A novel fibronectin binding site required for fibronectin fibril growth during matrix assembly

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Fibronectin (FN) assembly into a fibrillar extracellular matrix is a stepwise process requiring participation from multiple FN domains. Fibril formation is regulated in part by segments within the first seven type III repeats (III1–7). To define the specific function(s) of this region, recombinant FNs (recFNs) containing an overlapping set of deletions were tested for the ability to assemble into fibrils. Surprisingly, recFN lacking type III repeat III1 (FN/III1), which contains a cryptic FN binding site and has been suggested to be essential for fibril assembly, formed a matrix identical in all respects to a native FN matrix. Similarly, displacement of the cell binding domain in repeats IIIa–10 to a position close to the NH2-terminal assembly domain, as well as a large deletion spanning repeats III1–7, had no effect on assembly. In contrast, two deletions that included repeat III1, ΔIII1,2 and ΔIII1,5, caused significant reductions in fibril elongation, although binding of FN to the cell surface and initiation of assembly still proceeded. Using individual repeats in binding assays, we show that III1, but not III3, contains an FN binding site. Thus, these results pinpoint repeat III1 as an important module for FN–FN interactions during fibril growth.

Introduction

Fibronectin (FN)* functions from within a fibrillar matrix, and proper formation of matrix fibrils is crucial for controlling tissue structure and cell motility, growth, and differentiation (Mosher, 1989; Hynes, 1990; Schwarzbauer and Sechler, 1999). Multiple FN domains have been implicated in intermolecular interactions required for the assembly process, including FN’s dimer structure and NH2-terminal assembly domain (McKeown-Longo and Mosher, 1985; McDonald et al., 1987; Schwarzbauer, 1991). Integrin binding to the arg-gly-asp (RGD) cell binding sequence within the cell binding domain is necessary for initiation of fibril formation, but not for fibril elongation (Sechler et al., 1996). In the absence of the synergy site, α5β1-mediated assembly is stalled, suggesting that fibril growth requires strong interactions between FN and integrins (Sechler et al., 1997). This is further supported by the demonstration that activation of α4β1, αvβ3, and αIIbβ3 integrins can promote FN assembly (Wu et al., 1995, 1996; Wennerberg et al., 1996; Sechler et al., 2000).

FN–FN interactions are also important for fibril formation. The major site of interaction is the NH2-terminal assembly domain which consists of repeats I1–5 and binds FN and many other molecules (Mosher, 1989; Hynes, 1990; Schwarzbauer, 1991). Other FN binding sites have been localized to the first one or two type III repeats (Morla and Ruoslahti, 1992; Aguirre et al., 1994; Hocking et al., 1994; Ingham et al., 1997), the cell binding repeat III10 (Hocking et al., 1996), and the COOH-terminal heparin binding domain (III12–14) (Bultmann et al., 1998). Each of these sites interacts with the NH2-terminal assembly domain.

Results from binding, inhibition, and matrix assembly studies show that FN fibrils form via a multistep process (McKeown-Longo and Mosher, 1983; Schwarzbauer and Sechler, 1999). During the initiation stage of assembly, integrin binding immobilizes dimeric FN and promotes formation of deoxycholate (DOC)-soluble fibrils in a process that depends on the NH2-terminal assembly domain. Mutations that affect the RGD cell binding sequence or the NH2-terminal domain ablate fibril formation (Schwarzbauer, 1991; Sottile et al., 1991; Sechler et al., 1996; Sottile and Mosher, 1997). Assembly then progresses into a growth phase that involves...
 incorporation of additional FN dimers into nascent fibrils, fibril elongation, and conversion of fibrils into a DOC-insoluble form. The matrix is further stabilized as DOC-insoluble FN is formed into high molecular mass multimers.

We have shown previously that a recombinant FN (recFN) lacking the first seven type III repeats (FNΔIII1–7) is able to form a fibrillar matrix, albeit at an altered rate (Sechler et al., 1996). It appears that this set of seven repeats, or a subset of them, has a regulatory role in FN assembly. In this study, recFNs containing overlapping deletions across this region were tested for the ability to form fibrils, DOC-insoluble matrices, and high molecular mass multimers. Surprisingly, deletion of repeat III1, a site proposed to be essential for assembly, had no detrimental effects on assembly. Similarly, relatively large deletions of up to four type III repeats, as well as displacement of the cell binding domain to the NH2 terminus, caused no deficiencies in matrix formation. However, deletions that included repeat III2 reduced the assembly of a DOC-insoluble matrix and blocked fibril elongation. Binding studies using recombinant fragments showed that III2, but not III1, has FN binding activity. Our results indicate that repeat III2 is a key element in the regulation of FN–FN interactions during matrix assembly.

Results

Repeat III1 is not essential for FN assembly

To delineate the elements involved in the regulation of FN matrix assembly, a set of recFNs containing in-frame deletions within the first seven type III repeats was prepared (Fig. 1). Of particular interest was the first type III repeat, which has FN binding activity when in denatured form and has been implicated in FN matrix formation (Morla and Ruoslahti, 1992; Aguirre et al., 1994; Hocking et al., 1994; Ingham et al., 1997). Using the baculovirus expression system, FNΔIII1 was expressed from a rat FN cDNA mutated by PCR to eliminate the entire segment encoding repeat III1. Assembly was tested using CHO K1 cells transfected with human α5 integrin cDNA (CHOα5) that do not assemble an endogenous matrix (Sechler et al., 1996). FNΔIII1 was assembled into a fibrillar matrix morphologically identical to that formed by native FN (Fig. 2, A–D) or full-length recFN-A/B- (unpublished data and Sechler et al., 1996). No differences in native and FNΔIII1 matrices were detected at any of the time points studied. AtT-20 mouse pituitary cells transfected with human α5 integrin cDNA (AtT-20α5), a cell line that does not express any endogenous FN (Sechler et al., 1996), also assembled morphologically identical native and FNΔIII1 matrices (Fig. 2, E and F). Therefore, in two independent cell lines, FN matrix assembly was not altered by the deletion of repeat III1.

Biochemically, FNΔIII1 and native FN matrices were indistinguishable. Equivalent amounts of both proteins were associated with CHOα5 cells in the DOC-soluble fractions (Fig. 3 A). Similar proportions of DOC-insoluble material were formed from FN and FNΔIII1 during a 16-h incubation (Fig. 3 B). As has been shown previously for FN (McKeown-Longo and Mosher, 1983; Sechler et al., 1996), there was continued incorporation of FNΔIII1 into DOC-insoluble matrices and high molecular mass aggregates (Fig. 3 B, 48 h). These data show that III1 is neither required for the formation of fibrils, nor responsible for the altered rate of assembly observed with FNΔIII1–7.

Reduced matrix accumulation in the absence of repeats III2–5

In contrast to the normal matrix formed with FNΔIII1, deletion of III2–5 decreased matrix accumulation. Some fibril as-

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**Figure 1.** Schematic representation of FN and recFNs. The structural organization of FN consisting of type I (rectangles), type II (triangles), and type III (ovals) repeats is shown at top. Darkened ovals represent alternatively spliced EIIIA and EIIIB repeats that were not included in any recFNs. All recFNs contain the V120 variant of the alternatively spliced V region (cross-hatched box) as well as the COOH-terminal cysteine pair (S-S). The inverted triangle over FNIII2–5,9–10 indicates a deleted RGD sequence; white ovals are III9–10 in place of III4–5. All six recFNs were constructed from rat FN cDNA.

**Figure 2.** Assembly of FNΔIII1. CHOα5 cells were cultured in the presence of 50 μg/ml pFN (A and B) or 50 μg/ml FNΔIII1 (C and D) for 4 (A and C) or 16 (B and D) h. AtT-20α5 cells were incubated with 25 μg/ml pFN (E) or FNΔIII1 (F) for 16 h. Cells were then fixed and FN fibrils detected by indirect immunofluorescence with monoclonal anti-rat FN antibody IC3. Bar, 10 μm.
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assembly was initiated, but the amount of FN/III2–5 matrix assembled by CHOα5 cells was less than that of native FN, and the distribution of fibrils was more sparse than for native FN (Fig. 4 A). Furthermore, DOC-insoluble matrix was at least threefold less for FN/III2–5 than for native FN (Fig. 4 B). Limited conversion of DOC-soluble FN/III2–5 into DOC-insoluble matrix suggests that this recFN is impaired in its ability to participate in fibril growth.

Analyses of the assembly of two other deletion mutants, FN/III4–5 and FN/III4–7, showed formation of characteristic fibrils at all time points (unpublished data). Furthermore, biochemical analyses did not reveal any defects in incorporation into DOC-insoluble matrix or the formation of high molecular mass multimers, results similar to those observed for FN/III1. FN/III2–5 and FN/III4–7 both lack four type III repeats but differ in their capacity to be assembled. This indicates that alterations in the ability to assemble matrices cannot be attributed solely to the size of the deletion. Instead, there appear to be specific roles for individual type III repeats and normal assembly of FN/III4–5 compared with FN/III2–5 indicates that IIId and/or IIIe may be important.

Fibril growth depends on repeats III1,2
Because III1,2 has FN binding activity (Aguirre et al., 1994) and a proteolytic fragment containing III1 plus part of III2 inhibits incorporation of FN into matrix (Chernousov et al., 1991; Morla and Ruoslahti, 1992), we generated FN/III1–2 to test whether these two repeats together comprise a matrix regulatory region. Immunofluorescence analysis of FN assembled by CHOα5 cells showed that early during assem-

**Figure 3.** DOC-soluble and -insoluble FN/III1. CHOα5 cells were incubated with 50 μg/ml pFN or FN/III1 for the indicated times and lysed in buffered DOC. DOC-soluble (A) and -insoluble (B) fractions were separated in 5% polyacrylamide-SDS gels without reduction and transferred to nitrocellulose. FN was detected on immunoblots with monoclonal anti-FN antibody IC3 and chemiluminescence reagents. Dimeric pFN and FN/III1, are present (arrowhead) as well as high molecular mass multimers at the top of the stacking (bracket) and at the interface of the stacking and separating gels (arrow).

**Figure 4.** Assembly of FN/III2–5. (A) Native pFN and FN/III2–5 were added to CHOα5 cells at a concentration of 50 μg/ml and cultured for the indicated times. Fibrils were visualized by indirect immunofluorescence as in Fig. 2. (B) DOC-insoluble material isolated at the indicated times was analyzed under reducing conditions by immunoblotting with IC3 monoclonal antibody. Bar, 10 μm.

**Figure 5.** Assembly of FN/III1–2. (A) CHOα5 cells were incubated for either 4 or 24 h in the presence of 50 μg/ml FN/III1–2. Monoclonal antibody IC3 was used to detect recFN matrix by immunofluorescence. (B) DOC-soluble and -insoluble cell lysates were isolated from CHOα5 cells incubated in the presence of 50 μg/ml FN/III1–2 or pFN for 0.5, 4, 7, 16, 24, and 48 h. DOC-soluble and -insoluble material was analyzed as described in Fig. 3. Arrow and bracket indicate locations of high molecular mass multimers. Dash indicates location of 180-kD molecular mass standard. Bar, 10 μm.
ably, FNΔIII1–2 formed aggregates on the cell surface (Fig. 5A) similar to, but much smaller than, those formed by FNΔIII1–7 (Sechler et al., 1996). As assembly progressed, FNΔIII1–2 formed mainly short fibrils between cells although occasional long thin fibrils were also visible. An extensive fibrillar network was never observed even after prolonged incubations. Levels of DOC-insoluble material were significantly reduced, especially at later times of assembly, and no high molecular mass multimers were observed (Fig. 5B). Incubations with higher concentrations of FNΔIII1–2 did not increase the amount of DOC-insoluble matrix (unpublished data). Unlike FNs lacking the RGD sequence, FNΔIII1–2 was not deficient in binding to cells. In fact, substantially more FNΔIII1–2 than native FN was isolated as cell-associated DOC-soluble material. This indicates that FNΔIII1–2 can efficiently bind to the cell surface to initiate assembly, but is defective in the growth phase when conversion from DOC-soluble to -insoluble matrix occurs.

FN lacking the RGD sequence is unable to initiate matrix assembly but can be incorporated once assembly has been primed by native FN (Sechler et al., 1996). However, preinitiation by native FN would not be expected to rescue FNΔIII1–2 assembly, as this protein shows a defect in fibril growth. In fact, neither FNΔIII1–2 nor FNΔIII1–5 was able to efficiently incorporate into a preformed human FN matrix. A very few short fibrils were formed by FNΔIII1–2 (Fig. 6B) and FNΔIII1–5 fibrils were found in small patches distributed unevenly throughout the matrix (Fig. 6C). In contrast, an extensive fibrillar network was assembled by native FN (Fig. 6A). In all cases, a fibrillar human plasma FN (pFN) matrix could be readily detected with a human FN-specific monoclonal antibody (unpublished data). Thus, the presence of repeat III2 is required for the continued growth of FN fibrils.

Repeat III2 contains a FN binding site
To demonstrate a requirement for III2 in FN assembly, attempts were made to generate a recFN lacking repeat III2 or with another homologous repeat in its place. Unlike the recFNS reported here, secretion of mutant recFNS lacking III2 from infected insect cells was very inefficient, suggesting that in the absence of this repeat the proteins were not properly folded. We have shown previously that III1–2 purified in soluble form from bacterial lysates is able to bind FN (Aguirre et al., 1994). To identify the repeat responsible for FN binding activity, maltose-binding protein (MBP) fusion pro-
proteins containing either III₁ or III₂ were generated and tested for binding in solid phase binding assays. MBP-III₂, but not MBP-III₁, showed significant FN binding activity (Fig. 7 A) that was reversed by treatment with buffered SDS (unpublished data). The lack of MBP-III₁ binding confirms the results of Ingham et al. (1997) who showed that native III₁ does not bind to FN or its fragments. FN binding was concentration dependent and a complementary binding site was localized to the 70-kD region (Fig. 7 B). Apparent dissociation constants for FN and 70-kD binding to III₂ differ by only 3.5-fold, 28 nM, and 8 nM, respectively. The higher concentration constant for FN may reflect a difference in affinities. However, it may also be due to molecular differences between the relatively small 70-kD fragment and dimeric FN. In addition to the observed location of the binding sites as important contributors to this process. Specifically, deletion of the cell binding domain closer to the NH₂-terminal assembly domain and could affect recFN fibril formation. To eliminate the possibility that changes in the domain organization can alter FN assembly, we created FNrIII₄₋₅₉₋₁₀. Repeats III₄₋₅ were replaced with III₉₋₁₀ and the RGD sequence was deleted from its native position within the cell binding domain (Fig. 1). Therefore, the only functional III₉₋₁₀ pair is in the position normally occupied by III₉₋₁₀. CHOo5 cells efficiently assembled FNrIII₄₋₅₉₋₁₀ into a DOC-insoluble fibrillar matrix identical to that of native FN at all time points (Fig. 8, A–C). Cell cycle progression by CHOo5 cells assembling either FN or FNrIII₄₋₅₉₋₁₀ was identical, as were the levels of focal adhesion kinase phosphorylation (unpublished data). These results demonstrate that the location of the cell binding domain is not restricted to the center of the molecule and that displacement toward the NH₂ terminus does not reduce FN function in matrix assembly or its ability to influence cell cycle progression.

**Discussion**

FN fibril assembly is initiated by binding to integrin receptors and propagated by FN–FN interactions. The progression of FN conversion from soluble dimer into insoluble matrix fibrils is regulated in part by sequences within repeats III₁–₇ (Sechler et al., 1996). Using a set of overlapping deletions spanning this repeat position normally occupied by III₉₋₁₀. CHOo5 cells efficiently assembled FNrIII₄₋₅₉₋₁₀ into a DOC-insoluble fibrillar matrix identical to that of native FN at all time points (Fig. 8, A–C). Cell cycle progression by CHOo5 cells assembling either FN or FNrIII₄₋₅₉₋₁₀ was identical, as were the levels of focal adhesion kinase phosphorylation (unpublished data). These results demonstrate that the location of the cell binding domain is not restricted to the center of the molecule and that displacement toward the NH₂ terminus does not reduce FN function in matrix assembly or its ability to influence cell cycle progression.

These results identify repeat III₂ as a major FN binding site and suggest that the lack of fibril formation by FNΔIII₁₋₂ is due to the absence of this site.

**Repositioning of III₉₋₁₀ cell binding domain**

The cell binding domain consisting of an RGD sequence and synergy site in repeats III₉₋₁₀ is essential for initiation of FN matrix assembly by α5β₁ integrin (Sechler et al., 1996, 1997). Deletions within repeats III₁–₇ position the cell binding domain closer to the NH₂-terminal assembly domain and could affect recFN fibril formation. To eliminate the possibility that changes in the domain organization can alter FN assembly, we created FNrIII₄₋₅₉₋₁₀. Repeats III₄₋₅ were replaced with III₉₋₁₀ and the RGD sequence was deleted from its native position within the cell binding domain (Fig. 1). Therefore, the only functional III₉₋₁₀ pair is in the position normally occupied by III₉₋₁₀. CHOo5 cells efficiently assembled FNrIII₄₋₅₉₋₁₀ into a DOC-insoluble fibrillar matrix identical to that of native FN at all time points (Fig. 8, A–C). Cell cycle progression by CHOo5 cells assembling either FN or FNrIII₄₋₅₉₋₁₀ was identical, as were the levels of focal adhesion kinase phosphorylation (unpublished data). These results demonstrate that the location of the cell binding domain is not restricted to the center of the molecule and that displacement toward the NH₂ terminus does not reduce FN function in matrix assembly or its ability to influence cell cycle progression.
tion that appears to contribute to the formation of the compact conformation of soluble FN (Johnson et al., 1999). Thus, the III_2 module may participate in matrix assembly through interactions with several sites on FN. These interactions may promote elongation by aligning fibrils into a stable, uniform structure that can then be converted into a DOC-insoluble form. In the absence of this repeat, fibrils begin to form but become stalled during elongation and are inefficiently converted into the DOC-insoluble matrix.

DOC-insolubility of FN fibrils appears to occur through hydrophobic protein–protein interactions that resist SDS denaturation (Chen and Mosher, 1996). Perhaps III_2 participates in the formation of that hydrophobic interface.

Others have shown the presence of a cryptic FN binding site in III_1 that is exposed by denaturation (Hocking et al., 1994; Ingham et al., 1997). The apparent affinity of 70 kD for III_2 is higher than that reported for 70 kD binding to heat-denatured III_1 (Hocking et al., 1994). The identification of two distinct sites indicates that the II_1–II_2 segment contains more than one FN binding site, the site in III_1 that is critical for fibril assembly and a cryptic site in III_3 that is dispensable for this process. It is also possible that repeats II_1–II_2 act as a functional unit to regulate fibril assembly and promote elongation. For example, interactions between III_1 and III_2 could regulate the accessibility of an FN binding site. Previous studies lend support to the idea of cooperation between these repeats. Both repeats were required for formation of an in vitro ternary complex with heat-denatured III_3 and the NH_2-terminal 70-kD fragment (Hocking et al., 1996). III_3 has also been proposed to contribute to interactions between III_1 and the COOH-terminal heparin binding domain (Bultmann et al., 1998). Furthermore, the reduced secretion of recFNS lacking III_2 or carrying another type III repeat in place of III_2 indicates that interactions between adjacent repeats contribute to domain structure and stability. III-specific inhibitory peptides and antibodies have been described (Chernousov et al., 1987, 1991; Morla and Rusolati, 1992). If III_1 and III_2 do indeed function together, these inhibitory reagents may exert their effects indirectly through disruption of activities mediated by the adjacent III_2 module.

Regulated assembly depends in part on conformational changes in the FN molecule. Accumulating evidence indicates that soluble FN dimers must be converted from a compact inactive form into an “unfolded” activated form in order for assembly to proceed (Alexander et al., 1979; Williams et al., 1982; Erickson and Carrell, 1983; Rocco et al., 1983; Ugarova et al., 1995; Schwarzbauer and Sechler, 1999). In vivo, integrin binding induces FN activation and this may expose the III_2 binding site, allowing intermolecular interactions between cell surface–bound FNs. Whereas recFNS lacking III_2 are able to initiate assembly and form short fibrils, the significantly reduced levels of DOC-insoluble FNIII_1–II_2 suggest that in the absence of the III_2 binding site, this recFN cannot effectively participate in the essential FN–FN interactions needed for fibrillogenesis. FN molecules can also be induced to associate in solution by the addition of a peptide corresponding to part of the III_1 module (Morla et al., 1994). This treatment may expose the III_1 binding site by local perturbation of intramolecular interactions involving this region of the molecule.

Comparison of the progression of assembly by FNIII_1–2 with FNIII_1–7 shows that both initially form short stitches around cell peripheries and connect to adjacent cells. FNIII_1–2 then forms aggregates that prematurely become insoluble in DOC (Sechler et al., 1996). These aggregates can apparently be remodeled by binding to adjacent cells and getting stretched into fibrils. In this way, FNIII_1–7 forms a relatively normal-appearing fibrillar matrix. On the other hand, FNIII_1–2 forms only a few small, DOC-soluble aggregates that can be converted into predominantly short fibrils. FNIII_1–7 does not accumulate in DOC-insoluble material, nor does it form an extensive fibrillar matrix. The differences between assembly of FNIII_1–7 and FNIII_1–2 suggests that repeats III_1–III_2 contribute to the progression of FN fibril formation. A few activities have been mapped to the III_2 region. Repeats III_1–IV can bind to heparin and DNA under low salt conditions (Hynes, 1990). Cryptic binding sites within repeat III_1 have been reported for activated α4β1 and α4β7 integrins (Moyano et al., 1997) as well as for repeat III_1 (Hocking et al., 1996). Repeat III_1 can also bind to repeat III_4 (Ingham et al., 1997). Thus, it is possible that during assembly this region of FN interacts with cell surface or matrix proteins or glycosaminoglycans, and that these interactions may help to control fibril formation.

FNIII_1–2 and FNIII_1–7 probably also differ in the alignment of FN dimers into fibrils. For example, binding of the NH_2-terminal assembly domain of one FNIII_1–7 dimer to the COOH-terminal heparin domain of another (Bultmann et al., 1998) would align their cell binding domains relatively close to each other. This juxtaposition could result in increased clustering of integrins and more stable contacts between matrix and cytoskeleton, giving the strong connections needed to remodel aggregates into fibrils and form DOC-insoluble material. Tension applied to FN fibrils has been predicted to cause slight unfolding of type III repeats (Erickson, 1994; Krammer et al., 1999), and this might allow the formation of SDS-resistant protein–protein interactions (Chen and Mosher, 1996). On the other hand, the inclusion of III_3–7 may yield a potentially different organization of both cell surface receptors and cytoskeletal elements, thus precluding the formation of a stable matrix.

Clearly, multiple options exist for establishing FN–FN interactions during matrix assembly. For example, the NH_2-terminal assembly domain is required throughout the assembly process, whereas the III_2 module participates after initiation during a phase of fibril growth. This indicates that different FN binding sites have distinct temporal and spatial roles, and suggests that control of domain-specific FN interactions may play an important role in regulating the structural and functional organization of the FN matrix.

Materials and methods

Cell culture

CHOa5, clone 17, and AT-20a5, clone 11, transfected with a cDNA to the human a5 integrin subunit have been described previously (Sechler et al., 1996). For all experiments, CHOa5 cells were cultured in DME supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 μg/ml Genetin (Life Technologies/GIBCO BRL) and 10% fetal calf serum (HyClone Labs) depleted of FN. AT-20a5 cells were cultured in a 50:50 mixture of Ham’s F12 and DME, plus 20 mM Hepes, pH 7.4, 4 mM glutamine,
0.25 mg/ml Genetin (Life Technologies/GIBCO BRL), and 10% fetal calf serum (HyClone Labs) and 10% Nu-serum both depleted of FN.

**FN cDNA constructions and recombinant protein production**

All recFNs were expressed with baculovirus vector pVL1392. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. Oligonucleotide primers were prepared at the Synthesis and Sequencing Facility (Princeton University, Princeton, NJ).

All deletions were created by PCR amplification of rat FN cDNA. A KpnI site was engineered into each oligonucleotide to join the regions spanning each deletion. PCR amplification for 25 cycles was performed for all constructions under the following conditions: 95°C, 30 s; 60°C, 30 s; and 72°C, 60 s. Products were digested with flanking enzymes and inserted into the FN cDNA using convenient restriction sites. The sequencing of 5′ and 3′ primers were used to generate the indicated deletions (base positions of the primers within the FN cDNA are in parentheses and base changes to introduce the Kpn I sites are underlined): ΔII; GGGGTACCT- GTGCTGCTGCT (1831-1812), CTGTTGACCAAGCAGTGT (2130); ΔIII1; CCGGTACCTATCTGATCTGG (2271-2270), GCGTAC- CTCTCTCAGAGGAG (4655-4654); ΔIII2; GTTGCTGGCTGCTCGTGTG (2124-2105), CTTGAGTCCTGCGCTCAAG (3248-3267). pVL1392 FNΔIII1 was prepared by ligating a fragment from FNΔIII1 with a PCR-amplified fragment made using the primer CTGGTACCGACCTCTGATTGCGCCTCCAG (2424-2442). pVL1392 FNΔIII2 was created by ligating a 5′ fragment from pVL1392 FNΔIII1 with a 3′ fragment from pVL1392 FNΔIII1. To generate pVL1392 FNΔIII1ΔIII2, a segment spanning repeats IIIn-1 was amplified using 5′ primer GACCTGCCCCTGACCTGTTGCGTCC (4552-4570) and 3′ primer GAGGTAACGCGTGTGTAATGATGGAACACCGTC (5098-5073) containing KpnI sites (underlined). The resulting FN fragment was then inserted at the engineered KpnI site in pVL1392 FNΔIII1 and a segment encoding an RGD deletion was inserted into the cell binding domain (Schwarzbauer, 1991). All regions obtained from PCR products were verified by DNA sequence analysis.

Recombinant baculoviruses were created and recombinant proteins and rat pFN were purified as described (Sechler et al., 1996, 1997). Yields of PCR products were verified by DNA sequence analysis.

**Immunodetection**

Immunodetection was performed as described (Sechler et al., 1996) using immunofluorescence (Pierce Chemical Co.). Band intensities were quantified at 1:1,000. Immunoblots were developed with Super Signal chemiluminescence reagents (Pierce Chemical Co.). 8.8, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM iodoacetic acid, and 2 mM N-ethylmaleimide) per well. Lysates were separated into Triton X-100, 2 mM EDTA, 2 mM iodoacetic acid, and 2 mM N-ethylmaleimide) per well. Lysates were separated into 2130); ΔIII2; GGGGTACCTATCTGATCTGG (2271-2270), GCGTAC- CTCTCTCAGAGGAG (4655-4654); ΔIII2; GTTGCTGGCTGCTCGTGTG (2124-2105), CTTGAGTCCTGCGCTCAAG (3248-3267). pVL1392 FNΔIII1 was prepared by ligating a fragment from FNΔIII1 with a PCR-amplified fragment made using the primer CTGGTACCGACCTCTGATTGCGCCTCCAG (2424-2442). pVL1392 FNΔIII2 was created by ligating a 5′ fragment from pVL1392 FNΔIII1 with a 3′ fragment from pVL1392 FNΔIII1. To generate pVL1392 FNΔIII1ΔIII2, a segment spanning repeats IIIn-1 was amplified using 5′ primer GACCTGCCCCTGACCTGTTGCGTCC (4552-4570) and 3′ primer GAGGTAACGCGTGTGTAATGATGGAACACCGTC (5098-5073) containing KpnI sites (underlined). The resulting FN fragment was then inserted at the engineered KpnI site in pVL1392 FNΔIII1 and a segment encoding an RGD deletion was inserted into the cell binding domain (Schwarzbauer, 1991). All regions obtained from PCR products were verified by DNA sequence analysis.

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**Isolation and detection of DOC-soluble and -insoluble matrix**

DOC-soluble and -insoluble material was isolated from CHOα5 cells cultured in a 24-well dish with pFN or recFNs as described above. After the indicated time periods, cells were washed with serum-free DME and lysed with 200 μl of DOC lysis buffer (2% deoxycholate, 0.02 M Tris-HCl, pH 8.8, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM iodoacetate, and 2 mM N-ethylmaleimide) per well. Lysates were separated into DOC-soluble and -insoluble fractions that were analyzed by SDS-PAGE. Immunodetection was performed as described (Sechler et al., 1996) using ascites fluid from rat FN-specific monoclonal antibody IC3 at a dilution of 1:1,000. Immunoblots were developed with Super Signal chemiluminescence reagents (Pierce Chemical Co.). Band intensities were quantified at two exposure times using IPLab software (Mac v. 3.5; Scanco Inc.).

**Expression of bacterial fusion proteins and FN binding assays**

Rat FN cDNA fragments encoding repeat II, or III, were inserted into pMAL-cRI (New England Biolabs, Inc.) for expression as MBP fusion proteins. III, spanned amino acid positions 604–700 (TYP … TTS) and III2, extended from residue 701 to 808 (AST ... QT). BamHI sites and XhoI sites were engineered at the 5′ and 3′ ends, respectively. PCR amplification us-
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