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Inhibition of Cell Spreading by Expression of the C-Terminal Domain of Focal Adhesion Kinase (FAK) Is Rescued by Coexpression of Src or Catalytically Inactive FAK: a Role for Paxillin Tyrosine Phosphorylation

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pp125^{FAK} is a tyrosine kinase that appears to regulate the assembly of focal adhesions and thereby promotes cell spreading on the extracellular matrix. In some cells, the C terminus of pp125^{FAK} is expressed as a separate protein, pp41/43^{FRNK}. We have previously shown that overexpression of pp41/43^{FRNK} inhibits tyrosine phosphorylation of pp125^{FAK} and paxillin and, in addition, delays cell spreading and focal adhesion assembly. Thus, pp41/43^{FRNK} functions as a negative inhibitor of adhesion signaling and provides a tool to dissect the mechanism by which pp125^{FAK} promotes cell spreading. We report here that the inhibitory effects of pp41/43^{FRNK} expression can be rescued by the co-overexpression of wild-type pp125^{FAK} and partially rescued by catalytically inactive variants of pp125^{FAK}. However, coexpression of an autophosphorylation site mutant of pp125^{FAK}, which fails to bind the SH2 domain of pp60$^{c\text{-}Src}$, or a mutant that fails to bind paxillin did not promote cell spreading. In contrast, expression of pp41/43^{FRNK} and pp60$^{c\text{-}Src}$ reconstituted cell spreading and tyrosine phosphorylation of paxillin but did so without inducing tyrosine phosphorylation of pp125^{FAK}. These data provide additional support for a model whereby pp125^{FAK} acts as a "switchable adaptor" that recruits pp60$^{c\text{-}Src}$ to phosphorylate paxillin, promoting cell spreading. In addition, these data point to tyrosine phosphorylation of paxillin as being a critical step in focal adhesion assembly.

The ability of cells to migrate on the extracellular matrix (ECM) requires the formation of transient adhesive links between integrins and the components of the ECM, thereby allowing the cells to move by exerting force against the substrate (20, 26). Cultured cells form specialized adhesive structures termed focal adhesions (5, 6). The assembly of focal adhesions and the concomitant recruitment of actin stress fibers and cytoskeletal proteins appear to play an important role in cell migration in vivo (26). The integrins constitute a family of transmembrane heterodimeric proteins whose specificities for different ECM molecules are determined by the compositions of α and β subunits (21). Whereas binding to the ECM is mediated by the integrin extracellular domain, cytoplasmic domains interact with a number of cytoskeletal molecules thus providing a link to the actin cytoskeleton (5, 6). Migrating cells must continually remodel this actin cytoskeleton and form new adhesive links with the substratum (4, 20), suggesting that integrins may play a dual role, both as adhesive molecules and as receptors that transduce signals from the ECM that direct reorganization of the actin cytoskeleton. Thus, an understanding of the molecular events underlying cell motility requires analysis of the mechanisms by which integrins transduce signals from the ECM.

As integrins lack intrinsic catalytic activity, they recruit and activate other signaling molecules. One such signaling molecule is focal adhesion kinase (pp125^{FAK}), a protein tyrosine kinase that is enriched in focal adhesions (15, 37, 40, 44). Attachment and spreading of cells on a variety of ECM proteins lead to an increase in pp125^{FAK} phosphorylation on tyrosine and the concomitant activation of pp125^{FAK} catalytic activity, indicating a role for pp125^{FAK} in integrin signaling (27, 44). The importance of pp125^{FAK} in this pathway is further underscored by the observation that cells isolated from mice genetically deficient in focal adhesion kinase (FAK) show reduced rates of migration (22, 23). However, the forced overexpression of pp125^{FAK} in CHO cells results in an increase in cell migration (8). Furthermore, the increased expression of pp125^{FAK} noted in melanoma cell lines correlates with increased cell motility (1), and the elevated pp125^{FAK} expression observed in colonic and breast tumors appears to be restricted to invasive as opposed to noninvasive tumors (32, 49). Thus, considerable evidence points to a role for pp125^{FAK} in regulating the motility of both normal and malignant cells.

The structure of pp125^{FAK} has provided clues to how pp125^{FAK} transduces signals from integrins. pp125^{FAK} comprises a central catalytic domain flanked by N- and C-terminal domains (40). Clustering of cell surface integrins by using beads coated with noninhibitory monoclonal antibodies (MAbs) to integrin receptors induces intracellular accumulation of tyrosine-phosphorylated proteins, including pp125^{FAK} (29, 30). In vitro, the N-terminal domain of FAK binds directly to peptides corresponding to the cytoplasmic domain of integrin β subunits which have been covalently linked to beads (43). These data suggest that integrin clustering may provide a means of both recruiting and activating pp125^{FAK} in response to extracellular ligand binding.

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Integrin binding to the ECM results in activation of pp125^{FAK} and phosphorylation of Tyr-397 (44). The residues surrounding Tyr-397 constitute a sequence that efficiently binds to the SH2 domain of another protein tyrosine kinase, pp60^{Src} (12). The binding of phosphorylated Tyr-397 (*P*-Tyr-397) in pp125^{FAK} to the SH2 domain of pp60^{Src} activates pp60^{Src} catalytic activity, presumably by displacing the site of regulatory phosphorylation in pp60^{Src}, Tyr-527 (42). This model is supported by the observation that pp60^{Src} is activated following cell spreading on fibronectin (46, 47) as well as by the identification of activated Src present in FAK immune complexes (data not shown). Thus, integrin binding to the ECM creates and activates a bipartite kinase complex.

The C-terminal domain of pp125^{FAK} contains binding sites for a number of signaling molecules, including phosphoinositide 3-kinase (2, 9, 11, 14), the adapter proteins p130^{Cas} (16, 34, 35) and Grb2 (46), GTPase-activating protein GRAF (19), and the two cytoskeletal proteins paxillin and talin (10, 18). The C-terminal domain also contains a focal adhesion targeting sequence that is necessary and sufficient for recruiting pp125^{FAK} to focal adhesions (17). In some cells, the C-terminal domain of pp125^{FAK} is expressed as a separate protein, pp41/43^{FRNK} (FAK-related nonkinase) (41). We have previously shown that overexpression of pp41/43^{FRNK} functions to inhibit cell spreading and that ectopic expression of pp41/43^{FRNK} inhibits integrin-stimulated tyrosine phosphorylation of pp125^{FAK} as well as tyrosine phosphorylation of the focal adhesion-associated proteins, paxillin and tensin (38). These data support those from previous experiments implicating paxillin and tensin as downstream targets for either pp125^{FAK} or a kinase activated by pp125^{FAK} (25). Microinjection of a fusion protein corresponding to pp41/43^{FRNK} has been shown to reduce cell motility (13), and expression of pp41/43^{FRNK} delays the formation of focal adhesions and chicken embryo (CE) cell spreading on fibronectin (38), suggesting that pp125^{FAK} plays a direct role in promoting the assembly of focal adhesions.

Cell spreading is the process by which a cell in suspension adopts a flattened morphology when allowed to adhere to the ECM. This provides a convenient model for examining the formation of focal adhesions and provides a partial model of cell motility that is amenable to biochemical analysis. We have used overexpression of pp41/43^{FRNK} as a tool to examine the mechanism by which pp125^{FAK} promotes the cytoskeletal changes that are necessary for cell spreading on the ECM. Cells overexpressing pp41/43^{FRNK} exhibit an inhibition of the kinetics of cell spreading that can be "rescued" by co-overexpression of wild-type pp125^{FAK}. Using this assay we have ectopically expressed variants of pp125^{FAK} and pp60^{Src} that lack catalytic activity or binding sites for other proteins and have determined the abilities to promote cell spreading and tyrosine phosphorylation of paxillin and tensin in the presence or absence of the expression of pp41/43^{FRNK}. The data presented below indicate that the recruitment of pp60^{Src} and paxillin by pp125^{FAK} and the phosphorylation of paxillin may be limiting events in cell spreading. Dominant-negative inhibitors of pp125^{FAK} and pp60^{Src} also reduce cell migration, emphasizing the importance of pp125^{FAK} and pp60^{Src} in mediating molecular events that govern cell motility.

MATERIALS AND METHODS

Molecular and cell biology. The pp125^{FAK} cDNA was subcloned into pALTER, and site-directed mutagenesis was performed with the ALTERED SITES mutagenesis system (Promega). All mutations were confirmed by subsequent DNA sequencing. The mutated cDNA was subcloned into replication-competent retroviral vector RCAS (17, 38). RCAS constructs encoding pp60^{Src} variants were a kind gift from Joan Brugge (ARIAD, Inc.). CE cells were prepared as described previously (36), and pp125^{FAK}, pp41/43^{FRNK}, and pp60^{Src} variants were expressed by transfection with RCAS DNA. To coexpress two separate proteins, the individual coding sequences were inserted into RCAS viruses of different subgroups (either A or B). Cells were transfected with either RCAS (A) or RCAS (B) DNA and cultured for 4 to 6 days. The cells were then mixed and cultured for another 7 days, resulting in dual infection of the majority of the cells (38).

Measurement of cell spreading. Cell spreading was measured as described previously (38). Briefly, CE cells were collected by trypsinization and washed with 1 mg of soybean trypsin inhibitor per ml. Then, 10^6 cells were added to a 60-mm-diameter dish coated with fibronectin (1 $\mu g/cm^2$) and containing 2 ml of prewarmed L15 medium (Gibco). The cells were allowed to spread for 20 min at 37°C. For each experiment, five random fields were photographed. Between five and eight independent experiments, using at least four separately prepared populations of CE cells, were performed. A total of at least 500 cells were counted for each experimental condition. The photographs were coded, and the extent of cell spreading was assessed by three individuals. Unspread cells were defined as round phase-bright cells; spread cells were defined as those that had extended processes, that lacked a rounded morphology, and that were not phase bright. Fewer than 5% of the cells could not be categorized in either of these two groups.

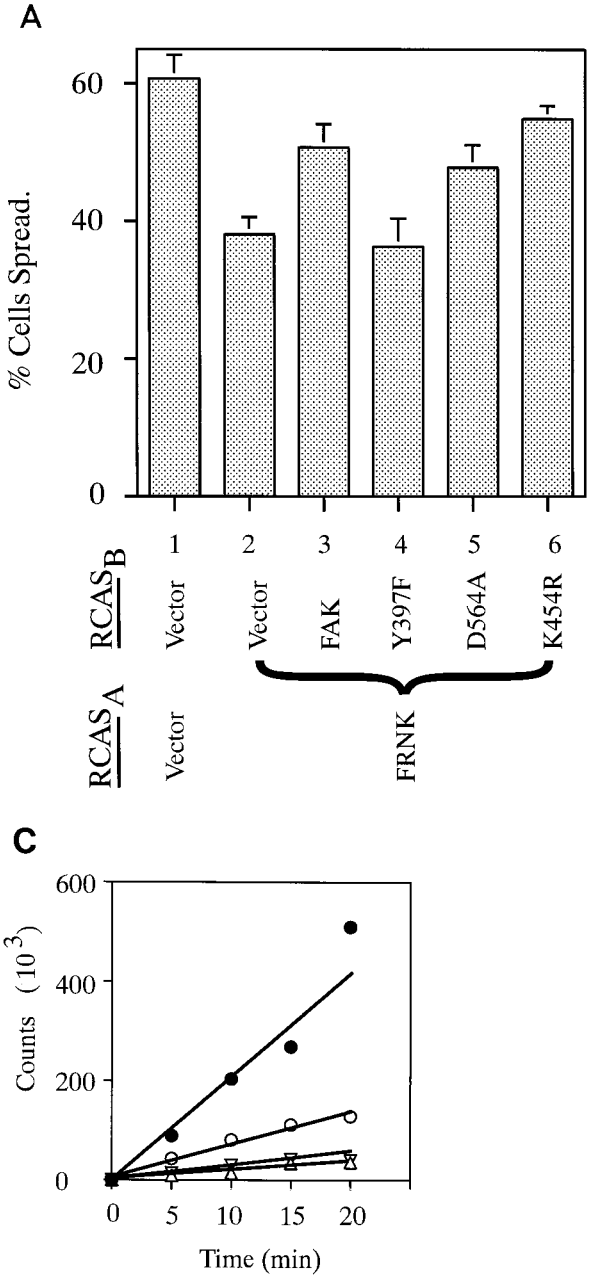
To measure cell migration, BIOCOAT cell culture inserts (8-μm pore size) were coated uniformly with fibronectin (1 $\mu g/cm^2$) for 2 h at 37°C. CE cells were grown to confluence and collected by trypsinization, and 200×10^3 were plated in the insert in conditioned media. After 12 h, the cells on both sides of the insert were collected by trypsinization and counted on a Coulter Counter. Under these conditions, the number of cells that migrated through the insert was linear for times up to 12 h. Additionally, staining with crystal violet indicated that a negligible fraction of the cells remained attached after trypsinization, and trypsinizing the cells immediately after allowing them to attach confirmed that a negligible proportion of the cells passed through the insert during trypsinization.

Immunoprecipitation and immunoblot analysis. Confluent CE cells were lysed in a supplemented radioimmunoprecipitation assay (S-RIPA) buffer containing 50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40 (pH 7.0), 100 μM leupeptin, 10 μM pepstatin, 0.05 TIU of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM benzamidine, 20 μg of soybean trypsin inhibitor per ml, 10 mM sodium pyrophosphate, 40 mM sodium-*p*-nitrophenylphosphate, and 40 mM NaF. The lysates were cleared by centrifugation at $15{,}000 \times g$ for 10 min at 4°C. Proteins were immunoprecipitated, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose. Paxillin was precipitated from 0.25 mg of lysate protein with 0.5 μg of MAb P13520 (Transduction Labs) and detected by immunoblotting with the same MAb (25 ng/ml). Tensin was immunoprecipitated from 0.8 mg of lysate protein with 5 μg of MAb 3C4 (24) and detected with the same MAb (1 μg/ml). Endogenous pp125^{FAK} was immunoprecipitated from 1.5 mg of lysate protein with 20 μl of polyclonal serum BC2 (an antibody to the catalytic domain of pp125^{FAK} [40]) and detected with serum BC3 (1:1,000 dilution) (40). Phosphotyrosine was detected with recombinant MAb RC-20 (1:2,500 dilution; Transduction Labs). All immunoblots were visualized by enhanced chemiluminescence (Amersham).

Immune complex kinase assays. pp125^{FAK} was immunoprecipitated (with 2 μg of MAb 2A7) (24, 40) from 0.2 mg of lysate prepared from CE cells infected with retroviruses encoding individual FAK variants. The immunoprecipitates were collected with 20 μl of protein A-Sepharose and were washed twice with S-RIPA, once with Tris-buffered saline, and once with kinase buffer (10 mM HEPES, 3 mM $MnCl_2$ [pH 7.0]). The immune precipitates were incubated with 1 μg of purified glutathione *S*-transferase (GST)–paxillin (N-term) in solution, 10 μCi of [γ-^{32}P]ATP, and 0.1 mM (unlabeled) ATP in a total volume of 20 μl. The reaction was terminated by the addition of sample buffer. The labeled GST-paxillin was separated by SDS-PAGE, and the gel was dried prior to analysis on a phosphorimager.

RESULTS

Rescue of FRNK-mediated inhibition of cell spreading by structural variants of FAK. We have previously shown (38) that overexpression of pp41/43^{FRNK} inhibits cell spreading on fibronectin and that this inhibition of spreading can be reversed by coexpression of wild-type pp125^{FAK}. To determine whether the catalytic activity or autophosphorylation of pp125^{FAK} was necessary for the reversal of the pp41/43^{FRNK} inhibition of spreading, variants of pp125^{FAK} that lacked the autophosphorylation site (Tyr-397 to Phe; pp125$^{FAK\ (Y397F)}$) were engineered or were catalytically inactivated by the mutation of residues predicted to function during catalysis [pp125$^{FAK\ (D564A)}$] or binding of ATP [pp125$^{FAK\ (K454R)}$]. To assess the ability of these FAK variants to rescue pp41/43^{FRNK}-induced inhibition of cell spreading, CE cells coexpressing pp41/43^{FRNK} and each of the individual pp125^{FAK} variants



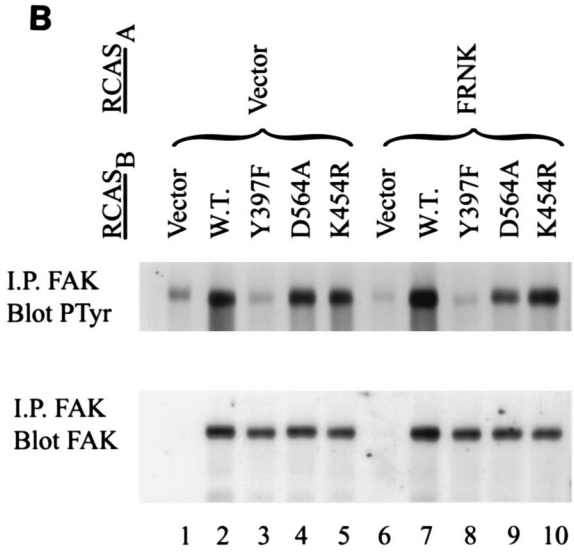


FIG. 1. Analysis of cell spreading and tyrosine phosphorylation of pp125^{FAK} following the coexpression of pp41/43^{FRNK} and either wild-type, autophosphorylation-defective, or kinase-deficient pp125^{FAK}. (A) CE cells transfected with empty vector (Vector) or cells expressing pp41/43^{FRNK} (FRNK) and/or pp125^{FAK}, pp125$^{FAK\ (Y397F)}$ (Y397F), pp125$^{FAK\ (D564A)}$ (D564A), or pp125$^{FAK\ (K454R)}$ (K454R) were allowed to spread on fibronectin-coated petri dishes, and the numbers of spread cells were assessed after 20 min. Cells transfected with pp41/43^{FRNK} showed significantly inhibited cell spreading ($P < 0.002$) compared to control cells. This was reversed by pp125^{FAK}, pp125$^{FAK\ (D564A)}$, and pp125$^{FAK\ (K454R)}$ ($P < 0.02$, $P < 0.05$, $P < 0.005$, respectively). Each result was expressed as the number of cells that were spread as a percentage of the total number of cells ± the standard error of the mean. (B) FAK variants were immunoprecipitated with antiserum BC2 from empty vector-transfected CE cells (Vector) or cells expressing either pp41/43^{FRNK}, pp125^{FAK}, pp125$^{FAK\ (Y397F)}$, pp125$^{FAK\ (D564A)}$, or pp125$^{FAK\ (K454R)}$. The immunoprecipitates were divided, and the extent of pp125^{FAK} tyrosine phosphorylation and the amount of pp125^{FAK} immunoprecipitated were assessed by immunoblotting as described in Materials and Methods. (C) pp125^{FAK} was immunoprecipitated, and the initial rates of phosphorylation of GST-paxillin (N-term) were determined as described in Materials and Methods. Immune complexes from pp125^{FAK}-expressing cells (solid circle), pp125$^{FAK\ (Y397F)}$-expressing cells (open circle), pp125$^{FAK\ (D564A)}$-expressing cells (inverted triangle), and pp125$^{FAK\ (K454R)}$-expressing cells (triangle) are shown.

were collected by trypsinization and replated onto dishes coated with fibronectin. After 20 min, the number of cells that had spread was determined as described in Materials and Methods (Fig. 1A). As reported previously, expression of pp41/43^{FRNK} delayed cell spreading and this inhibition was reversed by coexpression of wild-type pp125^{FAK} (Fig. 1A). In contrast to what was found for wild-type pp125^{FAK}, expression of the autophosphorylation-defective variant, pp125$^{FAK\ (Y397F)}$, was unable to restore the normal kinetics of cell spreading. However, expression of catalytically inactive variant pp125$^{FAK\ (D564A)}$ or pp125$^{FAK\ (K454R)}$ did efficiently rescue cell spreading (Fig. 1A, bars 3, 5, and 6).

To confirm the tyrosine phosphorylation of the pp125^{FAK} variants, each was expressed in CE cells and analyzed by immunoprecipitation and immunoblotting with anti-FAK and anti-*P*-Tyr antibodies. As shown in Fig. 1B, all variants were expressed at comparable levels, and as previously reported (42), pp125^{FAK} was tyrosine phosphorylated, whereas the tyrosine phosphorylation of pp125$^{FAK\ (Y397F)}$ was significantly reduced. Surprisingly, the catalytically inactive variants, pp125$^{FAK\ (D564A)}$ and pp125$^{FAK\ (K454R)}$, were also tyrosine phosphorylated, albeit to a level less than that of wild-type pp125^{FAK}. Additionally, phosphopeptide mapping of FAK expressed in CE cells indicated that pp125^{FAK}, pp125$^{FAK\ (D564A)}$, and pp125$^{FAK\ (K454R)}$ showed similar patterns of phosphorylation (data not shown). Immune complex kinase assays (Fig. 1C) using an exogenous substrate confirmed that the catalytic activities of pp125$^{FAK\ (D564A)}$ and pp125$^{FAK\ (K454R)}$ were impaired in vitro, and subsequent experiments suggested that these variants were also impaired in vivo (see below).

Rescue of pp41/43^{FRNK}-mediated inhibition of paxillin tyrosine phosphorylation by structural variants of FAK. Previous studies have shown that overexpression of pp125^{FAK} and concomitant treatment of CE cells with vanadate (a tyrosine phosphatase inhibitor) increased the extent of tyrosine phosphorylation of paxillin and tensin (45), suggesting that these

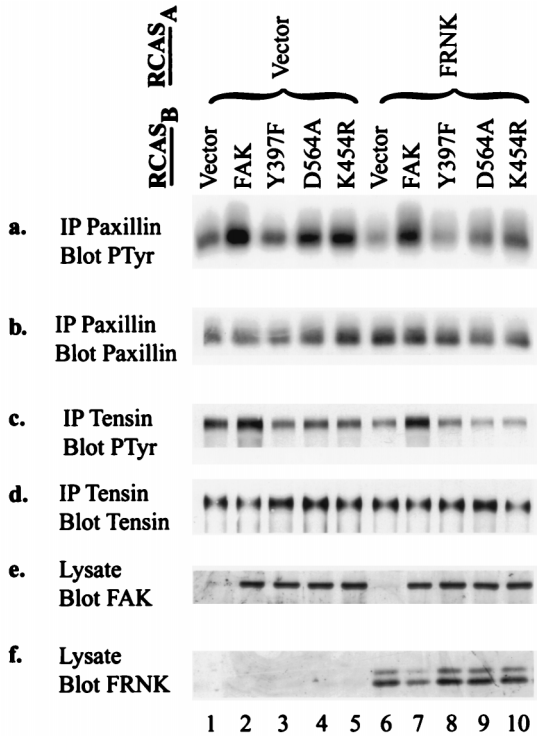


FIG. 2. Tyrosine phosphorylation of paxillin and tensin in cells coexpressing pp41/43^{FRNK} and mutants of pp125^{FAK}. Paxillin was immunoprecipitated from empty vector-transfected CE cells (Vector) or cells expressing pp41/43^{FRNK} and/or the pp125^{FAK} variants described in the legend for Fig. 1. The amount of paxillin in the immunoprecipitates (row b) and the extent of paxillin tyrosine phosphorylation (row a) were assessed by immunoblotting as described in Materials and Methods. Similar experiments were performed by immunoprecipitating and immunoblotting tensin (rows c and d). To confirm the expression of pp41/43^{FRNK} and of the pp125^{FAK} variants, 5 μg of cell lysate was blotted directly for pp125^{FAK} or pp41/43^{FRNK} with antiserum BC3 (rows e and f, respectively. Designations are as described in the legend for Fig. 1.

focal adhesion-associated proteins were targets for the FAK-Src bipartite kinase complex. Furthermore, overexpression of pp41/43^{FRNK} significantly inhibits the tyrosine phosphorylation of pp125^{FAK}, paxillin, and tensin (38). To determine if coexpression of wild-type, autophosphorylation-defective, or catalytically defective variants of pp125^{FAK} could restore the tyrosine phosphorylation of putative downstream targets of pp125^{FAK}, paxillin and tensin were immunoprecipitated from cells (cultured in the absence of vanadate) coexpressing pp41/43^{FRNK} and individual pp125^{FAK} variants and tyrosine phosphorylation of paxillin and tensin was assessed by immunoblotting. Expression of pp41/43^{FRNK}, as previously shown, inhibited tyrosine phosphorylation of paxillin and tensin, and this inhibition was reversed by coexpression of pp125^{FAK} (Fig. 2, rows a and c, lane 7). In contrast, overexpression of pp125$^{FAK\ (Y397F)}$ did not reverse the inhibitory effect of pp41/43^{FRNK} on paxillin tyrosine phosphorylation (Fig. 2, rows a and c, lane 8). Overexpression of catalytically inactive variants pp125$^{FAK\ (D564A)}$ and pp125$^{FAK\ (K454R)}$ reproducibly (in four separate experiments) reversed the reduction in tyrosine phosphorylation of paxillin induced by pp41/43^{FRNK} (Fig. 2, row a, lanes 9 and 10). Neither pp125$^{FAK\ (Y397F)}$ nor pp125$^{FAK\ (D564A)}$ nor pp125$^{FAK\ (K454R)}$ reversed the pp41/43^{FRNK}-induced inhibition of tyrosine phosphorylation of tensin (Fig. 2, row c, lanes 8 to 10). These data indicate that overexpression of either wild-type pp125^{FAK} or kinase-inactive pp125^{FAK} can restore most of the tyrosine phosphorylation of paxillin in cells coexpressing pp41/43^{FRNK}. However, only the expression of wild-type pp125^{FAK} restores the tyrosine phosphorylation of tensin, suggesting that different pp125^{FAK}-dependent pathways lead to paxillin and tensin phosphorylation. Control experiments confirmed that similar amounts of paxillin and tensin as well as of pp41/43^{FRNK} and pp125^{FAK} were immunoprecipitated from the various cells (Fig. 2, rows b, d, e, and f). As previously reported, overexpression of only pp125^{FAK} leads to a modest increase in paxillin and tensin phosphorylation, whereas the overexpression of the two catalytically inactive pp125^{FAK} variants modestly increased paxillin tyrosine phosphorylation but not the tyrosine phosphorylation of tensin (rows a and c). Overexpression of the autophosphorylation-defective variant, pp125$^{FAK\ (Y397F)}$, did not increase substantially tyrosine phosphorylation of either paxillin or tensin (rows a and c).

Expression of c-Src rescues the inhibition of cell spreading and paxillin tyrosine phosphorylation by pp41/43^{FRNK}. The data shown in Fig. 1 and 2 indicated that rescue of pp41/43^{FRNK}-induced inhibition of cell spreading and tyrosine phosphorylation of paxillin required FAK autophosphorylation but not FAK catalytic activity. We reasoned that the recruitment of a Src family kinase to pp125^{FAK} was likely to play an important role in restoring cell spreading and the formation of focal adhesions. To test this, we ectopically expressed pp60$^{c\text{-}Src}$ in

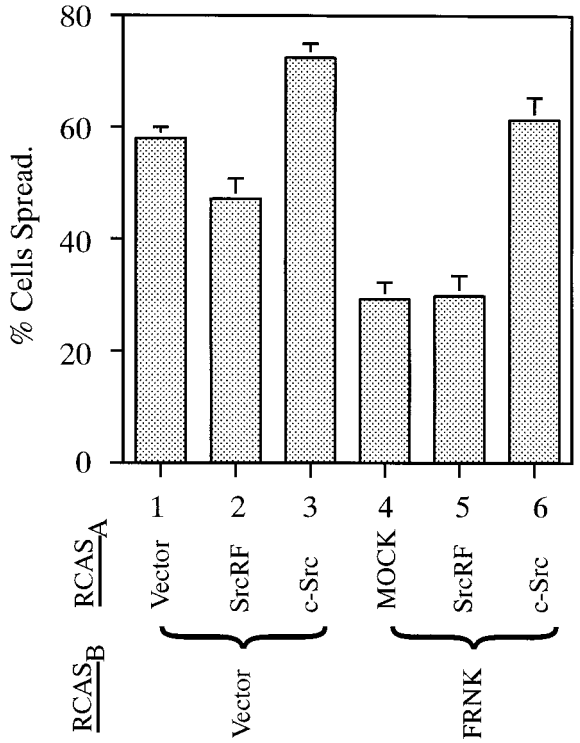


FIG. 3. The effect of pp60^{Src} on cell spreading. CE cells were transfected with empty vector (Vector) or retroviruses encoding pp60^{Src} (c-Src) or a variant of pp60^{Src} (SrcRF) containing mutations rendering it catalytically inactive (Lys-295→Arg) and in the "open" conformation (Tyr-527→Phe). The transfected cells were allowed to spread on fibronectin-coated petri dishes for 20 min, and the number of spread cells was determined in each case. Both pp60^{Src} and pp41/43^{FRNK} significantly delayed cell spreading ($P < 0.02$ and $P < 0.005$, respectively) and the inhibition by pp41/43^{FRNK} was overcome by coexpression of pp41/43^{FRNK} with pp60^{Src} ($P < 0.0002$). Each result was expressed as the number of cells that were spread as a percentage of the total number of cells ± the standard error of the mean. MOCK, cells infected with empty vector.

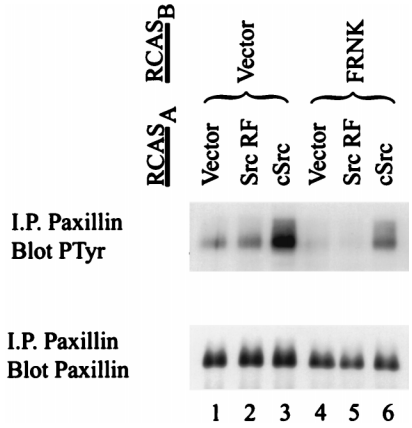


FIG. 4. Analysis of tyrosine phosphorylation of paxillin in cells expressing pp41/43^{FRNK} and pp60^{Src}. Paxillin was immunoprecipitated from CE cells expressing pp41/43^{FRNK} (FRNK) and pp60^{Src} (cSrc), or pp60$^{Src\ (RF)}$ (Src RF) as described in the legend for Fig. 3. The immunoprecipitates were divided, and the extent of paxillin tyrosine phosphorylation and the amount of paxillin immunoprecipitated were assessed by immunoblotting.

CE cells alone or in CE cells coexpressing pp41/43^{FRNK} (Fig. 3). Overexpression of pp60$^{c\text{-}Src}$ alone accelerated cell spreading. This increase in the apparent rate of cell spreading required the catalytic activity of pp60$^{c\text{-}Src}$, as a kinase-inactive variant of pp60$^{c\text{-}Src}$ [pp60$^{Src\ (RF)}$, containing mutations Lys-295→Arg and Tyr-527→Phe] failed to accelerate cell spreading (Fig. 3). As shown in Fig. 3, coexpression of pp60$^{c\text{-}Src}$ and pp41/43^{FRNK} reversed the inhibitory effects of pp41/43^{FRNK} on cell spreading, whereas the expression of pp60$^{Src\ (RF)}$ failed to rescue pp41/43^{FRNK}-induced inhibition of cell spreading, clearly showing that the catalytic activity of pp60$^{c\text{-}Src}$ was required. A pp60$^{c\text{-}Src}$ variant which contained only the Lys-295→Arg mutation, behaved similarly to pp60$^{Src\ (RF)}$ (data not shown) and was unable to rescue cell spreading and paxillin phosphorylation (see below).

We next considered whether pp60$^{c\text{-}Src}$ could reverse the inhibitory effects of pp41/43^{FRNK} by inducing tyrosine phosphorylation of paxillin. Immunoprecipitation and immunoblot analysis of paxillin indicated that, in the absence of pp41/43^{FRNK}, pp60$^{c\text{-}Src}$ induced significant tyrosine phosphorylation of paxillin (Fig. 4, upper row, lane 3). The inhibitory effects of pp41/43^{FRNK} on paxillin tyrosine phosphorylation were reversed by coexpression of pp60$^{c\text{-}Src}$ with pp41/43^{FRNK}, but not by coexpression of the catalytically inactive variant of c-Src, pp60$^{Src\ (RF)}$ (Fig. 4, lanes 4 to 6). Thus, there was a strong correlation between the regulation of cell spreading and tyrosine phosphorylation of paxillin.

A possible mechanism by which pp60$^{c\text{-}Src}$ might promote cell spreading and paxillin tyrosine phosphorylation is by stimulating FAK activity, perhaps by inducing tyrosine phosphorylation of pp125^{FAK}. Overexpression of Src with a vector control (Fig. 5, top row, lane 3) induced a modest increase in pp125^{FAK} tyrosine phosphorylation. However coexpression of Src and pp41/43^{FRNK} did not substantially reverse the pp41/43^{FRNK}-induced inhibition of tyrosine phosphorylation of pp125^{FAK} (Fig. 4, top row, lanes 4 and 6), whereas coexpression of Src and pp41/43^{FRNK} led to restoration of cell spreading (Fig. 3) and an increase in paxillin tyrosine phosphorylation (Fig. 4). Thus, we conclude that it is unlikely that pp60$^{c\text{-}Src}$ promotes cell spreading by inducing tyrosine phosphorylation of pp125^{FAK}.

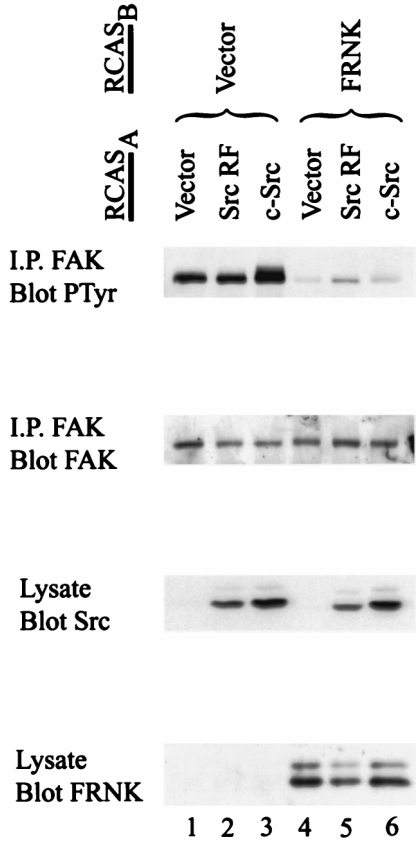


FIG. 5. Analysis of c-Src-induced tyrosine phosphorylation of FAK in cells coexpressing pp41/43^{FRNK}. pp125^{FAK} was immunoprecipitated with antiserum BC2 from CE cells transfected with empty vector (Vector) or with retroviruses encoding pp41/43^{FRNK} (FRNK) and/or pp60^{Src} (c-Src) or pp60$^{Src\ (RF)}$ (Src RF) as described in the legend for Fig. 3. The immunoprecipitates were divided, and the extent of tyrosine phosphorylation of pp125^{FAK} and the amount of pp125^{FAK} immunoprecipitated were assessed by immunoblotting. The ectopic expression of pp60^{Src} and pp41/43^{FRNK} was confirmed by direct immunoblotting of 5 μg of cell lysate with antibodies reactive with pp60^{Src} (MAb 2-17) and pp41/43^{FRNK} (BC3).

The paxillin binding site on FAK is required to promote cell spreading. Since the observations above point to the correlation between paxillin tyrosine phosphorylation and cell spreading, we examined the possible role of paxillin-pp125^{FAK} interactions by making use of the observation that deletion of the 13 C-terminal amino acids from pp125^{FAK} (residues 1041 to 1054) significantly reduces the binding of paxillin to pp125^{FAK} without affecting the localization to focal adhesions (18). Overexpression of pp41/43^{FRNK} inhibited cell spreading (Fig. 6, lane 4), and this was reversed by coexpression of pp125^{FAK} with pp41/43^{FRNK}, but not by coexpression of a pp125^{FAK} variant lacking the paxillin binding site (cFAK; Fig. 6, lanes 5 and 6). Similarly, coexpression of cFAK with pp41/43^{FRNK} did not reverse the inhibition of tyrosine phosphorylation of paxillin by pp41/43^{FRNK} (Fig. 7, upper row, lanes 4 and 6). These observations suggest that the binding of pp125^{FAK} to paxillin may promote paxillin tyrosine phosphorylation and may contribute to cell spreading.

These observations raised the possibility that pp41/43^{FRNK} acted as an inhibitor of pp125^{FAK} by sequestering paxillin and thereby reducing the amount of paxillin complexed with pp125^{FAK}. To test this, we determined the level of paxillin association with endogenous pp125^{FAK} in cells expressing a control vector or pp41/43^{FRNK}. As shown in Fig. 8A (upper

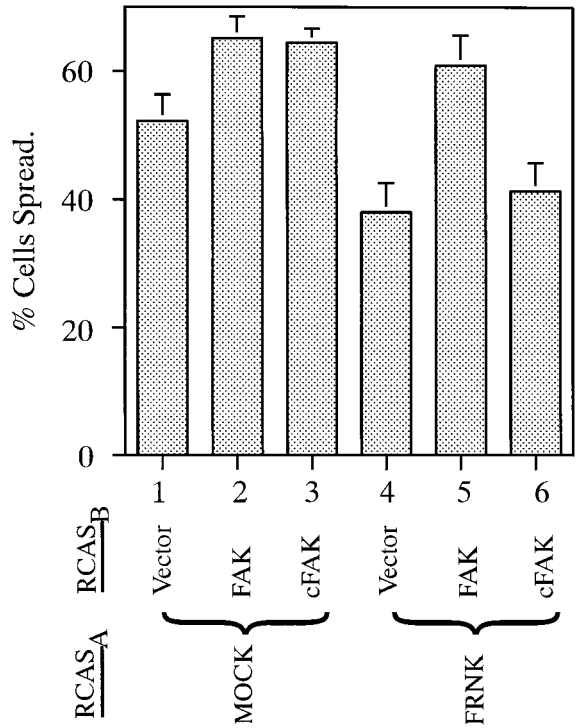


FIG. 6. Rescue of cell spreading by wild-type pp125FAK or mutants of pp125FAK that fail to bind to paxillin. CE cells were co-transfected with either empty vector (Mock; bars 1 to 3) or pp41/43FRNK (FRNK; bars 4 to 6) and pp125FAK (FAK; bars 2 and 5) or a pp125FAK variant lacking the C-terminal 11 amino acids which are essential for paxillin binding to pp125FAK (cFAK; bars 3 and 6). The transfected cells were allowed to spread on fibronectin-coated dishes, and the numbers of spread cells were determined after 20 min. pp41/43FRNK (bar 4) significantly delayed cell spreading ($P < 0.02$); this was reversed by pp125FAK (bar 5; $P < 0.0002$) but not by the pp125FAK variant (bar 6). Each result was expressed as the number of cells that were spread as a percentage of the total number of cells ± the standard error of the mean.

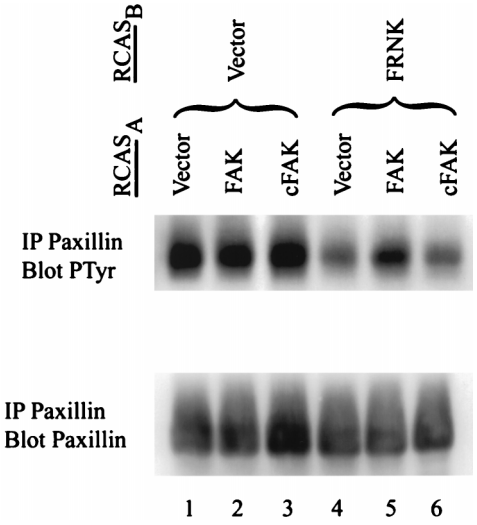


FIG. 7. Tyrosine phosphorylation of paxillin in cells coexpressing pp41/43FRNK and a FAK mutant. Paxillin was immunoprecipitated (IP) from CE cells transfected with empty vector (Vector; lanes 1 to 3), pp41/43FRNK (FRNK) (lanes 4 to 6), and/or pp125FAK (lanes 2 and 5) or a mutant of FAK that fails to bind paxillin (cFAK) (lanes 3 and 6). The immunoprecipitates were divided, and the extent of paxillin tyrosine phosphorylation and the amount of paxillin were determined by immunoblotting as described in Materials and Methods.

row, lanes 2 and 4), pp41/43FRNK expression did not substantially reduce the amount of paxillin coimmunoprecipitating with endogenous pp125FAK. In addition, when a variant of pp41/43FRNK, cFRNK, lacking the C-terminal 13 amino acids (residues 346 to 359) that correspond to residues in pp125FAK essential for the binding of paxillin was expressed in CE cells, the amount of paxillin coimmunoprecipitation with cFRNK was significantly reduced (Fig. 8B, row a). Overexpression of cFRNK inhibited the tyrosine phosphorylation of both pp125FAK (Fig. 8B, row c) and paxillin (data not shown). Overexpression of cFRNK also inhibited cell spreading (data not shown). Thus, the binding of paxillin to pp41/43FRNK does not appear to be necessary for the pp41/43FRNK-induced inhibition of cell spreading or the inhibition of FAK and paxillin tyrosine phosphorylation.

Src promotes and pp41/43FRNK inhibits cell motility. To extend the use of cell spreading as a paradigm for integrin signaling, we tested whether our observations of the effects of pp41/43FRNK and pp60c-Src extended to regulating cell motility. CE cells (17 ± 3%; defined as having a relative motility, *m*, of 1.00) migrated through a porous membrane coated with fibronectin. Although overexpression of pp60c-Src was unable to significantly increase motility ($m = 0.94 \pm 0.10$), pp60Src (RF) significantly reduced CE cell motility ($m = 0.58 \pm 0.06$; $P < 0.0005$). This was consistent with the role of pp60c-Src in cell spreading and the expectation that pp60Src (RF) would act as a dominant-negative inhibitor of pp60c-Src. pp41/43FRNK also reduced CE cell motility ($m = 0.73 \pm 0.08$; $P < 0.02$), suggesting a role for pp125FAK in regulating cell motility. However, the extent of inhibition by pp41/43FRNK was not sufficiently large to allow us to measure a reproducible increase in motility when pp125FAK was coexpressed with pp41/43FRNK. Thus, these results are consistent with a role for pp60c-Src and FAK in cell motility.

DISCUSSION

The assessment of the relative contributions of pp125FAK and pp60Src to promoting the cytoskeletal changes necessary for cell spreading is complicated by the presence of endogenous analogs of both Src and FAK. We report here a novel approach to studying signal transduction by integrins in promoting cytoskeletal rearrangement by taking advantage of our previous observations that ectopically expressed pp41/43FRNK, the autonomously expressed C-terminal domain of pp125FAK, inhibits both cell spreading and the tyrosine phosphorylation (activation) of endogenous pp125FAK and the tyrosine phosphorylation of the focal adhesion-associated protein paxillin. Previously, we have shown that coexpression of pp125FAK reverses the inhibitory effects of pp41/43FRNK (38). In this study we have used this rescue assay to measure the abilities of pp125FAK and pp60Src variants to reverse the inhibition by pp41/43FRNK of both cell spreading and the tyrosine phosphorylation of pp125FAK and paxillin. Using this assay we show that the rescue of pp41/43FRNK-induced inhibition of cell spreading is mediated by coexpression of catalytically inactive variants of pp125FAK, whereas coexpression of variants lacking the autophosphorylation site [pp125FAK (397F)] or of variants unable to stably bind paxillin is less efficient in rescuing pp41/43FRNK-induced inhibition. Overexpression of pp60Src also rescued pp41/43FRNK-mediated inhibition of cell spreading and paxillin tyrosine phosphorylation, but not the tyrosine phosphorylation of pp125FAK. These data suggest that activation of Src, either by interacting with FAK or by overexpression, is sufficient to override the pp41/43FRNK block.

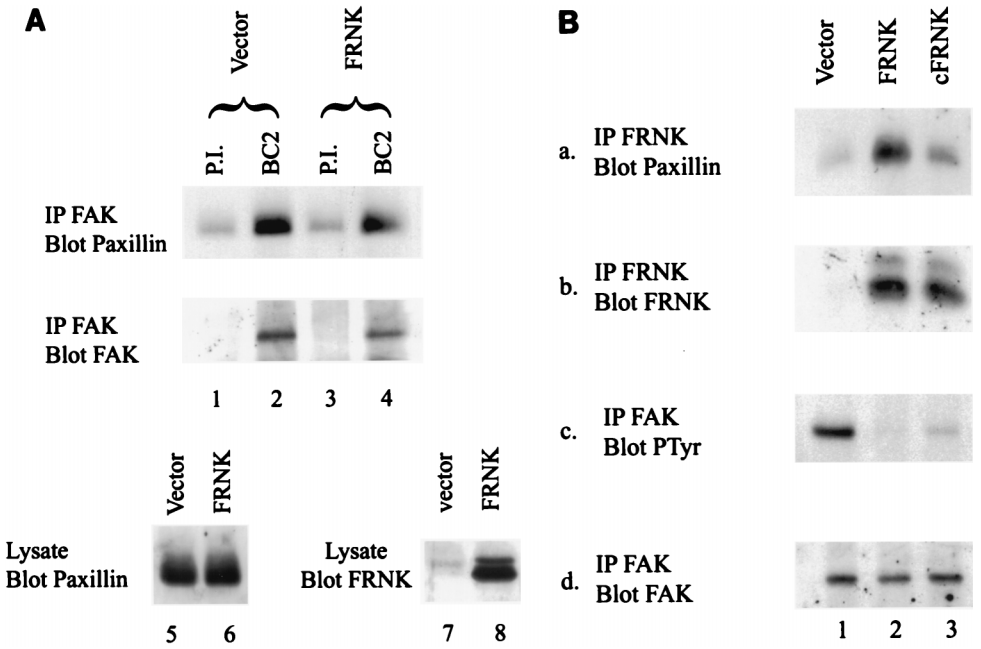


FIG. 8. Binding of paxillin to endogenous pp125^{FAK} in cells expressing pp41/43^{FRNK}. (A) CE cells were transfected with either empty vector (Vector) or pp41/43^{FRNK} (FRNK), and lysates were immunoprecipitated (IP) with either preimmune serum (P.I.; lanes 1 and 3) or the FAK-specific antibody, BC2 (BC2; lanes 2 and 4). The immune complexes were divided, and equal amounts were immunoblotted with antibodies to paxillin (top row) and FAK (middle row). An aliquot of cell lysate (5 μg) was subjected to SDS-PAGE and immunoblotted directly with an antibody to paxillin (lanes 5 and 6) or FRNK (lanes 7 and 8). The results shown are representative of four separate experiments. (B) CE cells were transfected with either empty vector (Vector), pp41/43^{FRNK} (FRNK), or a variant of pp41/43^{FRNK} lacking the residues corresponding to the paxillin binding site in pp125^{FAK} (cFRNK). FRNK was immunoprecipitated with antiserum BC3, and the immunoprecipitates were divided. The amounts of paxillin (row a) and FRNK (row b) were assessed by immunoblotting. pp125^{FAK} was immunoprecipitated from the same cells with antiserum BC2, and the immunoprecipitates were divided. The extent of pp125^{FAK} tyrosine phosphorylation (row c) and the amount of pp125^{FAK} (row d) in the immunoprecipitates were assessed by immunoblotting.

Our results point to a role for pp125^{FAK} and pp125^{FAK} autophosphorylation in the events required for cell spreading. Surprisingly, two separate pp125^{FAK} variants that were rendered catalytically inactive either by mutation of a residue predicted to be essential for catalysis or by mutation of a residue in the ATP binding site were able to reverse the inhibition of cell spreading by pp41/43^{FRNK}. In spite of a loss in catalytic activity these mutants of pp125^{FAK} were able to partially reverse the inhibition of tyrosine phosphorylation of paxillin by pp41/43^{FRNK}. In contrast pp125^{FAK} variants containing a mutation of the autophosphorylation site on pp125^{FAK} were incapable of promoting the tyrosine phosphorylation of paxillin. These results suggest that tyrosine phosphorylation of Tyr-397 is important and that in cells expressing the catalytically inactive pp125^{FAK}, tyrosine phosphorylation of ectopically expressed pp125^{FAK} may be mediated by endogenous pp125^{FAK} or, alternatively, by another protein tyrosine kinase (see below). The necessity for an intact pp60^{Src} binding site (e.g., Tyr-397) suggests that pp125^{FAK} is not the kinase responsible for the phosphorylation of paxillin and that a kinase recruited to pp125^{FAK} via Tyr-397 is a more likely candidate. In CE cells, this is likely to be pp59^{Fyn}, whereas in NIH 3T3 fibroblasts the kinase is likely to be pp60$^{c\text{-}Src}$, as these two kinases have been found to associate with pp125^{FAK} in these two cell types (7, 42, 46). It is also likely that Src and Fyn play redundant roles, consistent with the observation that both are capable of directing the phosphorylation of a range of substrates, including paxillin (48).

Additional evidence implicating a kinase recruited to pp125^{FAK} as playing a role in integrin-triggered cytoskeletal rearrangement comes from the observation that catalytically inactive variants of pp125^{FAK}, but not a variant in which Tyr-397 was mutated to Phe, have been shown to increase the motility of CHO cells (8). Despite being catalytically inactive, in our experiments both pp125$^{FAK\ (K454R)}$ and pp125$^{FAK\ (D564A)}$ were phosphorylated on Tyr-397, in agreement with the previous suggestions that tyrosine phosphorylation of pp125^{FAK} is not an intramolecular process (7). Thus, the pp125^{FAK} variants able to rescue cell spreading and phosphorylation of paxillin were themselves phosphorylated on tyrosine, whereas the variant unable to undergo phosphorylation on Tyr-397 was unable to rescue cell spreading and paxillin phosphorylation.

The foregoing observations suggested that phosphorylation of Tyr-397 and presumably the recruitment of pp60^{Src} to pp125^{FAK} were key to promoting cell spreading and tyrosine phosphorylation of paxillin. Previously, fibroblasts derived from Src (−/−) mice have been shown to spread more slowly on fibronectin than control cells (25), and this defect was rescued by expression of pp60$^{c\text{-}Src}$. Similarly, overexpression of Csk, which negatively regulates pp60$^{c\text{-}Src}$, has been shown to inhibit HeLa cell spreading (3). We observed that expression of pp60^{Src} in CE cells (in the absence of ectopic expression of pp41/43^{FRNK}) dramatically accelerated cell spreading. However, a variant of pp60$^{c\text{-}Src}$ which was rendered catalytically inactive by mutation [pp60$^{Src\ (RF)}$] was unable to increase the rate of cell spreading. When pp60$^{c\text{-}Src}$ was coexpressed with pp41/43^{FRNK}, pp60^{Src} was able to reverse the inhibitory effects of pp41/43^{FRNK} on cell spreading, and this too required the catalytic activity of pp60$^{c\text{-}Src}$. These observations suggest that the catalytic activity of pp60^{Src} is essential to promote cell spreading. In contrast, when pp60$^{c\text{-}Src}$ was expressed in Src (−/−) fibroblasts (25), the delay in cell spreading could be

rescued by catalytically inactive variants of pp60$^{c\text{-}Src}$. One possible explanation for the discrepancy with our results, in which the catalytic activity of pp60^{Src} is necessary to promote cell spreading, may be the different cellular backgrounds in which spreading is assessed. Thus, in cells expressing pp41/43^{FRNK}, the reduced tyrosine phosphorylation of pp125^{FAK} may also prevent activation of endogenous Src, thereby requiring the overexpression of catalytically active Src to bypass the effects of pp41/43^{FRNK}. In Src (−/−) cells, the catalytic activity of pp125^{FAK} may be sufficient to compensate for the lack of pp60^{Src} catalytic activity, as long as other domains of pp60^{Src} are expressed.

Our results point to a critical role for pp60$^{c\text{-}Src}$ in controlling cell spreading. Expression of pp60^{Src} was able to promote cell spreading in the presence of ectopically expressed pp41/43^{FRNK}. In addition, pp60^{Src} did not appear to induce tyrosine phosphorylation of pp125^{FAK}. Thus, the inhibitory effects of pp41/43^{FRNK} can be bypassed if pp60^{Src} is expressed at sufficiently high levels. As pp125^{FAK} is more abundant than pp60^{Src}, it is possible that the small amount of pp125^{FAK} that remains tyrosine phosphorylated in the presence of pp41/43^{FRNK} is sufficient to recruit and activate pp60^{Src} or that overexpression of pp60^{Src} leads to a higher level of basal activity of Src. An alternative explanation for the ability of pp60$^{c\text{-}Src}$ to promote and rescue cell spreading is that when pp60$^{c\text{-}Src}$ is expressed at high levels, it can bind directly to focal adhesion proteins and directly phosphorylate them. For example, pp60$^{c\text{-}Src}$ is reported to bind to paxillin (39). Expression of pp60$^{c\text{-}Src}$ induced strong tyrosine phosphorylation of paxillin. pp60$^{c\text{-}Src}$ also induced tyrosine phosphorylation of paxillin in cells in which pp41/43^{FRNK} was ectopically expressed, and in these cells pp60$^{c\text{-}Src}$ also reversed the inhibition of cell spreading by pp41/43^{FRNK}. This observation, along with our earlier observation that those pp125^{FAK} variants that were capable of overcoming the inhibition of cell spreading by pp41/43^{FRNK} were also capable of rescuing tyrosine phosphorylation of paxillin, makes paxillin an obvious candidate substrate for pp60$^{c\text{-}Src}$. Further evidence that pp60$^{c\text{-}Src}$ can phosphorylate paxillin comes from the observation that deregulation of pp60$^{c\text{-}Src}$ by rendering cells deficient in Csk leads to hyperphosphorylation of paxillin (48). This is partially reversed in cells which lack either Src or Fyn in addition to Csk (48). Thus, our data support the phosphorylation of paxillin by pp60^{Src} as being an important event during cell spreading.

A pp125^{FAK} variant lacking the binding site for paxillin failed to reverse the pp41/43^{FRNK}-mediated inhibition of both cell spreading and paxillin tyrosine phosphorylation. These data and the requirement for Tyr-397 phosphorylation provide additional support for a model whereby pp125^{FAK} functions as a "switchable adaptor". The C terminus of pp125^{FAK} binds paxillin, whereas activation of pp125^{FAK} by integrins induces tyrosine phosphorylation of pp125^{FAK} and leads to the recruitment of pp60^{Src}. Thus, pp125^{FAK} promotes the phosphorylation of paxillin by pp60$^{c\text{-}Src}$ by efficiently localizing both the substrate and the kinase.

We speculate that both the binding of pp125^{FAK} to paxillin and tyrosine phosphorylation of paxillin play important roles in cell spreading. A likely role for paxillin is the recruitment of additional proteins to the "early focal complex." Recruitment of additional paxillin binding proteins will likely be dependent on the tyrosine phosphorylation of paxillin, since the pp125^{FAK} variant unable to bind paxillin is unable to induce the tyrosine phosphorylation of paxillin and is unable to rescue cell spreading. However, it remains possible that signaling molecules that promote cell spreading bind to paxillin in a phosphotyrosine-independent fashion. In this case, pp60^{Src} would promote cell spreading by some mechanism other than by inducing tyrosine phosphorylation of paxillin. The foregoing observations suggest a central role for paxillin in promoting cell spreading. However, in addition to paxillin, tensin has also been suggested to be a substrate of pp125^{FAK}. Both the catalytically inactive and autophosphorylation-defective forms of pp125^{FAK} failed to reverse the pp41/43^{FRNK}-dependent inhibition of tyrosine phosphorylation of tensin. Thus, we suggest that tyrosine phosphorylation of tensin may be less critical for cell spreading.

Cell migration requires both the formation of new adhesive contacts at the leading edge and the disassembly of contacts at the trailing edge (26). When cells in suspension are allowed to spread on fibronectin, they initially form small focal complexes (31) which become progressively larger as the cells spread (38). This suggests that cells in suspension lack macromolecular focal complexes, so measuring cell spreading is likely to be biased towards reflecting events during the assembly rather than the disassembly of adhesive complexes. Therefore, we also examined the effects of pp60$^{c\text{-}Src}$ and pp41/43^{FRNK} on cell motility and found that pp60$^{Src\ (RF)}$ acted as an inhibitor of motility. The failure of this variant to display a measurable phenotype during cell spreading suggests that pp60^{Src} may play roles during motility in addition to those it plays during cell spreading. These roles need not be limited to the formation and disassembly of adhesive complexes, as cell motility involves numerous other events (28). In parallel to its effects on cell spreading, pp41/43^{FRNK} also functions to inhibit cell motility. Coexpression of pp60$^{c\text{-}Src}$ with pp41/43^{FRNK} restores cell motility and mirrors the ability of pp60$^{c\text{-}Src}$ to promote cell spreading. The effect of pp41/43^{FRNK} on motility was less pronounced than the inhibition of spreading by pp41/43^{FRNK}. Whether processes other than the formation of new adhesive contacts, which we presume are inhibited by pp41/43^{FRNK}, limit the rate of migration remain to be analyzed. In addition, interpretation of these results is complicated because the relationship between adhesive strength and motility is not simple—cells which adhere either too strongly or too weakly migrate more slowly (33).

We speculate that pp125^{FAK} promotes cell motility by catalyzing the formation of focal adhesions which act as anchor points against which the actin-myosin system can generate force. The activation of pp125^{FAK}, the recruitment and activation of pp60^{Src}, and the phosphorylation of paxillin appear to be the early key events during this process. It will be important to determine which molecules are subsequently recruited by paxillin and how this ultimately leads to reorganization of the actin cytoskeleton.

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A. Richardson and R. K. Malik contributed equally to this work.

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