Identification of the Fibronectin Sequences Required for Assembly of a Fibrillar Matrix

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Abstract. During extracellular matrix assembly, fibronectin (FN) binds to cell surface receptors and initiates fibrillogenesis. As described in this report, matrix assembly has been dissected using recombinant FN polypeptides (recFNs) expressed in mammalian cells via retroviral vectors. RecFNs were assayed for incorporation into the detergent-insoluble cell matrix fraction and for formation of fibrils at the cell surface as detected by indirect immunofluorescence. Biochemical and immunocytochemical data are presented defining the minimum domain requirements for FN fibrillogenesis. The smallest functional recFN is half the size of native FN and contains intact amino- and carboxy-terminal regions with a large internal deletion spanning the collagen binding domain and the first seven type III repeats. Five type I repeats at the amino terminus are required for assembly and have FN binding activity. The dimer structure mediated by the carboxy-terminal interchain disulfide bonds is also essential. Surprisingly, recFNs lacking the RGDS cell binding site formed a significant fibrillar matrix. Therefore, FN–FN interactions and dimeric structure appear to be the major determinants of fibrillogenesis.

FIBRONECTIN (FN)[1] is a major component of vertebrate tissues. FN, together with other glycoproteins and proteoglycans, forms a complex extracellular network that plays a crucial role in a wide variety of developmental and cellular processes including cell adhesion, migration, morphology, differentiation, and proliferation (reviewed in McDonald, 1988; Ruoslahti, 1988; Mosher, 1989; Hynes, 1990). At the molecular level, cell movement and behavior are mediated by FN fibrils extending between cells and to the substratum. Elaboration of this fibrillar network involves interactions between FN and a variety of extracellular matrix (ECM) molecules such as collagens and proteoglycans as well as with a number of different cell surface receptors, particularly integrins. This dynamic assembly process cannot be reproduced with purified matrix components.

In the initial stages of FN matrix assembly, the temporal and spatial order of association of cell surface molecules with extracellular proteins must be well-orchestrated in order to form a stable framework of FN fibrils. Subsequent interactions between matrix components at sites removed from the cell surface are probably involved in matrix accumulation and maintenance. As a complex, multi-component structure, the matrix does not readily lend itself to detailed molecular analysis. Consequently, many studies to date have focused on the FN domains required for the latter stages of assembly, namely, incorporation of FN into established matrices. Cells will assemble exogenously added FN into fibrils (Hayman and Ruoslahti, 1979; McKeown-Longo and Mosher, 1983; Chernousov et al., 1985; Millis et al., 1985; Guan et al., 1990). Proteolytic fragments containing the amino-terminal region of FN bind saturably to confluent monolayers of fibroblasts and can inhibit ECM assembly (McKeown-Longo and Mosher, 1985; McDonald et al., 1987; Allio and McKeown-Longo, 1988; Quade and McDonald, 1988; Barry and Mosher, 1989). Inhibition experiments have also been used to show a role for cell surface receptors. Reagents that block FN-cell interactions, i.e., anti-integrin and anti-FN antibodies and the arg-gly-asp-ser cell binding sequence (RGDS)-containing peptides, inhibit matrix assembly in culture systems (Akiyama et al., 1987; McDonald et al., 1987; Woods et al., 1988; Fogerty et al., 1990) and in vivo (Darribere et al., 1990). Finally, recombinant, truncated polypeptides, lacking the amino-terminus but containing the cell binding domain, are not incorporated into the matrix (Schwarzbauer et al., 1987). Although these studies implicate the amino-terminal region and the cell adhesive domain in ECM assembly, the complex nature of the matrix makes it difficult to define the activities contributed to this process by these domains or to distinguish between direct and indirect roles for these and other regions of FN.

To determine the sites directly involved in FN fibrillogenesis and matrix formation, I have elected to use recombinant polypeptides to dissect the domains required for this assembly process. A simplified system has been developed to analyze de novo assembly of secreted recombinant FNs (recFNs) in the absence of significant levels of other ECM proteins, thus, eliminating the complications inherent in formation of a complete matrix. Two criteria were applied to determine

1. *Abbreviations used in this paper*: DOC, deoxycholate; ECM, extracellular matrix; FN, fibronectin; pFN, plasma FN; recFN, recombinant FN; RGDS, arg-gly-asp-ser cell binding sequence; VT1, rat-specific antifibronectin mAb.

the competence of recFNs in matrix assembly: incorporation into the detergent-insoluble matrix fraction and formation of fibrils detectable by immunofluorescence. Recombinant polypeptides possessing the appropriate combination of domains are assembled into a fibrillar FN matrix. The amino-terminal region is indeed essential for this process and contains a site of direct FN–FN interaction. In addition, the recombinant polypeptides must be secreted as covalent disulfide-bonded dimers; monomeric polypeptides do not assemble. Interestingly, recFNs lacking the RGDS cell binding sequence are assembled, showing that this sequence is not essential for fibril formation. I propose that the two major steps in FN fibrillogenesis are intermolecular FN–FN interactions mediated by the amino-terminus followed by the multimerization of FN dimers into fibrils.

Materials and Methods

cDNA Constructions

The retroviral vector, pLJ (Schwarzbauer et al., 1989; Guan et al., 1990), was used for most of the constructions for expression. Restriction and other enzymes were purchased from New England Biolabs (Beverly, MA) or Boehringer-Mannheim Biochemicals (Indianapolis, IN). The majority of the 5′ untranslated sequence was removed from the rat cDNA by introducing a BamHI linker at a HinfI site 20 bp upstream of the ATG (Patel et al., 1987). For expression of the amino-terminal 70-kD fibrin/collagen binding fragment, a termination codon was inserted by adding an XbaI linker to a blunted PstI site at position 1810, located at the end of repeat I_9. The BamHI to XbaI fragment was cloned into SP73 (Promega-Biotech, Madison, WI), then excised with BamHI and SalI, which is adjacent to the XbaI site. The resulting 1.8-kb BamHI-SalI fragment was cloned directionally into pLJ.

For construction of the larger recombinant FN cDNA encoding both the amino-terminal region and the carboxy-terminal half of the molecule (recFN I_{1-9}/C110), a HindIII linker was added after the PstI site. To attach this to the rest of the molecule, a HindIII linker was also ligated onto an EagI site located at position 4070 in the rat cDNA. This corresponds to the junction between EIIIB and repeat III_8 and, therefore, I_{1-9}/C110 cDNA lacks residues 1810–4070 encoding the first seven type III repeats. The resulting amino acid sequence across this junction is QPLQPKLGAVPP, where the underlined residues were introduced by the HindIII linker and the flanking amino acids are present in rat pFN. The carboxy-terminal portion encoded the domains for binding to cells, heparin, and fibrin, along with the V region for efficient secretion and the pair of cysteines to form the interchain disulfide bonds and in the recFNs is termed C110. The other alternatively spliced regions, EIIIA and EIIIB, were omitted from these constructions. The 5′ end of the cDNA encoding ΔN80 kD is located at position 2400, and this recFN contains sequences from repeat III_3 to the carboxy terminus, including EIIIB and the V region in the vector, MSV-pLP (Schwarzbauer et al., 1989).

All deletions were made by using convenient restriction sites within the cDNA and were designed to remove individual or sets of repeats as units. For I_{1-4}/C110 and I_{1-5}/C110, the HindIII linker was added at a HpaII site (position 680) or a XhoII site (840), respectively, and the resultant BamHI-HindIII fragment encoding either the first four or five type I repeats was inserted in place of that encoding the 70-kD fragment (I_{1-9}). The amino acid sequences at these two junctions are CTSRPKLG for I_{1-4} and SAGSPKLG for I_{1-5} where underlined residues are encoded by the HindIII linker. Two other variations of the amino-terminal region, I_5-II_2 ($I_{5,6}$ + $II_{1,2}$) and I_{7-9}, required the addition of the FN signal sequence at the 5′ end. A 150-bp fragment extending from a HinfI site 20 bp upstream of the ATG to a BamHI linker inserted at a BstNI site at the end of exon 1 of the FN gene was subcloned between the BglII and BamHI sites in SP73 to generate the vector, SP73-FS. This fragment encodes the FN signal and putative pro sequences along with the first 19 amino acids of the mature protein. A BamHI linker chosen to maintain the correct reading frame was added to a blunted HpaII site (position 680) located between I_4 and I_5 and a HindIII linker was attached to a blunted BglII site between II_2 and I_7 at position 1400. This 720-bp BamHI-HindIII fragment was ligated into SP73-FS cut with BamHI and HindIII. From the resulting plasmid, a BglII-HindIII fragment containing sequences from the FN signal through II_2 was ligated to the C110 in the vector pLJ. I_{7-9}/C110 extends from the BglII site at 1,400 to the HindIII site added at PstI 1810, and was inserted into SP73-FS and then cloned into pLJ as for I_5-II_2/C110.

Two mutations were made within the C110 region of the cDNA. Deletion of the 20 amino acid segment containing the carboxy-terminal cysteines that form the interchain disulfide bonds has been described previously (Schwarzbauer et al., 1989). The segment encoding the RGDS cell binding peptide was removed using a HaeIII restriction site at 4840 and a Fnu4HI site at 4860. An 8-mer BamHI linker was added to the HaeIII and a 12-mer BamHI linker to the Fnu4HI site. After ligation and insertion into the larger cDNA, the final construction deletes seven residues, RGDSPAS, and replaces them with three new amino acids, GSA.

Cells and Viruses

SVT2 (SV40-transformed 3T3) and Ψ_2 cells were grown in DME plus 10% calf serum (Hyclone Laboratories, Logan, Utah). Ψ_2 cells were transfected with recombinant retroviral vectors and SVT2 cells were infected with recombinant retroviruses as described previously (Schwarzbauer et al., 1989). G418-resistant infectants were screened for expression of recombinant FNs by gelatin or heparin affinity chromatography and SDS-PAGE of metabolically labeled secreted proteins. For infected cell clones secreting recFNs, the presence of the correct recFN cDNA was confirmed by Southern analysis of isolated genomic DNA. The hybridoma cell line secreting the rat-specific anti-FN mAb, VT1, was kindly provided by Dr. K. Fukuda (University of Vermont) and was cultured in DME supplemented with 15% FCS (Hyclone Laboratories).

Metabolic Labeling and Immunoprecipitations

Cells were labeled with ^{35}S-methionine (Dupont-NEN, Boston, MA) at 25 μCi/ml for 24 h in DME minus methionine supplemented with unlabeled methionine (1.5 μg/ml) and 10% calf serum. For some experiments, serum was depleted of bovine pFN by gelatin-agarose affinity chromatography. Conditioned medium was collected, cells were lysed, and deoxycholate-insoluble material was fractionated as described (Choi and Hynes, 1979; Schwarzbauer et al., 1989). PMSF, EDTA, iodoacetic acid, and N-ethylmaleimide (NEM) were added as protease inhibitors. DOC-insoluble material was recovered by centrifugation and solubilized in 1% SDS, 25 mM Tris-HCl, pH 8.0, 2 mM EDTA. On average, 75% of the DOC-insoluble proteins were solubilized under these conditions.

RecFNs were immunoprecipitated using a rabbit anti-rat pFN antiserum (Schwarzbauer et al., 1989), samples were eluted from protein A–agarose beads (Repligen, Cambridge, MA) by boiling in SDS electrophoresis sample buffer, and samples were separated in a 5% SDS polyacrylamide gel either reduced, by adding DTT to 0.1 M, or nonreduced. Gels were treated, and proteins visualized and quantitated as described (Schwarzbauer et al., 1989).

Immunofluorescence

Rabbit anti-rat pFN antiserum was passed over a rat pFN-Sepharose column, and antibodies were eluted with 0.23 M glycine, pH 2.6, and immediately neutralized with 1 M Tris-HCl, pH 8.8. These affinity-purified antibodies exhibited lower background staining than the complete antiserum in immunofluorescence experiments, but retained the ability to recognize human, mouse, and bovine FNs. Polyclonal antibodies were diluted 1:25 and culture supernatant from VT1 hybridoma cells was used at a dilution of 1:10.

SVT2 cells expressing various recFNs were cultured on glass coverslips until almost confluent, washed with PBS, and fixed with 3.7% formaldehyde in PBS for 15 min at room temperature. Coverslips were washed several times with PBS and incubated with primary antiserum diluted in 2% ovalbumin in PBS in a moist chamber for 30 min at 37°C. Subsequent incubations were with goat anti-mouse or goat anti-rabbit biotinylated IgG (BRL, Gaithersburg, MD) diluted 1:100 in ovalbumin/PBS followed by rhodamine-avidin (ICN Biochemicals, Costa Mesa, CA) or fluorescein-avidin (Cappel Laboratories, Malvern, PA) at 1:400 or 1:300, respectively. Coverslips were washed three times with PBS between each incubation. A final wash with water was included prior to mounting in Fluorsave (Calbiochem-Behring Corp., La Jolla, CA). Stained fibrils and cells were visualized with a Nikon Optiphot microscope with epifluorescence using a 40× phase/fluorescence objective. Fibrils were photographed with Kodak Tri-X Pan 400 black and white print film which was developed with Diafine developer, and prints were prepared with an Ilford rapid print processor.

Collagenase Treatment

Cell-conditioned medium was collected, clarified by centrifugation, and treated with collagenase (Colstridium histolyticum; Sigma Chemical Co., St. Louis, MO) at 60 U/ml in the presence of 2 mM PMSF and 0.5 mM NEM for 1 h at 37°C. Digestion was stopped by adding EDTA to a final concentration of 10 mM, and samples were stored frozen or used immediately for immunoprecipitation or binding assays. Monolayers were treated with collagenase before lysis by incubating with DME plus PMSF and NEM as for medium with 120 U collagenase per ml of solution. Incubation was for 15 min at 37°C. EDTA was added and cells were washed twice with PBS and lysed with DOC as described above.

Affinity Binding Assays

Gelatin-agarose and heparin-agarose (Sigma Chemical Co.) affinity chromatography were performed batchwise by incubating ^{35}S-methionine–labeled conditioned medium from SVT2 cells lines with resin at 4°C for 1–2 h with end-over-end mixing. Bound proteins were separated from unbound by centrifugation and washing twice with cold PBS. Bound proteins were eluted by boiling in SDS electrophoresis sample buffer minus DTT.

Human pFN was purified from outdated plasma and rat pFN was purified from fresh plasma by gelatin-agarose affinity chromatography (Engvall and Ruoslahti, 1977). pFN was coupled to Sepharose (Pharmacia/LKB, Piscataway, NJ) following the manufacturer's recommendations. The batchwise FN-Sepharose incubation conditions were essentially the same as above except that 100 μl packed beads containing 100 μg coupled human FN was incubated with 0.5 ml labeled medium. Although this represents an excess of coupled FN over labeled protein, binding of labeled recFNs was never quantitative.

To assay the FN binding ability of recFNs in the absence of other secreted or serum proteins, particularly mouse and bovine FNs, cells were metabolically labeled in serum depleted of bovine pFN, and recFNs were purified using the following procedure. ^{35}S-methionine–labeled conditioned medium was passed through a gelatin-agarose column to remove secreted mouse FN and other gelatin binding proteins. The flowthrough fraction was put over a heparin-agarose column, which was then washed with PBS until the counts went down to background levels. Heparin-bound proteins were eluted with 0.8 M NaCl in 20 mM Tris-HCl, pH 8.0, 2 mM EDTA. Fractions containing the highest cpms were pooled and dialyzed into PBS. This sample was centrifuged to remove insolubles and applied to a FN-Sepharose column, washed with PBS, and eluted with 4 M urea/PBS. Fractions were counted and analyzed after electrophoresis by fluorography or by silver staining (Merril et al., 1984).

Results

A System for Dissecting FN Matrix Assembly

ECM assembly is a multicomponent, dynamic process that involves both secreted and cell surface molecules. Dissection of this process has been hampered by the complexity of the interactions, so a simplified system was designed whereby a single component could be introduced by cDNA expression and its activity tested. To minimize the effects of endogenous FN and other ECM components on matrix formation, SV40-transformed mouse 3T3 (SVT2) cells were used for these studies. SVT2 cells synthesize and secrete reduced levels of most ECM proteins, including FN (Yamada et al., 1976; Yamada and Kennedy, 1979; this report). As a consequence, these cells assemble little, if any, FN matrix. Only a very sparse, punctate pattern was observed after immunofluorescent staining with polyclonal anti-FN antibodies (Fig. 1 *A*). However, upon addition of purified rat plasma FN (pFN) to the culture medium, SVT2 cells accumulated the exogenous FN in the form of fibrils that were detectable with a rat-specific anti-FN mAb (VTI) (Fig. 1 *C*). Therefore, these cells can bind rat FN and assemble it into fibrils at the cell surface. No fluorescent signal was detected on SVT2 cells in the absence of added rat pFN (Fig. 1 *B*).

We have previously established that SVT2 cells will synthesize and secrete cDNA-encoded FN polypeptides at relatively high levels and in the form of disulfide-bonded dimers (Schwarzbauer et al., 1989). Together with the ability to assemble FN into fibrils in the absence of an extensive preexisting matrix, this provides a simplified system for analysis of the function of recombinant and mutant FN polypeptides during ECM formation.

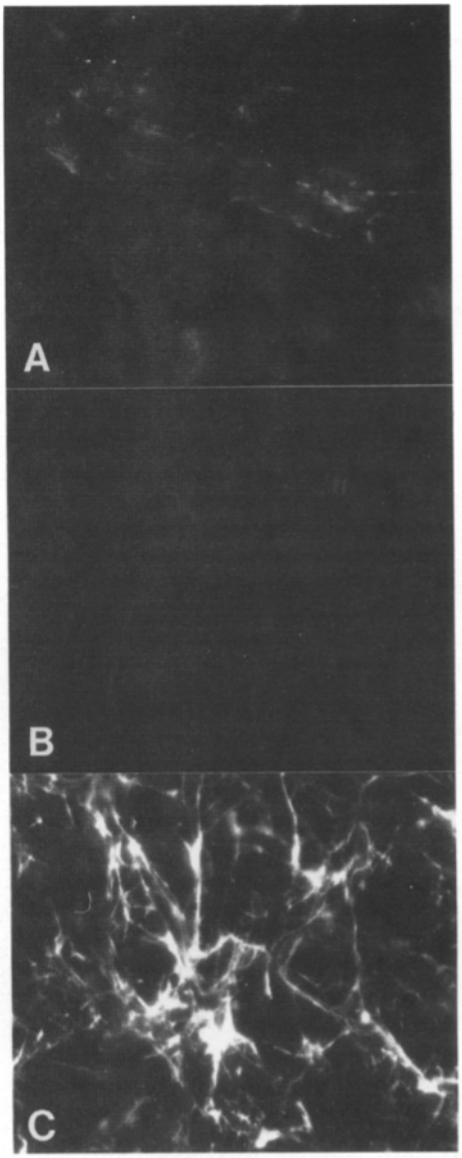

Figure 1. Immunofluorescence staining of SVT2 cells. To examine the endogenous SVT2 FN matrix, cells were cultured in medium containing FN-depleted calf serum and then fixed and stained with affinity-purified polyclonal anti-FN antibodies (*A*). A few faint FN fibrils can be seen. Similar staining was observed when cells were cultured in serum containing calf pFN (data not shown). There was no staining of SVT2 cells with the rat-specific mAb, VTI (*B*). When SVT2 cells were incubated with rat pFN for 24 h before fixing, an extensive fibrillar matrix of exogenous rat pFN was detected with the VTI mAb (*C*).

Construction and Expression of Recombinant FN Polypeptides

Truncated "deminectins," dimeric polypeptides composed of

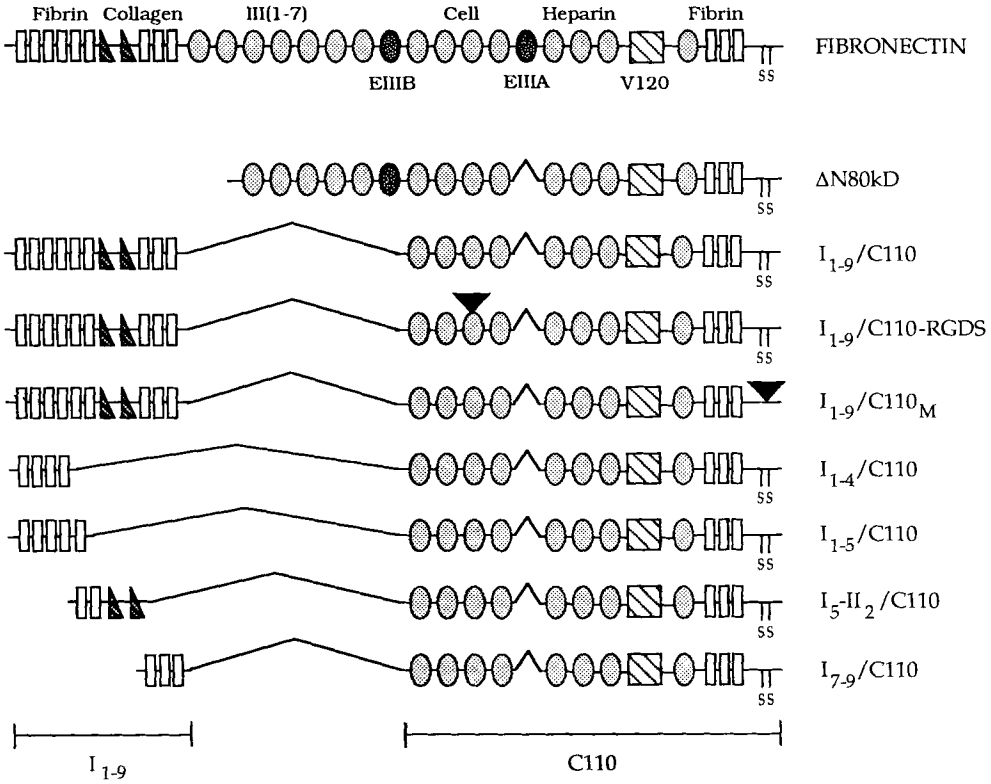

Figure 2. Schematic diagram of FN and recFNs expressed in SVT2 cells. The top diagram shows the structural organization of FN. Domains for binding fibrin, collagen, cells, and heparin are indicated as are the first seven type III repeats, III (1-7). The FN dimer is formed through the pair of cysteines at the very carboxy terminus (*SS*). FN is composed of three types of repeats, type I (*open rectangles*), type II (*crosshatched triangles*), and type III (*stippled ovals*). The alternatively spliced type III repeats, EIIIA and EIIIB, are darkened. A third region of alternative splicing, the V region (*crossed box*), has three different variants in rat FN. Only one of these was used in these studies and is shown as V120. Eight recombinant versions of FN were constructed from rat FN cDNAs and the repeats comprising each are shown. ΔN80 kD contains a continuous string of repeats, III_3 to the carboxy terminus. All other recFNs have repeats III_{1-7} deleted. All are EIIIA-, V120+. I_{1-9}/C110, I_{1-9}/C110-RGDS, and I_{1-9}/$C110_M$ are identical in the amino-terminal region (I_{1-9}) containing six type I repeats (I_{1-6}), two type II repeats ($II_{1,2}$), and three type I repeats (I_{7-9}). This segment is connected to the C110 half of the molecule beginning with repeat III_8. The deletions within C110 of the RGDS and of the carboxy-terminal cysteines are indicated by solid triangles. The next four recFNS are identical in the C110 segment but differ within repeats I_{1-9}. I_{1-4}/C110 and I_{1-5}/C110 contain the first four and five type I repeats, respectively. The next four repeats, $I_{5,6}$ and $II_{1,2}$ make up I_5–II_2/C110. The amino-terminus of I_{7-9}/C110 is composed of three type I repeats from the collagen binding domain. Each of the cDNAs was inserted into the retroviral vector, pLJ, and SVT2 cell lines expressing these polypeptides were produced by infection with recombinant retroviruses.

the carboxy-terminal half of FN, are not incorporated into the ECM (Schwarzbauer et al., 1987), suggesting that sequences within the amino-terminal half are required for incorporation. Therefore, a set of overlapping cDNAs was constructed that contained part or all of the amino-terminal 70-kD fibrin/collagen binding region spanning nine type I repeats and two type II repeats (I_{1-9}). Fig. 2 illustrates some of the rat recombinant polypeptides that have been expressed in SVT2 cells for these studies. All of these recFNs contain the carboxy-terminal half of FN, from repeat III_8 to the termination codon. This half of the molecule was given the name C110 to designate its carboxy-terminal location and its 110 kD molecular mass. The alternatively spliced V120 segment was also present in all cDNAs, as this region is required for efficient secretion of FN dimers (Schwarzbauer et al., 1989).

Two regions of FN implicated in matrix formation are the amino-terminal fibrin/collagen binding segment and the cell adhesive domain. RecFN I_{1-9}/C110 contains both of these regions but has a large internal deletion of repeats III_{1-7} and the collagen domain is directly connected to the cell binding domain (Fig. 2). The size difference between this protein and native FN, 180 and 250 kD, respectively, enabled the separation and unequivocal detection of recombinant polypeptides over the background of endogenous SVT2 proteins. ΔN80-kD is a large fragment of FN, covering sequences from the end of repeat III_2 to the carboxy terminus. This recFN is similar to I_{1-9}/C110 in size, but it lacks an intact amino-terminus.

To test the individual requirements for the fibrin and collagen domains or other segments of the amino-terminal region, deletions of sets of repeating units were made within the cDNA. Deletion boundaries were designed to fall precisely between repeats so as to have minimal disruptive effects on the folding of adjacent repeats. The resultant recombinant polypeptides contain repeats I_{1-4}, I_{1-5}, I_5-II_2, and I_{7-9} directly connected to C110 and are about half the size of full length FN at 132, 138, 134, and 127 kD, respectively. In addition, two sites within the carboxy-terminal half were mutated to determine their involvement in fibrillogenesis. I_{1-9}/C110-RGDS lacks the four amino acids, RGDS, that comprise the cell binding site in repeat III_{10}. In I_{1-9}/$C110_M$, the cysteines that form the interchain disulfide bonds were deleted, resulting in synthesis and secretion of a monomeric recFN.

The cDNAs were inserted into the retroviral vector, pLJ, and transfected into the Ψ_2 packaging cell line. SVT2 cell lines producing recFNs were generated by infection with the recombinant retroviruses, selection with the antibiotic G418, and screening of cell-conditioned medium for presence of

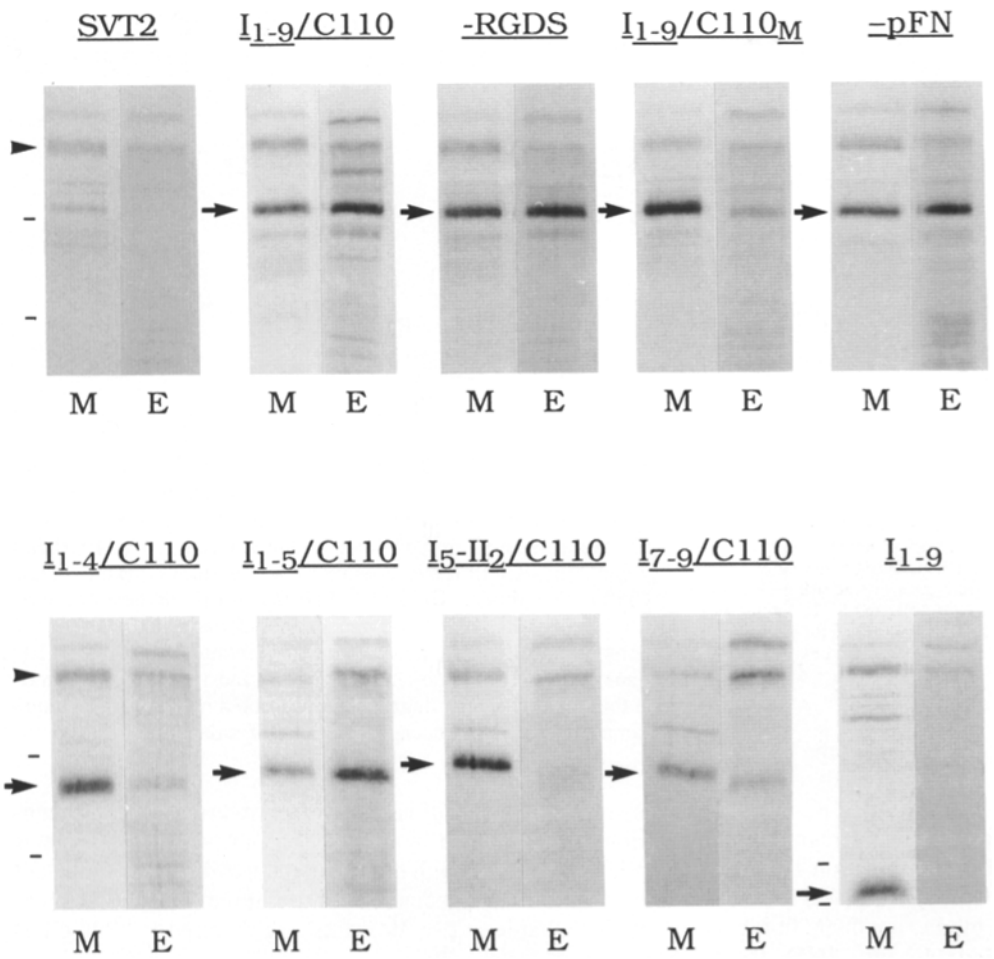

Figure 3. Comparison of secreted and ECM-associated recFNs. Cells were metabolically labeled with ^{35}S-methionine and aliquots of cell-conditioned medium (*M*) and the DOC-insoluble ECM fraction of the cell lysate (*E*) were immunoprecipitated and electrophoresed after reduction of disulfide bonds with DTT. Endogenous SVT2 mouse FN is present in both medium and matrix fractions (*arrowhead*). All recFNs were secreted at levels higher than that of SVT2 FN (*arrows*, lanes *M*), but only some of these polypeptides were incorporated into the ECM (lanes *E*). Similar levels of I_{1-9}/C110-RGDS were incorporated into the matrix in the absence of calf serum pFN (-pFN). Molecular mass standards of 180 and 116 kD are marked (*dashes at left*). The locations of 84- and 58-kD standards are indicated to the left of sample I_{1-9}.

high levels of recombinant polypeptides. SVT2 cell clones expressing different recombinant versions of rat FN were compared for their ability to form a recFN matrix.

Biochemical Analysis of ECM Components

ECM components form an insoluble network that is resistant to detergent extraction. As a consequence, stably incorporated molecules can be identified by cell lysis with buffered 2% deoxycholate (DOC) followed by isolation of the DOC-insoluble fraction. This technique was used to determine which of the expressed recombinant polypeptides were functional for incorporation into the ECM. ^{35}S-Methionine-labeled cells were lysed with 2% DOC in buffer, DOC-insoluble material was solubilized with SDS, and samples were analyzed after immunoprecipitation with anti-FN antiserum. Fig. 3 shows the levels of secreted recFNs in the cell-conditioned medium (lanes *M*) and assembled polypeptides in the ECM (lanes *E*). All recFNs were secreted at high levels relative to the SVT2 FN. The extent of incorporation of recFNs into the DOC-insoluble matrix fraction can be ascertained by comparing band intensities of recFN relative to SVT2 FN between medium and matrix samples. It is clear that only a subset of these proteins was capable of incorporation into the ECM. More specifically, the equivalent proportions of I_{1-9}/C110 in medium and ECM fractions shows that this recFN was fully competent for assembly. Within this context, absence of the RGDS cell binding peptide did not seem to reduce incorporation. On the other hand, the amino terminus and the dimer structure are essential for assembly. Polypeptides lacking the amino-terminal fibrin/collagen domains (ΔN80 kD, data not shown) were excluded from the DOC-insoluble material. Although I_{1-9}/C110$_M$ contains the amino terminus, the carboxy-terminal interchain disulfide bonds are absent and the monomeric form results in a greatly reduced proportion incorporated into the ECM. The presence of serum FN in the culture medium did not enhance the incorporation of recFNs into the matrix. Identical results were obtained when cells were cultured in FN-depleted serum as shown for the I_{1-9}/C110-RGDS recFN (Fig. 3, -pFN).

The essential site within the 70-kD region was further localized by expressing cDNAs with deletions within this region. I_{1-5}/C110, containing the amino-terminal first five type I repeats, was as active as I_{1-9}/C110 in FN matrix assembly (Fig. 3). ECM incorporation was ablated by removal of repeat I_5 from that polypeptide (I_{1-4}/C110). Thus, the first four type I repeats alone are insufficient for matrix assembly. This might suggest that I_5 is a critical component of a functional recFN by containing an essential sequence involved in fibril formation. To determine whether I_5 itself is the active site, the recFN consisting of this and the following three repeats, I_5–II_2/C110, was tested and found to lack the ability to be incorporated into the matrix (Fig. 3). These data suggest that an individual repeat is essential for fibril formation, but is itself insufficient for this activity.

Table I. Relative Proportions of Secreted and ECM-associated recFNs

		Molar ratios of RecFN/SVT2 FN	
		Medium	ECM
I_{1-9}/C110	1	3.8	4.4
	2	1.5	1.4
I_{1-9}/C110-RGDS	1	4.6	6.0
	2	2.8	3.1
$I_{1-9}/C110_M$	1	5.1	1.4
	2	6.4	2.0
I_{1-4}/C110	1	3.2	0.9
	2	5.1	1.5
I_{1-5}/C110	1	3.8	5.8
	2	2.5	2.5

RecFN and SVT2 FN levels in immunoprecipitates of medium and DOC-insoluble (ECM) samples were determined by densitometric scanning of fluorograms. RecFN values were corrected for difference in size using 180, 138, 132, and 250 kD for I_{1-9}/C110, I_{1-5}/C110, I_{1-4}/C110, and SVT2 FN, respectively, and molar ratios of recFN to SVT2 FN were calculated for each sample. The results for two independently isolated clones expressing different levels of recFN are listed. Note the correspondence between medium and ECM ratios for I_{1-9}/C110, I_{1-9}/C110-RGDS, and I_{1-5}/C110.

To test whether the carboxy-terminal half of the molecule is required for matrix incorporation, the levels of amino-terminal 70-kD (I_{1-9}) region alone in the DOC-insoluble fraction were determined. This recombinant fragment consists of type I and II repeats with a termination codon inserted at the end of repeat I_9. Although this fragment has collagen binding activity and can bind to confluent fibroblast monolayers (McKeown-Longo and Mosher, 1985), the de novo incorporation of newly synthesized rec70kD was negligible as determined by analysis of DOC-insoluble proteins (Fig. 3). Together with the fact that the carboxy-terminal half is incompetent independent of the amino-terminus, I conclude that at least two "domains" are required for stable formation of a FN matrix.

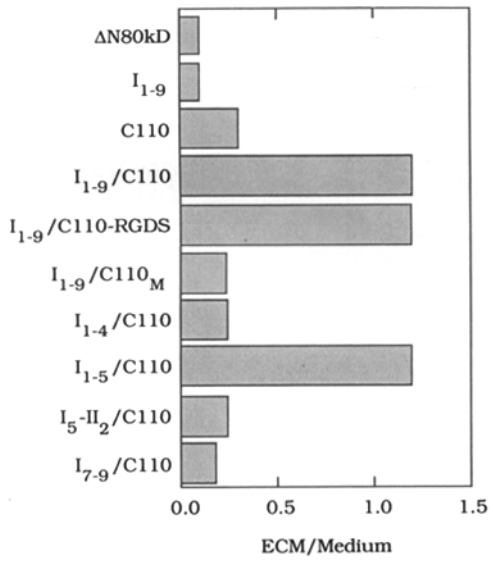

Figure 4. Quantitation of recFNs in the medium and matrix. Relative amounts of recFN were determined by densitometric scanning of fluorograms and normalization to endogenous FN in the same sample as in Table I. The ratio of normalized recFNs in representative ECM and medium samples was calculated and is shown here as a histogram. Values of 1.0 mean that the recFN was incorporated into the matrix, while values less than 1.0 indicate that the polypeptide was not functional.

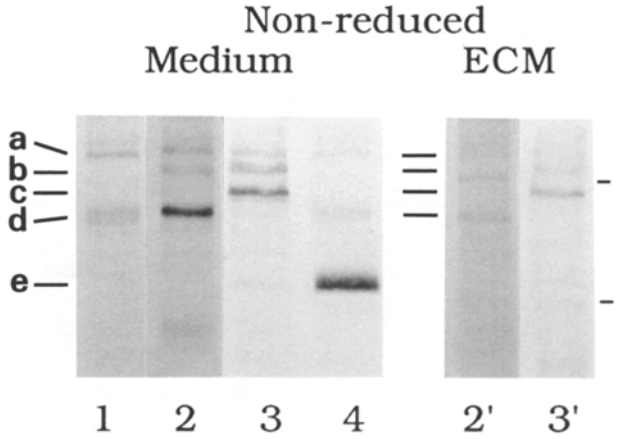

Figure 5. Dimer repertoire of recFNs. To determine the proportions of recFN homodimers and recFN-SVT2 FN heterodimers secreted into the medium (1–4) and incorporated into the matrix (2′,3′), samples were electrophoresed without reduction: (lane *1*) SVT2; (lanes *2* and *2′*) I_{1-5}/C110; (lanes *3* and *3′*) I_{1-9}/C110-RGDS; (lane *4*) $I_{1-9}/C110_M$. The dimers are: (*a*) FN-FN; (*b*) recFN-FN heterodimers; (*c*) I_{1-9}/C110-RGDS homodimers (lanes *3* and *3′*); (*d*) I_{1-5}/C110 homodimers (lanes *2* and *2′*); and (*e*) monomers of $I_{1-9}/C110_M$ (lane *4*). Molecular mass markers of 360 and 180 kD are indicated at the right. The band migrating at position d in the SVT2 medium sample (lane *1*) is a background band of unknown origin.

To compare directly the activities of different recFNs for fibril formation, levels of secretion and incorporation into DOC-insoluble material were quantitated. Ratios of recFN to endogenous SVT2 FN in the same sample were calculated using densitometric data for medium and ECM fractions. Molar ratios for two independently isolated clones expressing different levels of recFN are listed in Table I. Equivalent proportions of recFNs I_{1-9}/C110, I_{1-9}/C110-RGDS, and I_{1-5}/C110 were present in the medium and DOC-insoluble ECM fractions, while the ECM levels of $I_{1-9}/C110_M$ and I_{1-4}/C110 were reduced significantly. The ECM to medium ratios of these normalized values were calculated for each recFN (Fig. 4). A value of 1.0 means that equivalent proportions were present in matrix and medium and the recFN was as functional as endogenous FN. Values lower than 1.0 indicate that a recFN was only partially active or was excluded from the matrix. The results define a hierarchy of active proteins that fits with the qualitative data. I_{1-9}/C110, I_{1-9}/C110-RGDS, and I_{1-5}/C110 polypeptides, with values approaching 1.2, were at least as active as endogenous FN for assembly (Fig. 4). The others had values of 0.3 or less. Clearly, lack of either the interchain disulfide bonds or of repeats I_{1-5} greatly inhibits incorporation.

Three types of FN dimers are secreted when rat FN cDNAs are expressed in SVT2 cells: homodimers of two recombinant polypeptides, heterodimers of one recFN and one endogenous SVT2 FN subunit (recFN-FN), and dimers of two endogenous FN molecules (Schwarzbauer et al., 1989). To determine the representation of recFN-FN heterodimers and

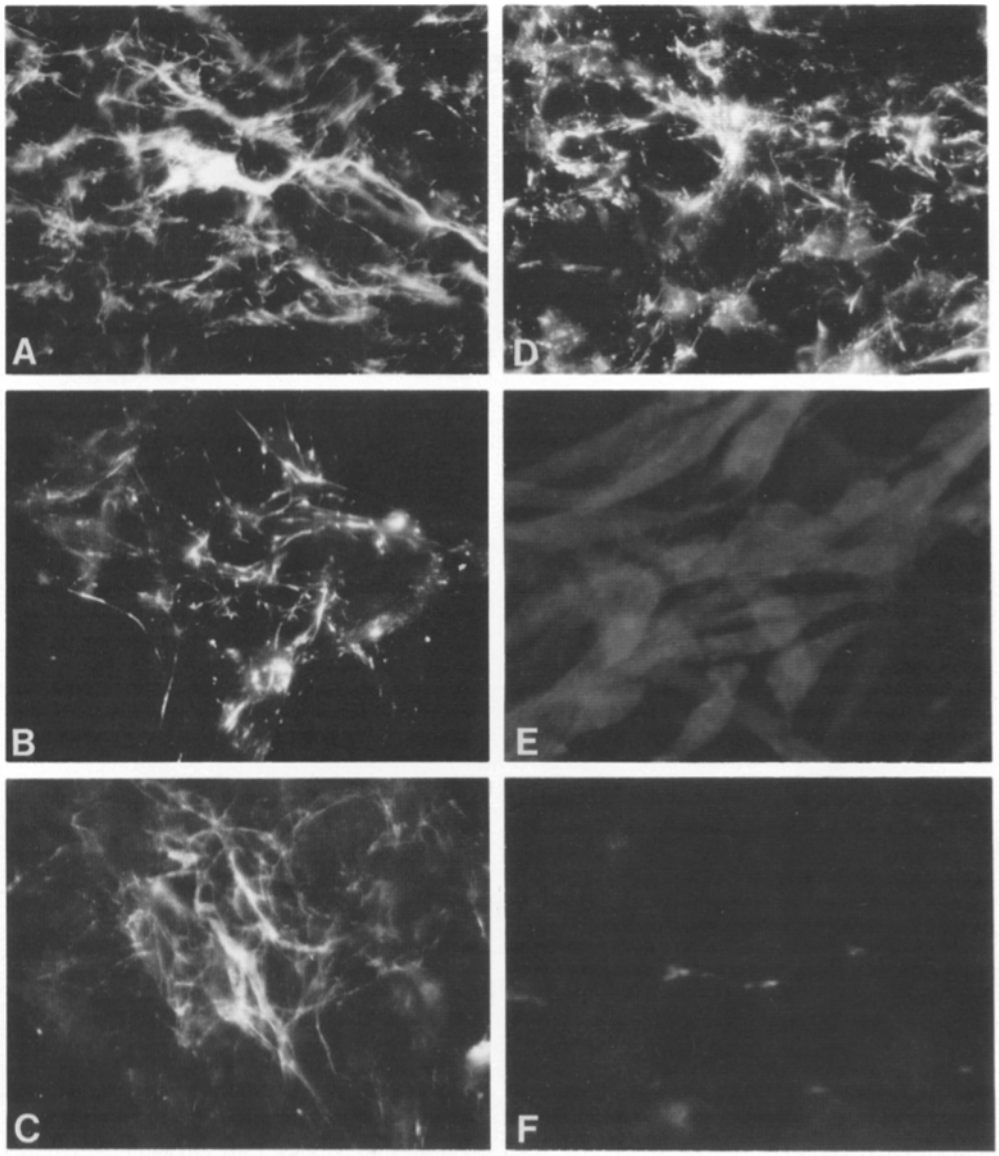

Figure 6. Immunofluorescence staining of recFN fibrils. SVT2 cell clones secreting different recFNs were analyzed for the presence of recFN fibrils by indirect immunofluorescence using the rat-specific mAb, VT1. I_{1-9}/C110 (*A* and *B*), I_{1-5}/C110 (*C*), and I_{1-9}/C110-RGDS (*D*) formed extensive fibrils. $I_{1-9}/C110_M$ (*E*) and I_{1-4}/C110 (*F*) were negative. Two fluors were used in these experiments: cells in *A*, *B*, *D*, and *E* were stained with rhodamine, and those in *C* and *F* with fluorescein. Background staining of cells by rhodamine-avidin is apparent in *E*.

recFN homodimers in medium and matrix fractions, the dimer repertoire was analyzed by electrophoresis of non-reduced immunoprecipitates of cell medium and DOC-insoluble samples (Fig. 5). SVT2 cell medium contained FN dimers only (lane *1*, band *a*). In cells expressing recombinant polypeptides, the major secreted forms were the recFN homodimers of I_{1-9}/C110-RGDS (lane *3*, band *c*) and I_{1-5}/C110 (lane *2*, band *d*), or recFN monomer in the case of $I_{1-9}/C110_M$ (lane *4*, band *e*), but recFN-FN heterodimers (band *b*) and FN dimers (band *a*) are also visible in the medium samples. In fact, homodimer levels exceeded heterodimers for all of the recFNs (data not shown). More importantly, the matrix was also composed primarily of recFN homodimers (lane *2'*, band *d* and lane *3'*, band *c*). This was not unexpected considering that the levels of recFN were significantly higher than SVT2 FN in reduced samples (see Fig. 3 and Table I). Although heterodimers are present in the ECM samples, the predominance of recFN homodimers demonstrates that these polypeptides are functional independent of full-length SVT2 FN. Therefore, matrix assembly activity is inherent in their structure.

Fibrillogenesis by Recombinant Fibronectins

The incorporation of large quantities of recombinant proteins into the DOC-insoluble cell fraction indicates that they exist as stable components of the matrix. However, this cell fractionation approach does not address the structure of the incorporated polypeptides. To determine whether the recFNs were assembled into fibrils, the ECM was examined by indirect immunofluorescence using the rat specific anti-FN mAb, VT1, to stain cell monolayers. The I_{1-9}/C110 recFN was formed into dense fibrils at the cell surface (Fig. 6 *A*) and extending between cells (Fig. 6 *B*). This recFN is clearly competent for fibrillogenesis, ruling out a major role for repeats III_{1-7} in this system. No fibrils were detected in cultures synthesizing $I_{1-9}/C110_M$ (Fig. 6 *E*) or in those lacking the amino-terminal 80-kD (ΔN80 kD) (data not shown). Therefore, the amino-terminal region and the dimer structure are essential for fibrillogenesis.

Biochemical comparisons showed that I_{1-9}/C110-RGDS was very efficiently incorporated into the ECM. By immunofluorescence analyses, fibrils composed of this protein were

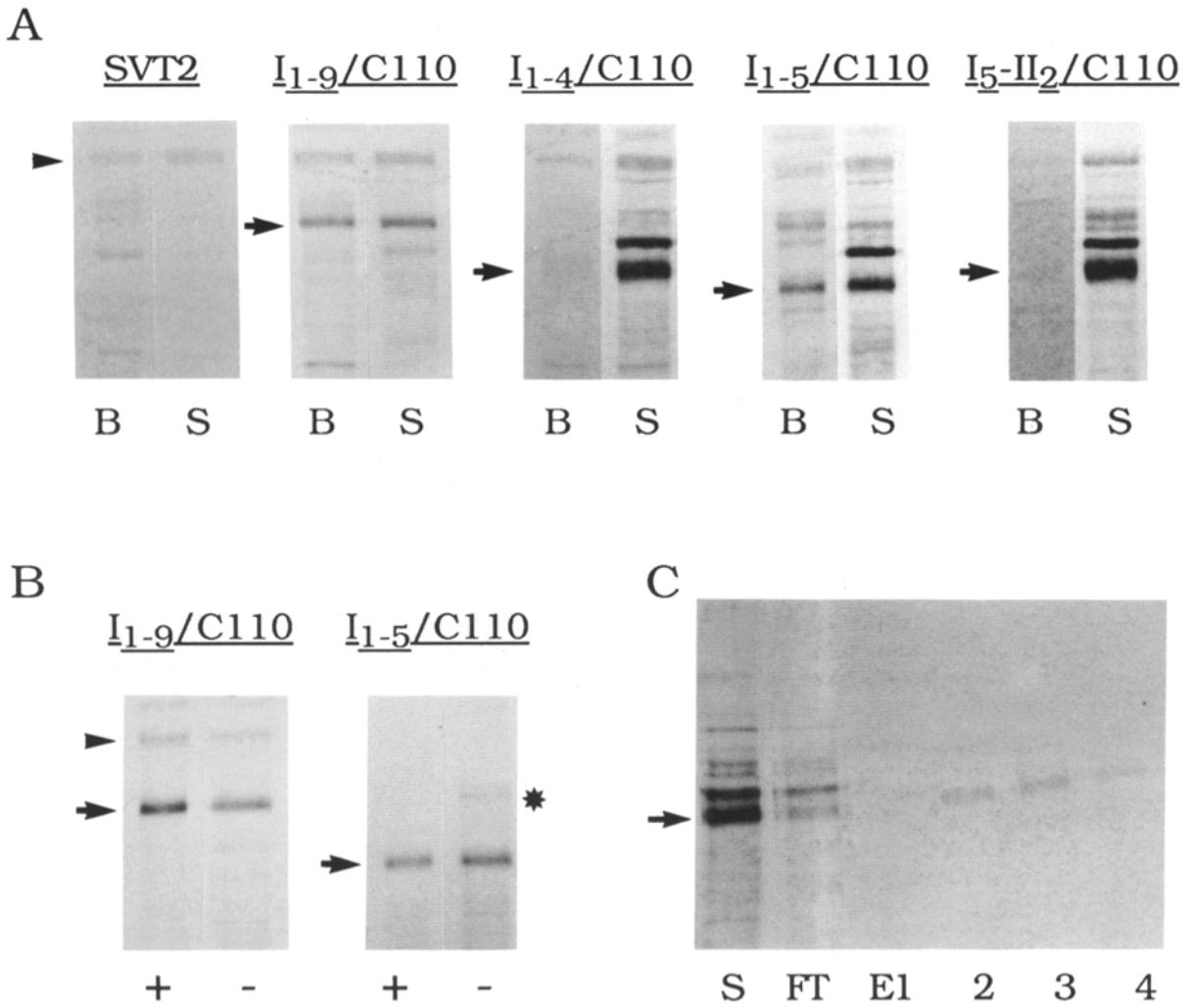

Figure 7. FN binding activity. Several recFNs were tested for binding to FN-Sepharose. (*A*) Labeled cell-conditioned media were incubated batchwise with human pFN-Sepharose. For direct comparison between bound (*B*) and supernatant (*S*) fractions, the supernatant was subsequently incubated with either gelatin-agarose (SVT2 and I_{1-9}/C110) or heparin-agarose (I_{1-4}/C110, I_{1-5}/C110 and I_5-II_2/C110) to recover recFNs (*arrows*). Proteins were eluted from FN-Sepharose and the other affinity resins in equal volumes of SDS sample buffer. Note that while SVT2 FN (*arrowhead*) is present in both bound and supernatant fractions, only two of the four recFNs, I_{1-9}/C110 and I_{1-5}/C110 (*arrows*), were retained by the FN-Sepharose. (*B*) Medium containing I_{1-9}/C110 was treated with collagenase to degrade endogenous collagens and then incubated with human pFN-Sepharose. Medium containing the I_{1-5}/C110 recFN was also treated with collagenase followed by incubation with gelatin-agarose to remove SVT2 FN (*arrowhead*) and finally incubated with rat pFN-Sepharose. Bound fractions from media with (+) and without (−) collagenase treatment are shown. The absence of collagen has no negative effects on recFN (*arrows*) binding to FN. Furthermore, removal of SVT2 FN does not hinder binding of I_{1-5}/C110. Note the low level of collagens synthesized by these cells and visible in the I_{1-5}/C110 (−) sample (*). (*C*) The I_{1-5}/C110 recFN was partially purified from metabolically labeled medium by gelatin and heparin affinity chromatography. The heparin eluate (*S*) was applied to a human FN-Sepharose column and flowthrough (*FT*), wash and eluted (*E1–4*) fractions were collected and analyzed. The amount of I_{1-5}/C110 (*arrow*) is reduced in the flowthrough fraction and can be seen in the eluted fractions E2–4. No other proteins, labeled or unlabeled, were detected in the eluted fractions.

indeed formed and detectable (Fig. 6 *D*). Again, in agreement with biochemical data, I_{1-5}/C110 formed an extensive fibrillar network, while I_{1-4}/C110 appeared totally incompetent (Fig. 6, *C* and *F*). Cells secreting I_5-II_2/C110 and I_{7-9}/C110 were also negative (data not shown).

Together these results localize two sites that are essential for efficient FN fibril formation. One site resides within amino-terminal repeats I_{1-5} and the other is the interchain disulfide bond cysteines. The RGD cell binding peptide probably plays a role in initiation of fibrillogenesis at the cell surface, but is not required for intermolecular interactions within fibrils away from the cell.

The Amino-terminal Repeats Comprise a Fibronectin Binding Site

A matrix composed primarily of FN must require intermolecular FN interactions and cannot rely on associations mediated by other ECM components for formation and stability. However, sites of FN-FN binding have not been precisely localized. Therefore, each of the recFNs was tested for binding to FN coupled to Sepharose in a batchwise assay. Radiolabeled conditioned media were incubated with either rat or human FN-Sepharose. Unbound material was subsequently incubated with heparin-agarose or gelatin-agarose to recover

any remaining recFNs. FN-, heparin-, or gelatin-bound proteins were eluted from the respective resins in equal volumes of SDS electrophoresis sample buffer, and equivalent samples were electrophoresed and compared. All samples contain the endogenous FN as an internal control. The data in Fig. 7 *A* clearly show that only some of these recombinant polypeptides can interact with FN. Specifically, I_{1-9}/C110 and I_{1-5}/C110 are present in both bound (*B*) and supernatant (*S*) fractions, while I_{1-4}/C110 and I_5-II_2/C110 are found entirely in the supernatant. Therefore, only those proteins containing repeats I_{1-5} bound to the FN-Sepharose and, by comparison with the unbound material, only a fraction (~25%) of the total recombinant and endogenous FNs was retained. RecFNs did not bind to Sepharose alone in parallel experiments.

A role for collagen as a mediator of this interaction was tested by pretreating the conditioned medium with bacterial collagenase. In addition, FN dimers and recFN-FN heterodimers were removed from the I_{1-5}/C110 sample before incubating with FN-Sepharose. Comparison of the FN-bound fractions in Fig. 7 *B* shows that removal of these collagens, and of SVT2 FN for the I_{1-5}/C110 sample, did not affect the quantity of recFN retained. Also note that SVT2 cells secrete relatively low levels of collagens which can be seen in the untreated I_{1-5}/C110 (−) sample.

It might be argued that the observed binding of recFNs from cell conditioned medium was mediated by secondary interactions with other FN binding proteins or was apparent binding due to insolubility. To rule out these explanations, I_{1-5}/C110, I_{1-4}/C110, and I_5-II_2/C110 recFNs were purified and reexamined by standard column chromatography. Endogenous SVT2 FN and heterodimers of recFNs with SVT2 FN were first removed by gelatin-agarose chromatography and a heparin-agarose column was used for partial purification. Proteins eluted from the heparin column were passed over a human FN-Sepharose column, and retained proteins were eluted with 4 M urea/PBS. The results obtained in this experiment were essentially the same as those from the batchwise assay (Fig. 7 *C*). I_{1-5}/C110 bound to the FN affinity resin and was eluted in fractions E2–4. All other labeled and unlabeled proteins in the original sample were found in the flowthrough fraction (silver stain data not shown). I_{1-4}/C110 and I_5-II_2/C110 were not retained by the FN-Sepharose column (data not shown). This result suggests that a FN binding site is contained within an intact domain consisting of repeats I_{1-5}. Together with the data showing a role for this domain in fibril formation, there is a strong correlation between FN binding activity and fibrillogenesis.

Collagen Is Not Required for Recombinant FN Matrix Incorporation

Many of the recFNs that were competent for ECM incorporation and fibril formation contain part or all of the collagen binding domain previously characterized by proteolysis and affinity chromatography on gelatin-coupled resins. Each of these polypeptides was tested for the ability to bind to collagen by batchwise incubation with gelatin-agarose. As expected, I_{1-9}/C110 and I_{7-9}/C110 bound gelatin, while I_{1-5}/C110, I_{1-4}/C110 and I_5-II_2/C110 did not (data not shown). That repeats I_{7-9} conferred gelatin-binding activity, while repeats I_5-II_2 did not, localizes the major binding site in the collagen domain to the type I repeats, in agreement with Ingham et al. (1989).

Although no apparent correlation exists between collagen binding activity and matrix incorporation, it is important to rule out a role for collagen in recFN matrix assembly. Collagenase treatment of cell-conditioned medium was used to identify secreted collagens and showed that SVT2 collagen levels are reduced compared to untransformed 3T3 cells. While collagens present at these low levels could perform catalytic or nucleation roles, they would not likely be able to play a major structural role in fibrillogenesis. To test whether collagen is involved in mediating fibril formation by recFNs, monolayers were treated with collagenase before lysis with DOC and levels of recFNs in the DOC-insoluble fractions from treated and untreated cells were compared. No differences in recFN quantities were seen, suggesting that disruption of collagen connections in the ECM does not release these proteins (data not shown). However, the strongest argument against a primary role for collagen is the inability of recFN I_{7-9}/C110, which maintains full gelatin binding activity, either to form fibrils or to be detected in the DOC-insoluble fraction (Figs. 3 and 4).

Discussion

The assembly of FN dimers into higher order structures at the cell surface is a dynamic process that requires both cells and extracellular components. In this report, I have shown that recombinant FN polypeptides, containing only a subset of the binding domains found in full-length FN, are fully functional in matrix assembly and fibrillogenesis. Retroviral vector–mediated expression of different versions of rat FN cDNA in transformed mouse SVT2 cells resulted in relatively high levels of secreted recFNs. Two assays were used to determine whether these recFNs were assembled into the matrix: incorporation into the DOC-insoluble cell fraction and detection of recFN fibrils by indirect immunofluorescence. Because SVT2 cells synthesize and secrete only low levels of ECM molecules, including FN, this system requires de novo fibril assembly in the absence of an extensive preexisting matrix. Furthermore, these assays require incorporation of mass amounts of protein, enough to detect by immunoprecipitation and immunofluorescence.

Recombinant polypeptides were designed to include carboxy-terminal sequences known to be required for dimer secretion (the alternatively spliced V region) and cell attachment (the cell binding domain). The first seven type III repeats (III_{1-7}) were deleted to generate a polypeptide distinct in size from endogenous SVT2 FN. A 180-kD recFN, I_{1-9}/C110, was constructed from this combination of domains together with the amino-terminal fibrin/collagen binding region. Expression of this cDNA resulted in formation of a dense fibrillar matrix of I_{1-9}/C110 surrounding the cells. This model system was used to determine the regions of FN involved in FN matrix assembly and fibrillogenesis. Of the eight recombinant polypeptides examined, three accumulated at the cell surface at high levels and in the form of fibrils.

One feature of FN that is absolutely required for fibrillogenesis is the disulfide-bonded dimer structure. The carboxy-terminal cysteines were deleted from the cDNA to generate a monomeric recFN, I_{1-9}/$C110_M$. This polypeptide was incapable of forming fibrils. This seems opposed to the results

of others showing binding of radiolabeled or fluorescent monomeric FN fragments to fibroblast monolayers (Chernousov et al., 1985; McKeown-Longo and Mosher, 1985; Quade and McDonald, 1989). However, their assays were designed to measure incorporation into preexisting matrices. The system described in this report requires de novo assembly of recFNs into fibrils independent of other matrix components. The results obtained with the monomeric recFN, I_{1-9}/$C110_M$, demonstrate that, along with any domain(s) involved in the intermolecular interactions within the fibrils, the functional recFNs must also possess a dimeric structure to propagate the multimeric fibrillar network.

Previous work in this system with shorter recombinant FNs (deminectins) demonstrated that the amino-terminal half of the protein was necessary for matrix incorporation (Schwarzbauer et al., 1987). By extending the FN sequence toward the 5′ end of the gene, I found that domains as close to the amino terminus as the collagen binding site, repeats I_{7-9}, were incapable of supporting matrix formation. Clearly, from the results with the I_{1-5}/C110 recFN, a site within the amino-terminal 247 amino acids is essential for this function. I_{1-5}/C110 was active in all assays, ECM incorporation, fibril formation, and FN binding. Related recFNs containing I_5 but lacking the first four type I repeats (I_5-II_2/C110) and containing the first four but lacking I_5 (I_{1-4}/C110) were inactive. Therefore, although repeat I_5 is necessary for these activities, it alone is not sufficient and requires flanking repeats. One plausible interpretation of these results is that an intact amino-terminal domain containing repeat I_5 is involved in both FN binding and fibrillogenesis. Subtle defects in the overall conformation and folding or in the proximity of repeats within this globular domain in the absence of repeat I_5 cannot be ruled out. However, deletion boundaries coincide precisely with the inter-repeat junctions so as to minimize conformational effects. In addition, the recFNs are synthesized and secreted at quite high levels, suggesting no disruption of the secretory process due to improper folding.

Deletion of the cell binding RGDS tetrapeptide from I_{1-9}/C110 had no effect on the overall incorporation of recFN into the matrix and had only a slight effect on fibrillogenesis. Fibrils still formed but they had a more punctate appearance than when the RGDS sequence was present. Possibly, frequent contacts with the $\alpha_5\beta_1$ integrins on the cell surface facilitate formation of stable, extended fibrils, while the intermolecular interactions within the matrix are not directly affected by the absence of this sequence. It seems likely that the presence of low levels of SVT2 cell FN (containing the RGDS sequence) aids in nucleation of fibril formation even though these molecules cannot serve a major structural role. Nucleation might also rely on the interactions of other FN domains with the cell surface, either complementing or replacing the cell binding region. We are in the process of further characterizing the roles of cell surface molecules in initiating matrix formation.

It follows from the results with recFN I_{1-9}/C110-RGDS that cell binding and fibrillogenesis are sequential, separable steps in the process of matrix assembly. This hypothesis provides an explanation for the inhibition of matrix assembly by anti-integrin antibodies and by antibodies against the cell binding domain of FN (McDonald et al., 1987; Woods et al., 1988; Akiyama et al., 1989; Fogerty et al., 1990). The antibodies would not directly affect FN fibrillogenesis, but would rather interfere with associations between FN fibrils and the cell surface. At high concentrations of antibody, FN-integrin interactions would be blocked and FN accumulation would be decreased. Similarly, a threshold level of cell surface integrins is necessary for deposition of a FN matrix by CHO cells (Giancotti and Ruoslahti, 1990).

The existence of a FN matrix assembly domain has been predicted by a number of approaches. Two amino-terminal proteolytic fragments, the 29-kD fibrin and the 70-kD fibrin/collagen binding segments, can bind to cell monolayers, can be crosslinked into the matrix, and, at high concentrations, can inhibit matrix assembly (McKeown-Longo and Mosher, 1985; McDonald et al., 1987; Quade and McDonald, 1988; Barry and Mosher, 1989). In accordance, I have demonstrated a requirement for amino-terminal repeats I_{1-5} in FN fibrillogenesis. In addition, a monoclonal antibody that recognizes repeats I_9/III_1 can block matrix assembly (Chernousov et al., 1987; Mosher et al., 1990). The smallest functional recFN, I_{1-5}/C110, lacks both of these repeats, suggesting that this region is not involved in fibrillogenesis. However, it is likely that multiple sites along the FN molecule are involved in interactions either with other FNs or with other matrix and/or cell surface proteins.

The results presented here localize a FN binding site to amino-terminal repeats I_{1-5}. Under the FN-binding assay conditions used, only 25% of the FN bound to the human pFN-Sepharose resin. This is not due to cross-species incompatibility, as identical results were obtained with rat pFN-Sepharose. Apparently, the interaction mediated by the amino-terminal domain is weak and the proteins dissociate. Cooperation among multiple sites could serve to increase the avidity of FN for itself and would strengthen and stabilize the associations. Interactions between fragments containing the amino-terminal 29-kD and the carboxy-terminal heparin domain as well as homophilic associations between carboxy-terminal heparin and fibrin binding fragments have been reported (Homandberg and Erickson, 1986). Furthermore, a 60-kD proteolytic fragment containing the collagen-binding domain has been shown to have FN binding activity (Ehrisman et al., 1981, 1982). Additional sites of interaction might exist in other regions of FN, for example, the cellular FN-specific alternatively spliced EIIIA and EIIIB repeats or a segment containing repeats I_9/III_1. Several approaches are currently being used to test other recombinant polypeptides for the ability to interact with FN.

I have shown that FN fibrils form via interactions between FN molecules, and multimerization relies on the FN dimer structure. The cell binding domain and the RGD sequence apparently initiate fibrillogenesis and stabilize the fibrils by interactions with cell surface integrins. Previous work has suggested the presence of a "matrix assembly receptor" that binds to the amino terminus of FN and initiates fibril formation (McKeown-Longo and Mosher, 1985). Although my results do not preclude the existence of such a receptor, they do provide an alternative model. Matrix assembly could proceed through three stages, initiation, fibrillogenesis, and stabilization. Upon secretion, FN dimers bind to the cell surface through the RGD sequence and other domains that interact with receptors. Due to the antiparallel orientation of the FN subunits within the dimer (Skorstengaard et al., 1986), the subunits are poised for head-to-tail associations with other FN dimers. Fibrillogenesis ensues where fibrils

are formed by associations between subunits from different dimers. Subsequent lengthening and bundling of fibrils generates a linear and branched extracellular network. Matrix stabilization then proceeds via a concerted set of interactions between FN fibrils and between FN and the cell surface. Clearly, other ECM components are involved in this process but their roles have not yet been defined. This system will prove useful for determining the functions of other regions of FN and other ECM proteins, such as proteoglycans, in this complex process.

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