Regulatory Role for Src and Phosphatidylinositol 3-Kinase in Initiation of Fibronectin Matrix Assembly*

Received for publication, January 10, 2002, and in revised form, March 8, 2002
Published, JBC Papers in Press, March 23, 2002, DOI 10.1074/jbc.M200270200

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Fibronectin (FN) matrix assembly is a tightly regulated stepwise process that is initiated by interactions between FN and cell surface integrin receptors. These interactions activate many intracellular signaling pathways that regulate processes such as cell adhesion, migration, and survival. Here we demonstrate that cells lacking Src family kinases showed reduced ability to assemble FN fibrils as detected by immunofluorescence and by analysis of detergent extracts. The amount of FN matrix was further reduced by treatment with the phosphatidylinositol 3 (PI 3-kinase) inhibitor, wortmannin. CHOα5 cells, which are dependent on exogenous FN to initiate fibril formation, also showed significant reductions in matrix when treated with inhibitors of Src and PI 3-kinase. Combination of both inhibitors showed an additive inhibitory effect on assembly, which was concomitant with a loss of focal adhesion kinase phosphorylation. Decreased binding of the 70-kDa amino-terminal FN fragment at matrix assembly sites further supports a role for these kinases early during the process. We propose that these two signaling molecules, which lie downstream of integrins and focal adhesion kinase, are essential for efficient initiation of FN matrix assembly.

The extracellular matrix (ECM)1 surrounds cells and dynamically regulates cellular functions such as adhesion, migration, growth, and differentiation. Composition of the matrix varies from tissue to tissue; however, FN is a major component of most matrices (1, 2). FN influences diverse processes including inflammation, wound repair, malignant metastasis, microorganism attachment, and thrombosis. It does this not as a soluble protein but as a major component of a fibrillar network. FN is assembled into fibrils through a regulated stepwise process (3). The initiation of matrix assembly depends on interaction of FN with its cell surface receptor α5β1 integrin. FN binding induces integrin interactions with the actin cytoskeleton inside the cell, whereas addition of FN dimers to growing multimers results in elongation of fibrils outside. Fibrils are then gradually converted into detergent-insoluble stable matrix.

Integrin-mediated interactions with FN-coated substrates induce reorganization of the actin cytoskeleton and associated proteins and lead to the formation of focal adhesions (4). These sites contain a multitude of cytoskeletal and adapter proteins such as vinculin, paxillin, and talin as well as signal transduction molecules such as focal adhesion kinase (FAK), Src family kinases, and protein kinase C. Many of these focal adhesion components are multiday molecules that can interact with several distinct partners. Focal adhesions have been shown to serve both structural and functional roles as the sites where activation of intracellular pathways takes place. Some of these pathways have been implicated in FN matrix assembly. For example, treatment of fibroblasts with phorbol esters or other protein kinase C activators resulted in increased FN binding to the cell surface (5). An FN fragment containing the first type III repeat, which can modify FN matrix assembly (6, 7), activates ERK/MAP kinase signaling (8) and affects vascular smooth muscle cell proliferation (7, 9).

Interaction of FN with integrins also results in activation of FAK, which then binds to the signaling proteins Src (10, 11), PI 3-kinase (12), and the Grb7 adapter protein (13). Src associates with FAK through direct interaction of its Src homology (SH)2 domain with the major autophosphorylation site Tyr-397 on FAK. Association of Src with FAK is believed to be important for reciprocal activation of those two kinases (14). Furthermore, Src binding to FAK leads to phosphorylation of additional tyrosine residues on FAK that not only create new binding sites but also increase FAK catalytic activity (15). Src/FAK complex formation results in phosphorylation of other proteins such as paxillin, tensin, and p130cas (16). The Tyr-397 site on FAK also serves as a binding site for PI 3-kinase. Integrin-induced PI 3-kinase association with FAK has been demonstrated in platelets (16) and fibroblasts (17).

We have demonstrated previously that a mutant FN, which forms a structurally altered FN matrix, inhibited phosphorylation of FAK and blocked cell cycle progression (18). The role of signaling effectors downstream of FAK in the assembly of FN into fibrils has not been extensively studied. Here we investigated the requirement for active Src and PI 3-kinase in FN matrix assembly. We demonstrate that both Src and PI 3-kinase regulate early stages of matrix assembly, suggesting that FAK acts through multiple pathways to regulate matrix formation.

EXPERIMENTAL PROCEDURES

Cell Culture—SYF and SYFwtSrc cells were a kind gift from Dr. Philippe Soriano (Fred Hutchinson Cancer Center, Seattle, WA). Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. CHOα5 cells (19) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (HyClone Laboratories), 2 mM glutamine, 1% nonessential amino acids, and 100 µM β-mercaptoethanol.
RESULTS

Decreased FN Matrix on Cells Lacking Src Family Kinases—SYF fibroblasts lacking three Src family kinases (Src, Yes, and Fyn) display no Src-related kinase activity but will attach and spread on FN-coated substrates (22). To examine the requirement for Src kinases in integrin-mediated assembly of FN matrix, assembly of FN fibrils was monitored by immunofluorescence. SYF cells were compared with SYFwtSrc cells, which are SYF cells transfected with wtSrc cDNA. Exogenous rat pFN was included in the medium to ensure a sufficient supply of FN. Both cell lines assembled FN into a fibrillar matrix; however, SYFwtSrc cells appeared to assemble more matrix than SYF cells (Fig. 1). Very early time points were examined to determine the ability of these cells to initiate assembly. To enhance our ability to examine assembly at early times, chemiluminescence exposure was increased for samples from shorter time points. A difference was detected at the earliest time point, 15 min after adding FN to the medium (Fig. 2B). SYFwtSrc cells produced detectable FN matrix by this time, whereas SYF cells showed very little FN matrix (even 30 min after addition of FN). This delayed assembly of FN matrix by SYF cells suggests that Src family kinases may regulate the early stages of FN matrix assembly.

PI 3-Kinase Participates in FN Matrix Assembly—Products of PI 3-kinase activity such as PIP2 are involved in integrin-related processes, and PI 3-kinase itself acts to integrate multiple pathways downstream of integrins (23–25). We therefore tested the requirement for active PI 3-kinase in SYF cells. SYF cells were incubated with FN in the presence of wortmannin, a PI 3-kinase inhibitor (26). As shown in Fig. 3A, wortmannin...
treatment resulted in almost complete inhibition of deoxycholate-insoluble FN matrix formation. No major effects of wortmannin on actin stress fibers were observed (Fig. 3B). These results show that together functional Src kinases and PI 3-kinase play a regulatory role in matrix formation by acting to enhance initiation of FN fibril assembly. A third pathway downstream of integrins leads to ERK/MAP kinase activation. Inhibition of this pathway with the MEK1 inhibitor PD98059 had no effect on initiation of fibril formation (data not shown).

Src and PI 3-Kinase Are Required for Assembly in CHO Cells—SYF cells produce endogenous FN, which makes it difficult to control the timing of assembly by addition of FN. Therefore, we used CHO cells transfected with human α5 cDNA (CHOα5 cells) (19) because these cells lack an endogenous FN matrix but are capable of assembling a matrix when provided with exogenous FN. CHOα5 cells were allowed to attach overnight and were then incubated in fresh medium containing 25 μg/ml rat pFN plus the Src inhibitor PP1 (27). CHOα5 cells in the absence of PP1 assembled an extensive matrix between and on top of the cells (Fig. 4A). In contrast, cells incubated with PP1 formed less matrix, with fibrils mainly between cells even after 6 h of incubation (Fig. 4A). A dramatic difference in deoxycholate-insoluble material was also observed at times up to 6 h (Fig. 4B). Treatment of CHOα5 cells with another Src inhibitor, radicicol (28), also inhibited FN matrix assembly similarly to PP1 (data not shown).

Similar to SYF cells, assembly was perturbed at a very early stage with reduced deoxycholate-insoluble material observed as early as 30 min (Fig. 5A). This process was also inhibited by wortmannin, although the level of inhibition appeared lower than with PP1 (Fig. 5A). Addition of Src and PI 3-kinase inhibitors together resulted in almost complete inhibition of FN matrix assembly over a 2-h incubation. No major effects of these inhibitors on actin stress fibers were noted (Fig. 5C). The effect of LY294002, another PI 3-kinase inhibitor (29), on FN matrix assembly was identical to that of wortmannin (data not shown).

In combination, inhibition of Src and PI 3-kinase caused a dramatic decrease in the amount of FN matrix. Quantitation of deoxycholate-insoluble fractions revealed that during 2 h of assembly both inhibitors together reduced matrix to about one-quarter of the control level (Fig. 5B). This level of inhibition was twice as high as when either inhibitor was added.
alone, suggesting that the combined effects of Src and PI 3-kinase on FN matrix assembly are additive. We also observed that after only 1 h the effect of wortmannin was much less than that of PP1, suggesting that Src may affect matrix formation earlier than PI 3-kinase (not shown).

**Src and PI 3-Kinase Affect the Formation of Matrix Assembly Sites**—FN assembly into fibrils is a stepwise process that is initiated through αβ5 integrin binding to FN. Clustering of receptors promotes fibroblastogenesis, which requires FN-FN interactions mediated by the amino-terminal domain and multimerization of FN dimers into fibrils (30). The 70-kDa amino-terminal fragment of FN (which contains a FN self-association site) can be used to identify sites of assembly within the matrix (6, 31). 70-kDa fragment binding is dependent on interactions with intact FN immobilized within newly formed matrix fibrils (32).

To determine whether Src and PI 3-kinase activities are required for formation of matrix assembly sites, the presence of these sites was detected with rhodamine-labeled 70-kDa fragment (Rh-70 kDa). CHOα5 cells were plated on FN-coated glass coverslips in medium containing Rh-70 kDa and in the presence or absence of PP1, wortmannin, or both inhibitors. CHOα5 cells reorganized the surface-coated FN into short fibrils that generated binding sites for Rh-70 kDa within 30 min with more extensive incorporation by 2 h (Fig. 6, A and A’). PP1 (6, B and B’) and wortmannin (6, C and C’) alone inhibited formation of Rh-70 kDa binding sites. The inhibitory effects were strongest, however, when PP1 and wortmannin were used in combination (Fig. 6, D and D’). These results demonstrate that active Src and PI 3-kinase are required for formation of FN matrix assembly sites and indicate that these enzymes regulate initiation of FN fibril assembly.

**Correlation of Src and PI 3-Kinase Activity with FAK Phosphorylation**—Both PI 3-kinase and Src family kinases interact with FAK and are involved in activation of downstream pathways. SYF cells plated on FN-coated substrate do not show FAK phosphorylation (22). To determine whether FAK activation correlates with FN matrix assembly, levels of FAK phosphorylation were analyzed. SYF cells showed no detectable FAK phosphorylation during the time course of this experiment (Fig. 7). In contrast, significant levels of FAK phosphorylation were observed in SYFwtSrc cells during the same period. Identical results were obtained when FAK phosphorylation was detected using FAK Tyr-577 phosphospecific antibody (anti-[Tyr(P)-577]FAK) (data not shown). Perturbation in FAK phosphorylation correlates with the timing of reduced FN assembly. Interestingly, FAK-null fibroblasts show very little detectable deoxocholate-insoluble FN matrix.2 Taken together, these results suggest that stimulation of Src and PI 3-kinase by FAK is required for efficient initiation of FN matrix assembly.

**DISCUSSION**

In this report, we show using two different cell systems that inhibition of Src by mutation of Src family kinase genes in SYF cells or with Src-specific inhibitors in CHOα5 cells results in significant reduction of FN matrix assembly. A further reduction in matrix was seen with concomitant inhibition of PI 3-kinase activity. The major effects of blocking kinase action occurred early during fibril formation and could be demonstrated by detergent insolubility as well as by monitoring development of matrix assembly sites with 70-kDa binding. Both Src and PI 3-kinase lie immediately downstream of FAK. Inhibition of MAP kinase signaling, a third pathway downstream of FAK, had no effect on initiation of fibril formation. Therefore, we propose that a subset of signaling pathways activated by FAK are essential for proper initiation of FN matrix assembly.

Src family kinases govern many cellular functions such as growth factor-induced proliferation and gene expression, ECM-promoted adhesion, spreading, migration, and protection from apoptosis (33–36). Mutation of three ubiquitous members (Src, Yes, and Fyn) leads to severe developmental defects and lethality by mid-gestation. Interestingly, the SYF triple mutant embryonic defects resemble those of FN-null embryos, suggesting overlapping functions during development (22, 37). SYF cells are able to adhere to FN substrata but show dramatically reduced FN-dependent tyrosine phosphorylation (22). Similarly, we found that FAK phosphorylation was ablated in SYF cells during matrix assembly and that this reduction in phosphorylation correlated with decreased fibril formation. In contrast, SYF cells overexpressing c-Src showed FN-induced tyrosine phosphorylation of focal adhesion proteins (including FAK) as well as normal levels of FN fibrils. These findings provide evidence for a link between Src kinase activity and FN matrix assembly.

PI 3-kinase phosphorylates phosphatidylinositol and together with its lipid derivatives acts as a second messenger in a variety of signaling pathways including cell survival through...
activation of PKB/Akt (23, 38–40) and cell migration (24, 25, 41, 42). PI 3-kinase binds to the same site on FAK as Src does (12) and has been shown to associate with FAK upon integrin activation in platelets (16) and fibroblasts (17). Our results indicate that PI 3-kinase also regulates FN fibril formation. In combination, inhibition of both Src and PI 3-kinase dramatically reduced FN matrix assembly. At 2 h the effect was twice as potent as with either inhibitor used alone, suggesting that the effects are additive. Interestingly, however, we consistently observed that Src activity was required at the earliest times tested, although PI 3-kinase inhibition had its major impact after 2 h. This suggests that although both kinases participate in this process, their roles are not identical.

A major mechanism of activation of Src and PI 3-kinase is through binding to phosphorylated FAK in response to integrin ligation by FN (43–45). FAK itself has been implicated in regulatory responses to FN matrix in that alterations in FN matrix structure modulate the level of FAK tyrosine phosphorylation (18). In addition, FAK-null cells show a dramatically reduced ability to assemble FN matrix3 although the ability to attach to immobilized FN is only slightly impaired (46). One plausible model for regulation of integrin-mediated FN fibril initiation is that active FAK recruits Src and PI 3-kinase, which in turn transduce downstream signals required to initiate and maintain propagation of FN fibrils. Inhibition of any one of these kinases, either through mutation or with specific inhibitors, reduces the extent of fibril formation. There is evidence that association of Src with FAK may further stimulate both kinases (14). Similarly, the inhibition of PI 3-kinase leads to partial inhibition of FAK tyrosine phosphorylation in COS7 cells (23). These observations suggest that there may be a feedback loop between FAK, Src, and PI 3-kinase that results in mutual activation of these signaling components through direct interactions. Both Src and PI 3-kinase bind to phosphorylated Tyr-397 on FAK, presenting an attractive idea that cells require phosphorylation of this tyrosine to regulate matrix assembly.

The initial interaction of FN dimers with cell surface receptors leads to receptor clustering, which not only promotes FN self-association but also connects FN to the actin cytoskeleton. Both an intact cytoskeleton and the β integrin cytoplasmic domain are required for FN matrix assembly (47, 48). Choquet et al. (49) showed that initial connections between FN, α5β1 integrin, and actin filaments are relatively weak in adherent cells but become reinforced in response to applied force. This strengthening mechanism appears to require phosphoproteins, although the specific components have not been reported. It seems likely that reinforcement of cell-FN connections is also required during fibril assembly. FAK, Src, and/or PI 3-kinase may contribute to reinforcement of transmembrane connections because integrins assemble FN fibrils and may thus represent some of the necessary phosphoproteins. We did not observe any major effects of Src and PI 3-kinase inhibitors on actin stress fibers, but it remains possible that these kinases exert effects by strengthening existing links between integrins and actin.

The deposition of FN into the ECM is a complex, integrin-dependent, and highly regulated process. The ECM has important effects on cell morphology, growth, and gene expression. Defects in matrix organization contribute to disease and developmental defects. Therefore, it is important to understand regulation of FN matrix formation. The data presented here dem-

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3 N. Sarraf and C. Damsky, manuscript in preparation; I. Wierzbicka-Patynowski, unpublished data.
onstrate that Src and PI 3-kinase, two downstream effectors of FAK, are important in this regulation, particularly during initiation. Assembly is a multistep process, and other signaling molecules have been implicated in both early and late stages, further demonstrating the complexities of regulating FN fibril formation. It remains possible that other signaling molecules downstream of FAK, such as Grb7 (50) or the γ, isoform of phospholipase C (51), may also participate. Clearly, further investigation is needed to sort out the intracellular components that control each step of FN matrix assembly.

Acknowledgments—We thank Drs. Hisaaki Kawakatsu and Dusko Ilic from UCSF for helpful discussions and Nedra Guckert for technical assistance.

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J. Biol. Chem. 2002, 277:19703-19708.
doi: 10.1074/jbc.M200270200 originally published online March 23, 2002

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