The LD4 motif of paxillin regulates cell spreading and motility through an interaction with paxillin kinase linker (PKL)

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The small GTPases of the Rho family are intimately involved in integrin-mediated changes in the actin cytoskeleton that accompany cell spreading and motility. The exact means by which the Rho family members elicit these changes is unclear. Here, we demonstrate that the interaction of paxillin via its LD4 motif with the putative ARF-GAP paxillin kinase linker (PKL) (Turner et al., 1999), is critically involved in the regulation of Rac-dependent changes in the actin cytoskeleton that accompany cell spreading and motility. Overexpression of a paxillin LD4 deletion mutant (paxillinLD4) in CHO.K1 fibroblasts caused the generation of multiple broad lamellipodia. These morphological changes were accompanied by an increase in cell protrusiveness and random motility, which correlated with prolonged activation of Rac. In contrast, directional motility was inhibited. These alterations in morphology and motility were dependent on a paxillin–PKL interaction. In cells overexpressing paxillinLD4 mutants, PKL localization to focal contacts was disrupted, whereas that of focal adhesion kinase (FAK) and vinculin was not. In addition, FAK activity during spreading was not compromised by deletion of the paxillin LD4 motif. Furthermore, overexpression of PKL mutants lacking the paxillin-binding site (PKLPBS2) induced phenotypic changes reminiscent of paxillinLD4 mutant cells. These data suggest that the paxillin association with PKL is essential for normal integrin-mediated cell spreading, and locomotion and that this interaction is necessary for the regulation of Rac activity during these events.

Introduction

Signals that derive from cell adhesion to the extracellular matrix (ECM)* regulate important physiological events including cell motility and growth, and most often involve changes in the organization of the actin cytoskeleton. Cells interact with the ECM via transmembrane receptors, termed integrins, located at the cell surface. Binding of integrins to the ECM is accompanied by a localized clustering of these receptors, with the subsequent recruitment of structural and signaling molecules to the sites of matrix attachment, focal contacts, providing links to the actin cytoskeleton (Schwartz et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996).

Regulation of the actin cytoskeletal dynamics that occur after the activation of integrins via engagement with the ECM is effected primarily by the Rho family of small GTPases, i.e., Cdc42, Rac, and Rho (Hall, 1994, 1998; Hotchin and Hall, 1995; Clark et al., 1998; Price et al., 1998). Activation of Cdc42 and Rac elicit the formation of filopodia, and lamellipodia and peripheral membrane ruffles respectively, as well as focal contacts (Ridley et al., 1992; Nobes and Hall, 1995). Rho activation propagates the formation of actin stress fibers and focal contacts (Ridley and Hall, 1992). Multiple effector molecules have been implicated in Rho family signaling including the p21-activated kinase (PAK), the partner of Rac 1 (POR1), the Wiskott-Aldrich syndrome protein, the Rho kinase family, and PI-4-P5 kinase (Bishop and Hall, 2000).

PAK binds to and is activated by both Cdc42 and Rac (Manser et al., 1994; Martin et al., 1995; Knaus and Bokoch, 1998) and has been implicated in both Cdc42 and Rac-dependent changes in the actin cytoskeleton (Sells et al., 1997, 1999; Obermeier et al., 1998; Zhao et al., 1998;
Kiosses et al., 1999). Interestingly, POR1, a scaffolding protein, has been shown to be involved in both Rac-mediated membrane ruffling, as well as ARF6-dependent cytoskeletal rearrangements (Van Aelst et al., 1996; D’Souza-Schorey et al., 1997). Furthermore, it is believed that the small GTPase ARF1 plays a role in regulating recruitment of paxillin to focal adhesions and in stimulating Rho activation leading to stress fiber formation (Norman et al., 1998). Thus, it appears that in certain cases the outcomes of Rho and ARF family activation are linked via the use of common downstream effectors.

Paxillin is a multidomain focal contact adapter protein involved in integrin and growth factor signaling (Turner, 2000). The NH₂ terminus of paxillin contains five leucine-rich domains, termed LD motifs, which mediate protein–protein interactions (Brown et al., 1996, 1998). The LD4 motif of paxillin binds a complex of proteins previously implicated in actin cytoskeletal regulation. These include the Cdc42/Rac guanine nucleotide exchange factor PIX, PAK, and the SH2-SH3 adapter protein Nck. This complex is linked to paxillin through the putative ARF-GTPase–activating protein (ARF-GAP) paxillin kinase linker (PKL) (Turner et al., 1999), although PAK has been suggested to also bind directly to paxillin via the LD4 motif (Hashimoto et al., 2001). Consequently, paxillin, through these associations, may serve as a point of integration in the control of actin cytoskeleton dynamics by both the Rho and ARF family GTPases.

In this study, we have evaluated the importance of the paxillin–PKL interaction in mediating cytoskeletal changes associated with cell adhesion and motility. We demonstrate that in CHO.K1 fibroblasts, perturbation of the interaction between paxillin and PKL interferes with the proper regulation of Rac activity and induces dramatic effects on cell spreading, morphology, and motility after plating on fibronectin.

**Results**

**Ectopic expression of paxillin lacking the LD4 motif promotes cell spreading and alters cell morphology**

The focal adhesion adapter protein paxillin participates in the assembly of a complex of proteins involved in Rac regulation of the cytoskeleton during cell spreading and migration (Turner et al., 1999). To further characterize the role of paxillin, and specifically the LD4 motif, in controlling these Rac-dependent processes, clonal cell lines expressing full-length avian paxillin engineered with a deletion of LD4 were transiently expressed in CHO.K1 cells. Analysis of these cells in spreading assays on fibronectin yielded results similar to those described above (data not shown). Since the paxillin WT and parental nontransfected cells behaved indistinguishably during spreading assays, paxillin WT cells were used for much of the remaining experimentation described herein.

To determine whether this morphological change was attributable solely to the absence of the paxillin LD4 motif, we introduced a full-length wild-type avian GFP–paxillin fusion (GFP–paxillin) into both paxillinΔLD4 and paxillin WT cells and examined the cell’s phenotype during spreading on fibronectin. Although GFP–paxillin had no effect on the morphology of paxillin WT cells, its introduction into paxillinΔLD4 cells reduced the generation of multiple broad lamellipodia to ~20%, as compared with ~60% in paxillinΔLD4 cells not containing GFP–paxillin (Fig. 2, B and c, and C). This effect was entirely due to the reintroduction of wild-type GFP–paxillin since expression of GFP alone did not disrupt the morphological changes observed in paxillinΔLD4 cells (data not shown). The above observations suggest that the LD4 motif of paxillin is involved in the regulation of actin cytoskeletal changes that occur during cell spreading.

**Deletion of paxillin LD4 increases random cell motility**

The formation of membrane ruffles and lamellipodia are characteristics of motile cells (Mitchison and Cramer, 1996). To test a role for paxillin’s LD4 motif in the regulation of cell motility, paxillinΔLD4, paxillin WT and parental nontransfected CHO.K1 cells were plated on fibronectin and then analyzed using time-lapse video microscopy. Interestingly, paxillinΔLD4 cells were highly active, extending multiple lamellipodia (Fig. 3 A, arrows, and B). In addition, long retraction fiber-like processes were observed (Fig. 3 A, arrowheads). In contrast, paxillin WT and parental nontransfected cells displayed little protrusive activity (Fig. 3 A and B). The exaggerated protrusive activity of the paxillinΔLD4 cells was accompanied by a significant increase in the rate of random motility of these cells over paxillin WT and parental non-
transfected cell populations (Fig. 3 C). To test the effects of the deletion of paxillin LD4 motif on haptotaxis, modified Boyden chamber migration assays were performed. In support of the time-lapse video microscopy data, paxillinΔLD4 cells migrated at a higher rate than both paxillin WT and parental nontransfected cells (Fig. 3 D). Interestingly, overexpressing paxillin constructs engineered with a deletion of the second LD motif (paxillinΔLD2) in CHO.K1 cells did not affect migration (Fig. 3 D). Focal adhesion kinase (FAK), a protein implicated in cell motility, has been shown to associate with paxillin through both LD2 and LD4 (Turner and Miller, 1994; Ilic et al., 1995; Brown et al., 1996; Cary et al., 1996; Turner et al., 1999). This suggests that the increases in random motility caused by deletion of the LD4 motif were not due to the uncoupling of FAK and paxillin.

We have previously demonstrated that microinjection of a glutathione S-transferase (GST)–LD4 fusion protein into NIH 3T3 cells inhibited cell migration into scrape wounds (Turner et al., 1999). To address this potential discrepancy, wound assays were performed with paxillinΔLD4 and paxillin WT cells. The ability of each cell type to close the wound was then assessed over a 24-h time period (24h) using time-lapse video microscopy. 24 h after wounding, the paxillin WT cells had filled in the wound area (Fig. 3 E, d), identical to parental CHO.K1 cells (data not shown), whereas the paxillinΔLD4 cells were significantly retarded in their ability to close the wound (Fig. 3 E, c). Together, these data demonstrate that although overexpression of paxillinΔLD4 leads to an increase in cell protrusive activity and random cell motility, it inhibits persistent cell migration as assessed by wound assays.

The generation of broad lamellipodia-like structures in paxillinΔLD4 cells correlates with a prolonged elevation in Rac activity

Activation of the Rho family member Rac induces the formation of lamellipodia and membrane ruffles and has been
implicated in cell spreading and motility (Ridley et al., 1992; Clark et al., 1998; Price et al., 1998; Rottner et al., 1999). In paxillinΔLD4 cells, a dramatic increase in broad lamellipodia-like structures was observed during cell spreading along with increases in cell protrusive activity and random motility. This suggested a potentiation of Rac activity. To determine whether or not Rac plays a role in these processes, paxillinΔLD4 and paxillin WT cells were transiently transfected with GFP-paxillinΔLD4 and subjected to spreading assays on fibronectin for 60, 240, and 360 min, and GFP-paxillin transfectants were visualized by GFP fluorescence (a and b), and actin, by RITC-phalloidin (c and d). Arrows in c indicate paxillinΔLD4 cells expressing GFP-paxillin lacking broad lamellipodia, whereas arrowheads in c demonstrate the presence of these structures in a cell lacking GFP-paxillin. Images of the cells were captured at the 240-min time point and are representative of the differences in cell morphology observed at all time points. (C) The number of cells exhibiting multiple broad lamellipodia was quantified by counting >200 cells per time point. Values are the average of experiments performed in triplicate.

The amount of activated endogenous Rac bound was analyzed by Western blotting using anti-Rac antibodies. Paxillin WT cells spreading on fibronectin showed an increase in Rac activity at 5 min, peaking at 15 min, followed by a gradual return to baseline (Fig. 4, A and B). In contrast, paxillinΔLD4 cells exhibited a steady and prolonged activation of Rac that was maintained out to 360 min (Fig 4, A and B). The Rac activation profile observed in paxillin WT cells is similar to what has been reported for cells stimulated with exogenous growth factors, and to the activation of PAK during cell spreading (Price et al., 1998; Sander et al., 1999). However, this is the first study in which the time course of

Figure 2. Reintroduction of wild-type paxillin into paxillinΔLD4 cells rescues the normal spreading phenotype. (A) Western blot analysis using GFP polyclonal antisera was used to confirm the presence of the ectopic GFP-paxillin protein. (B) PaxillinΔLD4 and paxillin wild-type cells were transiently transfected with GFP-paxillinΔLD4 and subjected to spreading assays on fibronectin for 60, 240, and 360 min, and GFP-paxillin transfectants were visualized by GFP fluorescence (a and b), and actin, by RITC-phalloidin (c and d). Arrows in c indicate paxillinΔLD4 cells expressing GFP-paxillin lacking broad lamellipodia, whereas arrowheads in c demonstrate the presence of these structures in a cell lacking GFP-paxillin. Images of the cells were captured at the 240-min time point and are representative of the differences in cell morphology observed at all time points. (C) The number of cells exhibiting multiple broad lamellipodia was quantified by counting >200 cells per time point. Values are the average of experiments performed in triplicate.
Rac activity during cell spreading has been directly measured.

To test if the elevated and prolonged Rac activation was responsible for the additional protrusive activity and broad lamellipodia exhibited by the paxillinΔLD4 cells, dominant-negative forms of Rac (Myc-N17 Rac) were transiently expressed in paxillinΔLD4 and paxillin WT cells. Introduction of N17 Rac into paxillinΔLD4 cells completely abolished the generation of the multiple broad lamellipodia-like structures characteristic of these cells (Fig. 5, B and C). N17 Rac also inhibited lamellipodia formation in the paxillin WT cells (Fig. 5, B and C). Similarly, expression of dominant-negative Cdc42, which is upstream of Rac and is critically involved in cell spreading, also severely inhibited broad lamellipodia formation in paxillinΔLD4 cells (Fig. 5 D) (Ridley et al., 1992; Clark et al., 1998; Price et al., 1998). Together, these data indicate that elevated and prolonged Rac activation is involved in the generation of the phenotype observed in paxillinΔLD4 cells and that paxillin can regulate normal Rac activity during cell spreading. In addition, deleting the paxillin LD4 motif appears to prevent the normal progression from a Rac-dependent phenotype, to an angular elongated phenotype during cell spreading.

Deletion of paxillin LD4 perturbs the localization of endogenous PKL, but not FAK or vinculin, in paxillinΔLD4 cells

We have previously shown that the LD4 motif of paxillin binds to both FAK and vinculin as well as to the newly characterized putative ARF-GAP protein PKL (Turner and Miller, 1994; Brown et al., 1996; Turner et al., 1999). The association of paxillin with PKL mediates an interaction with a protein complex comprised of PIX/PAK/Nck, which has been implicated in Rac-dependent cytoskeletal rearrangements (Manser et al., 1997; Sells et al., 1997; Obermeier et al., 1998; Zhao et al., 1998). The involvement of the LD4 motif of paxillin in the signaling pathway(s) controlling actin-driven cytoskeletal dynamics during cell spreading and motility led us to investigate a possible role for the interaction between paxillin and PKL in these events. To evaluate the significance of the paxillin–PKL association, the distribution of endogenous PKL was first determined in paxillinΔLD4, paxillin WT, and parental CHO.K1 cells.

Although colocalization of paxillin and PKL in focal contacts was observed in paxillin WT and parental CHO.K1 cells (Fig. 6 A, b and e, c and f, respectively), the distribution of PKL in paxillinΔLD4 cells was almost exclusively cytoplasmic (Fig. 6 A, a). In contrast, the ectopic paxillin in paxillinΔLD4 cells was found in peripheral and central focal contacts, as well as the cytoplasm (Fig. 6 A, d).

The failure of paxillin and PKL to colocalize in focal contacts suggests a disruption of the interaction between these proteins. To demonstrate this in vivo, parental CHO.K1 cells were transiently transfected with GFP fused to either full-length wild-type paxillin (GFP–paxillin) or paxillinΔLD4 (GFP–paxillinΔLD4). Cell lysates from transfected cells spread on fibronectin for 60 min were immunoprecipitated with anti-GFP antibody. GFP–paxillin, but not GFP–paxillinΔLD4 was able to precipitate endogenous PKL (Fig. 6 B).

Although the localization of PKL to focal contacts was disrupted by the deletion of paxillin LD4, expression of this mutant had no affect on the distribution of either FAK or vinculin, two other focal contact proteins shown to associate with paxillin via multiple LD motif interactions (Fig. 6 C) (Turner and Miller, 1994; Brown et al., 1996; Turner et al., 1999).

To further demonstrate that the phenotypic changes observed in paxillinΔLD4 cells were not due to perturbation of normal FAK function through the disruption of a paxillin–LD4–FAK interaction, the change in FAK activity in these cells during spreading was assessed by measuring phosphorylation of the FAK autophosphorylation site Y397 (Schaller et al., 1994; Ruest et al., 2000). Parental CHO.K1, paxillin WT, or paxillinΔLD4 cells respread on fibronectin for the times indicated demonstrated similar general changes in phosphorytosine phosphorylation levels (Fig. 6 D, top). Moreover, immunoblotting of FAK immunoprecipitates from these same cell lysates with FAK Y397 phosphospecific antiseras indicated equivalent FAK activation in all three cell lines (Fig. 6 D, middle).

Introduction of PKL mutants defective in paxillin-binding, but not wild-type PKL, induce paxillin WT cells to exhibit a morphology characteristic of paxillinΔLD4 cells

We have previously localized the binding site of PIX to the NH2 terminus of PKL, and that of paxillin to the COOH terminus (Turner et al., 1999). However, PKL contains two putative paxillin-binding subdomains (PBSs), based on their homology with the paxillin-binding sites of vinculin and FAK (Wood et al., 1994; Tachibana et al., 1995; Brown et al., 1996). To identify the PKL PBS that mediates paxillin LD4 binding, radiolabeled full-length PKL proteins, as well as PKL proteins containing either a deletion of PBS1 or PBS2 (Fig. 7 A), were produced by transcription/translation-coupled reactions and used in in vitro binding assays with GST-coupled fusion proteins, GST–PIX and GST–LD4. Although GST–PIX was found to bind the wild-type and both mutant PKL proteins, GST–LD4 bound to both full-length PKL and the PBS1 deletion mutant, but no binding was observed to the PBS2 deletion mutant (Fig. 7 B). These results identify the PBS2 region of PKL as the paxillin–binding site.

To demonstrate the importance of the PBS2 domain of PKL in mediating the binding to paxillin in vivo, parental CHO.K1 cells were transiently transfected with either GFP–PKL or GFP–PKLΔPBS2. Cell lysates from cells spread on fibronectin for 60 min were immunoprecipitated with anti-GFP antibody, and the ability to coprecipitate endogenous paxillin was analyzed. Endogenous paxillin coimmunoprecipitated only with full-length GFP–PKL, whereas GFP–PKLΔPBS2 failed to precipitate paxillin (Fig. 7 C).

To test whether PKL binding to the paxillin LD4 motif via the PBS2 domain was necessary for normal cell spreading both paxillinΔLD4 and paxillin WT cells were transiently transfected with GFP–PKL or GFP–PKLΔPBS2 and evaluated for spreading on fibronectin. Although the introduction of GFP–PKLΔPBS2 into paxillinΔLD4 cells had no effect on cell morphology, expression of this construct in paxillin WT cells caused the generation of a paxillinΔLD4-
like phenotype in ~50% of cells (Fig. 8, B and C). Expression of GFP–PKL had no effect on the morphology of either paxillinΔLD4 or paxillin WT cells (Fig. 8, B and C). However, although it can be seen that paxillin and PKL colocalize in paxillin WT cells expressing GFP–PKL (Fig. 9 A, b and f), in paxillinΔLD4 cells expressing either GFP–PKL or GFP–PKLΔPBS2, PKL is cytoplasmic (Fig. 9 A, a and c), whereas paxillin is found in focal contacts (Fig. 9 A, e and g). In paxillin WT cells, GFP–PKLΔPBS2 remained cytoplasmic (Fig. 9 A, d), whereas paxillin was found in focal contacts (Fig. 9 A, h). Thus, the introduction of wild-type PKL or PKL lacking the paxillin-binding site does not affect paxillin localization. Similar effects on morphology and paxillin and PKL subcellular localization were also obtained by the introduction of either GFP–PKL or GFP–PKLΔPBS2 into parental CHO.K1 cells (data not shown).

To further address the role of the paxillin–PKL interaction in the phenotypic changes observed in paxillinΔLD4...
Discussion

Paxillin is a multidomain focal contact adapter protein involved in integrin and growth factor-mediated signaling (Turner, 2000). In this study, we demonstrate the importance of the interaction between paxillin and PKL in controlling morphologic and cytoskeletal changes associated with cell adhesion and motility.

Cell spreading on ECM involves coordinated regulation of the activity of various Rho family members. In particular, previous studies using NIH3T3 cells have shown that early spreading events are characterized by the extension of lamellipodia, which correlates with a transient increase in Cdc42/Rac-dependent PAK activity. Maximum activity is achieved between 10 and 20 min after plating, followed by a steady decline in kinase activity to baseline levels (Price et al., 1998). A transient reduction in Rho activity during the initial phase of cell spreading has also been reported (Ren et al., 1999). The decline in PAK, and presumably Cdc42/Rac activity, is generally accompanied by the development of a more elongated angular phenotype associated with the activation of Rho (Rottner et al., 1999; Sander et al., 1999; Arthur et al., 2000). Our results indicate that in parental CHO.K1 cells, or CHO.K1 cells overexpressing wild-type...
paxillin (paxillin WT), there is a similar sequence of morphologic changes and time course of Rac activation/inactivation, reaching a maximum at 15 min after plating. In striking contrast, overexpression in CHO.K1 cells of a paxillin mutant lacking the LD4 motif (paxillin/H9004LD4) resulted in a dramatic increase in membrane activity during cell spreading. This included the generation of multiple broad lamellipodia-like structures and enhanced membrane protrusive activity that persisted for several hours; i.e., the cells failed to transition to the more typical angular phenotype. Interestingly, the phenotypic changes induced by the paxillin/H9004LD4 were accompanied by a prolonged elevation in Rac activity, suggesting that paxillin, through its LD4 adapter function, is critical for the tight regulation of Rac activity during the spreading process.

We have previously demonstrated that the paxillin LD4 motif is capable of binding directly to several proteins including the focal adhesion tyrosine kinase FAK, the actin-binding protein vinculin, and more recently PKL, a putative ARF-GAP (Turner and Miller, 1994; Brown et al., 1996; Turner et al., 1999). The paxillin/LD4 mutant protein localized as efficiently as wild-type paxillin to focal complexes and focal contacts (Fig. 1 B), consistent with the use of the focal contact targeting sequence within the paxillin LIM domains (Brown et al., 1996). Importantly, overexpression of the paxillin/LD4 mutant failed to affect the localization to focal contacts of either FAK or vinculin (Fig. 6 C). In contrast, PKL localization to these structures was lost in the paxillin/LD4-expressing cells (Fig. 6 A). This difference is likely due to the fact that, although FAK and vinculin can bind to additional LD motifs within the NH2 terminus of paxillin, PKL binds exclusively to the LD4 motif (Turner and Miller, 1994; Brown et al., 1996; Turner et al., 1999).

In addition to increased cell spreading, enhanced protrusiveness, and multiple lamellipodia, paxillin/LD4 mutant cells exhibit long tail-like retraction fibers suggestive of highly motile cells and/or a defect in rear release (Figs. 1 B and 3 A). We found that these cells display an increased random motile capacity (Fig. 3, A–D). This stimulation of motility is consistent with data demonstrating that perturbation of the normal pathway of PAK function by expression of either kinase-dead or constitutively active PAK mutants increases basal cell motility (Sells et al., 1999). However, PAK kinase activity primarily functions to effect directional motility (Sells et al., 1999). Previously we demonstrated that microinjection of GST–LD4 into NIH 3T3 cells inhibited directed migration in a scrape wound assay (Turner et al., 1999). We confirm in this report that although deletion of paxillin LD4 increases random motility, directional motility as measured by scrape wound assay is profoundly attenuated (Fig. 3 E). The existence of long retraction fibers in paxillin/LD4 mutant cells is consistent with impaired tail release. Efficient release is necessary for cell migration (Small et al., 1996; Horwitz and Parsons, 1999) and has been proposed to require proper PAK activity (Kiosses et al., 1999).

However, in addition to mediating PKL binding, paxillin LD4, as well as LD2, supports FAK association (Turner and Miller, 1994; Brown et al., 1996; Turner et al., 1999). A role for FAK in cell motility is well characterized (Ilic et al., 1995; Cary et al., 1996; Hauck et al., 2000; Sieg et al.,}

**Figure 4.** Rac activity is prolonged in paxillinΔLD4 cells. (A) PaxillinΔLD4 and paxillin WT cells were detached and allowed to spread on fibronectin for 0, 5, 15, 60, 240, and 360 min. At each time point, cell lysates were prepared, and then activated Rac was precipitated using GST fusion proteins of the p21-binding domain of PAK (GST–PBD). Total and active Rac was assessed by immunoblot analysis with anti-Rac monoclonal antibodies and revealed a gradual prolonged Rac activation in paxillinΔLD4 cells compared with paxillin WT cells, which exhibit an initial rise in activity before returning to baseline activity. (B) The activation of Rac in paxillinΔLD4 and paxillin WT cells during spreading was quantified by measuring the ratio of GST–PBD-bound Rac to total Rac. The t0 value of paxillin WT cells was set to 1, and all other values were measured against it. Values are the average of experiments performed in triplicate. The inset depicts Rac activation in paxillinΔLD4 and paxillin WT cells between 0 and 70 min.
Introduction of dominant-negative forms of Rac and Cdc42 abrogate the morphological changes observed in paxillinLD4 cells. (A) Western blot analysis using monoclonal anti-Myc antibodies was used to confirm the presence of the ectopic Myc-tagged N17 Rac in paxillinLD4 and wild-type paxillin-transfected cells. (B) PaxillinΔLD4 and paxillin WT cells were transiently transfected with either Myc-tagged forms of dominant-negative Rac (N17 Rac) or dominant-negative Cdc42 (N17 Cdc42), detached and then allowed to spread on fibronectin for 60, 240, and 360 min. N17 Rac–transfected cells were then visualized by anti-Myc (a and b) and actin by RITC-phalloidin (c and d) and demonstrate that the introduction of N17 Rac is able to completely inhibit the formation of multiple broad lamellipodia in paxillinΔLD4 cells. Images of the cells were captured at the 240-min time point and are representative of the differences in cell morphology observed at all time points. (C and D) Quantification of the ability of N17 Rac and N17 Cdc42 to abolish the morphological changes observed in paxillinΔLD4 cells demonstrates that both Rho family small GT-Pases affect the generation of multiple broad lamellipodia in paxillinΔLD4 cells. >200 cells were counted per time point, and values are the average of experiments performed in duplicate.

Figure 5. Introduction of dominant-negative forms of Rac and Cdc42 abrogate the morphological changes observed in paxillinLD4 cells. (A) Western blot analysis using monoclonal anti-Myc antibodies was used to confirm the presence of the ectopic Myc-tagged N17 Rac in paxillinLD4 and wild-type paxillin-transfected cells. (B) PaxillinΔLD4 and paxillin WT cells were transiently transfected with either Myc-tagged forms of dominant-negative Rac (N17 Rac) or dominant-negative Cdc42 (N17 Cdc42), detached and then allowed to spread on fibronectin for 60, 240, and 360 min. N17 Rac–transfected cells were then visualized by anti-Myc (a and b) and actin by RITC-phalloidin (c and d) and demonstrate that the introduction of N17 Rac is able to completely inhibit the formation of multiple broad lamellipodia in paxillinΔLD4 cells. Images of the cells were captured at the 240-min time point and are representative of the differences in cell morphology observed at all time points. (C and D) Quantification of the ability of N17 Rac and N17 Cdc42 to abolish the morphological changes observed in paxillinΔLD4 cells demonstrates that both Rho family small GT-Pases affect the generation of multiple broad lamellipodia in paxillinΔLD4 cells. >200 cells were counted per time point, and values are the average of experiments performed in duplicate.

2000, 1999; Klingbeil et al., 2001). Further, a FAK–paxillin interaction has been implicated directly and indirectly (as measured by paxillin tyrosine phosphorylation) in cell spreading, neurite outgrowth, and cell migration (Ivankovic-Dikic et al., 2000). In addition, expression of the PKL-related protein GIT1 promotes cell motility in a FAK- and paxillin-dependent manner (Zhao et al., 2000). It has been proposed that GIT1 increases cell motility by paxillin-dependent recruitment to focal contacts where it causes loss of paxillin to increase focal complex dynamics, and through PIX and FAK binding, activates Rac and antagonizes Rho, respectively (Zhao et al., 2000). Importantly, PKL, unlike GIT1, does not contain the Spa2 homology domain that mediates FAK binding (Turner et al., 1999; Zhao et al., 2000). In addition, neither a change in FAK localization or activity was apparent in paxillinΔLD4 cells (Fig. 6, C and D, respectively), nor was any defect in motility observed with paxillinΔLD2 mutant cells (Fig. 3 D). These data combined with the demonstration that PKLΔPBS2 recapitulates the paxillinΔLD4 phenotype (Figs. 8 and 9) are consistent with a role for paxillin–PKL in regulating PAK function. Moreover, these data indicate a direct PAK–paxillin interaction (Hashimoto et al., 2001) cannot compensate for the perturbation of the PKL–paxillin connection. Thus, interference with the normal physiologic paxillin–PKL–PIX–PAK cascade causes the profound phenotypic alterations we report in this study.

Although the role for FAK in cell motility is well characterized, the precise role for paxillin is less clear. Paxillin, and its tyrosine phosphorylation, has been implicated in the regulation of cell migration (Liu et al., 1999; Riedy et al., 1999; Salgia et al., 1999; Turner et al., 1999; Ito et al., 2000; Nakamura et al., 2000; Petit et al., 2000; Yano et al., 2000). However, overexpression of wild-type paxillin α has been shown to stimulate (Nakamura et al., 2000; Yano et al., 2000), inhibit (Salgia et al., 1999), and also fail to affect cell motility (Petit et al., 2000). These apparent discrepancies may be due to differences in cell systems and assays, but also likely point to the complex interactions that occur between paxillin and its binding partners that serve to regulate cell motility. That notwithstanding, this study demonstrates that the interaction between paxillin and PKL is critical in regulating Rac-dependent cell shape change and motility. How might the interaction between paxillin and PKL regulate this cytoskeletal reorganization and Rac activity? Paxillin is linked, via PKL, to a PIX–PAK–Nck complex (Turner et al., 1999), each member of which is critically involved in the regulation of actin cytoskeleton dynamics. The precise temporal activation and spatial distribution of these proteins has been shown to be critical for proper Rac and Cdc42 signaling to the cytoskeleton (Bokoch et al., 1996; Galisteo et al., 1996; Lu et al., 1997; Manser et al., 1996; Manser et al., 1997, 1998; Bagrodia et al., 1998; Frost et al., 1998; Obermeier et al., 1998; Sells et al., 1999, 1997). A major means of regulating PAK function is through its restricted capacity to localize to focal complexes. Wild-type PAK normally maintains a diffuse cytoplasmic localization and transiently translocates to focal contacts stimulated by active Cdc42 or Rac, but not RhoA (Manser et al., 1997). The primary mechanism by which PAK localizes to focal contacts is not clear. However,
overexpression of either PAK mutants (G191 and A192) that cannot bind PIX or PIX SH3 mutants that can no longer bind PAK prevent the recruitment of PAK to G12V/Cdc42 focal contacts (Manser et al., 1998). Our previous results suggest that PAK may be targeted to these sites through an interaction with PKL (Turner et al., 1999). In fact, we have found that PAK localization to focal contacts is entirely dependent on the precise interaction between paxillin and PKL (unpublished data).

Paxillin–PKL-mediated localization of PIX and PAK to focal contacts may be essential for the proper function of PAK as an upstream regulator of Rac (Obermeier et al., 1998). It had been suggested that PAK and an as yet unidentified 90-kD protein (PKL?) may regulate PIX activity (Obermeier et al., 1998). Subsequent work has confirmed that both PAK membrane targeting and PAK binding to PIX increases PIX GEF activity towards Rac (Obermeier et al., 1998). Furthermore, overexpression of βPIX produced active Rac-like changes in cell morphology presumably through increased GEF activity (Manser et al., 1998). It is not yet known if PKL binding to PIX influences GEF activity directly, although it is plausible that improper localization and assembly of a PKL–PIX–PAK complex in the context of paxillinΔLD4 leads to aberrant Rac activity.

Finally, the ability of Rac to control actin cytoskeletal dynamics has been linked to its activation via translocation to the membrane (Bokoch and Knaus, 1994; Michaely et al., 1999; Kraynov et al., 2000). This process of Rac membrane localization is believed to be under the control of the ARF family of proteins, in particular ARF6 (Radhakrishna et al., 1997).
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brane and the sustained recruitment and activation of Rac, ter cell morphology, it is interesting to speculate that mislo-

gation of a PKL–PIX–PAK complex may indirectly result

2000). Several groups have recently demonstrated that overexpression of ARF-GAP mutants of the ASAP/PAPα/PKL families resulted in marked effects on cell morphology (Di Cesare et al., 2000; Kondo et al., 2000; Randazzo et al., 2000). In light of the ability of the PKLΔPBS2 mutant to alter cell morphology, it is interesting to speculate that mislocalization of a PKL–PIX–PAK complex may indirectly result in the maintenance of ARF6 activity at the plasma membrane and the sustained recruitment and activation of Rac, conceivably through an inability to control the nucleotide state of ARF6 via PKL–ARF–GAP activity.

In conclusion, our observations demonstrate that proper paxillin adapter function is critical to the regulation of the ac-

tin cytoskeletal changes that accompany integrin engagement with the ECM, and subsequent cell spreading and motility. This involvement is a result of paxillin’s ability to associate with the PKL–ARF–GAP via the LD4 motif, and entails the modulation of Rac activity. Thus, paxillin’s ability to influence Rac activation through PKL binding conceivably places paxillin at a junction between Rho- and ARF-family–mediated changes in the actin cytoskeleton. Future investigation will be aimed at elucidating how paxillin, PKL, and their associated proteins act in concert to regulate these processes.

Materials and methods

Cell culture and transfection

CHO.K1 cells were cultured in modified Ham’s F-12 (Mediatech, Inc.) supplemented with 10% (vol/vol) heat-inactivated, certified FBS (Summit Biotechnologies), 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma-Aldrich) at 37°C in a humidified chamber with 5% CO2.

CHO.K1 cells expressing full-length wild-type avian paxillin (paxillin WT), wild-type paxillin engineered with a deletion of LD4 (amino acids 263–282) (paxillinΔLD4), or wild-type paxillin engineered with a deletion of LD2 (amino acids 141–160) (paxillinΔLD2) was generated as previously described (Brown et al., 1996; Riedy et al., 1999b). Clonal paxillinΔLD4, paxillinΔLD2, and paxillin WT cell lines were maintained in the presence of 100 μg/ml G418. Transient transfections of paxillinΔLD4 and paxillin WT clonal cell lines, as well as parental CHO.K1 cells, were performed using lipofectamine (GIBCO BRL). Plasmids used in this study include GFP fused to full-length wild-type avian paxillin α, (GFP–paxillin), wild-type paxillin engineered with a deletion of LD4 (amino acids 263–282), GFP– paxillinΔLD4 full-length wild-type PKL (GFP–PKL), PKL containing a deletion of the second paxillin-binding subdomain (PBS2) (amino acids 643–679) (GFP–PKLΔPBS2), Myc-tagged N17 Rac, and N17 Cdc42 (gifts from Dr. Pam Silver, Dana Farber Institute, Boston, MA). Ectopic expression was confirmed by indirect immunofluorescent analysis as described elsewhere (Turner and Miller, 1994).

Immunofluorescence microscopy

For immunofluorescence of endogenous PKL, cells were plated on fibronectin-coated 13-mm glass coverslips in serum-free media for the times described in the spreading assays. Cells were then fixed for 8 min in PBS containing 3.7% formaldehyde, washed in 20 mM Tris-Pe-HCl pH 7.6, 150 mM NaCl, 0.01% NaN3 (TBS), permeabilized in TBS containing 0.2% Triton X-100 (Sigma-Aldrich), and processed as previously described (Nikolopoulou and Turner, 2000). Photographs were taken using a Zeiss Axios- phot photomicroscope equipped with epifluorescence illumination using either T-max 400 film (Eastman Kodak Co.), or a SPOT™ RT slider camera (Diagnostic Instruments). Images were scanned using Coolscan IIHQ (Eastman Kodak Co.) and processed using Adobe® Photoshop® v3.0.5 or SPOT® RT software v3.0 (Diagnostic Instruments).

Antibodies used in this study include avian-specific antipaxillin polyclonal antibody (Pax1), which has been described previously (Turner and Miller, 1994). Other antibodies used were antipaxillin (clone 349), anti-PKL (clone 13, generated in collaboration with Transduction Labs using a GST–PKL [amino acids 140–472] fusion protein as an antigen), anti-Rac (clone 102) anti-p130<sup>crk</sup> (clone 21), and anti-FAK (clone 77) monoclonal antibodies (Transduction Labs), antivinculin (Vin 11-5) monoclonal antibody (Sigma-Aldrich), anti-phosphoY118 paxillin and anti-phosphoY397 FAK polyclonal antibodies (Biosource International), and anti-GFP polyclonal antibody (a gift from Dr. Pam Silver, Dana Farber Institute, Boston, MA). The anti-Myc monoclonal antibody (9E10) developed by Dr. J. Michael Bishop (University of California at San Francisco, San Francisco, CA) was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA.

Spreading and motility assays

Cells were removed from tissue culture dishes by washing the cells once with PBS containing 1 mM EDTA, followed by incubation for 3–5 min at 37°C with 0.2 ml trypsin-EDTA (Sigma-Aldrich) in 1 ml PBS (1 mM EDTA). Cells were harvested and then washed twice with complete Ham’s F-12 media (see above) containing 10 μg/ml soybean trypsin inhibitor (Sigma-Aldrich), and once with serum-free Ham’s F-12 containing 10 μg/ml soybean trypsin inhibitor, and 1.1 × 10<sup>5</sup> cells were placed on a rocker in suspen-
sion for 1 h at 37°C in serum-free media containing 1% BSA. Cells were re-plated on fibronectin-coated (10 μg/mL Sigma-Aldrich) coverslips in serum-free media and then processed for indirect immunofluorescence. For transiently transfected cells, assays were performed 15–18 h after transfection.

Modified Boyden chamber migration assays were performed as previously described (Riedy et al., 1999a). Wound Assays were performed as follows. In brief, 2.5 × 10^5 cells were plated on 35-mm tissue culture dishes for 12 h at 37°C in complete media. Scrape wounds were generated in the confluent cell monolayers using a micropipet tip. Cells were then washed extensively and refed complete media. Phase–contrast images were acquired with a Nikon TE-200 inverted microscope fitted with a charge-coupled device camera (DAGE MT1). The images were taken using a 10× objective at 5-min intervals for 5–6 hours and organized into time-lapse movies using the NIH Image software. For immunofluorescence studies, a Nikon TE-300 inverted microscope with a Hamamatsu OrcaII cooled charge-coupled device camera (Hamamatsu-City, Japan) was used. The microscope was also equipped with a Ludl Electronic Products motorized XYZ stage and heating insert. Time-lapse images were acquired with 10× objective using the iSee software (Inovision). At the end of filming, fields were observed by immunofluorescence of GFP using a 40× objective to identify transfected cells whereupon images were analyzed using NIH Image software. For migration speed, the cell centroid was tracked and the average speed for the cell determined by computing the average net displacement of the cell centroid divided by the time interval at each time point. For protrusiveness

Figure 7. Characterization of the paxillin-binding site on PKL. (A) Schematic representations of full-length wild-type (PKL), deletion of PBS1 (PKLΔPBS1), and deletion of PBS2 (PKLΔPBS2) PKL proteins, respectively. (B) Radiolabeled wild-type PKL proteins and PKL protein containing either a deletion of PBS1 or PBS2 were produced by transcription/translation-coupled reactions and used in vitro binding assays with GST–PIX or GST–LD4 to identify PBS2 as the paxillin-binding site on PKL. PIX binding to PKL was unaffected by either deletion. (C) Parental CHO.K1 cells were transiently transfected with either GFP–PKL or GFP–PKLΔPBS2, detached from tissue culture dishes, and respread on fibronectin for 60 min. Coimmunoprecipitation assays were then performed followed by Western blot analysis and demonstrate that GFP–PKL is capable of associating with paxillin in vivo, whereas GFP–PKLΔPBS2 is not. Immunoblot analysis with an antibody to p130Cas was used to demonstrate the specificity of PKL–paxillin interaction.

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analysis, cells were outlined at two time points separated by 10 min; the two images were thresholded and then subtracted to estimate the new area. The area measurements were calibrated using a micrometer scale.

Cell lysate preparation, immunoprecipitation, and Western immunoblotting

Cell lysates were prepared using assay-specific lysis buffers: (a) standard lysis buffer (150 mM NaCl, 10 mM Tris-Cl, pH 7.6, 1 mM EDTA, 1% Triton X-100, and 0.1% sodium deoxycholic acid, 20 mM sodium pyrophosphate, 2 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 1 mM Na2VO3, 1 mM p-nitrophenylphosphate; Sigma-Aldrich); (b) coimmunoprecipitation lysis buffer (10 mM Tris-Cl, pH 7.6, 50 mM NaCl, 1% NP-40 [Sigma-Aldrich] and 10% glycerol [Sigma-Aldrich]); and (c) denaturing lysis buffer for FAK as- say (1% SDS, 1% Triton-X 100, 0.1% sodium deoxycholic acid, 10% glycerol, 1% sodium deoxycholic acid, 20 mM Hepes, pH 7.4, 150 mM NaCl, 2.5 mM EDTA, 10% glycerol, 1% sodium deoxycholic acid, 25 mM NaF, 25 mM p-nitrophenylphosphate; Sigma-Aldrich). Cell lysates were cleared of insoluble material by centrifugation at 14,000 rpm for 10 min. Protein concentrations were determined using the Dc™ protein assay (Bio-Rad Laboratories). Immunoprecipitation was performed by incubating the appropriate primary antibody and protein A/G PLUS-agarose (Santa Cruz Biotechnology) with cell lysate (for standard or denaturing immunoprecipitation, 200–300 μg of protein was used; for coimmunoprecipitation, 800–1,000 μg of protein was used) for 1–2 h at 4°C, rotating. Protein from detergent-soluble cell lysates (for cell lysates, 20–30 μg of protein was mixed 1:1 with 2× SDS-PAGE loading buffer) and immunoprecipitates were separated by SDS-PAGE using 10% polyacrylamide gels and transferred to Immobilon-NC (Millipore). Western immunoblotting analysis using the appropriate primary antibodies, followed by incubation with either antimouse or –rabbit secondary antibodies conjugated to horseradish peroxidase (Bio-Rad Laboratories) was performed to detect the proteins of interest. Chemiluminescence was used to visualize the specific proteins (Amersham Pharmacia Biotech) by exposure to blue-sensitive autoradiographic film (Marsh Bio Products, Inc.).

GST fusion protein binding assays

GST fusion proteins of BPIX (a gift from Dr. R. Cerione, Cornell University, Ithaca, NY) and paxillin LD4 motif were expressed in Escherichia coli (DH5α) and purified on glutathione–agarose beads as previously described (Turner and Miller, 1994; Riedy et al., 1999b; Turner et al., 1999). Radiolabeled full-length PKL and PKL deletion mutants of either PBS1 (amino acids 119–155) or PBS2 (amino acids 643–679) were generated by coupled transcription/translation reaction in a cell-free reticulocyte lysate system (TNT and Promega) as previously described (Nikolopoulos and Turner, 2000; Turner et al., 1999).

Rac activation assays were a modified version of that designed by Ren et al. (1999) using a GST fusion protein containing the p21-binding domain of PAK (GST–PB) (a gift from Dr. R. Cerione). Immunodetection of total and active Rac was performed using Rac-specific antisera (Transduction Labs). Densitometry analysis was performed on a Macintosh computer.
Figure 9. Introduction of PKL and PKLΔPBS2 mutants does not affect paxillin localization to focal contacts, but PKLΔPBS2 does affect cell protrusiveness. (A) PaxillinΔLD4 and paxillin WT cells were transiently transfected separately with GFP–PKL or GFP–PKLΔPBS2, detached, and subjected to spreading assays on fibronectin for 60, 240, and 360 min. Transfected cells were then visualized by GFP fluorescence (a–d) and ectopic paxillin with GFP–PKL or GFP–PKLΔPBS2 into paxillinΔLD4 and paxillin WT cells does not affect paxillin localization to focal contacts. Images of the cells were captured at the 240-min time point and are representative of the differences in morphology observed at all time points. (B) Quantification of protrusiveness demonstrates an increase in cell area (μm²) of CHO.K1 cells transfected with PKLΔPBS2 (left), as compared with parental nontransfected cells (Fig. 3 B, bottom). This observed increase in cell area is elevated by the cotransduction of paxillin and PKLΔPBS2 (right).

using the public domain NIH Image program (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image). The amount of PBD-bound Rac was normalized to the total amount of Rac in the lysates for comparison of Rac activity in different samples. Depending on the different cell types used (either paxillinΔLD4 or paxillin WT cells), the PBD-bound Rac accounts for ~0.4–3.2% of total Rac.

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