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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 ($III_{1–7}$). 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 III_1 ($FN\Delta III_1$), 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 $III_{9–10}$ to a position close to the NH_2-terminal assembly domain, as well as a large deletion spanning repeats $III_{4–7}$, had no effect on assembly. In contrast, two deletions that included repeat III_2, $\Delta III_{1–2}$ and $\Delta III_{2–5}$, 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 III_2 but not III_1 contains an FN binding site. Thus, these results pinpoint repeat III_2 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 NH_2-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 NH_2-terminal assembly domain which consists of repeats $I_{1–5}$ 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 III_{10} (Hocking et al., 1996), and the COOH-terminal heparin binding domain ($III_{12–14}$) (Bultmann et al., 1998). Each of these sites interacts with the NH_2-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 NH_2-terminal assembly domain. Mutations that affect the RGD cell binding sequence or the NH_2-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

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*Abbreviations used in this paper: DOC, deoxycholate; FN, fibronectin; pFN, plasma FN; recFN, recombinant FN; MBP, maltose-binding protein; RGD, arg-gly-asp.

Key words: fibronectin; matrix assembly; type III repeats; RGD sequence; self-association

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 (FNΔIII$_{1-7}$) 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 III$_1$, 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 toward the NH_2 terminus, caused no deficiencies in matrix formation. However, deletions that included repeat III$_2$ reduced the assembly of a DOC-insoluble matrix and blocked fibril elongation. Binding studies using recombinant fragments showed that III$_2$, but not III$_1$, has FN binding activity. Our results indicate that repeat III$_2$ is a key element in the regulation of FN–FN interactions during matrix assembly.

Results

Repeat III$_1$ 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, FNΔIII$_1$ was expressed from a rat FN cDNA mutated by PCR to eliminate the entire segment encoding repeat III$_1$. 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). FNΔIII$_1$ was assembled into a fibrillar matrix morphologically identical to that formed by native FN (Fig. 2, A–D) or full-length recFNA-B- (unpublished data and Sechler et al., 1996). No differences in native and FNΔIII$_1$ 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 FNΔIII$_1$ matrices (Fig. 2, E and F). Therefore, in two independent cell lines, FN matrix assembly was not altered by the deletion of repeat III$_1$.

Biochemically, FNΔIII$_1$ 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 FNΔIII$_1$ 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 FNΔIII$_1$ into DOC-insoluble matrices and high molecular mass aggregates (Fig. 3 B, 48 h). These data show that III$_1$ is neither required for the formation of fibrils, nor responsible for the altered rate of assembly observed with FNΔIII$_{1-7}$.

Reduced matrix accumulation in the absence of repeats III$_{2-5}$

In contrast to the normal matrix formed with FNΔIII$_1$, deletion of III$_{2-5}$ decreased matrix accumulation. Some fibril as-

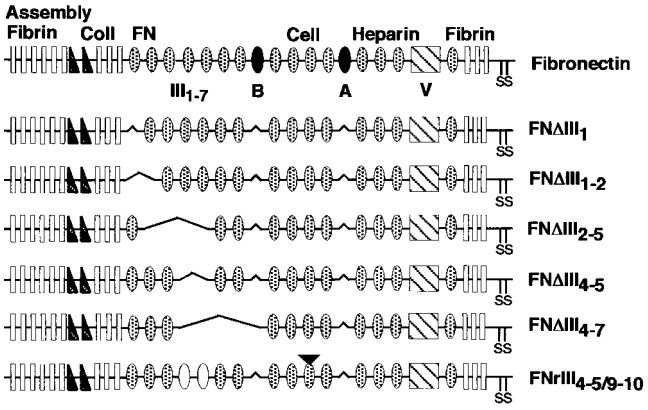

Figure 1. **Schematic representation of FN and recFNs.** The structural organization of FN consisting of type I (rectangles), type II (triangles), and type III (ovals) repeats is shown at top. Darkened ovals represent alternatively spliced EIIIA and EIIIB repeats that were not included in any recFNs. 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 FNrIII$_{4-5/9-10}$ indicates a deleted RGD sequence; white ovals are III$_{9-10}$ in place of III$_{4-5}$. All six recFNs were constructed from rat FN cDNA.

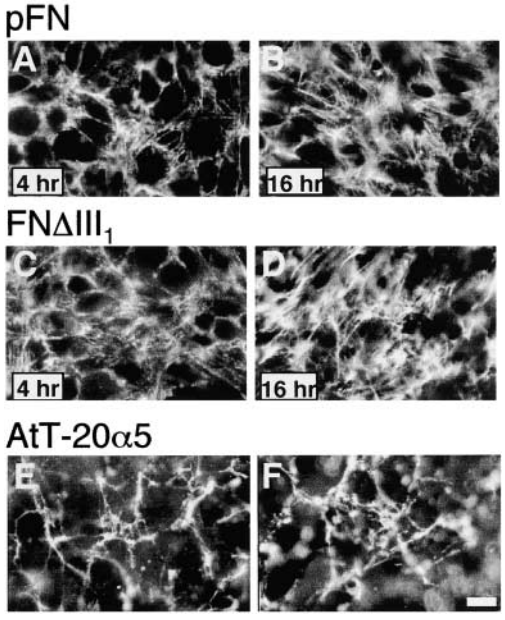

Figure 2. **Assembly of FNΔIII$_1$.** CHOα5 cells were cultured in the presence of 50 μg/ml pFN (A and B) or 50 μg/ml FNΔIII$_1$ (C and D) for 4 (A and C) or 16 (B and D) h. AtT-20α5 cells were incubated with 25 μg/ml pFN (E) or FNΔIII$_1$ (F) for 16 h. Cells were then fixed and FN fibrils detected by indirect immunofluorescence with monoclonal anti–rat FN antibody IC3. Bar, 10 μm.

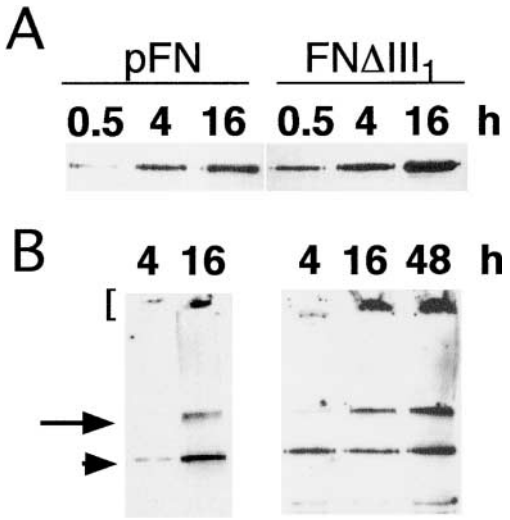

Figure 3. **DOC-soluble and -insoluble FNΔIII$_1$.** CHOα5 cells were incubated with 50 μg/ml pFN or FNΔIII$_1$ for the indicated times and lysed in buffered DOC. DOC-soluble (A) and -insoluble (B) fractions were separated in 5% polyacrylamide-SDS gels without reduction and transferred to nitrocellulose. FN was detected on immunoblots with monoclonal anti-FN antibody IC3 and chemiluminescence reagents. Dimeric pFN and FNΔIII$_1$ 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).

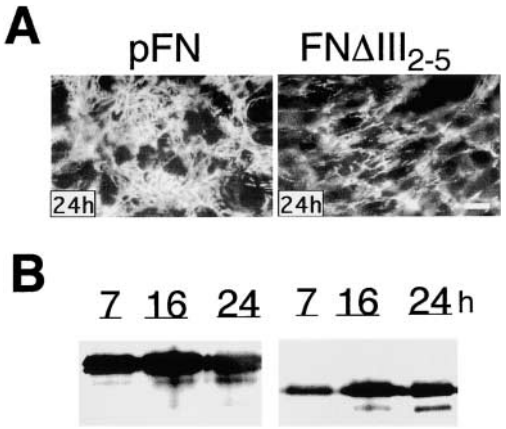

Figure 4. **Assembly of FNΔIII$_{2–5}$.** (A) Native pFN and FNΔIII$_{2–5}$ were added to CHOα5 cells at a concentration of 50 μg/ml and cultured for the indicated times. Fibrils were visualized by indirect immunofluorescence as in Fig. 2. (B) DOC-insoluble material isolated at the indicated times was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

sembly was initiated, but the amount of FNΔIII$_{2–5}$ 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 FNΔIII$_{2–5}$ than for native FN (Fig. 4 B). Limited conversion of DOC-soluble FNΔIII$_{2–5}$ 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, FNΔIII$_{4–5}$ and FNΔIII$_{4–7}$, 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 FNΔIII$_1$. FNΔIII$_{2–5}$ and FNΔIII$_{4–7}$ 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 FNΔIII$_{4–5}$ compared with FNΔIII$_{2–5}$ indicates that III$_2$ and/or III$_3$ may be important.

Fibril growth depends on repeats III$_{1–2}$

Because III$_{1–2}$ has FN binding activity (Aguirre et al., 1994) and a proteolytic fragment containing III$_1$ plus part of III$_2$ inhibits incorporation of FN into matrix (Chernousov et al., 1991; Morla and Ruoslahti, 1992), we generated FNΔIII$_{1–2}$ 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-

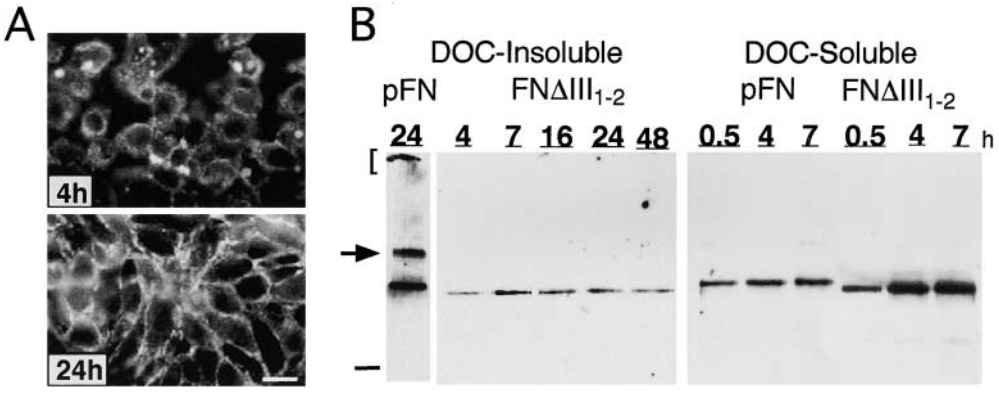

Figure 5. **Assembly of FNΔIII$_{1–2}$.** (A) CHOα5 cells were incubated for either 4 or 24 h in the presence of 50 μg/ml FNΔIII$_{1–2}$. Monoclonal antibody IC3 was used to detect recFN matrix by immunofluorescence. (B) DOC-soluble and -insoluble cell lysates were isolated from CHOα5 cells incubated in the presence of 50 μg/ml FNΔIII$_{1–2}$ 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.

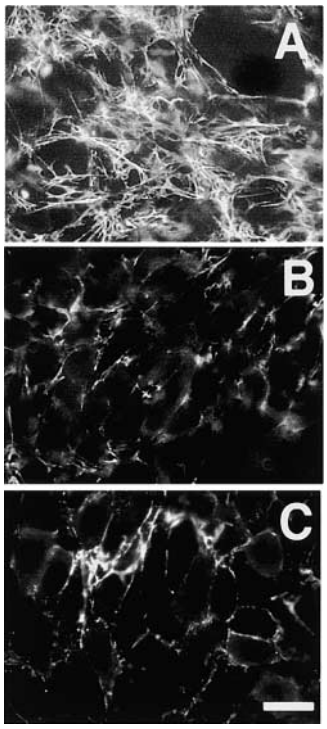

Figure 6. **RecFN assembly in the presence of human FN matrix.** CHOα5 cells were incubated with 50 μg/ml human pFN for 8 h. Medium was then removed, cell layers were washed to remove unbound FN, and cells were fed with fresh medium containing 25 μg/ml full-length recFN (A), FNΔIII$_{1-2}$ (B), or FNΔIII$_{2-5}$ (C). After incubation for an additional 16 h, cells were fixed and recFN fibrils detected with an anti–rat FN-specific monoclonal antibody. Bar, 20 μm.

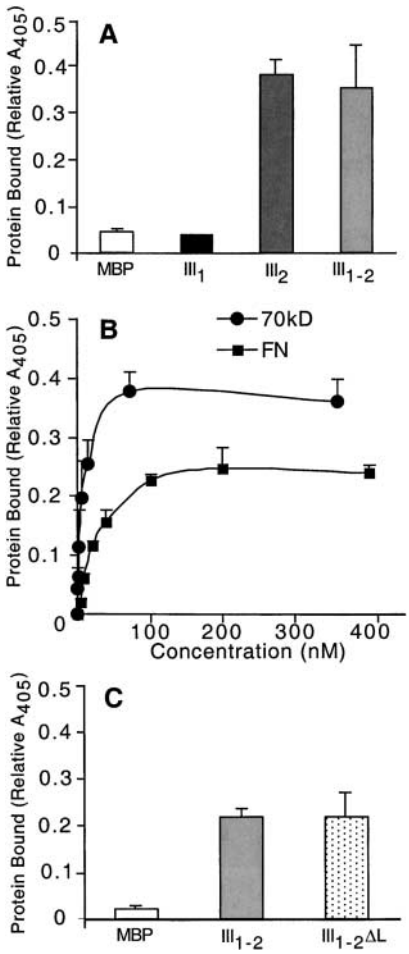

Figure 7. **Repeat III$_2$ contains a FN binding site.** Solid phase binding assays were used to examine binding of FN (A–C) and NH_2-terminal 70-kD fragment (B) to immobilized fusion proteins. (A and C) MBP and fusion proteins were coated at 2 μg/ml and incubated with 50 μg/ml rat pFN. (B) MBP-III$_2$ coated at 4 μg/ml was incubated with rat pFN or 70-kD fragment at increasing concentrations. Background binding to MBP was subtracted from these data. Binding was detected with 5G4 anti-FN monoclonal antibody for FN (A and C) or R457 anti–70-kD polyclonal antiserum (B). Results are presented as the average of normalized values from two to three experiments.

bly, FNΔIII$_{1-2}$ formed aggregates on the cell surface (Fig. 5 A) similar to, but much smaller than, those formed by FNΔIII$_{1-7}$ (Sechler et al., 1996). As assembly progressed, FNΔIII$_{1-2}$ 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. 5 B). Incubations with higher concentrations of FNΔIII$_{1-2}$ did not increase the amount of DOC-insoluble matrix (unpublished data). Unlike FNs lacking the RGD sequence, FNΔIII$_{1-2}$ was not deficient in binding to cells. In fact, substantially more FNΔIII$_{1-2}$ than native FN was isolated as cell-associated DOC-soluble material. This indicates that FNΔIII$_{1-2}$ 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 FNΔIII$_{1-2}$ assembly, as this protein shows a defect in fibril growth. In fact, neither FNΔIII$_{1-2}$ nor FNΔIII$_{2-5}$ was able to efficiently incorporate into a preformed human FN matrix. A very few short fibrils were formed by FNΔIII$_{1-2}$ (Fig. 6 B) and FNΔIII$_{2-5}$ fibrils were found in small patches distributed unevenly throughout the matrix (Fig. 6 C). In contrast, an extensive fibrillar network was assembled by native FN (Fig. 6 A). 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 III$_2$ is required for the continued growth of FN fibrils.

Repeat III$_2$ contains an FN binding site

To demonstrate a requirement for III$_2$ in FN assembly, attempts were made to generate a recFN lacking repeat III$_2$ or with another homologous repeat in its place. Unlike the recFNs reported here, secretion of mutant recFNs lacking III$_2$ 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 III$_{1-2}$ 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-

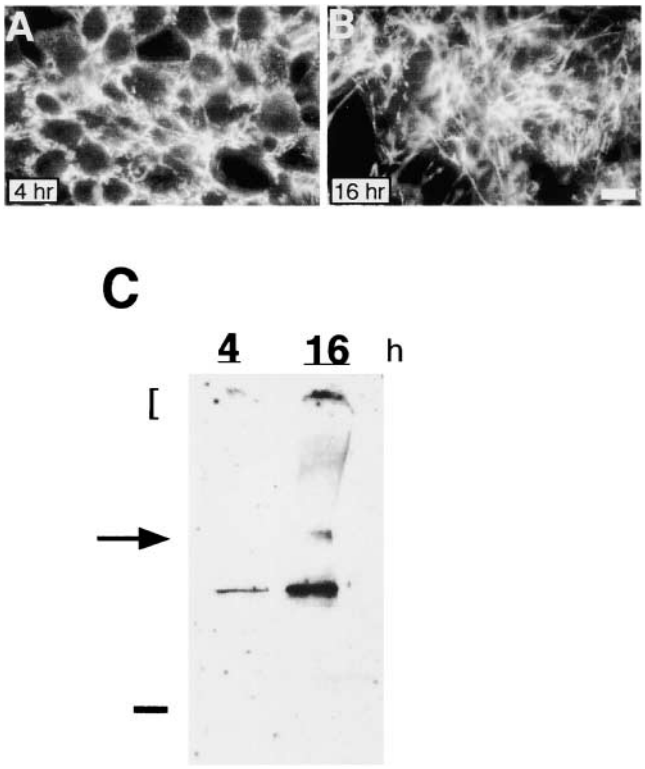

Figure 8. **Assembly of FNrIII$_{4–5/9–10}$.** CHOα5 cells were cultured in the presence of 50 μg/ml FNrIII$_{4–5/9–10}$ for 4 (A) and 16 (B) h. (C) DOC-insoluble material was isolated from CHOα5 cells 4 and 16 h after incubation with 50 μg/ml FNrIII$_{4–5/9–10}$ and analyzed as in Fig. 3. Locations of high molecular mass multimers are indicated with arrow and bracket. 180-kD molecular mass standard is indicated by dash. Bar, 10 μm.

teins containing either III$_1$ or III$_2$ were generated and tested for binding in solid phase binding assays. MBP-III$_2$, but not MBP-III$_1$, showed significant FN binding activity (Fig. 7 A) that was reversed by treatment with buffered SDS (unpublished data). The lack of MBP-III$_1$ binding confirms the results of Ingham et al. (1997) who showed that native III$_1$ 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 III$_2$ differ by only 3.5-fold, 28 nM, and 8 nM, respectively. The higher dissociation constant for FN may reflect a difference in affinity. However, it may also be due to molecular differences between the relatively small 70-kD fragment and dimeric pFN, which has a compact conformation that may reduce accessibility to III$_2$ binding sites.

Between III$_1$ and III$_2$ is a 17–amino acid "linker" segment that was present in the MBP-III$_2$ protein. To determine whether the binding site was located in the type III repeat or the linker, an MBP protein was generated containing III$_{1–2}$ but lacking the linker (MBP-III$_{1–2}$ΔL) and tested for FN binding. MBP-III$_{1–2}$ΔL bound to FN (Fig. 7 C) and the 70-kD fragment (unpublished data) to an extent identical to MBP-III$_{1–2}$. Similar III$_{1–2}$ and III$_{1–2}$ΔL fragments prepared from human FN also showed concentration-dependent binding to 70 kD (unpublished data). Therefore, the site resides in III$_2$ itself and is present in both rat and human FNs. These results identify repeat III$_2$ as a major FN binding site and suggest that the lack of fibril formation by FNΔIII$_{1–2}$ is due to the absence of this site.

Repositioning of III$_{9–10}$ cell binding domain

The cell binding domain consisting of an RGD sequence and synergy site in repeats III$_{9–10}$ is essential for initiation of FN matrix assembly by α5β1 integrin (Sechler et al., 1996, 1997). Deletions within repeats III$_{1–7}$ position the cell binding domain closer to the NH$_2$-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 FNrIII$_{4–5/9–10}$. Repeats III$_{4–5}$ were replaced with III$_{9–10}$ and the RGD sequence was deleted from its native position within the cell binding domain (Fig. 1). Therefore, the only functional III$_{9–10}$ pair is in the position normally occupied by III$_{4–5}$. CHOα5 cells efficiently assembled FNrIII$_{4–5/9–10}$ into a DOC-insoluble fibrillar matrix identical to that of native FN at all time points (Fig. 8, A–C). Cell cycle progression by CHOα5 cells assembling either FN or FNrIII$_{4–5/9–10}$ 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 NH$_2$ 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 III$_{1–7}$ (Sechler et al., 1996). Using a set of overlapping deletions within this region, we have identified repeat III$_2$ as an important FN binding site involved in fibril elongation. Deletions spanning this repeat severely limit fibril assembly and block formation of DOC-insoluble material. In addition to implicating III$_2$, our results also eliminate several candidate sites as important contributors to this process. Specifically, we have shown that: (a) the III$_1$ repeat and its cryptic FN binding site are not needed for fibril formation; (b) relatively large deletions of as many as four repeats do not affect fibril assembly; and (c) there is considerable pliability in the location of the cell binding domain. None of the deletions tested here had an obvious effect on FN binding to integrins or initiation of fibril assembly, indicating that reported interactions between III$_1$ and the cell binding domain (Hocking et al., 1996) are not required in the early stages of FN matrix assembly.

Our data with FNΔIII$_{1–2}$ and FNΔIII$_{2–5}$ show that repeat III$_2$ participates in the growth phase of FN assembly. Assembly was initiated by both of these recFNs but DOC-insoluble matrix formation was blocked. Together with data showing a direct interaction between III$_2$ and the NH$_2$-terminal assembly domain of FN, our matrix assembly results indicate that this FN binding site plays a key role in FN–FN interactions during fibril assembly. Repeats III$_{2–3}$ have been shown previously to interact with repeats III$_{12–14}$, an interac-

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 $III_{1–2}$ segment contains more than one FN binding site, the site in III_2 that is critical for fibril assembly and a cryptic site in III_1 that is dispensable for this process. It is also possible that repeats $III_{1–2}$ 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_{10} and the NH_2-terminal 70-kD fragment (Hocking et al., 1996). III_2 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_1-specific inhibitory peptides and antibodies have been described (Chernousov et al., 1987, 1991; Morla and Ruoslahti, 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 $FN\Delta III_{1–2}$ 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_2 binding site by local perturbation of intramolecular interactions involving this region of the molecule.

Comparison of the progression of assembly by $FN\Delta III_{1–2}$ with $FN\Delta III_{1–7}$ shows that both initially form short stitches around cell peripheries and connect to adjacent cells. $FN\Delta III_{1–7}$ 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, $FN\Delta III_{1–7}$ forms a relatively normal-appearing fibrillar matrix. On the other hand, $FN\Delta III_{1–2}$ forms only a few small, DOC-soluble aggregates that can be converted into predominantly short fibrils. $FN\Delta III_{1–2}$ does not accumulate in DOC-insoluble material, nor does it form an extensive fibrillar matrix. The differences between assembly of $FN\Delta III_{1–2}$ and $FN\Delta III_{1–7}$ suggests that repeats $III_{3–7}$ contribute to the progression of FN fibril formation. A few activities have been mapped to the $III_{3–7}$ region. Repeats $III_{4–6}$ can bind to heparin and DNA under low salt conditions (Hynes, 1990). Cryptic binding sites within repeat III_5 have been reported for activated $\alpha4\beta1$ and $\alpha4\beta7$ 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_7 (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.

$FN\Delta III_{1–2}$ and $FN\Delta III_{1–7}$ probably also differ in the alignment of FN dimers into fibrils. For example, binding of the NH_2-terminal assembly domain of one $FN\Delta III_{1–7}$ 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

CHOα5, clone 17, and AtT-20α5, clone 11, transfected with a cDNA to the human $\alpha5$ integrin subunit have been described previously (Sechler et al., 1996). For all experiments, CHOα5 cells were cultured in DME supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 μg/ml Geneticin (Life Technologies/GIBCO BRL), and 10% fetal calf serum (Hyclone Labs) depleted of FN. AtT-20α5 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 Geneticin (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 by 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, 60 s; and 72°C, 60 s. PCR products were digested with flanking enzymes and inserted into the FN cDNA using convenient restriction sites. The following 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): ΔIII_1: GGGGTACCTGTGCCTGGGTA (1831-1812), CTGGTACCAGCAACACAGTG (2116-2130); ΔIII_{4-7}: CGGGTACCTCATCGGATCGT (2721-2702), GCGGTACCTCCTCCCACGGA (4065-4084); ΔIII_{2-5}: TGTTGCTGGGTACCGGTGTG (2124-2105), CCTGGTACCTCTGCGCTCCA (3248-3267). pVL1392 $FN\Delta III_{1-2}$ was prepared by ligating a fragment from $FN\Delta III_1$ with a PCR-amplified fragment made using the primer CTGGTACCGCACCTGATGCGCCTCCAG (2424-2442). pVL1392 $FN\Delta III_{4-5}$ was created by ligating a 5′ fragment from pVL1392 $FN\Delta III_{4-7}$ with a 3′ fragment from pVL1392 $FN\Delta III_{2-5}$. To generate pVL1392 $FNrIII_{4-5/9-10}$, a segment spanning repeats III_{9-10} was amplified using 5′ primer GAGGTACCGGACTCCCCAACTGGTTTTG (4552-4570) and 3′ primer GAGGTACCGCTGTTTGATAATTGATGGAAACTGGC (5098-5073) containing KpnI sites (underlined). The resulting KpnI fragment was then inserted at the engineered KpnI site in pVL1392 $FN\Delta III_{4-5}$ 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 recFNs were at least 0.8 mg/100 ml of culture supernatant. The identity of $FN\Delta III_1$ and $FN\Delta III_{1-2}$ recombinant baculoviruses was further confirmed using DNA isolated from infected High Five insect cells as template for PCR amplification with flanking primers. Correctly sized PCR products were obtained as analyzed by PAGE before and after restriction with KpnI.

Immunofluorescence

As described in Sechler et al. (1996), CHOα5 and AtT-20α5 cells were seeded in medium containing FN-depleted serum and plated onto glass coverslips in a 24-well dish or four-well chamber slides at a concentration of 1.5×10^5 and 4×10^5, respectively. After an overnight incubation, fresh medium was added along with either pFN or purified recFN and incubated for the times indicated. Cell layers were fixed with 3.7% formaldehyde and stained with a 1:1,000 dilution of IC3 ascites in PBS with 2% ovalbumin followed by a 1:400 dilution of fluorescein-conjugated goat anti–mouse secondary antibody. Stained cells were mounted with FluoroGuard (Bio-Rad Laboratories), and fibrils were visualized with a Nikon Optiphot-2 microscope. Images were collected with a DEI-750 cooled CCD camera (Optronics Engineering) and transferred to a Macintosh G3 computer with an LG3 frame grabber (Scion Corp.) and Adobe Photoshop v. 5.0.

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 iodoacetic acid, 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; Scanalytics, Inc.).

Expression of bacterial fusion proteins and FN binding assays

Rat FN cDNA fragments encoding repeat III_1 or III_2 were inserted into pMAL-cRI (New England Biolabs, Inc.) for expression as MBP fusion proteins. III_1 spanned amino acid positions 604–700 (TYP … TTS) and III_2 extended from residue 701 to 808 (AST … QTT). BamHI sites and XbaI sites were engineered at the 5′ and 3′ ends, respectively. PCR amplification using primers homologous to sequences flanking the 17–amino acid linker (701–717) was used to replace these residues (AST … APF) with a KpnI site to generate $III_{1-2}\Delta L$. *Escherichia coli* TB1 cells expressing individual MBP fusion proteins were lysed with B-PER (Pierce Chemical Co.) and proteins were purified by amylose resin affinity chromatography following the manufacturer's recommendations. Rat pFN, recombinant 70-kD fragment, MBP, and MBP-III_{1-2} were purified as described previously (Aguirre et al., 1994).

Solid phase binding assays were performed essentially as described by Aguirre et al. (1994). Fusion proteins containing III_1, III_2, III_{1-2}, and $III_{1-2}\Delta L$ were immobilized on Nunc Maxisorp microtiter plates by overnight incubation at the indicated concentrations. Relative amounts of immobilized proteins were determined by ELISA with an anti-MBP antiserum. After washing and blocking with 1% BSA in PBS, wells were incubated with rat pFN at 50 μg/ml or NH_2-terminal 70-kD fragment of FN at 30 μg/ml for 2 h at room temperature. Bound FN and 70 kD were detected and quantified by ELISA using anti–rat FN monoclonal antibody 5G4 or anti–70-kD polyclonal antiserum R457.

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