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Invited Review

Roles of integrins in fibronectin matrix assembly

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Summary. Fibronectin (Fn) matrix assembly is a dynamic cellular process in which the soluble dimeric Fn molecules are assembled into insoluble, disulfide bond stabilized fibrillar polymeric matrix. Fn matrix assembly requires specific Fn binding integrins. Several Fn binding integrins that are capable of mediating Fn matrix assembly have been identified. They include α5ß1, αIIbß3 and αvß3 integrins. Cells regulate the matrix assembly process not only by controlling cell surface expression level of the Fn binding integrins but also by modulating Fn binding and cytoskeleton binding activities of the integrins. A major challenge of future studies is to delineate the signal transduction pathway that regulates Fn matrix assembly.

Key words: Integrins, Fibronectins, Extracellular matrix assembly, Signal transduction

Introduction

Fibronectins (Fns) are cell adhesive glycoproteins that play critical roles in many biological and pathological processes including embryogenesis, wound healing, metastasis, fibrosis and thrombosis (Hynes, 1990). During organogenesis, Fns are major constituents of the extracellular matrix. Inactivation of the mouse Fn gene caused early embryonic lethality (George et al., 1993), demonstrating that Fn is indispensable for vertebrate embryogenesis. Fn matrix assembly is a dynamic cellular process in which the soluble dimeric Fn molecules are assembled into insoluble, disulfide bond stabilized fibrillar polymeric matrix (McDonald, 1988; Mosher et al., 1992). Matrix Fn promotes cell adhesion, migration and differentiation, and thus is the primary functional form of the protein. The assembly of a Fn matrix is both temporally and spatially regulated in vivo. For example, in adult mammals, most Fn is synthesized by the liver and exists primarily as a soluble plasma protein present at high concentration (about 300 mg/ml in man). Only with wounding or during hemostasis is Fn expressed locally and deposited into extracellular matrix. Cells not only respond to signals from Fn matrix but also actively participate in the formation of a Fn matrix. Recent studies suggest that specific cell surface Fn binding integrins play important roles in initiation and regulation of fibronectin matrix assembly. In this review, I will provide a brief overview of recent advances in our understanding of the roles of integrins in Fn matrix assembly.

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Fn domains involved in Fn matrix assembly

Fns are modular, multidomain glycoproteins of approximately 500,000 daltons consisting of repeating homologies of three types (Type I, II and III) (Fig. 1) (Hynes, 1990). Each Fn molecule contains two similar disulfide bonded subunits with differences created by RNA splicing, and by glycosylation and other post-translational modifications (Hynes, 1990). The disulfide-bonded dimer structure of Fn is critical for Fn matrix assembly (Schwarzbauer, 1991; Ichihara et al., 1995). In addition, the first five type I repeats (29 kDa Matrix Assembly Site, Fig. 1) (McKeown-Longo and Mosher, 1985; Quade and McDonald, 1988; Schwarzbauer, 1991; Sottile and Wiley, 1994), the first type III repeat (Homophilic Interactive Site, Fig. 1) (Chernousov et al., 1991; Morla and Ruoslahti, 1992), and the arg-gly-asp (RGD) containing integrin binding domain are important for Fn matrix assembly (McDonald, 1988; McDonald et al., 1987; Mosher et al., 1992; Hocking et al., 1996). The first five type I repeats of Fn that apparently fold to form a high affinity binding site for specific components on the surface of matrix forming cells (Limper et al., 1991; Sottile et al., 1991; Moon et al., 1994). Fn fragments that include this domain inhibit matrix formation by fibroblasts (McKeown-Longo and Mosher, 1985; McDonald et al., 1987; Quade and McDonald, 1988), resulting in a specific phenotype characterized by short stitches of cell surface Fn associated with α5ß1 integrin receptors. The first five type I repeats of Fn are also required for de novo assembly of recombinant Fns into the extracellular matrix (Schwarzbauer, 1991; Sottile and Wiley, 1994). Antibodies recognizing the first type III

repeat inhibit Fn binding and matrix assembly by fibroblasts (Chernousov et al., 1991). However, recombinant Fn lacking the first type III repeat can be assembled into the matrix (Schwarzbauer, 1991; Ichihara-Tanaka et al., 1992), suggesting that this site is not absolutely required for Fn matrix assembly. The first five type I repeats bind to the first several type III repeats and it has been proposed that this interaction may be involved in regulation of Fn-Fn interactions (Aguirre et al., 1994). Indeed, a specific peptide sequence derived from the first type III repeat induces disulfide crosslinking of Fn into high molecular weight multimers in vitro (Morla and Ruoslahti, 1992; Morla et al., 1994). However, attempts to demonstrate the presence of a Fn fragment equivalent to this peptide under physiological condition have been unsuccessful (Morla et al., 1994). In addition, it has been shown that the heat-denatured first type III module, but not the native form of the module, binds to the 70 kDa amino terminal fragment of Fn (Hocking et al., 1994). These results suggest that during Fn matrix assembly in cultured cells and in vivo, one or more earlier events are required to unmask putative homophilic binding sites on Fn. One of the earliest events in Fn matrix assembly by cultured cells appears to be the binding of Fn to cell surface mediated by interactions between the RGD containing cell binding domain of Fn and specific Fn binding integrins (Wu et al., 1993, 1995c, 1996; Hocking et al., 1996). Antibodies to the RGD containing cell binding domain and Fn fragments containing the cell binding domain inhibit Fn matrix assembly by fibroblasts, resulting in fewer fibrils that are of normal length, consistent with a role for this site in initiation of Fn matrix assembly in these cells (McDonald et al., 1987; Roman et al., 1989).

Fn-binding integrins

Integrins are αß heterodimeric transmembrane glycoproteins that interact with extracellular (or other cell surface) molecules and cytoplasmic molecules including cytoskeletal and catalytic signaling proteins (Ruoslahti, 1988; Hynes, 1992; Schwartz et al., 1995). Integrins mediate cell-cell and cell-extracellular matrix interactions and have been implicated in many biological and pathological processes including embryogenesis, wound healing, inflammation and cancer. Several integrins, including α5ß1, α4ß1, αvß1, αIIbß3 and αvß3, bind Fns. Most of the Fn-binding integrins (α5ß1, αvß1, αIIbß3 and αvß3) recognize the RGD containing cell binding domain. In addition to the common RGD sequence, distinctive sequences (synergy regions) located amino-terminal to the RGD sequence also contribute to the binding of Fn to α5ß1 integrin (Aota et al., 1991, 1994) and αIIbß3 integrin (Bowditch et al., 1991, 1994). In contrast to α5ß1, αvß1, αIIbß3 and αvß3, α4ß1 integrin recognizes the CS1 (or V25) site within the alternatively spliced IIICS (or V) region of Fn (Fig. 1) (Wayner et al., 1989; Guan and Hynes, 1990; Mould et al., 1990).

The ligand binding affinity of a given integrin may vary depending on its cellular environment. For example, α5ß1 integrin binds Fn with high affinity (Kd~0.1 μM) in CHO cells but it binds Fn with low affinity (Kd >1 μM) in human K562 erythroleukemia cells (O'Toole et al., 1994). The effect of cellular environment on integrin ligand binding affinity is dependent on specific types of integrins, as another Fn-binding integrin, αIIbß3, has low affinity ligand binding in CHO cells (O'Toole et al., 1994). This is similar to αIIbß3 integrin on resting circulation platelets, which

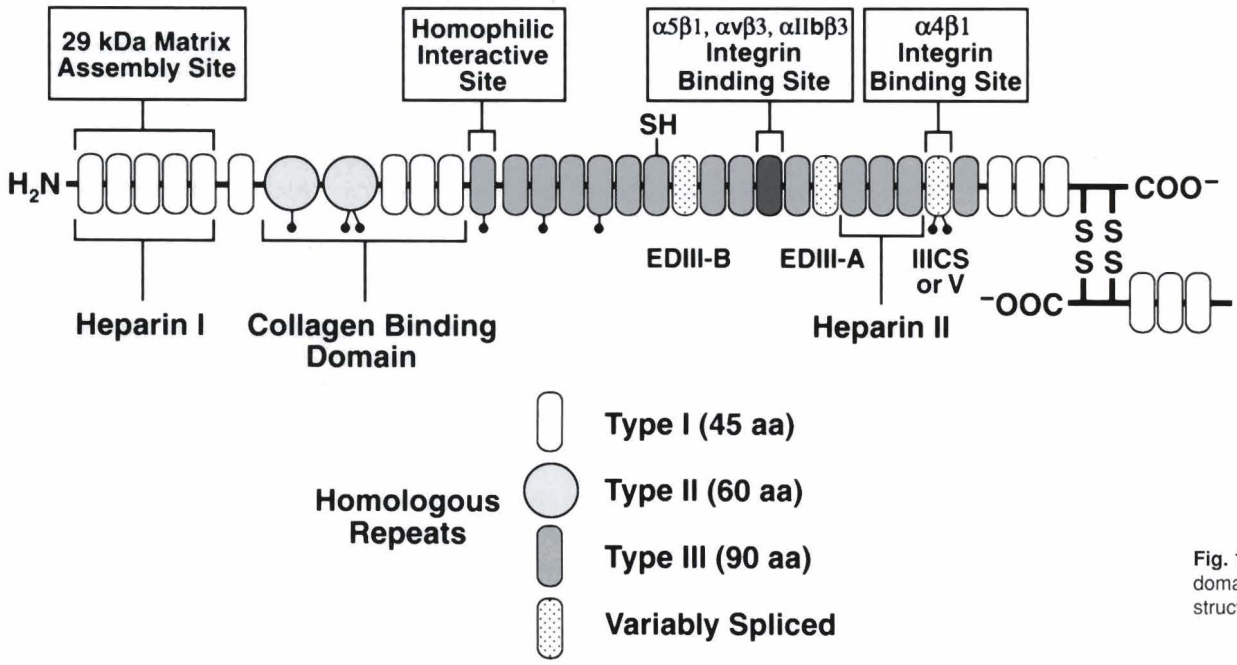

Fig. 1. Fn domain structure.

also has a low affinity binding state. The ligand binding affinity of integrins can be controlled from within the cells ("inside-out signaling") (Ginsberg et al., 1992; Hynes, 1992; Schwartz et al., 1995). Recent studies have demonstrated that the integrin cytoplasmic domains mediate inside-out signal transduction. One of the best studied integrins in term of inside-out signal transduction is αIIbß3 (Faull and Ginsberg, 1995). The α cytoplasmic domain designates integrin-specific differences in affinity state. Replacement of the cytoplasmic domain of αIIb with that of certain a subunits (e.g., α5) but not others (e.g., αL) conferred the high ligand binding state (O'Toole et al., 1991, 1994) in CHO cells. Moreover, activation of αIIbß3 integrin can be achieved by truncation of the aIIb cytoplasmic domain before but not after the highly conserved GFFKR sequence (O'Toole et al., 1991). Replacement of αIIb cytoplasmic domain with a mutated αL cytoplasmic domain in which the VGFFK sequence was deleted also activated ligand binding of αIIbß3 integrin (O'Toole et al., 1994). These results suggest that the GFFKR sequence has a negative effect on ligand binding affinity of αIIbß3. The ß cytoplasmic domain also plays critical roles in modulation of ligand binding affinity of αIIbß3. Recent studies suggest that a potential salt-bridge between R995 on the membrane-proximal region of the αIIb cytoplasmic domain and D723 on the membrane-proximal region of the ß3 cytoplasmic domain may confer the low affinity state of the integrin (Hughes et al., 1996). In addition, a Ser^{752} to Pro mutation in the ß3 cytoplasmic domain was associated with an apparent αIIbß3 activation defect in a variant of Glanzmann thrombasthenia (Chen et al., 1992).

Integrin-cytoplasmic molecule interactions

Integrins are capable of interacting with various cytoskeletal and catalytic signaling proteins molecules (Fig. 2). The major cytoskeleton-binding sites have been located to the ß cytoplasmic domain of integrins (Sastry and Horwitz, 1993). Analyses of single subunit chimeric integrins and other integrin ß subunit mutants have indicated that the ß cytoplasmic domains are necessary and sufficient to target integrins to focal adhesions (Sastry and Horwitz, 1993; LaFlamme et al., 1994), presumably via interactions with cytoskeletal proteins. Direct in vitro binding studies have demonstrated that two actin-binding proteins, α-actinin (Otey et al., 1990; Pavalko and LaRoche, 1993) and talin (Horwitz et al., 1986), interact specifically with the ß cytoplasmic domains. The primary ß1 segment involved in α-actinin binding is localized in the FAKFEKEKMN sequence on the ß1 cytoplasmic domain (Fig. 2) (Otey et al., 1993). A second α-actinin binding site located near the C terminus of the ß1 cytoplasmic domain has also been identified (Otey et al., 1993). The talin binding sequence appears to be localized near the C terminal α-actinin binding site on ß1 cytoplasmic domain (Buck and Horwitz, 1987; Tapley et al., 1989; Lewis and Schwartz, 1995). Analysis of ß1 mutants revealed that many of the residues within the α-actinin and talin binding regions were critical for localization of the integrins to focal adhesions (Reszka et al., 1992).

In addition to α-actinin and talin, several other cytoplasmic molecules have been shown to interact with the α or the ß cytoplasmic domain of integrins (Fig. 2). Calreticulin, a calcium-binding protein that is also

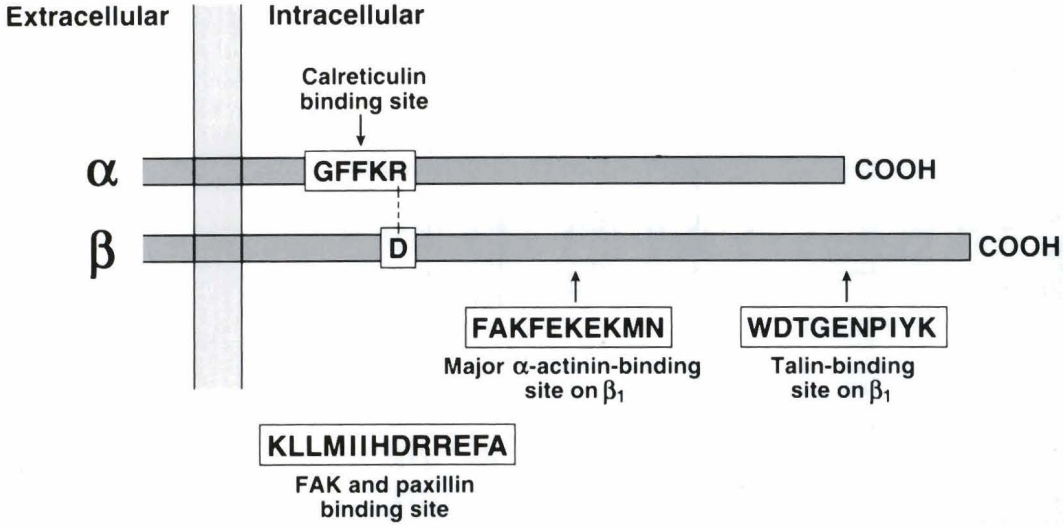

Fig. 2. Integrin-cytoplasmic molecule interactions. The dashed line represents a potential salt bridge between the highly conserved R and D residues in the membrane proximal regions of the integrin α and ß cytoplasmic domains (Hughes et al., 1996).

Additional β cytoplasmic domain binding proteins:

$β_3$-endonexin: Specific for membrane distal portion of the $β_3$ cytoplasmic domain

Integrin-linked kinase (ILK)

involved in modulation of nuclear hormone-receptor activities (Burns et al., 1994; Dedhar et al., 1994), binds to the highly conserved GFFKR sequence on the a cytoplasmic domain (Rojiani et al., 1991). The binding of calreticulin to integrins appears to play a role in modulating the functional status of integrins (Leung et al., 1994; Coppolino et al., 1995). Binding sites for focal adhesion kinase (FAK) and paxillin have been localized to the membrane proximal region of the ß1 cytoplasmic domain (Schaller et al., 1995). Recently, two novel ß cytoplasmic domain-binding proteins were identified using a yeast two-hybrid screen system. One of them, ß3-endonexin, interacts specifically with the ß3 cytoplasmic domain (Shattil et al., 1995). The other one, ß1-integrin-linked kinase (ILK), is a serine/threonine kinase which may be involved in integrin mediated signal transduction (Hannigan et al., 1996).

Initiation of Fn matrix assembly by integrins

A number of studies have established that the binding of Fn by cell surface integrins is critical in initiating Fn matrix assembly. Fn fragments containing the RGD-containing integrin binding site or antibodies recognizing the integrin binding site inhibited Fn matrix assembly in cultured cells and developing amphibian embryos (McDonald et al., 1987; Darribere et al., 1992; Wu et al., 1995c, 1996). In addition, anti-bodies to ß1 integrin, and antibodies to α5 integrin to a lesser degree, reduced the deposition of Fn into extracellular matrix by fibroblasts (Akiyama et al., 1989; Roman et al., 1989; Fogerty et al., 1990). The participation of α5ß1 integrins in Fn matrix assembly has been extensively studied in CHO cells. Over-expressing α5ß1 in CHO cells with endogenous α5ß1increased Fn deposition in extracellular matrix (Giancotti and Ruoslahti, 1990). The CHO B2 line is a particularly useful model, as it does not express α5ß1 integrin, and attaches and migrates poorly on Fn (Schreiner et al., 1989). CHO B2 cells do not assemble plasma Fn into the extracellular matrix (Wu et al., 1993). Reconstituting α5ß1 integrin expression by transfecting the cells with a full length cDNA encoding the human α5 chain completely restored fibrillar Fn matrix assembly (Wu et al., 1993). The primary role of α5ß1 integrin in Fn matrix assembly appears to initiate the assembly, as Fn mutants lacking the α5ß1 integrin binding site could be incorporated into Fn matrix (Schwarzbauer, 1991).

Although compelling evidence links α5ß1 integrin with Fn matrix assembly, mechanisms for assembling a Fn matrix in the absence of α5ß1clearly exist. Certain cells, such as fibroblastic cells derived from α5 integrin null mutant embryos (Yang et al., 1993), assemble a Fn matrix in the absence of α5ß1. To identify other Fn binding integrins that support Fn matrix assembly, we have expressed α3ß1 (Wu et al., 1995a), α4ß1 (Wu et al., 1995b), αIIbß3 (Wu et al., 1995c) and αvß3 (Wu et al., 1996) in the α5 deficient CHO B2 cells. Another Fn

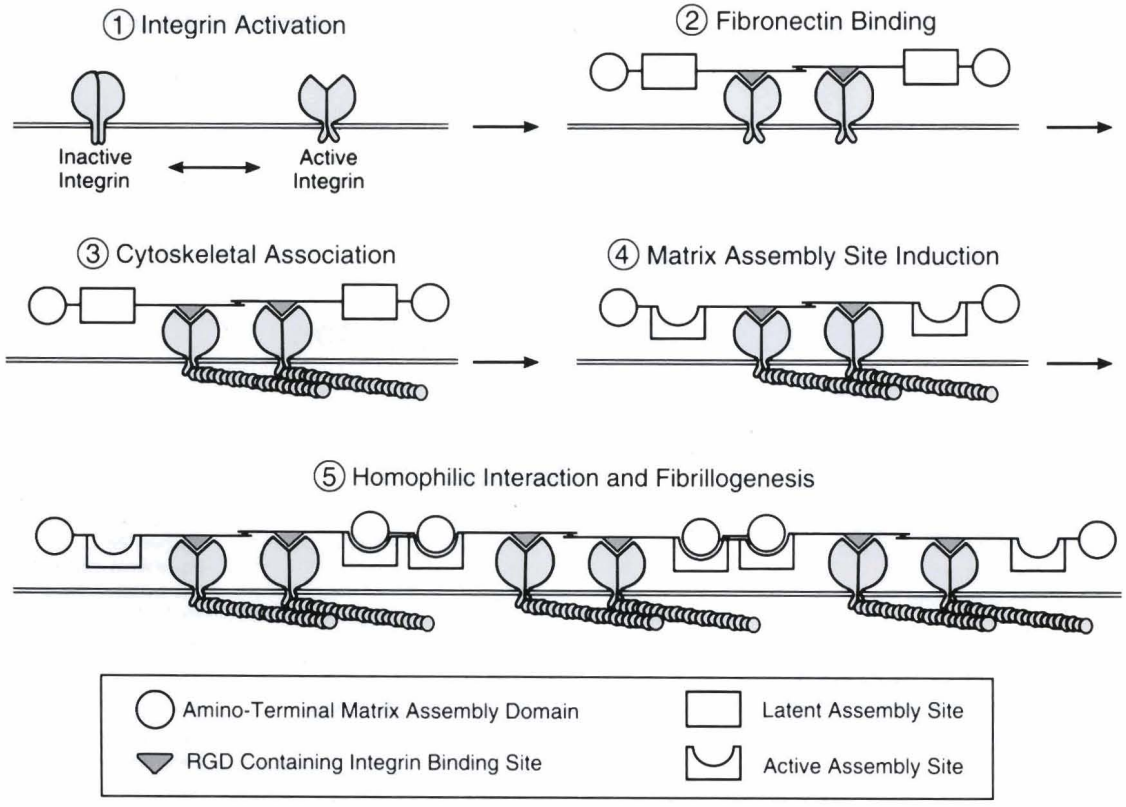

Fig. 3. A model of integrin mediated Fn matrix assembly

binding integrin, αvß1, has been expressed in CHO B2 cells by Zhang et al. (1993). A summary of the activities of the Fn-binding integrins in supporting Fn matrix assembly in CHO cells is presented in Table 1. Expression of α4ß1 in CHO B2 cells enabled the cells to adhere and migrate in response to Fn, but not to assemble a Fn matrix (Wu et al., 1995b). On the other hand, although CHO cells bearing αvß1 adhere to Fn, they neither migrate on Fn nor assemble a Fn matrix (Zhang et al., 1993). Overexpressing α3ß1 integrins in these cells conferred the ability to attach to entactin, but not to Fn, and also dramatically increased the deposition of entactin and Fn into the pericellular matrix (Wu et al., 1995a). The deposition of Fn in these cells most likely is promoted indirectly by α3ß1 integrin, probably via entactin. Expression of activated αIIbß3 integrin, but not wild type αIIbß3 integrin, in CHO B2 cells resulted in Fn matrix assembly (Wu et al., 1995c). Similarly, activation of αvß3 dramatically stimulated Fn matrix assembly (Wu et al., 1996). These results indicate that the ability of cells to assemble a Fn matrix is controlled by both the types and the activation states of the cell surface integrins.

Based on previous studies from our and other laboratories, we recently proposed a model of integrin initiation of Fn matrix assembly (Fig. 3) (Wu et al., 1995c). This model has the following important features:

1. Multiple Fn binding integrins can initiate Fn matrix assembly.

2. Integrins must bind the RGD containing cell binding domain of Fn with high affinity to initiate Fn matrix assembly (integrin activation results in high affinity Fn binding).

3. High affinity binding of Fn to integrins is necessary but not sufficient for Fn matrix assembly. Integrin-cytoskeleton interaction is critical for Fn matrix assembly (cytoskeletal association).

4. The binding of the integrin to both the extracellular Fn and the intracellular cytoskeletal proteins induces Fn-Fn interactions and promotes Fn fibril elongation.

Table 1. Fn matrix assembly activities of integrins in CHO cells.

INTEGRIN	Fn MATRIX ASSEMBLY
α5ß1 (Wu et al., 1994)	+
α4ß1 (Wu et al., 1995b)	-
α3ß1 (Wu et al., 1995a)	+*
αvß1 (Zhang et al., 1993)	-
αIIbß3 (Wu et al., 1995c)	-
Activated αIIbß3 with full length ß3 cytoplasmic domain (Wu et al., 1995c)	+
Activated αIIbß3 with truncated ß3 cytoplasmic domain (Wu et al., 1995c)	-
αvß3 (Wu et al., 1996)	±
Activated αvß3 (Wu et al., 1996)	+

*: α3ß1 integrin promotes Fn deposition into the entactin-rich pericellular matrix by a mechanism distinct from α5ß1 integrin mediated Fn matrix assembly, probably via entactin (Wu et al., 1995a).

Regulation of Fn Matrix Assembly

A number of cell surface and intracellular molecules may be involved in regulation of Fn matrix assembly (Fig. 4). Recent studies suggest that integrin cytoplasmic domains play important roles in cellular regulation of Fn

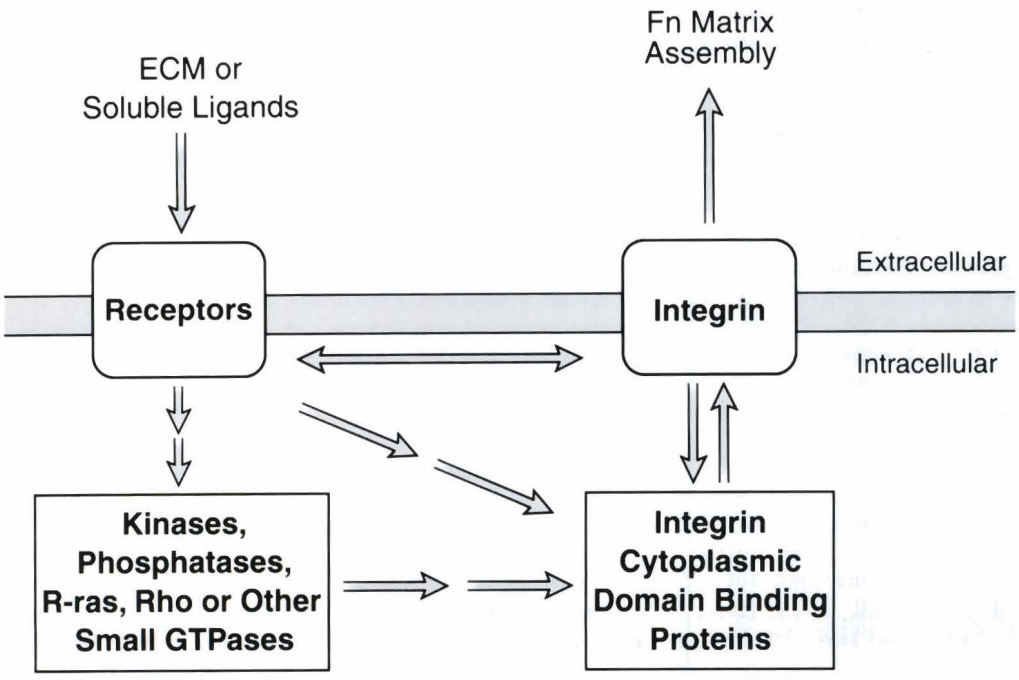

Fig. 4. Hypothetical cellular signalling pathways that regulate Fn matrix assembly.

matrix assembly (Wu et al., 1993, 1995c, 1996; Hughes et al., 1996). At least three regions of integrin cytoplasmic domains are important in regulation of Fn matrix assembly. They are (1) the highly conserved GFFKR sequence in the membrane-proximal region of integrin α cytoplasmic domain, (2) the membrane-proximal region of integrin ß cytoplasmic domain, and (3) the membrane-distal region of integrin ß cytoplasmic domain. The membrane-proximal regions of the α and ß cytoplasmic domains are involved in regulation of extracellular ligand binding affinity of the integrins (inside-out signalling). Deletion of the highly conserved GFFKR sequence in the membrane-proximal region of αIIß (O'Toole et al., 1991) or α5 (Wu, unpublished observation) cytoplasmic domain increases the extracellular ligand binding affinities of the integrins and stimulates Fn matrix assembly (Wu et al., 1993, 1995c). Moreover, a single point mutation within the GFFKR sequence in the αIIß cytoplasmic domain (F992A) or a point mutation (D723➢R) in the membrane-proximal region of the ß3 cytoplasmic domain enhances the ligand binding and promotes the Fn matrix assembly activities of the αIIbß3 integrin (Hughes et al., 1996) and the αvß3 integrin (Wu et al., 1996). Thus, one mechanism by which cells control extracellular Fn matrix assembly is to regulate Fn binding affinity of integrins. Indeed, activation of aIIbß3 integrin (Hughes et al., 1996) or αvß3 integrin (Wu et al., 1996) to a higher Fn binding affinity state with an activating anti-ß3 antibody stimulated Fn matrix assembly. Expression of a constitutively active R-ras in CHO cells bearing αIIbß3 integrin enhanced ligand binding activity of the aIIbß3 integrin and resulted in several fold more Fn assembled into extracellular matrix (Zhang et al., 1996).

In addition to high Fn binding affinity, interactions between Fn binding integrins and an intact actin cytoskeleton are critical for the assembly of a Fn matrix. Fn fibrils often coalign with actin cytoskeleton. Drugs that disrupt actin cytoskeleton also disorganize Fn fibrils and release Fn from cell surface to the culture medium (Hynes, 1990). The membrane-distal region of integrin ß cytoplasmic domain contains binding sites for several actin associated proteins (Fig. 2). Deletion of these binding sites from the integrin ß cytoplasmic domain abolished Fn matrix assembly (Wu et al., 1995c). Expression of ß1 or ß3 cytoplasmic domain containing the cytoskeleton binding sites in cultured cells inhibited Fn matrix assembly (LaFlamme et al., 1994). Moreover, intracellular injection of anti-ß1 cytoplasmic domain antibodies inhibited Fn matrix assembly in vivo (Darribere et al., 1990). Thus, another mechanism by which cells may control extracellular Fn matrix assembly is to regulate the assembly of actin cytoskeleton and integrin-actin cytoskeleton interaction. Because several members of the Rho family of small GTPases (Rho, Rac and Cdc42) regulates actin cytoskeleton formation and possibly integrin-actin cytoskeleton interaction (Ridley and Hall, 1992; Hall, 1994; Hotchin and Hall, 1995; Nobes and Hall, 1995), it will be very interesting to determine whether the Rho family of small GTPases regulates extracellular Fn matrix assembly.

A number of extracellular and intracellular regulatory factors of Fn matrix assembly have been identified. One of them is lysophosphatidic acid, a product of activated platelets which has diverse actions on cells. Lysophosphatidic acid rapidly stimulates stress fiber and focal adhesion formation in serum-starved fibroblasts via Rho-mediated signal transduction pathway (Ridley and Hall, 1992). It also stimulates the binding of Fn and its amino terminal fragment to cells and Fn matrix assembly (Checovich and Mosher, 1993; Zhang et al., 1994). Other Fn matrix assembly modulators include protein kinase C inhibitors. In contrast to lysophosphatidic acid, inhibitors of protein kinase C reduce stress fiber and focal adhesion formation (Woods and Couchman, 1992), and rapidly decrease the binding of Fn and its amino terminal fragment to cells (Somers and Mosher, 1993). This suggests that protein kinase C is involved in regulation of Fn matrix assembly. Apparently, an important area of future investigation is to delineate the signa transduction pathway that regulates Fn matrix assembly.

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