Paxillin is essential for PTP-PEST-dependent regulation of cell spreading and motility: a role for paxillin kinase linker

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Summary
The tyrosine phosphatase PTP-PEST has been implicated in the regulation of cell spreading and migration through dephosphorylation of focal adhesion proteins and inhibition of Rac GTPase activity. The focal adhesion adaptor protein paxillin is also necessary for normal cell migration and binds directly to PTP-PEST. In this study, we have utilized PTP-PEST−/− and paxillin−/− fibroblasts to demonstrate that paxillin is essential for PTP-PEST inhibition of cell spreading and membrane protrusion as well as inhibition of adhesion-induced Rac activation. Furthermore, we show that paxillin-binding is necessary for PTP-PEST stimulation of cell migration. Mutation analysis indicates that PTP-PEST function involves binding to the paxillin C-terminal LIM domains, and signaling through the tyrosine 31 and 118 phosphorylation sites, as well as the LD4 motif of the paxillin N-terminus. Using ‘substrate trapping’ approaches and immunoprecipitation, we show that the ARF GAP paxillin kinase linker PKL/GIT2, a paxillin LD4 binding partner, is a substrate for PTP-PEST. Additionally, the PKL-paxillin interaction was necessary for PTP-PEST inhibition of cell spreading. These data provide mechanistic insight into how the paxillin-PTP-PEST interaction contributes to integrin signaling events associated with the spatiotemporal regulation of key modulators of the cytoskeleton and cell motility machinery.

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Key words: Rac, Cytoskeleton, Phosphatase, Focal adhesions, Tyrosine phosphorylation, Cell migration

Introduction
Regulated cell attachment, spreading and motility are essential for the normal processes of tissue remodeling during embryogenesis, immune surveillance and wound healing (Juliano, 2002). Perturbation in cell adhesion signaling contributes to tissue scarring, restenosis, tumorigenesis and metastasis (Juliano, 2002). Transmembrane integrin receptors provide a structural link between the extracellular matrix (ECM) and the actin cytoskeleton (DeMali et al., 2003). Sites of cell-ECM contact, termed focal complexes or focal adhesions, also mediate extracellular-intracellular communication and cell responsiveness through the dynamic recruitment of an array of structural and signaling proteins (Brown and Turner, 2004; Wozniak et al., 2004). These proteins serve to coordinate the changes in cell shape and cytoskeletal organization necessary for productive cell locomotion and also provide a platform for efficient signaling to the nucleus to effect changes in gene expression (Brown and Turner, 2004; Wozniak et al., 2004).

Paxillin is a 68 kDa multi-domain focal adhesion protein that functions as a molecular adaptor or scaffold to facilitate integrin signaling (Brown and Turner, 2004; Playford and Schaller, 2004). It is essential for embryonic development and plays an important role in the regulation of cell attachment, spreading and motility (Brown and Turner, 2004; Hagel et al., 2002). Paxillin contains five leucine-rich LD motifs in its N-terminus that function as interaction sites for actin-binding proteins – such as vinculin and actopaxin – and for signaling proteins – such as integrin-linked kinase (ILK), focal adhesion kinase (FAK), and ADP-ribosylation-factor–GTPase-activating proteins (ARF-GAPs). The latter include the G protein coupled receptor kinase (GRK) interacting ARF GAP (GIT1) and the paxillin kinase linker (PKL)/GIT2 (Brown and Turner, 2004). The N-terminus also contains several tyrosine residues that, when phosphorylated in response to adhesion or growth factor stimulation, provide docking sites for the Src homology 2 (SH2) domains of Crk and p120RasGAP (Petit et al., 2000; Tsubouchi et al., 2002). The C-terminus of paxillin comprises four LIM domains that are required for paxillin localization to focal adhesions and also serve as binding sites for the protein tyrosine phosphatase (PTP)-PEST and tubulin (Brown and Turner, 2002; Brown and Turner, 2004; Côté et al., 1999; Shen et al., 1998).

Tyrosine phosphatases such as PTP-PEST, along with the tyrosine kinases FAK and Src, are necessary for coordinating the dephosphorylation/phosphorylation of focal adhesion proteins to promote focal adhesion turnover and thereby
stimulate cell migration (Mitra et al., 2005; von Wichert et al., 2003; Webb et al., 2004). Both the over-expression and the genetic ablation of PTP-PEST causes a profound inhibition of cell motility (Angers-Loustau et al., 1999; Garton and Tonks, 1999) consistent with a requirement for a precise balance of PTP-PEST function in regulating cell adhesion. PTP-PEST interacts with several focal adhesion proteins including p130Cas, FAK/Pyk2, and paxillin (Brown and Turner, 2002; Côté et al., 1999; Garton et al., 1996; Shen et al., 2000; Shen et al., 1998; Spencer et al., 1997) and contributes to the dephosphorylation of these proteins, although only p130 Cas and Pyk2 appear to be direct substrates (Angers-Loustau et al., 1999; Lyons et al., 2001; Shen et al., 2000).

The Rho family GTPases Cdc42, Rac and RhoA are activated by integrin engagement with the ECM, and control the formation of filopodia, lamellipodia and stress fibers, respectively (Burridge and Wennerberg, 2004). Coordination of Rho GTPase activity is required for efficient migration; regulating membrane protrusion at the leading edge, focal adhesion turnover, cytoskeletal contractility and cell retraction (Burridge and Wennerberg, 2004; Schmitz et al., 2000; Wittchen et al., 2005). Both paxillin and PTP-PEST have been linked to the regulation of Rho family signaling. Paxillin contributes to the regulation of Rho GTPase function, in part via the protein networks that signal through the LD4 motif and tyrosine residues 31 and 118 (Petit et al., 2000; West et al., 2001). Assembly of a paxillin LD4-PKL-PIX-Pak-Nck complex at focal adhesions is crucial for normal Rac activation, cell spreading, polarization and directed migration (Brown et al., 2002; Turner et al., 1999; West et al., 2001; Zhao et al., 2000). Interestingly, PKL was shown to be tyrosine phosphorylated in response to cell adhesion (Bagrodia et al., 1999) and recent studies in our lab have shown that PKL tyrosine phosphorylation facilitates its focal adhesion localization in response to Rac activation (Brown et al., 2005). Tyrosine phosphorylation of paxillin residues 31 and 118 and subsequent interaction with the Crk/Cas/DOCK180 complex is also implicated in Rac activation and cell motility in certain cell types (Lamorte et al., 2003; Petit et al., 2000; Valles et al., 2004). Finally, a role for PTP-PEST in the suppression of cell spreading and control of cell motility through inhibition of Rac was identified (Sastry et al., 2002), although the pathway through which this regulation occurred has not been determined.

In view of the direct interaction between paxillin and PTP-PEST, we have examined the importance of this association in the regulation of integrin-mediated signaling events. Herein we show, through the reconstitution of paxillin+/– mouse embryo fibroblast (MEF) cells and PTP-PEST+/– MEF cells, that PTP-PEST-dependent regulation of cell spreading and migration depends on a direct interaction with paxillin and involves a complex relationship that requires binding of PTP-PEST to the paxillin C-terminal LIM domains and signaling through the tyrosine 31 and 118 phosphorylation sites and the LD4 motif of the paxillin N-terminus. Furthermore, we show that paxillin is necessary for PTP-PEST suppression of Rac activity during cell spreading. Finally, we identified the paxillin LD4 motif binding partner PKL as a new PTP-PEST substrate and show that a functional PKL-paxillin interaction is required for PTP-PEST to regulate cell spreading. These data provide mechanistic insight into how the paxillin-PTP-PEST interaction contributes to the regulation of cell migration.

Materials and Methods

Plasmids and antibodies

PTP-PEST wild-type and the PTP-PEST Pro II deletion mutant (355-374), pEGFPc1-paxillin wild-type and pCDNA31 paxillin wild-type (1-559), ΔLD4, C523S and Y31/118F have been described previously (Côté et al., 1999; Petit et al., 2000; West et al., 2001). Plasmids encoding paxillin ΔLD1 (deletion 2-20), ΔLD2 (deletion 134-158), LIM1-4 (326-553) and Y31/118FΔLD4 (del 263-282), which all had their internal Kozak sequence mutated at position 132 to prevent the generation of paxillin y at the alternate start site (Brown and Turner, 2004; Tumbarello et al., 2005), were generated using the QuikChange site-directed mutagenesis kit (Stratagene). Sequences were verified by the Upstate Medical University DNA sequencing core facility. Plasmids encoding avian wild-type PKL (1-757 amino acids), ΔPBS2 (deletion 643-679), and Triple YF (Y286/392/592F) were cloned into pEgFPCl (Clontech). Anti-paxillin (clones 165), anti-PKL and Hic-5 monoclonal antibodies were from BD Biosciences. Paxillin PXClO, anti-α-actinin and anti-vinculin were from Sigma. Anti-paxillin (clone H114) was from Santa Cruz. Primary antibody to the HA-tag of PTP-PEST (12CA5) was from Roche. Anti-green fluorescent protein (GFP)-purified rabbit antibody was from Molecular Probes. Anti-phosphotyrosine 4G10 was from Upstate Biotechnology. The anti-Myc monoclonal antibody (9E10) was from the Developmental Studies Hybridoma Bank, University of Iowa, IA. Human plasma fibroeclin was from Sigma-Aldrich.

Cell culture and transfection

Normal mouse embryo fibroblasts (MEF) and paxillin+/– MEF cells (Hagel et al., 2002) (a gift from Sheila Thomas, Harvard University, Boston, MA), and PTP-PEST−/− cells (Angers-Loustau et al., 1999; Côté et al., 1998) have been previously described. MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Atlas Biologicals), 50 U/ml penicillin, 50 μg/ml streptomycin and kanamycin (complete medium) at 37°C in a humidified chamber with 5% CO2. Chinese hamster ovary (CHO) K1 cells were cultured in modified Ham's F-12 medium supplemented with 10% (v/v) heat-inactivated FBS (Atlas Biologicals), 50 μM penicillin, 50 μg/ml streptomycin and kanamycin. Cells were transfected using Fugene6 (Roche) or Metafectene (Biontex) at a 3:1 ratio with 1 μg of total DNA according to the manufacturer's instructions. Transfection efficiencies were monitored by calculating the percentage of GFP-positive cells 24 hours post transfection and was approximately 60% for paxillin+/– cells and greater than 80% for PTP-PEST−/− cells. PTP-PEST re-expression was approximately fourfold endogenous levels. The expression level with the median impact on cell spreading area was quantified for 50 cells in three independent experiments.

Cell spreading and area analysis

Cells were resuspended in 1 ml PBS-EDTA solution washed twice in serum-free DMEM containing 0.025% trypsin inhibitor and 0.1% BSA. Cells were maintained in suspension in the same media at 150,000 cells/ml for 1 hour at 37°C. Cells (approximately 75,000) were plated for the times indicated on 10 μg/ml fibronectin-coated coverslips. Serum was reintroduced for cells maintained overnight. Cells were fixed at indicated time points and processed for indirect immunofluorescence microscopy as described previously (West et al., 2001). For quantification of cell spreading, cells were scored as round (unspread) when they were attached to the substrate but had not extended stable lamellipodia. At least 100 GFP-positive cells from three independent experiments were counted for each condition. Spreading area was quantified for 50 cells in three independent experiments.
Protrusive analysis

Transfected cells were spread on fibronectin-coated 35-mm dishes as described above. 3 hours post replating, cells were washed and transferred to prewarmed serum-, sodium bicarbonate-, phenol-red-free DMEM supplemented with 25 mM Heps pH 7.5 and 0.1% BSA. Plates, covered with mineral oil, were maintained at 37°C in a Harvard Apparatus PDMI 35-mm dish microincubation chamber mounted on a Nikon E600 microscope. GFP-expressing cells were identified and images were captured every 5 minutes for up to 2 hours using Compix Simple PCI software and a Spot RT CCD camera. Cell protrusion was quantified for 1 hour at 10-minute intervals as described previously (Kinley et al., 2003; West et al., 2001) by comparing two images (10 minutes apart) thresholding images and subtracting overlapping regions. Protrusions were quantified as percentage of cell area and averaged over ten cells.

Modified Boyden chamber migration assays

PTP-PEST−/− cells were transfected with GFP, PTP-PEST and paxillin constructs as indicated. Transfection efficiency, as measured by the percentage of GFP-positive cells 24 hours post transfection, was approximately equivalent for all cell populations and was routinely greater than 80%. Cells were harvested and modified Boyden chamber migration assays were performed as previously described (Riedy et al., 1999) using 8-μm pore membranes pre-coated with 100 μg/ml gelatin (Sigma-Aldrich). Twenty-thousand cells were added to the top well and the chamber was incubated for 12 hours at 37°C in 5% CO2 before the filter was fixed and evaluated (Riedy et al., 1999). Assays were performed in triplicate. The motility of PTP-PEST+/− cells was evaluated in parallel.

Rac-activity assay

Transfected cells were either held in suspension or plated onto fibronectin-coated (10 μg/ml) 100-mm plates. Cells (1×10⁶ paxillin−/− or 1.5×10⁶ PTP-PEST−/− cells) were lysed [50 mM Tris pH 7.6, 1% Triton X-100, 150 mM NaCl, 5 mM MgCl₂, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and cellular debris was removed. Equivalent amounts (500 μg protein) of lysates were incubated with 10 μg of glutathione-S-transferase–PAK-binding domain of PTP-PEST (wild type or C231S mutant) for 2 hours at 4°C. Lysates were precleared at 21,000 g for 15 minutes and immunoprecipitated by incubating 250 μg of cell lysate with the anti-GFP (purified IgG, Molecular Probes) antibody for 3 hours at 4°C following by protein A/G agarose beads (Santa Cruz) for 1 hour at 4°C. Immunoprecipitates were washed extensively with lysis buffer then processed for SDS-PAGE analysis and western blotting.

Substrate trapping

PTP-PEST substrate trapping experiments were performed as described previously (Blanchetot et al., 2005; Côté et al., 1998).

Briefly, PTP-PEST−/− cells were lysed in 20 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 5 mM iodoacetamide and proteases inhibitors. Dithiothreitol (DTT, 10 mM final) was added for 15 minutes at 4°C and lysates were precleared by centrifugation at 21,000 g for 15 minutes. One mg of cell lysate was incubated with 5 μg of GST protein or GST fused to the catalytic domain of PTP-PEST (wild type or C231S mutant) for 2 hours at 4°C. Beads were washed several times in lysis buffer and processed for SDS-PAGE and western blotting.

Results

Paxillin is required for PTP-PEST-dependent regulation of cell spreading

PTP-PEST has been identified as a negative regulator of Rac and to inhibit cell spreading and motility (Sastry et al., 2002). PTP-PEST has also been shown to bind to the LIM domains of the adaptor protein paxillin; an interaction proposed to be important in regulating focal adhesion dynamics (Côté et al., 1999). We were interested in evaluating a requirement for paxillin in mediating PTP-PEST function. Using paxillin−/− MEF cells (paxillin−/− cells) in conjunction with control paxillin+/+ MEF cells (paxillin+/+ cells), we examined the effect of PTP-PEST overexpression on cell spreading (Fig. 1A,B). Consistent with previous studies (Sastry et al., 2002), PTP-PEST caused a significant inhibition of cell spreading in paxillin+/+ cells as indicated by the large number of round cells (87%) at 45 minutes post plating on fibronectin (Fig. 1B). By contrast, overexpression of PTP-PEST in paxillin−/− cells had no inhibitory effect on cell spreading. Spreading was fully rescued in both cell lines after overnight incubation in the presence of serum (Fig. 1B). Interestingly, although the paxillin−/− cells exhibit elevated expression of Hic-5, a paxillin family member (Fig. 1C), this protein failed to compensate for the absence of paxillin despite its ability to also interact directly with PTP-PEST (Nishiya et al., 1999). PTP-PEST inhibition of spreading was abrogated when the catalytically inactive PTP-PEST mutant was introduced into PTP-PEST+/− cells (data not shown) consistent with the requirement for PTP-PEST phosphatase activity (Sastry et al., 2002).

To determine whether the absence of paxillin was responsible for the failure of PTP-PEST to inhibit cell spreading in the paxillin−/− cells, full length paxillin was reintroduced into these cells and the effect of PTP-PEST overexpression on spreading was evaluated (Fig. 2). At 45 minutes posts replating 70% of cells remained round, compared to 25% of paxillin−/− cells expressing only PTP-PEST (Fig. 1B, Fig. 2B), confirming a requirement for paxillin in PTP-PEST function. The interaction between paxillin and PTP-PEST requires the LIM-3-4 domains of paxillin and the Pro II domain of PTP-PEST (Côté et al., 1999). To test whether a physical interaction between paxillin and PTP-PEST was required for PTP-PEST inhibition of cell spreading, we co-transfected paxillin−/− cells with either PTP-PEST and a paxillin C523S mutant, or wild-type paxillin with the PTP-PEST ΔPro II mutant. Both the paxillin C523S and PTP-PEST ΔPro II mutants have previously been shown to abolish paxillin-PTP-PEST binding (Côté et al., 1999). Neither of these combinations resulted in reduced cell spreading when compared to GFP-transfected control cells (Fig. 2A,B), indicating a functional paxillin-PTP-PEST interaction must be maintained for PTP-PEST to inhibit cell spreading. All cell
populations exhibited similar levels of spreading after two hours (Fig. 2D). Western blot analysis of these cell populations confirmed approximately equivalent expression of PTP-PEST and paxillin constructs (Fig. 2C). Additionally, indirect immunofluorescence microscopy demonstrated that the paxillin C523S mutant localized efficiently to focal adhesions (Fig. 2D).

To further examine the requirement for paxillin binding to PTP-PEST for its effects on cell spreading, these experiments were repeated in PTP-PEST–/– cells. Re-expression of PTP-PEST, with or without over-expression of paxillin, inhibited spreading up to three hours (Fig. 3A,B). These results suggest that endogenous levels of paxillin are sufficient for PTP-PEST inhibition of spreading in these cells. However, to determine whether paxillin-binding to PTP-PEST was necessary for PTP-PEST to inhibit spreading in this cell type, the paxillin C523S mutant was introduced along with PTP-PEST. Cells co-expressing PTP-PEST and paxillin C523S spread at a rate similar to the GFP-transfected population (Fig. 3A,B). Western blot analysis confirmed similar expression of all constructs (Fig. 3C). The original analysis of PTP-PEST effects on cell spreading was performed in CHO.K1 cells (Sastry et al., 2002). To evaluate a requirement for paxillin, spreading experiments were repeated in this cell type. As in the paxillin–/– and PTP-PEST–/– cells, CHO.K1 cells expressing PTP-PEST alone or PTP-PEST together with wild-type paxillin exhibited a significant delay in spreading. However, spreading was not inhibited when PTP-PEST was co-expressed with paxillin C523S (data not shown).

Paxillin facilitates PTP-PEST inhibition of spreading through its LD4 motif and tyrosine phosphorylation sites Y31 and Y118

PTP-PEST interacts with the C-terminus LIM domains of paxillin, a region of the protein that is also required for targeting paxillin to focal adhesions (Brown and Turner, 2004; Côté et al., 1999; Shen et al., 1998). To determine whether the C-terminus of paxillin is sufficient to restore PTP-PEST inhibition of cell spreading in paxillin–/– cells, the paxillin LIM1-4 domains were overexpressed with PTP-PEST. Interestingly, this resulted in only a modest (10%) restoration in PTP-PEST inhibition of spreading as compared to the introduction of paxillin plus PTP-PEST (Fig. 4A). These data suggest that the paxillin N-terminus is essential for PTP-PEST to effectively inhibit cell spreading.

Several regions within the N-terminus of paxillin have been implicated in the regulation of cell spreading, including the LD1, LD2 and LD4 motifs, through the binding of actopaxin, FAK and PKL (Nikolopoulos and Turner, 2000; Turner et al., 1999; West et al., 2001). To determine the involvement of these domains in PTP-PEST function, paxillin LD deletion mutants (ΔLD1, ΔLD2 and ΔLD4) were expressed in paxillin–/– cells in the presence or absence of PTP-PEST. Expression of the paxillin ΔLD4 mutant alone had no effect on the number of cells spread at 45 minutes post plating on fibronectin, whereas the ΔLD1 or ΔLD2 mutants exerted a modest inhibition of spreading (Fig. 4B,C). Co-expression of paxillin ΔLD1 or ΔLD2 mutants with PTP-PEST showed significant spreading inhibition, similar to wild-type paxillin.
PTP-PEST-dependent regulation of cell migration requires paxillin

(Fig. 4C), indicating that these domains were not crucial to PTP-PEST function. By contrast, expression of the paxillin ΔLD4 mutant blocked PTP-PEST inhibition of spreading (Fig. 4B). Phosphorylation of the paxillin N-terminal tyrosine residues Y31 and Y118 has been shown to influence cell spreading and migration in certain cell types through binding of Crk and p120RasGAP and modulation of Rac and RhoA activity (Petit et al., 2000; Tsubouchi et al., 2002; Valles et al., 2004). To determine whether the phosphorylation of these sites is required for PTP-PEST to negatively regulate spreading, we examined the effect of introducing a paxillin double tyrosine mutant, Y31/118F into the paxillin−/− cells. In cells expressing paxillin Y31/118F, PTP-PEST over-expression was unable to inhibit cell spreading (Fig. 4D). Similarly, expression of paxillin Y31/118F or paxillin ΔLD4 with PTP-PEST in paxillin−/− cells blocked inhibition of cell spreading (data not shown).

To further examine the effects of the paxillin-PTP-PEST interaction on cell spreading, we calculated the spreading area of transfected populations of paxillin−/− cells at 45 minutes and 120 minutes post replating on a fibronectin matrix. Spreading areas of all populations were compared with the area of GFP-transfected cells. At 45 minutes, the area of paxillin−/− cells co-expressing PTP-PEST and paxillin was 43% of the GFP-expressing population, whereas cells transfected with PTP-PEST alone or with PTP-PEST and paxillin C523S spread to 92% and 93% of GFP-transfected cells, respectively (Fig. 4E). Notably, cells expressing PTP-PEST in combination with either the paxillin LD4 deletion mutant or the paxillin Y31/118F mutant spread to 68% and 69% of GFP-transfected cells, respectively (Fig. 4E). Furthermore, when PTP-PEST was expressed with a paxillin Y31/118FΔLD4 mutant, cells spread to 100% of GFP-transfected cells. Taken together, these results support a mechanism in which both the LD4 motif, and...
phosphorylation of the paxillin residues Y31 and Y118 are involved in PTP-PEST-dependent regulation of spreading.

PTP-PEST inhibits membrane protrusion through a paxillin interaction

Both paxillin and PTP-PEST are involved in the regulation of membrane dynamics. PTP-PEST–/– cells spread at an increased rate (Angers-Loustau et al., 1999) and the expression of the catalytically inactive PTP-PEST mutant C231S has been shown to cause prolonged protrusions in CHO.K1 cells (Sastry et al., 2002). Paxillin–/– cells also have irregular membrane dynamics (Hagel et al., 2002) and introduction of paxillin small interference RNA (siRNA) into HeLa cells results in abnormal membrane activity with an increase in protrusion formation (Yano et al., 2004). To determine whether the paxillin-PTP-PEST interaction is involved in the regulation of membrane protrusion after cells have spread, we quantified protrusive activity in paxillin–/– cells at 180 minutes post replating on fibronectin using time-lapse video microscopy. Cell images were taken every 10 minutes for 60 minutes, consecutive images were overlaid and protrusions were quantified by excluding overlapping regions (see Materials and Methods for details). Protrusive activity of the cell was averaged over the 60-minute period and results are displayed in a box-and-whisker plot format (Fig. 5). The expression of PTP-PEST had very little effect on protrusions, whereas the expression of paxillin alone resulted in a significant reduction in protrusions. Interestingly, co-expression of paxillin and PTP-PEST caused a significant additional reduction in protrusive activity when compared to paxillin alone. To determine whether the direct interaction of paxillin and PTP-PEST was necessary for the regulation of protrusion, we expressed the paxillin C523S mutant with PTP-PEST. In this case, protrusive activity returned to levels observed in GFP-transfected paxillin–/– cells (Fig. 5). (For representative movies of GFP-transfected, PTP-PEST plus wild-type paxillin transfected, and PTP-PEST plus C523S paxillin transfected paxillin–/– cells see supplementary material Fig. S2.)

To extend our examination of the role of the paxillin N-terminus in PTP-PEST function, we expressed the paxillin ΔLD4, Y31/118F and Y31/118FΔLD4 mutants in paxillin–/– cells in the presence or absence of PTP-PEST, and examined their effect on membrane dynamics (Fig. 5 and supplementary material Fig. S3). Expression of PTP-PEST together with the paxillin Y31/118FΔLD4 mutant completely ablated the decrease in protrusive activity observed in cells expressing PTP-PEST and wild-type paxillin, whereas expression of PTP-PEST together with paxillin Y31/118F or ΔLD4 mutants resulted in only partial reduction in protrusive activity (Fig. 5). Together, these results indicate that, after cell spreading, PTP-PEST requires both the direct interaction with the paxillin C-terminus as well as functional domains in the N terminus to regulate membrane protrusion.
A paxillin-PTP-PEST interaction is required for PTP-PEST-regulated cell migration

Paxillin−/− and PTP-PEST−/− cells both exhibit defects in cell migration (Angers-Loustau et al., 1999; Hagel et al., 2002). Since the phenotypic changes and signaling events accompanying cell spreading are considered a relevant model for the events occurring during extension of lamellipodia at the leading edge of migrating cells, we employed modified Boyden chamber assays to determine whether the paxillin-PTP-PEST interaction is important for the regulation of cell migration. Prior to these experiments, we performed a migration assay of PTP-PEST−/− cells transfected with increasing amounts of PTP-PEST to determine the optimal rescue concentration (supplementary material Fig. S4). PTP-PEST−/− cells exhibit reduced migration rates compared with PTP-PEST+/− cells (Fig. 6) (Angers-Loustau et al., 1999). This defect was partially rescued by the overexpression of PTP-PEST (Fig. 6) to a level consistent with published reports (Angers-Loustau et al., 1999). Results show the mean±s.d. A minimum of 100 cells were counted in three independent experiments. **P<0.001, *P<0.05 (E) GFP-transfected paxillin−/− cells that had also been transfected with PTP-PEST, paxillin and PTP-PEST, PTP-PEST and Pax C523S, PTP-PEST and Pax ΔLD4, PTP-PEST and Pax Y31/118F, or PTP-PEST and Pax Y31/118F ΔLD4 were plated on fibronectin-coated coverslips for 45 and 120 minutes. Cells were fixed and processed for indirect immunofluorescence as described above. Total cell area was obtained from these images using Spot™ RT software. Results represent the mean±s.d. of a minimum of 50 cells for each time point and three independent experiments. **P<0.001.

Fig. 4. Paxillin N-terminus, LD4 motif and tyrosine phosphorylation sites Y31 and Y118 are required for PTP-PEST-dependent inhibition of spreading. Paxillin−/− cells were transiently transfected with GFP, paxillin alone or together with PTP-PEST, or the following paxillin constructs with and without PTP-PEST: (A) LIM1-4, (B) ΔLD4, (C) ΔLD1 or ΔLD2 and (D) Y31/118F, and plated on fibronectin-coated coverslips. Cells were fixed after 45 minutes and processed for indirect immunofluorescence. Results represent the percentage of round GFP-positive cells.

Fig. 5. Paxillin is required for PTP-PEST to inhibit cell protrusiveness. Paxillin−/− cells transfected with GFP and the PTP-PEST and paxillin constructs as indicated were plated on 10 µg/ml fibronectin-coated coverslips for 180 minutes; thereafter, time-lapse images were taken every 10 minutes. Changes in cell protrusion area of a minimum of 10 transfected cells were quantified at 10-minute intervals for 1 hour as described in Materials and Methods. **P<0.001, *P<0.05.
al., 1999) and was unaffected by the co-expression of paxillin with PTP-PEST. However, this rescue was completely abolished when PTP-PEST was co-expressed with the paxillin C523S mutant, which is defective for PTP-PEST binding (Fig. 6). Thus PTP-PEST-dependent cell migration requires a functional interaction with paxillin.

PTP-PEST influences Rac activity through paxillin

PTP-PEST and paxillin have both been coupled to the regulation of the Rho GTPase Rac (Brown and Turner, 2004; Sastry et al., 2002; West et al., 2001). Rac is activated during cell spreading and is required for lamellipodia extension (Price et al., 1998). The role of the paxillin-PTP-PEST interaction in the regulation of Rac activity was tested using Pak-binding-domain (PBD) pull-down assays. PTP-PEST−/− cells were transfected with PTP-PEST or with PTP-PEST and the paxillin C523S mutant defective for PTP-PEST binding, and compared with a GFP-transfected control population. Rac activity was measured in cells that had either been held in suspension or

Fig. 6. A productive paxillin–PTP-PEST interaction is required for PTP-PEST-regulated cell migration. Modified Boyden chamber assays were performed as described in Materials and Methods to determine whether the paxillin–PTP-PEST interaction was important for the regulation of cell migration. PTP-PEST−/− cells were transiently transfected with GFP alone or with PTP-PEST, PTP-PEST and paxillin, or PTP-PEST and paxillin C523S. PTP-PEST−/− cells exhibit reduced migration rates compared with PTP-PEST+/− cells. This defect was partially rescued by overexpression of PTP-PEST. However, this rescue was completely abolished when PTP-PEST was co-expressed with the paxillin C523S mutant. Results show the mean ± s.d. and represent three independent experiments. **P<0.001.

Fig. 7. PTP-PEST regulates Rac1 activation through an interaction with paxillin. (A) PTP-PEST−/− cells or (B) paxillin−/− cells were co-transfected with Myc-tagged wild-type Rac1 together with GFP, PTP-PEST, PTP-PEST and paxillin, or PTP-PEST and paxillin C523S, and either held in suspension or plated on fibronectin-coated culture dishes for 60 minutes. The level of activated Rac was determined using a GST-PAK-PBD pull-down assay as described in Materials and Methods. Total and active Rac levels were visualized by western blotting and quantitative densitometry was performed. (C,D) Results show the mean ± s.d. of three independent experiments. PTP-PEST inhibited adhesion-induced Rac activation only in the presence of paxillin. This effect was reversed by introduction of the paxillin C523S mutant defective in PTP-PEST binding. **P<0.001.
had been replated for 60 minutes on fibronectin. Consistent with previous studies (Sastry et al., 2002), Rac activity was elevated when PTP-PEST−/− cells were plated on fibronectin, and this increase in Rac activity was suppressed when PTP-PEST was reintroduced (Fig. 7A,C). Interestingly, disruption of the paxillin-PTP-PEST interaction by co-transfecting the paxillin C523S mutant with PTP-PEST resulted in elevated Rac activity in response to cell spreading, similar to GFP-transfected control cells (Fig. 7A,C). Parallel experiments were performed in paxillin−/− cells to examine the role of paxillin in the PTP-PEST-dependent regulation of Rac in a paxillin−/− background. Cells transfected with GFP exhibited increased Rac activity during cell spreading on fibronectin (Fig. 7B,D). There was still an induction of Rac activity when PTP-PEST was expressed alone or with the paxillin C523S mutant. However, the adhesion-induced increase in Rac activity was effectively blocked following the introduction of wild-type paxillin and PTP-PEST (Fig. 7B,D), consistent with the ability of this combination to inhibit cell spreading (Fig. 2). PTP-PEST also failed to inhibit Rac activity when expressed with the paxillin Y31/118F/LD4 mutant (data not shown).

Rac has previously been shown to function downstream of paxillin and PTP-PEST (Brown and Turner, 2004; Sastry et al., 2002). To determine whether Rac is a downstream effector of the paxillin-PTP-PEST interaction, we co-transfected constitutively-active Rac (G12V Rac) or dominant-negative Rac (T17N Rac) with PTP-PEST, and also in combination with wild-type or mutant paxillin, and analysed cell spreading. In paxillin−/− cells that re-express wild-type paxillin and PTP-PEST, the introduction of G12V Rac promoted spreading despite a functional paxillin-PTP-PEST interaction (Fig. 8). By contrast, T17N Rac suppressed spreading in all cell populations not inhibited by PTP-PEST alone (Fig. 8). Experiments performed in PTP-PEST−/− cells showed similar results (data not shown). Together, these data suggest that Rac is a downstream effector of the paxillin-PTP-PEST interaction and that this association must be maintained for PTP-PEST to regulate Rac activity during cell spreading.

PTP-PEST mediates PKL dephosphorylation

Previous reports have indicated that tyrosine phosphorylation of an unidentified, 95-kDa protein is regulated by PTP-PEST (Côté et al., 1998). Interestingly, the LD4 motif of paxillin, which we have shown here is involved in PTP-PEST signaling, binds PKL/GIT2, a 95-kDa protein that is tyrosine phosphorylated during adhesion and also in response to Rac activation (Bagrodia et al., 1999; Brown et al., 2005). We therefore used a substrate-trapping mutant of PTP-PEST (C231S), to determine whether PKL is a bona fide substrate for this phosphatase. Consistent with previous studies, the catalytically inactive PTP-PEST substrate-trapping mutant (C231S) precipitated several phosphorylated proteins from PTP-PEST−/− cell lysate, including protein(s) of 95 kDa (Fig. 9A). Reblotting with an anti-PKL antibody confirmed that a significant amount of PKL was precipitated by the substrate-trapping mutant and substantially less by the wild-type PTP-PEST GST-fusion protein (Fig. 9A). In addition, GFP-WT PKL, introduced into PTP-PEST−/− cells, was tyrosine phosphorylated in response to adhesion (Fig. 9B) and this phosphorylation was substantially reduced following the reintroduction of PTP-PEST (Fig. 9B). Conversely, a GFP-PKL phosphorylation mutant, Y286/392/492F (GFP-PKL triple YF) (Brown et al., 2005), was not phosphorylated during adhesion and the addition of PTP-PEST had no effect, suggesting one or more of these tyrosine residues are dephosphorylated by PTP-PEST. Taken together, these data strongly suggest PKL as a direct substrate for PTP-PEST. However, as reported here and previously (Sastry et al., 2002), PTP-PEST inhibits Rac activation. Since PKL phosphorylation is regulated by Rac (Brown et al., 2005), it is plausible that PTP-PEST indirectly blocks PKL tyrosine phosphorylation by decreasing Rac...
activity. To examine this, we expressed PTP-PEST with PKL in PTP-PEST−/− cells in the presence and absence of active G12V Rac (Fig. 9C). PKL phosphorylation was elevated in cells expressing active Rac. However, the phosphorylation was still decreased in the presence of PTP-PEST, suggesting that PTP-PEST-dependent regulation of PKL phosphorylation was not caused by the downregulation of Rac activity.

To determine whether PKL-binding can account for the role of the paxillin LD4 motif in PTP-PEST-dependent regulation of cell spreading (Fig. 4), we introduced wild-type PKL and a PKL mutant that had previously shown to be defective in paxillin binding (PKL ΔPBS2) (West et al., 2001) into PTP-PEST−/− cells expressing active Rac. However, the phosphorylation was still decreased in the presence of PTP-PEST, suggesting that PTP-PEST-dependent regulation of PKL phosphorylation was not caused by the downregulation of Rac activity.

Discussion
Cell migration is a multi-step, precisely coordinated, process involving membrane extension at the leading edge, followed by cell adhesion to the ECM; stabilization of these adhesions permits the contraction of the actin-based cytoskeleton to pull the cell forward and, finally, disassembly of adhesions at the rear of the cell in tail release (Ridley et al., 2003). It is well established that tyrosine phosphorylation and/or dephosphorylation of focal adhesion proteins, combined with appropriate spatiotemporal regulation of the Rho GTPase family members, plays an important role in regulating these events (Burridge and Wennerberg, 2004; Kurokawa et al., 2005; Lim et al., 1996). Previous studies have shown that the focal adhesion adaptor protein, paxillin and one of its binding partners, the tyrosine phosphatase PTP-PEST, are both necessary for normal cell migration as well as for integrin-dependent Rac signaling (Angers-Loustau et al., 1999; Brown and Turner, 2002; Brown and Turner, 2004; Garton and Tonks, 1999; Sastry et al., 2002; West et al., 2001). In this report, we have used paxillin−/− and PTP-PEST−/− cells to demonstrate that a paxillin−PTP-PEST interaction is essential for PTP-PEST-dependent regulation of cell spreading, associated Rac activity, membrane protrusion and cell migration. PTP-PEST signaling requires the functional tyrosine phosphorylation sites Y31 and Y118 as well as the LD4 motif of paxillin. Finally, we identified the LD4-binding protein PKL/GIT2 as a substrate for PTP-PEST and have showed, through expression of a PKL mutant defective for paxillin binding, that PKL recruitment to focal adhesions plays an integral role in PTP-PEST function.
Fig. 10. PKL-paxillin interaction is necessary for PTP-PEST to regulate cell spreading. PTP-PEST<sup>−/−</sup> cells were transiently transected with GFP with or without PTP-PEST, together with paxillin ΔLD4, PKL or the PKL ΔPBS2 mutant, plated on fibronectin-coated coverslips for 60 minutes and processed for immunofluorescence. Results represent the percentage of round GFP-positive cells. Results show mean±s.d. A minimum of 100 cells were counted in three independent experiments. **P<0.001, *P<0.05.

PTP-PEST<sup>−/−</sup> cells exhibit enlarged focal adhesions and elevated tyrosine phosphorylation of the focal adhesion proteins paxillin, p130 Cas and FAK, leading to the speculation that PTP-PEST stimulates cell migration by promoting dephosphorylation of these proteins, which, in turn, contributes to increased focal adhesion turnover (Angers-Loustau et al., 1999; Garton et al., 1996; Shen et al., 2000). Interestingly, focal adhesion turnover and cell migration are also reduced in paxillin<sup>−/−</sup> cells (Hagel et al., 2002; Webb et al., 2004), indicating that these cells might be deficient in PTP-PEST signaling. The failure of paxillin<sup>−/−</sup> cells, when compared to paxillin<sup>+/+</sup> cells, to exhibit impaired cell spreading following overexpression of PTP-PEST (Fig. 1) confirmed this speculation. This result was somewhat surprising because the paxillin<sup>−/−</sup> cells have elevated levels of the paxillin family member Hic-5 (Fig. 1). Clearly, although Hic-5 shares a similar domain-structure to paxillin (Brown and Turner, 2004) and has also been shown to share many paxillin-binding partners including PTP-PEST (Nishiya et al., 1999), Hic-5 is unable to compensate for the loss of paxillin in the regulation of spreading, consistent with the embryonic lethality of paxillin<sup>−/−</sup> mice (Hagel et al., 2002). The lack of well-conserved tyrosine residues in Hic-5 that correspond to Y31 and Y118 of paxillin, and the different spatial organization of the LD motifs within the N-terminus might account for this difference (Brown and Turner, 2004).

PTP-PEST binds to paxillin through the LIM3-4 domains (Côté et al., 1999). In a previous study, we showed that overexpression of a paxillin mutant lacking LIM4 resulted in reduced cell migration of CHO.K1 cells. Additionally, introduction of the LIM3-4 domain only, which is primarily cytosolic in its distribution, caused increased cell spreading (Brown and Turner, 2002). It is now evident that both of these phenotypes can be accounted for by a perturbation in paxillin-mediated recruitment of PTP-PEST to focal adhesions. However, in paxillin<sup>−/−</sup> cells the expression of the LIM1-4 domains of paxillin, which contain both focal adhesion targeting and PTP-PEST binding sites (Brown et al., 1996; Côté et al., 1999), was not sufficient for PTP-PEST to inhibit cell spreading (Fig. 4A), demonstrating that the role of the interaction with paxillin is not exclusively to target PTP-PEST to the focal adhesions. Instead, the LD4 motif and the tyrosine phosphorylation sites Y31 and Y118 within the paxillin N-terminus, are also required for PTP-PEST function (Figs 4 and 5).

The LD4 motif of paxillin has several binding partners, including the ARF GAP PKL/GIT2 and actopaxin, through which PTP-PEST might regulate adhesion-induced Rac signaling, and thus spreading, protrusion and migration (Brown and Turner, 2004; Clarke et al., 2004; Turner et al., 1999). Interestingly, PKL is tyrosine phosphorylated during cell spreading (Bagrodia et al., 1999; Brown et al., 2005) and recently we have shown that this phosphorylation, which is stimulated in response to Rac activation, is necessary for efficient recruitment of PKL to focal adhesions (Brown et al., 2005). Herein, using a substrate-trapping mutant, we show that PKL is a substrate of PTP-PEST (Fig. 9) and might indeed correspond to the 95-kDa protein that was reported to exhibit elevated tyrosine phosphorylation in PTP-PEST<sup>−/−</sup> cells (Côté et al., 1998). Importantly, by using the PKL ΔPBS2 mutant that is defective for paxillin binding and focal adhesion targeting (West et al., 2001), we show that the paxillin-PKL interaction is necessary for PTP-PEST to inhibit spreading (Fig. 10). Since overexpression of either a paxillin LD4-deletion mutant or PKL ΔPBS2 results in abnormal cell spreading and sustained activation of Rac (Brown et al., 2002; West et al., 2001), we speculate that PTP-PEST, by dephosphorylating PKL present in focal adhesions, provides a mechanism to terminate localized Rac activity. Whether this occurs through dissociation of, as yet unidentified, SH2-domain-containing binding partners for phosphorylated PKL, activation of PKL ARF GAP activity – which has been linked to Rac inhibition in the related GIT1 protein (Nishiya et al., 2005) – or through modulation of the guanine nucleotide exchange factor (GEF) activity of the PKL-associated PAK-interacting exchange factor (PIX) (Manser et al., 1998; Turner et al., 1999) will require further research.

How do tyrosine residues 31 and 118 of paxillin facilitate PTP-PEST function? Phosphorylation of these sites is induced during cell spreading (Brown and Turner, 2004; Petit et al., 2000). Phosphorylated paxillin binds to CrkII, which, in turn, can bind to the atypical Rac GEF DOCK180/ELMO complex along with p130 Cas, a PTP-PEST substrate (Gumienny et al., 2001; Klemke et al., 1998), to stimulate spreading and cell migration by activating Rac (Feller, 2001; Valles et al., 2004). In the context of cell spreading, PTP-PEST that binds to the paxillin C-terminus in focal adhesions might therefore suppress Rac activation by dephosphorylating p130 Cas and thus inhibit DOCK180 Rac GEF activity. Indeed, p130 Cas was dephosphorylated more efficiently in PTP-PEST<sup>−/−</sup> cells when PTP-PEST was reintroduced together with wild-type paxillin as opposed to the paxillin C523S mutant, which is defective for PTP-PEST binding (data not shown). Alternatively, PTP-PEST might also interfere with this signaling axis at the level of the paxillin-Crk interaction, because overexpression of PTP-PEST results in a decrease in the phosphorylation levels of paxillin (Shen et al., 2000). However, if this is the case, interference is probably indirect, because the use of a substrate-trapping mutant of PTP-PEST
indicated that paxillin itself is not a direct substrate of PTP-PEST (Côté et al., 1999).

Finally, although this study focused on dissecting the contribution of paxillin in the control of cell spreading, membrane protrusion and Rac activation by PTP-PEST, we have also shown that binding to paxillin is essential for reintroduced PTP-PEST to rescue cell migration in PTP-PEST−/− cells. It is generally accepted that, the signaling events that control lamellipodia extension in a spreading cell represent the molecular events that occur during the membrane extension and adhesion phases of cell migration. The translation of these events into productive cell movement undoubtedly requires successive rounds of activation and inactivation of relevant signaling pathways, including phosphorylation and dephosphorylation, and cycling of Rho family GTPase activities. Our current study suggests that, PTP-PEST contributes to this process through binding to the focal adhesion protein paxillin and modulates signaling to Rac by obtaining access to other proteins, such as PKL, that bind through multiple domains within the N-terminus of paxillin. Future studies will be directed towards understanding the complex interrelationship of these various signaling moieties.

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