The ins and outs of fibronectin matrix assembly

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Summary

Cell phenotype is specified by environmental cues embedded in the architecture and composition of the extracellular matrix (ECM). Much has been learned about matrix organization and assembly through analyses of the ECM protein fibronectin (FN). FN matrix assembly is a cell-mediated process in which soluble dimeric FN is converted into a fibrillar network. Binding of cell surface integrin receptors to FN converts it to an active form, which promotes fibril formation through interactions with other cell-associated FN dimers. As FN fibrils form on the outside of the cell, cytoplasmic domains of integrin receptors organize cytoplasmic proteins into functional complexes inside. Intracellular connections to the actin cytoskeletal network and stimulation of certain key intracellular signaling pathways are essential for FN–integrin interactions and propagation of FN fibril formation. Thus, assembly of native functional ECM depends on exquisite coordination between extracellular events and intracellular pathways.

Key words: Extracellular matrix, Fibronectin, Integrin, Fibril, Receptor, Intracellular signaling

Introduction

Fibronectin (FN), a ubiquitous and abundant extracellular matrix (ECM) protein, is secreted by cells as a soluble dimer and is subsequently assembled into insoluble multimeric fibrils at the cell surface. Assembly of a fibrillar FN network is a complex process, the mechanics of which are an area of active research. Fibril formation is cell mediated and depends on interactions between FN and integrin receptors on the cell surface. Subsequent steps convert FN into a dense meshwork of interconnected fibrils that provide a dynamic environment for cells. We know quite a bit about the major steps that comprise the FN matrix assembly reaction. However, as we learn more about FN and its receptors, new and interesting mechanistic questions have arisen regarding the molecular changes that convert soluble FN into insoluble fibrils and how assembly is modulated by intracellular signals. What we know and some of what we don't know are summarized in this Commentary.

Major steps in the FN matrix assembly pathway

FN is secreted as a disulfide-bonded dimer composed primarily of three types of repeating module (I, II and III) (Fig. 1) (Hynes, 1990; Pankov and Yamada, 2002). Sets of modules make up domains for binding to a variety of extracellular and cell surface molecules, including collagen, glycosaminoglycans, fibrin, integrins and FN itself. Although the diagram in Fig. 1 implies an extended structure for FN subunits, FN in solution is a compact dimer, folded into a conformation that does not undergo fibril assembly (Erickson and Carrell, 1983; Johnson et al., 1999; Rocco et al., 1983; Williams et al., 1982). Substantial evidence supports the idea that FN must be activated to assemble into fibrils (Johnson et al., 1999; Mosher, 1993; Schwarzbauer and Sechler, 1999; Sechler et al., 1996). Activation is induced by interactions with cell surface receptors, usually through binding of the α5β1 integrin receptor. Specific recognition of FN by this integrin requires the Arg-Gly-Asp (RGD) cell-binding sequence in the type III_9 module (Hynes, 1992; Ruoslahti and Pierschbacher, 1987) along with the synergy sequence located in the adjacent type III_{10} module (Bowditch et al., 1994; Nagai et al., 1991) (Fig. 1)[†]. Cell-associated FN is initially distributed diffusely over the cell surface. As assembly progresses, dimeric FN forms short deoxycholate-soluble fibrils, which are subsequently converted into a dense detergent-insoluble fibrillar network (Fig. 2).

As a dimeric ligand, FN induces integrin clustering, which brings together bound FN and increases its local concentration. The cysteines that constitute the dimerization site at the C-terminus are, therefore, essential for assembly, promoting receptor clustering and FN–FN interactions. There are at least four sites for FN–FN binding, and these are distributed across the length of each subunit (Fig. 1). Interestingly, many of these sites act as partners for the site in the N-terminal assembly domain, which perhaps explains why this is the only FN-binding site that is essential for fibril formation. Fragments containing the assembly domain, such as the 70 kDa N-terminal fragment (Fig. 1), can inhibit fibrillogenesis without affecting FN–integrin interactions (McDonald et al., 1987; McKeown-Longo and Mosher, 1985; Sechler and Schwarzbauer, 1998), and FN lacking this domain is incapable of assembly (Schwarzbauer, 1991; Sottile and Mosher, 1997; Sottile et al., 1991). In addition, during the early stages of fibril formation, the 70 kDa fragment can be used to identify sites

[†]Individual modules are numbered according to their positions from N- to C-terminus along the FN molecule. For example, III_9 is the ninth type III module. Alternatively spliced modules that are occasionally included in the protein are not numbered but are instead called EIIIA and EIIIB. The alternatively spliced segment between III_{14} and III_{15} is called the V region or IIICS. The N-terminal 25 amino acids of the V region contain the α4β1 integrin-binding site called CS1.

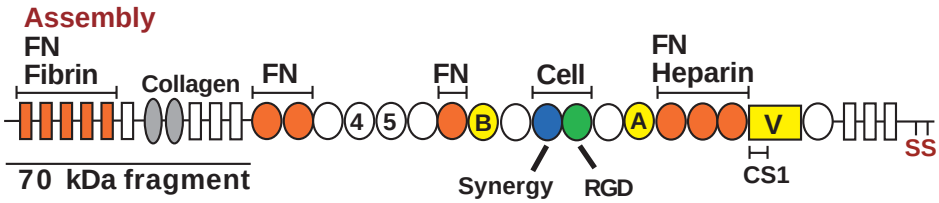

Fig. 1. Domain structure of fibronectin (FN). FN consists of type I (rectangles), type II (ovals) and type III (circles) repeats. Sets of repeats constitute binding domains for fibrin, FN, collagen, cells and heparin, as indicated. The three alternatively spliced segments, EIIIA, EIIIB and V (or IIICS), are in yellow. The assembly domain and FN-binding sites are highlighted in orange. SS indicates the C-terminal cysteines that form the dimer.

of assembly, where it binds to FN and colocalizes with clustered α5β1 integrin (Dzamba et al., 1994; Wierzbicka-Patynowski and Schwarzbauer, 2002). The existence of one essential FN-binding site enhances control of the assembly process since all interactions depend on accessibility to this single site. The fact that there are multiple partners for this site suggests that the alignment of FN dimers within fibrils varies depending on which partners are available for assembly domain binding. Variable alignment would place the other binding domains (for heparin, cells, collagen, etc.) into different molecular contexts and close to different 'near-neighbors' on adjacent dimers. In this way, dimer alignment would have a significant impact on fibril complexity.

In most instances, FN assembly is initiated by integrins that recognize the RGD and synergy sequences. Surprisingly, the specific location of the cell-binding site within FN is not critical. Placement of repeats $III_{9\text{-}10}$ more N-terminal in place of $III_{4\text{-}5}$ (Fig. 1) generated a recombinant FN that assembled normally (Sechler et al., 2001). An RGD-independent mechanism acts through binding of α4β1 integrin to the CS1 site† within the alternatively spliced V region near the C-terminus (Sechler et al., 2000). Clearly, the integrin-binding site does not need to be centrally located for initiation and propagation of FN fibril formation.

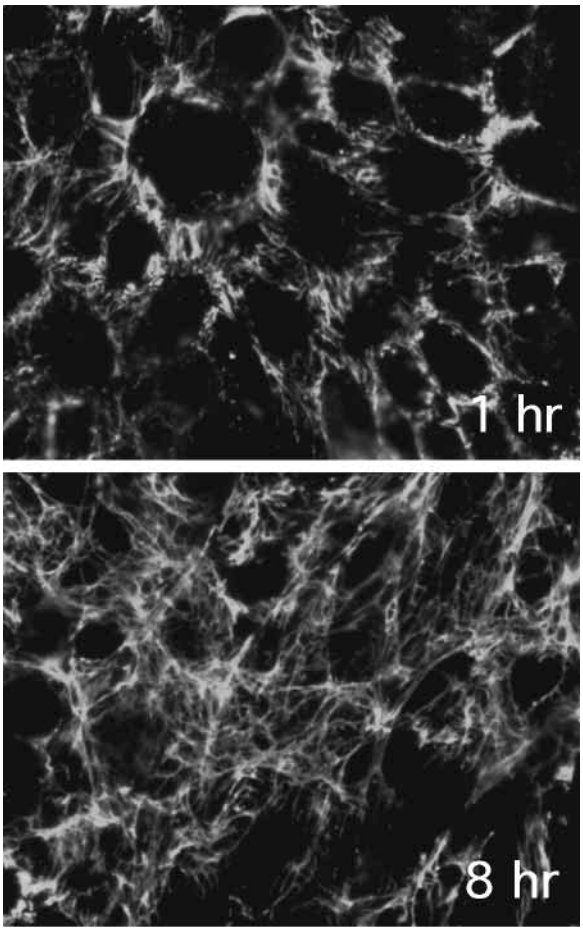

Fig. 2. Progression of fibronectin (FN) fibril formation. CHOα5 cells were incubated with rat plasma FN (25 μg ml^{-1}) for 1 hour or 8 hours. Matrix was visualized by staining with IC3 anti-rat FN antibody followed by fluorescein-labeled secondary antibody. FN fibrils accumulate over time to form a dense network around the cells.

At early stages of de novo assembly, FN fibrils are short and usually extend between adjacent cells or from the cell to nearby substrate (Fig. 2). These fibrils are soluble in buffers containing 2% deoxycholate detergent. As more FN accumulates at the cell surface, fibrils are gradually converted into a detergent-insoluble form, and a significant proportion of these exist as high-molecular-weight multimers (McKeown-Longo and Mosher, 1983). Insolubility and multimerization might involve intermolecular disulfide bonding catalyzed by the intrinsic protein disulfide isomerase activity of FN (Langenbach and Sottile, 1999) or might result from highly stable protein–protein interactions (Chen and Mosher, 1996). Partial unfolding of the III_9 module of FN promotes formation of amyloid-like fibrils in vitro (Litvinovich et al., 1998); so perhaps a similar process of β-strand exchange contributes to the detergent insolubility of the FN matrix. Further investigation of this and other potential mechanisms is needed to decipher the process by which FN fibrils become insoluble.

Fibronectin activation by conformational change

A key feature of the matrix assembly model is a conformational change that converts soluble FN into an activated dimer. In vitro manipulation of soluble FN has provided compelling evidence for conformational changes that take FN from a compact to an extended form. Changes in pH or ionic strength, addition of mild denaturants, as well as interactions with heparin or collagen fragments, can induce conformational changes as measured by a variety of biophysical, biochemical and microscopic approaches (Bushuev et al., 1985; Erickson and Carrell, 1983; Khan et al., 1990; Ugarova et al., 1996; Williams et al., 1982). Many of the models for the structure of soluble FN include folding over of the N-terminal region to allow interdomain interactions with other parts of the molecule (Homandberg and Erickson, 1986; Ingham et al., 1988; Johnson et al., 1999; Khan et al., 1990; Rocco et al., 1983; Sechler et al., 1996). Thus, one outcome of integrin binding is induced expansion of the compact dimer. This expansion exposes FN-binding domains that are hidden in the compact form and allows them to participate in FN–FN interactions.

Such a model provides a satisfying explanation for regulated FN assembly. Experiments using a recombinant FN lacking the first seven type III repeats support this idea. This protein shows an increased rate of fibril assembly characterized by accelerated formation of detergent-insoluble material, which is consistent with increased access to FN-interacting domains (Sechler et al., 1996). Regulated activation by induced expansion has particular significance for circulating plasma FN, providing a mechanism to prevent formation of insoluble fibrils in the bloodstream.

Many different sites can participate in FN–FN interactions (Fig. 1), and some of these may confer the compact conformation on the soluble protein. The N-terminal assembly domain has the most binding partners and is able to interact with native III_{1-2} (Aguirre et al., 1994), heat-denatured III_1 (Hocking et al., 1994), the heparin-binding domain (III_{12-14}) (Bultmann et al., 1998) and a combination of III_{1-2} plus heat-denatured III_{10} (Hocking et al., 1996). Interactions have also been reported between native III_1 and III_7 (Ingham et al., 1997), as well as between III_{12-14} and III_{2-3} (Johnson et al., 1999). Thus, there are numerous permutations of FN–FN interactions that can occur. It remains to be determined whether certain sites are preferentially used in soluble versus fibrillar FN.

Availability of integrin-binding sites also appears to be regulated. Epitopes within the cell-binding domain and the V (IIICS) region are exposed by adsorption of FN to a solid surface or by binding of heparin, treatment with proteases or changes in salt concentration (Ugarova et al., 1996; Ugarova et al., 1995). Alternative splicing modulates cell interactions as well. For example, increased cell spreading and migration of HT1080 cells occurs on FN containing the EIIIA module, suggesting that inclusion of this repeat, which resides near the RGD and synergy sequences, improves access to cell-binding sites (Manabe et al., 1999).

Exposure of binding sites

Treatments that induced protein expansion in vitro, as well as mutations that affect the conformation of the molecule, increase FN dimer incorporation into the matrix (McKeown-Longo and Mosher, 1985; Sechler et al., 2001; Sechler et al., 1996; Sottile and Mosher, 1993). This indicates that FN conformation controls the progression of assembly. In addition, mechanical stretching of FN adsorbed onto a silicone rubber substrate increases binding of a 70 kDa fragment containing the assembly domain (Zhong et al., 1998). This latter result demonstrates that FN assembly sites can become exposed by application of tension to immobilized FN.

Cryptic binding sites, those that are exposed by unfolding of individual type III modules, have also been implicated in FN assembly. The most dramatic examples of cryptic sites are those that are detected only in denatured fragments or peptides. Incubation of soluble FN with the III-1C peptide, which spans about two-thirds of the III_1 module, induced formation of a multimeric form of FN that could be stretched into a fibrillar-like network (Morla et al., 1994). One plausible mechanism of III-1C action is that it disrupts interdomain interactions in soluble FN and thus promotes intermolecular associations in solution. That this peptide is able to bind to many different parts of FN (Ingham et al., 1997) indicates that it might expose multiple FN-binding sites. Hocking et al. have also identified a cryptic binding site for FN and the 70 kDa fragment in intact III_1 by heat denaturation (Hocking et al., 1994). Similarly, heat-denatured III_{10} can bind to III_1 (Hocking et al., 1996). In both the peptide and heat-denatured modules, the β-sandwich structure of the type III repeat is unfolded, and hydrophobic core residues are exposed. Possibly, exposure of hydrophobic sequences is sufficient to provide a platform for FN binding. Or perhaps the effects are less specific, since the III-1C peptide also induces aggregation of fibrinogen (Yi and Ruoslahti, 2001), and heat-denatured III_5 is as active as heat-denatured III_{10} in III_1 binding (Hocking et al., 1996). Although III_1 fragments have some assembly-related activities in vitro, it is now clear that III_1 is not essential for matrix assembly, since a recombinant FN lacking this module forms a perfectly normal matrix (Sechler et al., 2001). Although there remain questions about which cryptic sites are relevant to assembly, it is clear that binding sites are sequestered in soluble FN and must be exposed for FN to form fibrils.

Elasticity of FN fibrils

FN fibrils are not static but are rearranged and recycled by cell movements, cell density and degradative processes (Hynes, 1990; Hynes, 1999). Tools are now available for determining the extent of FN rearrangements in real time. Erickson and co-workers have used cells expressing a GFP-tagged FN to show that FN fibrils are quite elastic and highly stretched (Ohashi et al., 1999). For example, detachment of one end of a fibril from a cell surface resulted in fibril shortening as it snapped back to its site of attachment on another cell. In some cases, detached fibrils contracted to less than one-quarter of their extended length. Changes in cell shape induced by EDTA treatment also caused fibril rearrangements. This elasticity provides a dynamic and pliable ECM environment to accommodate cell activities within tissues and also provides the potential for regulation of fibril organization and availability of binding sites. However, extensive fibril contraction may not occur very frequently in vivo since FN fibrils are attached both to cells and to other ECM proteins.

Several mechanisms have been proposed to explain fibril elasticity (Erickson, 1994; Ohashi et al., 1999). Straightening of FN subunits provides one mode of extension. Disruption of interdomain interactions that contribute to the compact conformation of soluble FN would expand the protein. This may be facilitated by ‘hinge’ sequences within FN. In fact, sequence gazing at the repeating FN structure shows several sites where extra residues are inserted between repeats. The largest of these is the alternatively spliced V region (see Fig. 1). Interestingly, this segment is always present in tissue FN (Schwarzbauer et al., 1985). Further straightening of the zigzag connections between type III modules would provide additional extension from 160 nm to ~175 nm for each dimer (Erickson, 1994). Another level of unfolding could result from unraveling of individual type III repeats, which, unlike type I and II repeats, are not stabilized by disulfide bonds. Modeling studies have suggested that application of sufficient force promotes gradual unfolding of individual repeats. Breakage of hydrogen bonds between β-strands in type III modules could lead to partial or eventually complete unraveling (Krammer et al., 1999). This has important ramifications for regulation of cell–matrix interactions because

unfolding of III_{10} would include concomitant flattening of the RGD loop. Experimental support for type III module unraveling has been provided by atomic force microscopy (AFM) analyses where individual fragments from titin, tenascin or fibronectin have been stretched (Oberhauser et al., 2002; Oberhauser et al., 1998; Rief et al., 1997).

If unfolding can occur in vivo, which parts of the molecule are likely to be affected? FN studies show variation in the stability of individual type III repeats in that different amounts of force are needed to unfold them. AFM data suggest that the III_{1-2} pair is quite stable and unlikely to unfold in the native protein (Oberhauser et al., 2002), raising a question about the nature of the cryptic site in III_1. Other AFM work and computer modeling, by contrast, support III_{10} unfolding at relatively low force (Craig et al., 2001; Krammer et al., 1999; Oberhauser et al., 2002). This may have functional significance given that heat-denatured III_{10} can form a ternary complex with the 70 kDa fragment and heat-denatured III_1 (Hocking et al., 1996). Other regions that may be prone to unravel include III_9 (Litvinovich et al., 1998) and III_{12-13} (Oberhauser et al., 2002). Thus, variation in type III stability may limit the unfolded regions to specific parts of the protein, in particular the cell- and heparin-binding domains.

Baneyx et al. have used fluorescence resonance energy transfer (FRET) to determine the contribution of FN elasticity to formation of matrix fibrils (Baneyx et al., 2001; Baneyx et al., 2002). They attached different fluorescent tags to free sulfhydryls in repeats III_7 and III_{15} and to free amines randomly along the length of FN. Variations in FRET provide convincing evidence for conformational changes within an FN dimer as it goes from solution to cell-associated to fibrillar. Differences along the length of fibrils indicated that FN dimers vary in their degrees of expansion from the compact to the extended form. The level of FRET at some locations may also suggest module unraveling but this is open to interpretation. Erickson has recently argued that the majority of the FRET signals in these experiments can be attributed to expansion of the compact conformation and not to module unraveling (Erickson, 2003). Thus, although there is general agreement that FN dimers are compact in solution and expand to an extended conformation during matrix assembly, it is still unclear whether unfolding of individual modules contributes to fibril elasticity.

Elasticity and cytoskeletal contractility

Integrins link FN fibrils to the actin cytoskeleton, and this connection is important for FN fibrillogenesis (Ali and Hynes, 1977; Wu et al., 1995). The organization of the actin cytoskeleton is regulated by signaling through Rho family GTPases (Hall and Nobes, 2000). Treatment of cells with serum or its component lysophosphatidic acid (LPA) activates Rho, inducing changes in cell shape and actin organization and enhancing FN matrix assembly (Zhang et al., 1994). Similar effects have been observed after microinjection of constitutively active Rho (Zhong et al., 1998), treatment of cells with the microtubule-disrupting agent nocodazole (Liu et al., 1998; Zhang et al., 1997) or culturing cells under tension within a stabilized, stressed collagen gel (Halliday and Tomasek, 1995). All of these treatments are associated with actomyosin-induced cell contraction. Not surprisingly, inhibitors of contractility have the opposite effect and block assembly of FN matrix induced by LPA (Zhong et al., 1998). Clearly, RhoA-mediated contractility can regulate FN assembly and acts, at least in part, by generating the tension required for expansion of compact dimers and exposure of FN-binding sites.

Control of FN matrix by contractility may have physiological ramifications in the vasculature. Varicose human saphenous veins show reduced deposition of FN matrix and decreased Rho kinase expression (Cario-Toumaniantz et al., 2002). Platelets assemble FN fibrils when stimulated by LPA or sphingosine 1-phosphate (Olorundare et al., 2001). Interactions between FN and $\alpha5\beta1$ integrin contribute to full activation of Rho, whereas $\alpha v\beta3$ and other integrins do not substantially activate Rho (Danen et al., 2002) and are less able to form dense matrix (Wennerberg et al., 1996; Wu et al., 1996). In vivo, LPA stimulation might allow integrins other than $\alpha5\beta1$ to support matrix assembly.

How do FN, integrins and cytoskeletal components come together to promote fibrillogenesis? Integrins affect intracellular processes through a variety of cytoskeletal, adapter and signaling molecules, including paxillin, vinculin, talin, focal adhesion kinase (FAK) and Src (Miranti and Brugge, 2002; Schwartz et al., 1995). In response to integrin–FN interactions in culture, some of these proteins are differentially incorporated into two distinct protein assemblies: focal adhesions and fibrillar adhesions (Geiger et al., 2001; Zamir et al., 1999). Focal adhesions, which are paxillin- and vinculin-rich structures, provide cells with firm substrate attachment and points of anchor for actin stress fibers. Fibrillar adhesions, by contrast, are rich in tensin but not paxillin or vinculin. They form by FN-dependent movement of ligated $\alpha5\beta1$ integrins along stress fibers towards the cell center (Ohashi et al., 2002; Pankov et al., 2000; Zamir et al., 2000). This process may mediate matrix assembly by stretching FN into fibrils from the pool of dimers that are clustered at focal adhesions. In cultured cells, tensin appears to be an important component of this process, because expression of a tensin fragment blocks integrin translocation and FN fibrillogenesis (Pankov et al., 2000). However, although focal adhesions and fibrillar adhesions participate in fibril formation in vitro, matrix assembly within tissues might use yet another type of paxillin-positive matrix contact (Cukierman et al., 2001; Sechler and Schwarzbauer, 1997). Clearly, integrins are essential mediators of FN fibrillogenesis through their connections between FN and the actin cytoskeleton and their effects on Rho activity. However, questions remain about the composition of functional integrin-based connections and the effects of extracellular environment on recruitment of intracellular components.

Integrin signaling controls assembly

Cell–ECM interactions regulate gene expression, intracellular pH and calcium levels, phospholipid metabolism, small GTPases, kinases and phosphatases (Miranti and Brugge, 2002; Schwartz et al., 1995). The ability of integrins to link the ECM with cytoplasmic molecules and the actin cytoskeleton is critical in initiating a variety of intracellular signaling pathways. Cell adhesion and spreading, migration, cell survival and proliferation all depend on integrin signaling through FAK,

which directly interacts with downstream signaling molecules such as Src, phosphatidylinositol 3-kinase (PI3-kinase) and Grb7 (Schlaepfer et al., 1999; Zhao and Guan, 2000). FAK has been implicated in cell growth responses to altered FN matrix structure (Sechler and Schwarzbauer, 1998). In addition, FAK and its downstream effectors Src and PI3-kinase play essential regulatory roles in the early stages of FN fibrillogenesis. SYF cells (which lack Src family kinases Src, Yes and Fyn), cells treated with Src and PI3-kinase inhibitors, and FAK-null cells all show significant decreases in FN matrix assembly (Wierzbicka-Patynowski and Schwarzbauer, 2002) (D. Ilic and C. Damsky, personal communication). SYF cells also show dramatically reduced phosphorylation of FAK. The recruitment of Src and PI3-kinase by active FAK thus probably transduces signals required to initiate and maintain propagation of FN fibrils. This hypothesis is further supported by the similarity in embryonic defects of mice lacking FN (George et al., 1993), FAK (Furuta et al., 1995) or the three kinases Src, Yes and Fyn (Klinghoffer et al., 1999). Because Src can affect FAK phosphorylation and thus its activity (Miranti and Brugge, 2002; Zhao and Guan, 2000), there is probably a feedback loop among these kinases resulting in mutual activation and reinforcement, which in turn promotes FN matrix assembly. Such a loop might activate intracellular signaling in response to cell–ECM interactions while it also regulates cell binding to ECM and organization of FN into fibrils.

Perturbation of FN assembly by changes in intracellular pathways through activation of Src and other oncogenes makes a significant contribution to tumor cell phenotype. For example, increased Src expression and activity are associated with a decrease in the amount of FN matrix (Hynes, 1990; Olden and Yamada, 1977) and with changes in cell–FN interactions in human colon cancer (Jones et al., 2002). In at least some cells, the ERK/MAP kinase pathway mediates the inhibitory effects of v-Src (Ladeda et al., 2001), thus implicating the Ras oncogene pathway in FN matrix regulation.

Oncogenes also affect integrin function and localization, which further exacerbates defects resulting from loss of FN. Activation of Raf-1 downstream of H-Ras suppresses the ability of α5β1 integrin to mediate FN matrix assembly (Hughes et al., 1997). The suppression correlates with activation of ERK, which is similar to *v-src*-transformed cells. HT1080 human fibrosarcoma cells, which have one activated *N-ras* allele, can be stimulated to assemble FN matrix by activation of integrins using Mn^{2+} or β1 integrin-activating antibody or by inhibition of Ras signaling through ERK (Brenner et al., 2000). Thus, mutations in at least two oncogenes, *ras* and *src*, have detrimental effects on FN matrix and, in some cells, exert their effects through a common downstream effector, ERK.

Integrin signals do not act alone

Cells that have impaired proteoglycan synthesis exhibit defective FN matrix assembly (Chung and Erickson, 1997). This defect may be due, in part, to reduced activity of syndecans, transmembrane proteoglycans that can bind to FN (Mercurius and Morla, 2001; Woods et al., 1988). Syndecan-2, the major syndecan in fibroblasts, appears to have a regulatory, albeit indirect, effect on matrix assembly (Klass et al., 2000). Truncated syndecan-2 lacking part of the cytoplasmic tail acts in a dominant-negative fashion to ablate assembly of both FN and laminin matrices. By contrast, syndecan-2 lacking the entire tail has no effect on matrix formation.

Syndecan-4 may play a more direct role in FN fibrillogenesis. It cooperates with integrins to regulate Rho-dependent cell adhesion, spreading and actin organization (Saoncella et al., 1999). Concomitant ligation of α5β1 integrin and syndecan-4 increases levels of active Rho and phosphorylated FAK (Wilcox-Adelman et al., 2002). Conditions that stimulate both Rho and FAK have been shown to favor FN matrix assembly (Midwood and Schwarzbauer, 2002).

Syndecan-4 may also act on assembly through protein kinase C (PKC). The cytoplasmic tails of clustered syndecan-4 bind to PKC (Horowitz and Simons, 1998; Oh et al., 1997). This kinase can associate with focal adhesions in fibroblasts (Barry and Critchley, 1994). In addition, PKC activation improves cell spreading and FAK phosphorylation on an FN substrate (Vuori and Ruoslahti, 1993) and increases binding of FN to fibroblast cell surfaces (Somers and Mosher, 1993). Thus, PKC localization and activation affect processes required for cell-mediated FN matrix assembly. In addition, PKC activation has been shown to increase FN production and fibrillogenesis in a variety of cell types, including pulmonary fibroblasts, retinal pigment epithelial cells, vascular smooth muscle cells, osteoblasts, hyperglycemic mesangial cells and *Xenopus* cells (Kaiura et al., 1999; Lee et al., 1996; Lin et al., 2002a; Lin et al., 2002b; Osusky et al., 1994; Singh et al., 2001; Yang et al., 2002). Together, these findings implicate PKC signaling in modulation of FN assembly.

An integrated model of FN assembly

The work discussed above allows us to generate an integrated model of FN assembly in vivo. In this model, FN matrix assembly is initiated when inactive, compactly folded FN binds to α5β1 integrins. FAK co-localizes with integrins and is rapidly phosphorylated in response to ligand binding (Fig. 3A). Phospho-FAK recruits Src, and these two kinases regulate the very early steps of assembly. Although initially distributed diffusely over the cell surface, integrin complexes soon become clustered in response to bivalent FN. Integrin clustering recruits signaling and cytoskeletal proteins into focal complexes (Fig. 3B). Kinase cascades downstream of activated Ras and Rho GTPases stimulate a number of distinct intracellular responses, including reorganization of the actin cytoskeleton and changes in gene expression. Concomitantly, FN is converted from an inactive, compact form to an active, expanded form through a process that depends on cell contractility (Fig. 3B). Tension applied to FN dimers can expand and unfold the subunits to expose sites for FN–FN interaction, thus initiating FN fibril formation.

Co-localization of syndecan-4 in focal adhesions increases the levels of active Rho GTPase and PKC, further reinforcing focal adhesion function. Many of these signaling pathways appear to feedback on integrins, strengthening connections through recruitment of additional components and sustained activation of signals. Together the combination of cytoskeleton

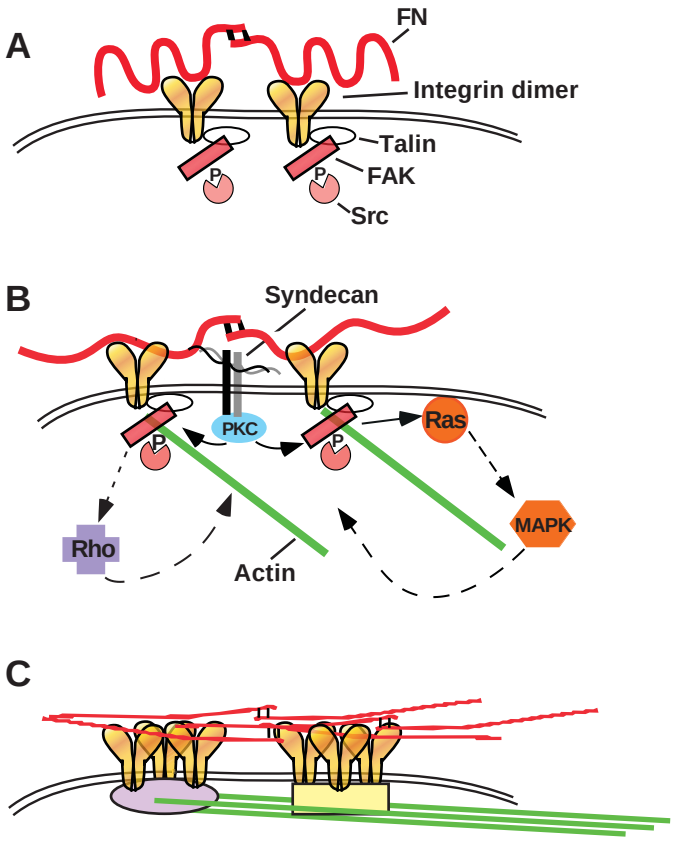

Fig. 3. Fibronectin (FN) matrix assembly model. (A) Binding of compactly folded, inactive FN to diffusely distributed integrins induces receptor clustering and co-localization of talin (white ovals) and focal adhesion kinase (FAK) (red rectangles). FAK autophosphorylation (P) recruits Src (pink circles). (B) Clustered integrins with co-localized syndecan (gray and black bars) organize the actin cytoskeleton (green lines) and activate signaling molecules including Ras/MAP kinase (orange), Rho GTPase (violet) and protein kinase C (PKC) (blue). Signals downstream of these pathways further reinforce organization of actin and focal complexes. Contractile forces aid in converting inactive FN into the active extended form. (C) Concentration of active FN dimers at integrin clusters promotes FN–FN interactions and fibril assembly. Movement of α5β1 integrins and associated proteins along stress fibers towards the cell center redistributes intracellular components into paxillin-rich focal adhesions (pink oval) and tensin-rich fibrillar adhesions (yellow rectangle). This movement may facilitate fibril formation.

and signaling inside promotes propagation of FN fibrils outside.

As FN is expanded and additional dimers are incorporated into fibrils, intracellular components are redistributed into focal adhesions and fibrillar adhesions (Fig. 3C). In culture, these can be distinguished by the presence of paxillin versus tensin, respectively. Movement of α5β1 integrin and associated proteins along stress fibers toward the center of the cell may aid in activating FN. In this way, intracellular complexes might contribute to the formation of a dense fibrillar network. Matrix complexity is derived, in part, from multiple binding partners for the FN assembly domain, which provide different dimer alignment options and thus lead to combinatorial variation in fibril organization.

Perspectives

We have made significant headway in determining the major molecular steps of FN matrix assembly. Understanding the molecular changes as FN goes from the inactive to the active form will be aided by high-resolution structures of larger pieces of the protein and of these in various activation states. In addition, there are certainly other ECM proteins that modulate or facilitate fibril assembly and these need to be identified, perhaps by application of genome-wide approaches. We must also investigate further the role of intracellular pathways. We need to learn more about not only the components that co-localize with integrins during assembly but also their stoichiometries and nearest neighbors within focal and fibrillar adhesions. Issues such as reinforcement of integrin–FN connections and regulation of fibril density also need to be tackled. We still have much to learn about matrix assembly.

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