to detect any FN(syn$^-$) at the cell surface (Figure 1D). By 16 h, FN(syn$^-$) was assembled into very sparse short fibrils (Figure 1E), suggesting that mutation of the synergy site delays the assembly of this recFN. Increasing the concentration of FN(syn$^-$) to 50 $\mu$g/ml also gave a similar sparse matrix. There was no significant increase in length or number of fibrils after 48 h of incubation (Figure 1F), demonstrating that the absence of a synergy site prevents rather than delays assembly.

During FN matrix assembly, fibrils are gradually converted from a DOC-soluble into a DOC-insoluble form. The amount of DOC-insoluble matrix after 16 h of incubation with FN(syn$^-$) was similar to DOC-insoluble pFN after only 2 h (Figure 2,A and B). Little change was observed in FN(syn$^-$) accumulation after 16 h. Furthermore, the amount of FN(syn$^-$) DOC-insoluble matrix was only slightly greater than that isolated from cells with FN(RGD$^-$) (Figure 2B). These results show that FN(syn$^-$) is defective in the initiation and progression of assembly.

### *Activated $\alpha v\beta 3$ Integrin Supports FN(syn$^-$) Matrix Assembly*

Function blocking anti-$\alpha 5$ integrin antibodies inhibit FN fibril formation by CHO$\alpha 5$ cells, indicating that the $\alpha 5\beta 1$ integrin is the principle receptor supporting matrix assembly in these cells (our unpublished observations). However, activated $\alpha v\beta 3$ and $\alpha IIb\beta 3$ integrins can also participate in FN matrix assembly (Wu *et al.*, 1995, 1996). Unlike $\alpha 5\beta 1$ which requires both the RGD and synergy sites for maximal cell adhesion to FN, $\alpha v\beta 3$ recognizes only the RGD sequence (Bowditch *et al.*, 1994; Danen *et al.*, 1995). Although CHO$\alpha 5$ cells showed a 50% reduction in adhesion to FN(syn$^-$) compared with pFN, $\alpha v\beta 3$-mediated adhesion of CHO-$\alpha v\beta 3$ cells was not significantly different. CHO-$\alpha v\beta 3$ cells were used to determine whether FN(syn$^-$) is capable of forming fibrils. An anti-$\beta 3$-activating antibody (LIBS6) was included to enhance $\alpha v\beta 3$ activity. As shown in Figure 3, when $\alpha v\beta 3$ integrin was used as the FN receptor, FN(syn$^-$) and pFN were assembled into comparable matrices as assessed by both the

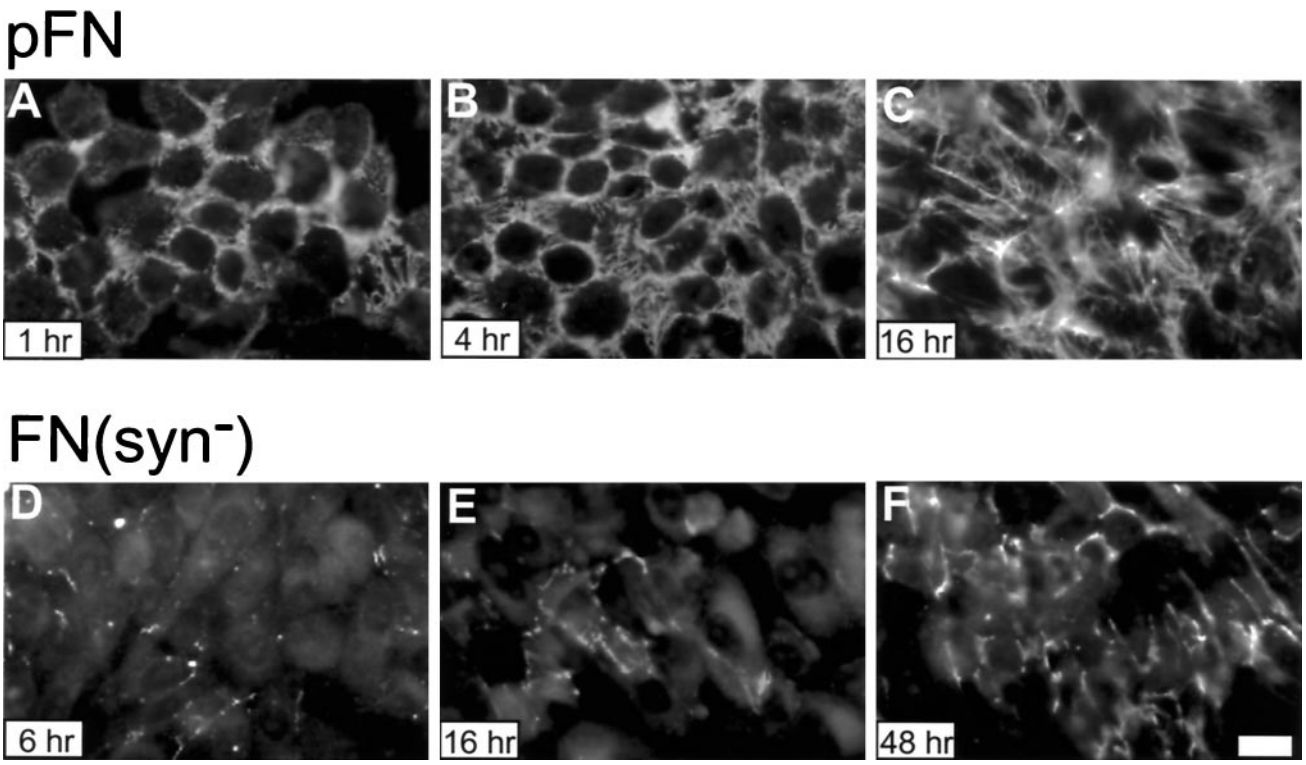


**Figure 1.** Time course of fibril formation by CHO$\alpha$5 cells. CHO$\alpha$5 cells were cultured in medium containing FN-depleted serum and incubated with 25 $\mu$g/ml pFN (A–C) and FN(syn$^-$) (D–F) for the times indicated. FN fibrils were visualized by indirect immunofluorescence with monoclonal antibody IC3 specific for rat FN. Bar, 10 $\mu$m.

amount and morphology of fibrils formed. Therefore, the synergy site is not essential for fibril assembly by activated $\alpha v\beta 3$ receptor.

### *Rate of FN Assembly Can Be Increased with the Addition of $Mn^{2+}$*

Activating antibodies can induce high-affinity binding of integrins to their ligands (O'Toole *et al.*, 1990; Faull *et al.*, 1993). Similarly, divalent cations such as $Mn^{2+}$ have been shown to change integrin binding affinity (Gailit and Ruoslahti, 1988; Bazzoni *et al.*, 1995) and $Mn^{2+}$ can increase the binding of $\alpha 5\beta 1$ integrin receptor to fibronectin (Gailit and Ruoslahti, 1988; Mould *et al.*, 1995). To determine whether $Mn^{2+}$ stimulation of $\alpha 5\beta 1$ integrin can enhance FN assembly, $MnCl_2$ was added to CHO$\alpha$5 culture medium along with 25

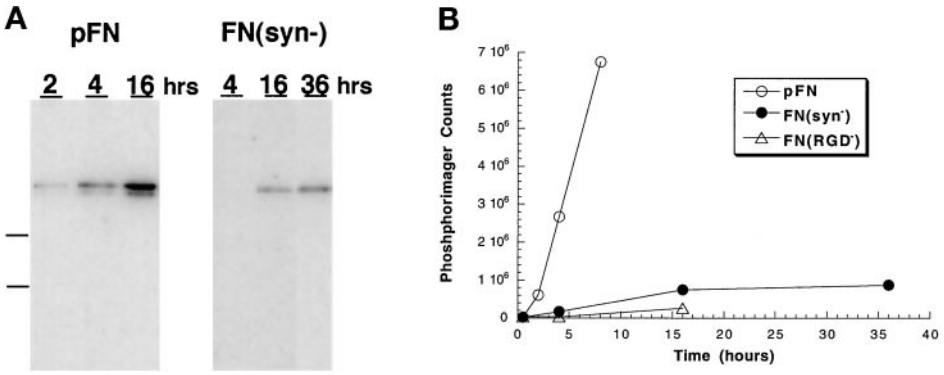


**Figure 2.** Native and recFN incorporation into DOC-insoluble matrix. (A) Immunoblot analysis of incorporation of pFN and FN(syn$^-$) into DOC-insoluble matrix. DOC-insoluble material was extracted from CHO$\alpha$5 cells incubated with 25 $\mu$g/ml pFN or FN(syn$^-$) at the times indicated (h) and separated by 5% SDS-PAGE under reduced conditions. FN was detected with monoclonal antibody IC3. Molecular weight markers 180 and 116 kDa are indicated by dashed lines. (B) Quantification of DOC-insoluble FN isolated from CHO$\alpha$5 cells incubated with 25 $\mu$g/ml pFN (open circles), FN(syn$^-$) (closed circles), or FN(RGD$^-$) (open triangles). The amount of DOC-insoluble FN was quantitated using a Molecular Dynamics PhosphorImager, and values are expressed as total phosphorimager counts. Data are from a single experiment and are representative of results from at least four independent experiments as described in MATERIALS AND METHODS.

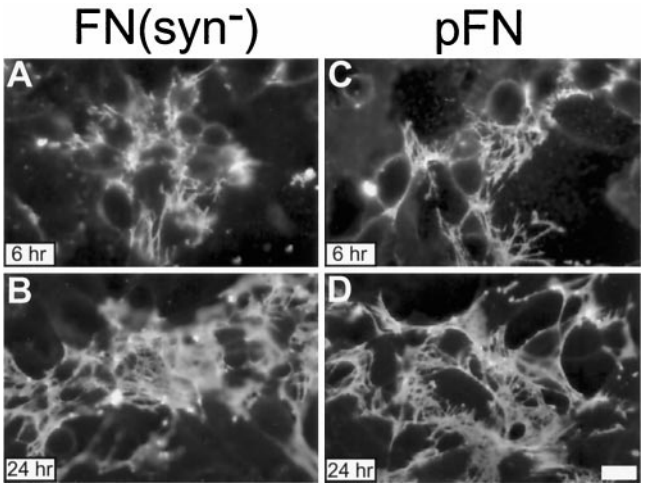


**Figure 3.** FN(syn$^-$) and pFN matrix assembly supported by $\alpha v\beta 3$. $\alpha v\beta 3$-transfected CHO K1 cells were incubated with 20 $\mu$g/ml cycloheximide to inhibit endogenous FN synthesis, monoclonal antihamster $\alpha 5$ integrin function blocking antibody, activating LIBS6 anti-$\beta 3$ antibody, and 25 $\mu$g/ml of either FN(syn$^-$) (A and B) or pFN (C and D) for 6 and 24 h. FN fibrils were visualized by indirect immunofluorescence using monoclonal antibody IC3. Bar, 10 $\mu$m.

$\mu$g/ml pFN. No noticeable difference in assembly between $Mn^{2+}$-treated and untreated cells was observed after 0.5 h of incubation (Figure 4, A and C). However, by 4 h, the pFN matrix assembled by $Mn^{2+}$-treated cells appeared to be more dense than that of untreated cells (Figure 4, B and D). Wild-type recFN (FNA$^-$B$^-$; Sechler *et al.*, 1996) showed the same $Mn^{2+}$-stimulated increase in matrix assembly as pFN. The presence of $Mn^{2+}$ was not required for the entire 4-h incubation. A matrix comparable to that shown in Figure 4D was assembled when $Mn^{2+}$ was added 2 h after pFN and incubated for 2 additional h.

De novo FN assembly into DOC-insoluble matrix follows a pattern similar to formation of a linear polymer with a slow initiation phase followed by a more rapid growth phase. In untreated CHO$\alpha$5 cells, the accumulation of total cell-associated FN increased steadily over time in direct proportion to the input FN concentration (Figure 5A). Incorporation into DOC-insoluble matrix, however, occurred slowly at early times but showed an increase after a lag period of several hours (Figure 5B). The initiation phase is more clearly evident with the addition of $Mn^{2+}$ which accelerated total FN binding and incorporation into DOC-insoluble material after 2 h but had no apparent effect before that time (Figure 5B). $Mn^{2+}$ stimulation had a more dramatic effect on the proportion of DOC-insoluble FN. More than 80% of the total FN was DOC insoluble with $Mn^{2+}$ as compared with 35% without $Mn^{2+}$ (Figure 5C). Therefore, $Mn^{2+}$ speeds up the conversion from DOC-soluble to DOC-insoluble matrix. This is not simply due to an increase in the amount of FN bound to the cell surface because doubling the FN concentration did not significantly increase the proportion of DOC-insoluble matrix.

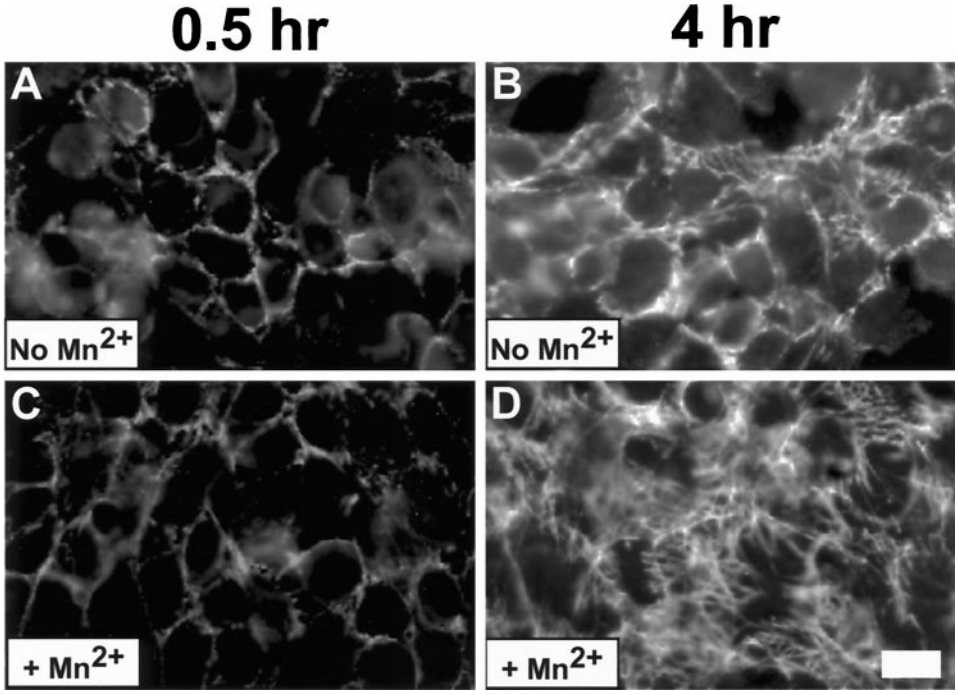


**Figure 4.** Effect of $Mn^{2+}$ on FN matrix assembly. CHO$\alpha$5 cells were incubated with 25 $\mu$g/ml pFN in the presence or absence of 1 mM $MnCl_2$ for 0.5 h (A and C) and 4 h (B and D) followed by staining for FN fibrils by immunofluorescence. Bar, 10 $\mu$m.

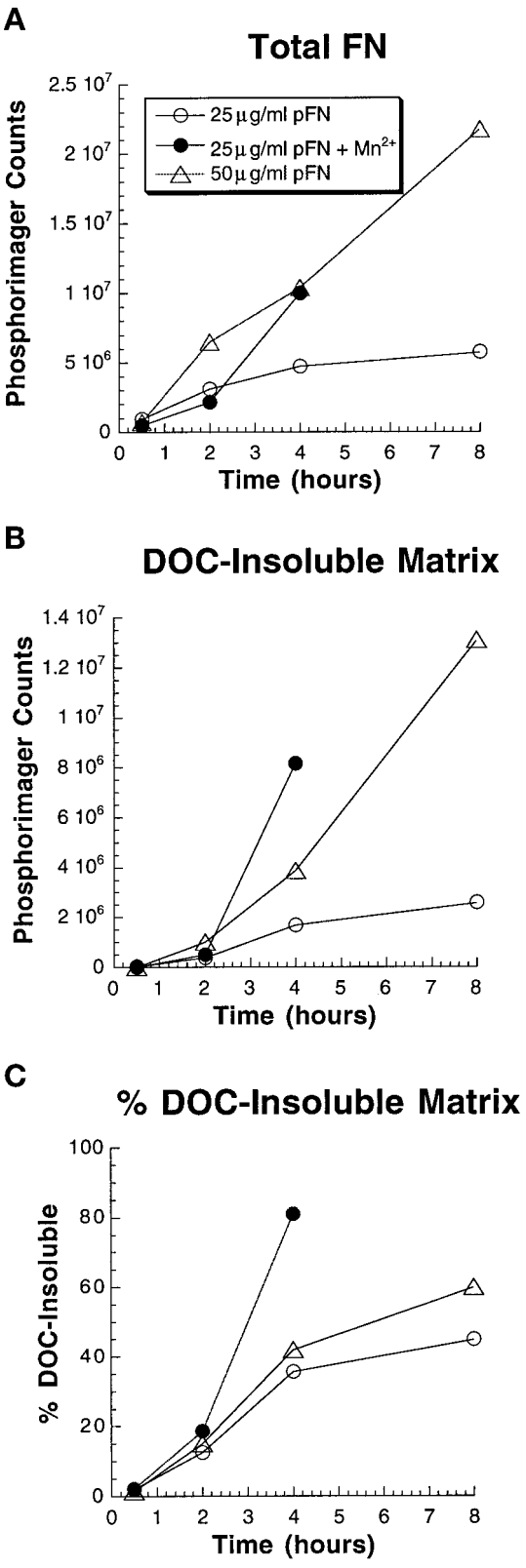


**Figure 5.** Rate of incorporation of FN into DOC-insoluble matrix in the presence or absence of $Mn^{2+}$. Quantitative immunoblot analysis was performed on DOC-insoluble and -soluble extracts isolated from CHO$\alpha$5 cells incubated with 25 $\mu$g/ml pFN alone (open circles), 25 $\mu$g/ml with 1 mM $MnCl_2$ (closed circles), or 50 $\mu$g/ml pFN alone (open triangles) over the indicated periods of time. Accumulation of total cell-associated FN (A), DOC-insoluble FN matrix (B), and DOC-insoluble FN as percentage of total (C) was determined. Data are from a single experiment and are representative of resuts from at least four independent experiments as described in MATERIALS AND METHODS.

### *$Mn^{2+}$ Activation of $\alpha5\beta1$ Allows Assembly of FN(syn$^-$)*

An initiation phase of several hours is observed before significant conversion of FN fibrils into DOC-insoluble matrix. FN(syn$^-$) appears to be arrested in this phase, since little DOC-insoluble material is recovered even after prolonged incubation (see Figure 2). Because $Mn^{2+}$ can increase matrix accumulation of pFN by CHO$\alpha$5 cells, we tested its effects on FN(syn$^-$) assembly. Incubation for up to 4 h in the presence of 1 mM $MnCl_2$ did not increase incorporation of FN(syn$^-$) into fibrils or DOC-insoluble material. However, a longer 16-h incubation with 0.2 mM $MnCl_2$ did stimulate assembly of FN(syn$^-$) into a fibrillar matrix (Figure 6). Apparently, $Mn^{2+}$ cannot exert an effect on FN(syn$^-$) assembly until significantly later due to the extended initiation phase observed with the mutant protein.

To test the effects of $Mn^{2+}$ after initiation of FN(syn$^-$) fibril assembly, 1 mM $MnCl_2$ was added to CHO$\alpha$5 cells that had already formed sparse fibrils of FN(syn$^-$). A substantial increase in the amount of fibrillar FN(syn$^-$) was evident after only 4 h of incubation with $Mn^{2+}$ (Figure 7A). The increase in fibrillar FN(syn$^-$) also corresponded to a significant accumulation of DOC-insoluble material (Figure 7B). $Mn^{2+}$ stimulation failed to enhance the assembly of FN(RGD$^-$) into a matrix.

To confirm that $\alpha5\beta1$ is responsible for $Mn^{2+}$ stimulation of FN(syn$^-$) matrix assembly, function blocking anti-integrin antibodies and 1 mM $MnCl_2$ were added simultaneously to CHO$\alpha$5 cells with FN(syn$^-$) matrix. As shown in Figure 8, antihuman and antihamster $\alpha5$ integrin antibodies inhibited FN(syn$^-$) matrix formation. Together, these results demonstrate that the block in progression of FN(syn$^-$) matrix assembly can be reversed by altering $\alpha5\beta1$ integrin affinity by $Mn^{2+}$ stimulation.

### *PMA Does Not Stimulate FN Matrix Assembly by CHO$\alpha$5 Cells*

Other agents such as phorbol esters have been shown to increase cell adhesion (Danilov and Juliano, 1989; Vuori and Ruoslahti, 1993; Faull *et al.*, 1994) and matrix assembly (Somers and Mosher, 1993) by integrin receptors. Phorbol 12-myristate 13-acetate (PMA) increases cell adhesion to FN without inducing an in-

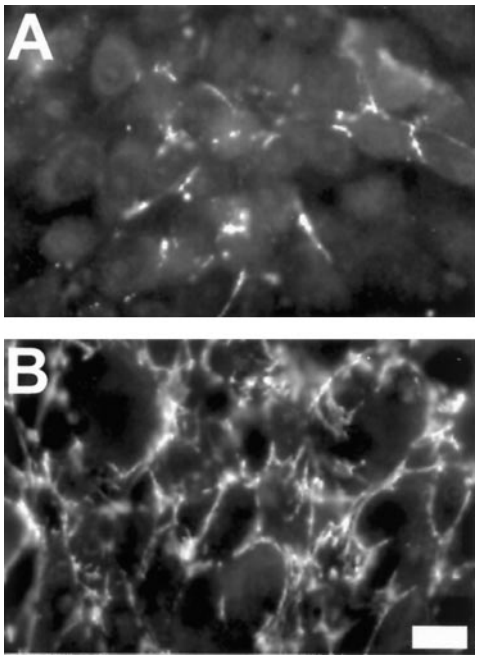


**Figure 6.** FN(syn$^-$) assembly in response to $Mn^{2+}$ addition. Twenty-five micrograms per milliliter FN(syn$^-$) were added to CHO$\alpha$5 cells along with no $MnCl_2$ (A) or 0.2 mM $MnCl_2$ (B) and incubated for 16 h. Cells were then fixed and FN fibrils were detected by immunofluorescence. Bar, 10 $\mu$m.

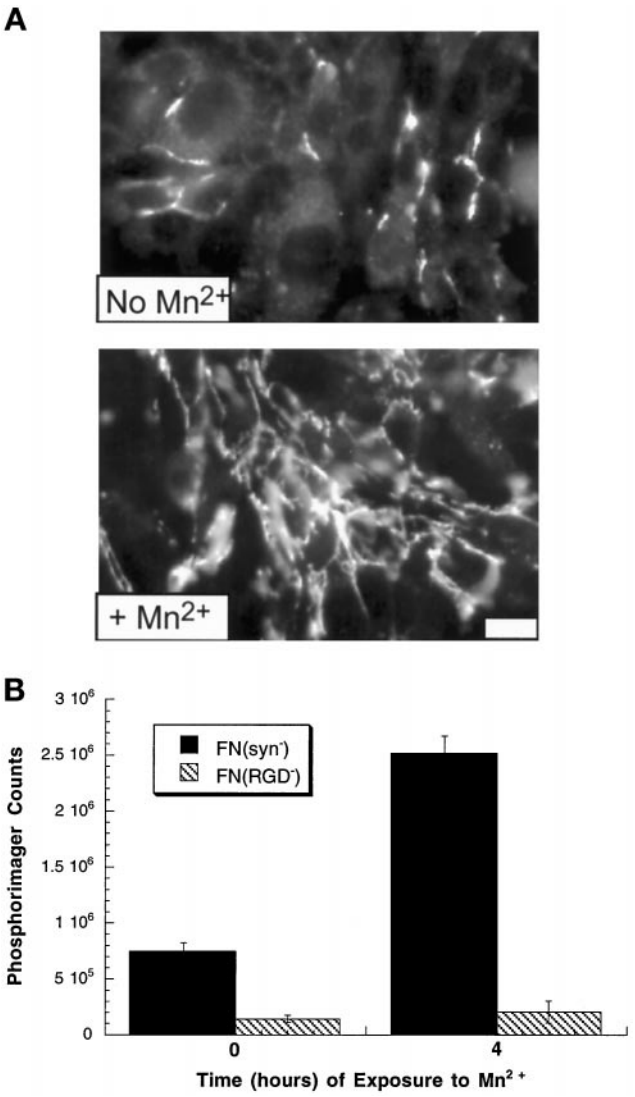


**Figure 7.** $Mn^{2+}$ stimulation of assembly after initiation of a FN(syn$^-$) matrix. (A) Twenty-five micrograms per milliliter FN(syn$^-$) were added to CHO$\alpha$5 cells and incubated for 16 h to allow for the formation of FN(syn$^-$) fibrils. Cells were then incubated for an additional 4 h in either the absence (top) or presence (bottom) of 1 mM $MnCl_2$. FN fibrils were detected by immunofluorescence with IC3 antibody. Bar, 10 $\mu$m. (B) Twenty-five micrograms per milliliter FN(syn$^-$) and FN(RGD$^-$) were added to CHO$\alpha$5 cells and cultured under conditions described in A. DOC-insoluble matrix was isolated and the amount of FN detected by quantitative immunoblot analysis. Results are the average of three experiments for FN(syn$^-$) and two experiments for FN(RGD$^-$).

crease in the affinity of integrin receptors for ligand (Faull *et al.*, 1994). Although treatment of CHO$\alpha$5 cells with PMA resulted in an increase in cell spreading (our unpublished observations), it had no effect on the assembly of pFN into fibrils (Figure 9,A and B). PMA was also unable to enhance the incorporation of FN(syn$^-$) into a matrix (Figure 9C). Furthermore, addition of $Mn^{2+}$ and PMA together did not result in an increase in stimulation over $Mn^{2+}$ alone. Therefore, agents which alter integrin function without modulating affinity do not affect matrix assembly in this system.

## DISCUSSION

These analyses provide new insights into the process of FN assembly into a fibrillar matrix. First, FN assembly into DOC-insoluble matrix begins with a slow initiation phase followed by a more rapid accumulation phase. This biphasic process is very similar to the nucleation and growth phases during assembly of linear polymers such as actin filaments. Second, $Mn^{2+}$ activation of integrins does not increase the amount of FN binding during the initiation phase but instead increases the rate of subsequent matrix growth, suggesting that a higher level of integrin activation is required during the later stages of the assembly process. Third, $\alpha5\beta1$-mediated assembly is dependent on the presence of the synergy site and, as we showed previously, also requires the RGD sequence. Fourth, integrins activated by external agents such as antibodies or $Mn^{2+}$ are able to assemble FN without the need for a synergy site. Together, our results demonstrate that FN assembly proceeds through at least two different stages and each stage has different requirements for integrin activation state and FN sequences. Ini-

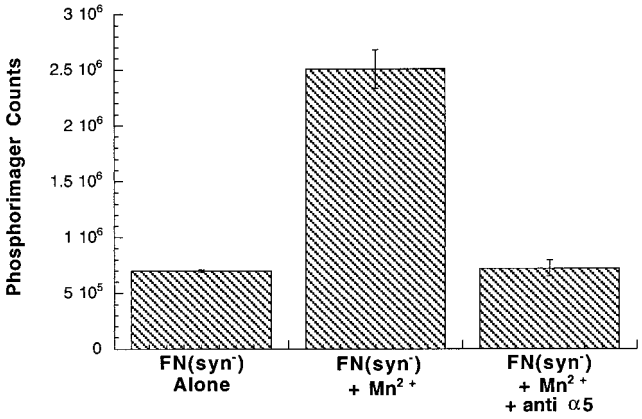


**Figure 8.** $Mn^{2+}$ stimulates $\alpha5\beta1$ integrin and enhances FN(syn$^-$) matrix assembly. CHO$\alpha$5 cells were incubated with FN(syn$^-$) for 16 h followed by an additional 4 h of incubation under the following conditions: no $MnCl_2$ or antibodies [FN(syn$^-$) alone], 1 mM $MnCl_2$ [FN(syn$^-$) + $Mn^{2+}$], and 1 mM $MnCl_2$ and function blocking anti-$\alpha$5 integrin antibodies [FN(syn$^-$) + $Mn^{2+}$ + anti-$\alpha$5]. DOC-insoluble matrix was isolated and the amount of FN present was determined by quantitative immunoblot analysis. Results are the average of two experiments.

tially, integrins bind sufficient FN for fibril formation to begin. Later in the process, the level of integrin activation determines the rate of accumulation of a dense, DOC-insoluble matrix. The synergy site is required in this second phase where contact with the receptor may serve to enhance integrin activity.

Ligand binding induces a conformational change in the extracellular domain of integrin receptors, and this change is linked to an alteration in receptor activity (reviewed in Ginsberg *et al.*, 1992; Humphries, 1996; Mould, 1996). $Mn^{2+}$ also alters the conformation of integrins (Bazzoni *et al.*, 1995; Humphries, 1996) and promotes high levels of ligand binding (Gailit and Ruoslahti, 1988; Mould *et al.*, 1995). Bazzoni *et al.* (1995) have shown that the anti-$\beta$1 antibody 9EG7 recognizes an epitope which is induced on the integrin upon binding to ligand or stimulation with $Mn^{2+}$. This observation suggests that $Mn^{2+}$ increases the rate of conversion of FN to DOC-insoluble matrix and enhances assembly of FN(syn$^-$) by stabilizing a more active $\alpha5\beta1$ integrin conformation. Although $Mn^{2+}$ can significantly increase the rate of FN assembly, the effect does not occur immediately. With both native FN and FN(syn$^-$), enhancement of matrix assembly only occurred after fibril formation had been initiated. One interpretation of these results is that initiation of assembly has different integrin activation requirements than accumulation into DOC-insoluble matrix. $Mn^{2+}$ may induce an integrin conformation particularly favorable for this later stage. $Mn^{2+}$ is probably not acting by allowing $\alpha5\beta1$ interactions with the mutant synergy site since synergy site activity is dependent on the arginine residue (Aota *et al.*, 1994) which in our mutation has been changed to a glutamate.

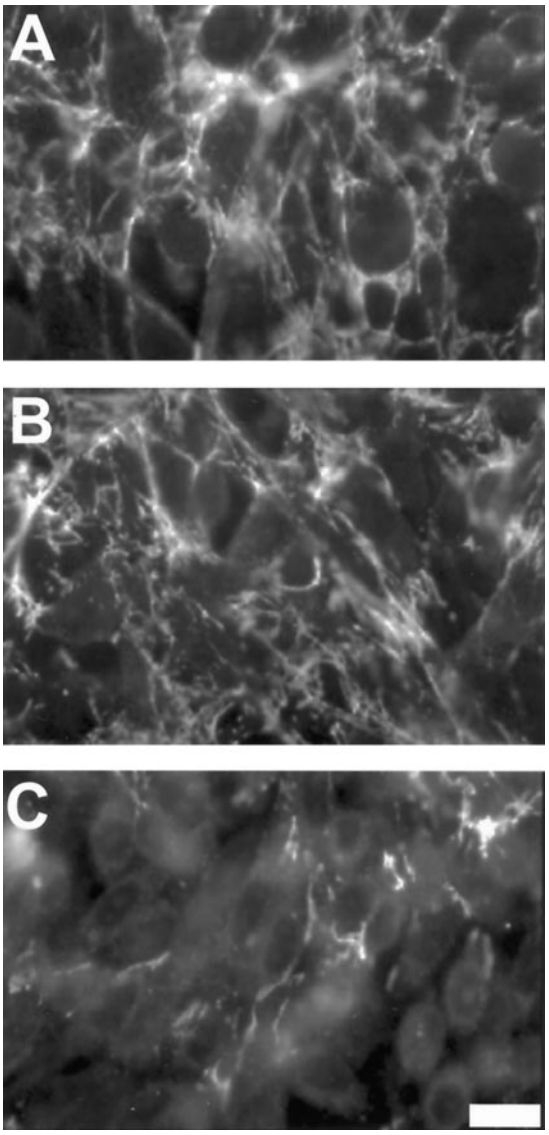


**Figure 9.** Matrix assembly in response to PMA treatment. pFN was incubated with CHO$\alpha$5 cells along with 100 nM PMA (A) or no PMA (B) for 4 h followed by immunofluorescence staining of FN fibrils. (C) FN(syn$^-$) was incubated with CHO$\alpha$5 cells for 16 h. An additional 4-h incubation was then performed in the presence of 100 nM PMA followed by detection of FN fibrils by immunofluorescence. Bar, 10 $\mu$m.

FN(syn$^-$) assembly differs from that of FN(RGD$^-$). A minimal matrix consisting of short sparse fibrils of FN(syn$^-$) could be detected with both CHO$\alpha$5 cells as well as At-T20$\alpha$5 cells (our unpublished observations). The initiation step was slowed considerably and progression beyond that phase was prevented. FN(RGD$^-$), in contrast, was not assembled (Sechler *et al.*, 1996). In addition, $Mn^{2+}$ was able to stimulate

FN(syn$^-$) assembly but had no effect on FN(RGD$^-$). Therefore, under certain conditions it may be possible to make a matrix without a synergy site but not without an RGD sequence. A dual requirement for both the synergy site and the RGD sequence in matrix assembly is also consistent with the results of Nagai *et al.* (1991) who showed that monoclonal anti-FN antibodies that map near the RGD or synergy sites inhibited the formation of a FN matrix.

Not all agents that stimulate cell adhesion can affect matrix assembly. Treatment of CHO$\alpha$5 cells with PMA did not alter the assembly of either FN or FN(syn$^-$) matrix. In contrast, PMA did increase $\alpha5\beta1$-mediated adhesion to FN fragments lacking a synergy site (Danen *et al.*, 1995). Adhesion to these fragments was maximal when cells were treated with a combination of activating antibodies, $Mn^{2+}$, and PMA. Therefore, the requirements for cell adhesion are different from the requirements for FN matrix assembly. PMA was not able to induce exposure of the 9EG7 epitope in $\alpha5\beta1$ integrin, indicating that the conformational change in response to $Mn^{2+}$ stimulation is not induced by PMA (Bazzoni *et al.*, 1995). PMA has been shown to affect the binding of $^{125}$I-labeled FN to fibroblasts with an established FN matrix (Somers and Mosher, 1993) which may be representative of the later stages of assembly. Our data show that PMA does not influence the early events of assembly initiation and accumulation but do not rule out the possibility that it could modulate later stages of fibril formation.

We have recently proposed a model for FN matrix assembly in which FN is converted from an inactive form in solution to an active form at the cell surface (Sechler *et al.*, 1996). In this model, FN is activated for assembly by binding to integin receptors, thus exposing sites required for the FN–FN interactions needed for fibril formation. Consistent with this model, Ugarova *et al.* (1995) have demonstrated the presence of reversible conformational states within FN fragments containing the cell-binding domain. Different epitopes were exposed depending on whether the fragment was in a "compact" or "extended" form. This type of regulation is not unique to FN. The focal adhesion protein vinculin, for example, has been shown to undergo changes in its conformation which serve to expose ligand-binding sites (reviewed in Jockusch and Rudiger, 1996). The results presented in this report show that, along with FN, integrins also play a dynamic role in multiple stages of matrix assembly. During initiation, integrin-FN binding and receptor clustering provide a nucleus of activated FNs (Figure 10A). As in other polymerization reactions, this phase is slow. Ligation of FN by $\alpha5\beta1$ may induce the appropriate conformational changes within the integrin to stabilize the interaction and allow accumulation of additional FN dimers and conversion to DOC-insoluble matrix (Figure 10B). This step can be accelerated by

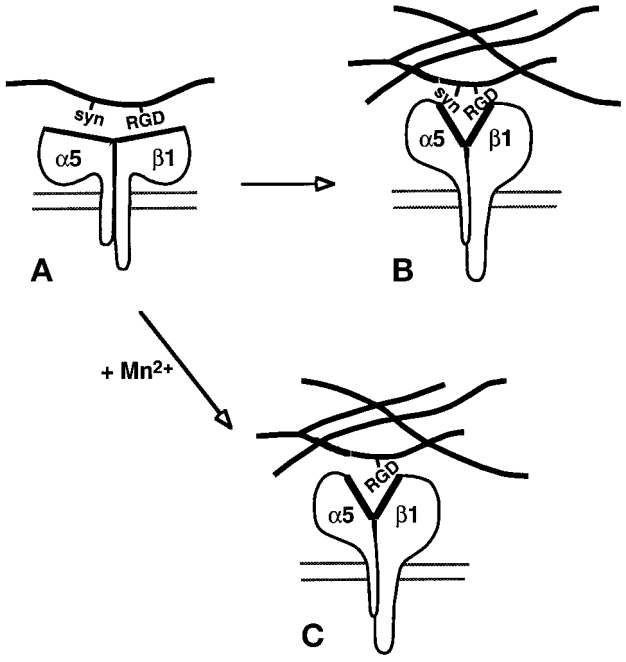


**Figure 10.** Model for $Mn^{2+}$ activation of $\alpha5\beta1$ integrin and FN matrix assembly. (A) FN binding to $\alpha5\beta1$ results in receptor clustering and unfolding of the FN molecule, exposing FN-FN binding sites required for initiation of matrix assembly. (B) A different $\alpha5\beta1$ conformation is induced, thus allowing the formation of FN fibrils. $Mn^{2+}$ can increase the rate of conversion from conformation A to conformation B. (C) In the absence of the synergy region of FN, conformation B is not induced and matrix assembly does not proceed. $Mn^{2+}$ stimulation of $\alpha5\beta1$ results in an activated receptor that can support FN assembly in the absence of the synergy site.

stimulating the integrins with $Mn^{2+}$. In the absence of the synergy site, the RGD region alone is sufficient for some initial binding but the assembly process becomes stalled, which explains the extended lag phase observed with the mutant protein. $Mn^{2+}$ apparently induces a conformation that further activates $\alpha5\beta1$ and allows the assembly of FN(syn$^-$) to proceed (Figure 10C).

Until recently, only $\alpha5\beta1$ integrin had been reported to function in initiating the formation of a FN matrix. However, $\alpha5$-null and $\beta1$-null cells assemble a FN matrix, indicating that other receptors must be able to compensate for the loss of $\alpha5\beta1$ (Yang *et al.*, 1993; Wennerberg *et al.*, 1996; Yang and Hynes, 1996). $\alpha$IIb$\beta3$ and $\alpha v\beta3$ integins have been shown to initiate and sustain FN fibril polymerization in transfected cell lines (Wu *et al.*, 1995, 1996; this report). However, these integrins require exogenous activation by antibodies to achieve maximal matrix incorporation. FN assembly by antibody-activated $\beta3$ integrins is similar to FN(syn$^-$) assembly by $Mn^{2+}$-stimulated $\alpha5\beta1$. Neither process requires the synergy site but both are dependent on exogenous activators. Apparently, the unique interaction between the synergy-RGD sites of

FN and $\alpha5\beta1$ integrin is designed to be particularly favorable for the polymerization of fibrils.

We have shown that the ability of $\alpha5\beta1$ integrin to support FN matrix assembly is dependent on the presence of both the RGD sequence and the synergy site. $Mn^{2+}$ activation of $\alpha5\beta1$ speeds up the conversion from DOC-soluble to -insoluble matrix and abrogates the need for a synergy site. The $Mn^{2+}$ effect is not seen until the formation of fibrils has been initiated, indicating that the affinity requirements of FN-$\alpha5\beta1$ interactions change during the later stages of assembly.

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