

PTP-PEST controls motility through regulation of Rac1

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Summary

The cytoplasmic protein tyrosine phosphatase, PTP-PEST, associates with the focal adhesion proteins p130cas and paxillin and has recently been implicated in cell migration. In this study, we investigated the mechanism by which PTP-PEST regulates this phenomenon. We find that PTP-PEST is activated in an adhesion-dependent manner and localizes to the tips of membrane protrusions in spreading fibroblasts. We show that the catalytic activity of PTP-PEST is a key determinant for its effects on motility. Overexpression of PTP-PEST, but not a catalytically inactive form, impairs haptotaxis, cell spreading and formation of membrane protrusions in CHOK1 cells. In addition, overexpression of PTP-PEST in Rat1 fibroblasts perturbs membrane ruffling and motility in response to PDGF stimulation. The expression level of PTP-PEST

modulates the activity of the small GTPase, Rac1. PTP-PEST overexpression suppresses activation of Rac1 in response to both integrin-mediated adhesion or growth factor stimulation. In contrast, fibroblasts that lack PTP-PEST expression show enhanced Rac1 activity. Co-expression of constitutively active Rac1 with PTP-PEST overcomes the inhibition of cell spreading and migration indicating that PTP-PEST acts by antagonizing Rac1 activation. Our data suggest a model in which PTP-PEST is activated by integrins and localized to regions where it can control motile events at the leading edge through inhibition of the small GTPase Rac1.

Key words: Cell migration, Rho family GTPases, Tyrosine phosphatase

Introduction

Cell motility is integral to numerous biological processes including embryonic development, wound healing, angiogenesis, inflammation, and neuronal outgrowth (Stossel, 1993). Aberrant migration contributes to pathologies such as atherosclerosis and tumor cell invasion and metastasis. Thus, the molecular and cellular mechanisms that control migration are of considerable relevance to both normal and pathological processes.

Motility is controlled by multiple external cues. Both the extracellular matrix (ECM), upon which cells adhere via integrin receptors, and soluble factors such as growth factors or cytokines transmit signals to control cell morphology and cytoskeletal organization (Huttenlocher et al., 1995). In response to migratory stimuli, cells adopt a polarized morphology consisting of a highly protrusive, spread leading edge and a thin tail. At the leading edge, actin-rich membrane ruffles form and nascent adhesive contacts with the ECM, focal complexes, are established through membrane extensions like filopodia and lamellipodia. Focal adhesions, which mature from focal complexes, link integrins to contractile actin stress fibers which exert traction on the substrate (Burridge and Chrzanowska-Wodnicka, 1996). At the rear of a migrating cell, focal adhesions disperse and the tail retracts. The concerted processes of extension, contraction, and retraction result in forward translocation of the cell (Stossel, 1993; Lauffenburger and Horwitz, 1996).

Significant progress has been made in defining the

intracellular signal transduction pathways activated by integrins to control motility. Most notably, in response to clustering or ligation by ECM proteins, integrins activate tyrosine kinases (Clark and Brugge, 1995; Schwartz et al., 1995; Parsons, 1996) and the Rho family of small GTP-binding proteins (Ren et al., 1999; Arthur et al., 2000; del Pozo et al., 2000; O'Connor et al., 2000). The activation of these GTPases is partly controlled by tyrosine kinases (Ridley and Hall, 1994). Cdc42 controls cell polarity and filopodia formation, Rac1 stimulates membrane ruffling, lamellipodia, and focal complex formation (Ridley et al., 1992). RhoA induces focal adhesion and stress fiber assembly (Ridley and Hall, 1992). The activity of Cdc42, Rac1 and RhoA all are required for cells to efficiently migrate (Nobes and Hall, 1999).

Tyrosine kinase signaling in response to integrin-mediated cell attachment plays a major role in motility. Several focal adhesion proteins become tyrosine phosphorylated upon integrin engagement including FAK, paxillin, and p130cas (Clark and Brugge, 1995; Schwartz et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996). In migrating cells, many of these proteins concentrate at the leading edge in membrane ruffles. Inhibition or enhancement of FAK function reveals a role for FAK in cell migration (Ilic et al., 1995; Gilmore and Romer, 1996; Cary et al., 1996). The interaction of FAK with p130cas and c-src appear to be critical for this process (Cary et al., 1996; Cary et al., 1998). Tyrosine phosphorylation of p130cas creates a binding site for the adaptor protein crk (Klemke et al., 1998). Together, the p130cas/crk complex

stimulates cell migration. Importantly, many of these focal adhesion proteins are hyperphosphorylated in transformed cells correlating with increased invasiveness (Kanner et al., 1990; Owens et al., 1995).

Protein tyrosine phosphatases (PTPase) counteract the tyrosine kinase signaling emanating from integrin receptors to limit inappropriate cell motility. Early work demonstrated that the level of PTPase activity is suppressed in response to cell-ECM adhesion (Maher, 1993); although this change in activity was not mapped to a specific phosphatase. Direct evidence that integrins regulate specific PTPases is lacking thus far. Some PTPases localize in focal adhesions (Serra-Pages et al., 1995) while others may affect focal adhesion assembly (Yu et al., 1998). A number of PTPases have been implicated in regulating cell motility (Angers-Lousteau et al., 1999b). Many of these PTPases demonstrate overlapping substrate specificities as well as functional redundancies. Thus, identification of the factors regulating different PTPases and the mechanisms they utilize to control motility are key questions.

The widely distributed PTPase, PTP-PEST, is particularly intriguing with respect to cell motility. The cellular and molecular mechanisms regarding its role in motility remain largely unexplored. Both the overexpression of PTP-PEST in fibroblasts (Garton and Tonks, 1999) or its targeted deletion (Angers-Lousteau et al., 1999a) inhibit cell motility. These results suggest that at normal expression levels, the localization of PTP-PEST or its activity are regulated to coordinate the balance between forward protrusion and formation of new adhesions at the leading edge versus detachment and release of the tail. The knockout study implicates PTP-PEST in cell spreading and focal adhesion disassembly suggesting that this phosphatase acts at multiple steps during migration (Angers-Lousteau et al., 1999a).

It is unclear whether the effects on motility are due to a catalytic or scaffolding function. PTP-PEST contains an N-terminal catalytic domain and a C-terminal scaffolding domain with binding sites for numerous signaling proteins. A major physiologic substrate of PTP-PEST is p130cas (Garton and Tonks, 1994; Garton et al., 1997; Cote et al., 1998). Overexpression of PTP-PEST leads to dephosphorylation of p130cas and disruption of its interaction with crk (Garton and Tonks, 1999). In contrast, PTP-PEST null cells contain hyperphosphorylated p130cas whose association with crk is enhanced, yet these cells are unable to migrate (Angers-Lousteau et al., 1999a). In addition to p130cas, PTP-PEST binds to and acts on the focal adhesion protein paxillin (Shen et al., 1998; Cote et al., 1999; Shen et al., 2000). Other substrates include PYK2 (Lyons et al., 2001; Davidson and Veillette, 2001), c-abl (Cong et al., 2000), and PSTPIP (Spencer et al., 1997; Wu et al., 1998b) a WASp-binding protein (Wu et al., 1998a; Cote et al., 2002). PTP-PEST also interacts with the src inhibitory kinase, csk (Davidson et al., 1997), and the adaptor proteins shc (Habib et al., 1994; Charest et al., 1996) and grb2 (Charest et al., 1997). Through its interactions, PTP-PEST likely exerts its effects on motility via multiple pathways.

In this study, we addressed the cellular and molecular mechanisms by which PTP-PEST controls motility. We determined whether PTP-PEST activity or localization are regulated by cell adhesion, which step of migration PTP-PEST

affects, and if these effects are due to a catalytic or scaffolding function. We demonstrate that PTP-PEST activity is stimulated by cell adhesion and that PTP-PEST localizes to the tips of membrane protrusions. Through its catalytic activity, PTP-PEST regulates events at the leading edge of a migrating cell such as membrane ruffling and protrusion. PTP-PEST mediates these effects through the regulation of Rac1.

Materials and Methods

Antibodies, plasmids, and reagents

Fibronectin (FN) was purified from human plasma as described (Ruoslahti et al., 1982). Poly-L-lysine was purchased from Sigma (St Louis, MO). [γ - 32 P]ATP was purchased from Dupont-NEN (Boston, MA). PDGF-BB was purchased from R&D Systems (Minneapolis, MN). The AU-tagged constitutively activate Rac1 plasmid, pCEVAUQ61LRac1 (Teramoto et al., 1996) was a generous gift of J. Silvio Gutkind (NIH; Bethesda, MD). Constitutively active Rho expression constructs fused to GFP, pEGFP-Q61LRac1, pEGFPQ61Lcdc42, and pEGFPQ63LrhoA, were a gift of Krister Wennerberg. The pGEX vector encoding the GST-PBD fusion protein (Glaven et al., 1999) was a generous gift of Rick Cerione (Cornell University, Ithaca, NY). The GFP expression plasmid, pGreen Lantern was purchased from Gibco-BRL.

The anti-phosphotyrosine mAb, PY20, and anti-Rac1 mAb were purchased from Transduction Labs (Lexington, KY). The anti-GFP polyclonal antibody was purchased from Clontech (Palo Alto, CA). A mAb antibody recognizing the AU epitope tag was purchased from Babco (Richmond, CA). The anti-vinculin mAb, 7F9 (Glukhova et al., 1990), and the KT3 mAb against an SV40 epitope tag were described previously (Schaller et al., 1993). Polyclonal antisera against PTP-PEST were generated as described (Davidson et al., 1997) or by immunization of rabbits with a GST fusion protein encoding amino acids 441-775 of mouse PTP-PEST (Shen et al., 2000).

Cell culture, transfections and plasmid construction

CHOK1 cells were obtained from ATCC (Rockville, MD). Cells were maintained in DMEM (Gibco-BRL; Gaithersburg, MD) containing 10% Fetal Bovine Serum (BioWhittaker, Walkersville, MD), 0.1 mM non-essential amino acids (GIBCO-BRL), and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μ g/ml amphotericin B; Sigma, St Louis, MO) in a 10% CO₂ incubator. Cells were passaged with trypsin-EDTA (Gibco-BRL) and used between passages 2 and 10. Parental Rat1 fibroblasts were maintained in DMEM containing 10% FBS. Stable Rat1 cell lines expressing PTP-PEST or a mock vector (Garton and Tonks, 1999) were a generous gift of N. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) and were maintained in DMEM with 10% FBS and 100 μ g/ml hygromycin. PTP-PEST knockout cell lines (PTP-PEST $-/-$) and control cell lines (+/- and reexpressors) (Cote et al., 1998; Angers-Lousteau, 1999a) were a generous gift of Michel Tremblay (McGill University, Montreal, Quebec) and were maintained in DMEM containing 10% FBS.

A pcDNA expression plasmid containing PTP-PEST was constructed by engineering a C-terminal epitope tag (KT3) in the mouse PTP-PEST cDNA (Davidson et al., 1997) using PCR. A 3' primer that created a *Bam*HI and that destroyed the termination codon was used. The PCR product was inserted into pC-tag (Schaller et al., 1993) such that PTP-PEST was in frame with the KT3 sequence. The tagged PTP-PEST cDNA was then subcloned into pcDNA3.0 (Invitrogen; Carlsbad, CA). To generate a catalytically inactive PTP-PEST, a point mutation within the catalytic domain of PTP-PEST, C231S, was introduced by PCR mutagenesis based on the Stratagene Quick Change protocol (Stratagene; La Jolla, CA). The primers used

were: CS1 5'-ATTTGTATTTCATTCCAGTGCAGGCTG-3' AND CS2 5'-CAGCCTGCACTGGAATGAATACAAAT-3'. The template was a KT3-tagged PTP-PEST cDNA in pBluescript (Stratagene). Correct clones were verified by sequencing and then subcloned into pcDNA3.1 (Invitrogen). The C231S mutant is referred to as PTP-PESTC231S.

For transient transfections, cells were seeded onto 60 mm (4×10^5 cells) or 100 mm (10^6 cells) tissue culture plates for 18–20 hours in growth medium. Transfections were performed using Lipofectamine Plus (Gibco-BRL) according to manufacturer's recommendations. 2 μ g of plasmid DNA were used for 60 mm plates and 4 μ g of plasmid for 100 mm plates. For co-expression of PTP-PEST and Q61LRac1, transfections were performed in 100 mm plates with a 1:1 ratio of the two plasmids. DNA complexes were incubated with the cells for 3 hours in serum-free medium, cells were washed, and maintained in growth medium for 18–24 hours prior to use in experiments.

PTPase assays

Assays of PTP-PEST catalytic activity were performed using an immune complex PTPase assay. Briefly, PTP-PEST immunoprecipitates were incubated with a tyrosine phosphorylated peptide substrate and the extent of dephosphorylation by PTP-PEST determined under different cell conditions. CHOK1 cells were trypsinized, washed twice in serum-free medium containing 2% BSA (SFM), maintained in suspension in SFM for 1 hour and then cell aliquots were kept suspended or plated onto tissue culture plates coated with either 10 μ g/ml FN or 10 μ g/ml poly-L-lysine diluted in phosphate buffered saline (PBS) for times indicated. Cells were lysed in ice cold buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100 plus 10 μ g/ml of the protease inhibitors aprotinin and leupeptin (Sigma). PTP-PEST was immunoprecipitated by incubating 400–500 μ g of lysate with an anti-PTP-PEST antiserum at a 1:250 dilution for 1 hour at 4°C followed by 50 μ l of a 50% slurry of protein A sepharose beads (Sigma) for an additional hour. Immune complexes were washed three times in lysis buffer and then twice in PTP assay buffer (50 mM Hepes, pH 7.3, 4 mM DTT, 2 mg/ml BSA). Beads were suspended in PTP buffer and 50,000 cpm of a 32 P labeled poly-Glu-Tyr substrate was added. The phosphatase reaction was rotated at room temperature for 15 minutes and terminated by addition of an equal volume of ice cold 20% TCA. The amount of free 32 P released into the supernatant was measured in a scintillation counter. The background in the absence of primary antibody was subtracted. Assays were performed at least three times for different cell conditions. The activity of PTP-PEST in suspension cultures was arbitrarily set at 1 to compare different experiments. An aliquot of the beads was saved prior to the PTP assay for western blot analysis (see below) to ensure that equal amounts of PTP-PEST were precipitated. For some experiments an alternate method for PTPase activity was also employed. PTP-PEST activity was assessed in PTP-PEST immunoprecipitations using an ELISA based Tyrosine Phosphatase Assay Kit from Boehringer-Mannheim (Indianapolis, IN) according to the manufacturer's protocol. Activity was determined within a linear range of detection.

Cell spreading, membrane ruffling, and immunofluorescence

For cell spreading, control CHOK1 cells or transfected cells were trypsinized, washed in SFM twice and plated on FN-coated coverslips (10 μ g/ml; 5×10^4 cells/12 mm coverslip) in SFM. Cells were allowed to attach and spread for 45 minutes, 90 minutes, and 3 hours. Coverslips were fixed in 3.7% formaldehyde in PBS then permeabilized in 0.5% Triton X-100 in TBS (50 mM Tris, pH 7.6; 150 mM NaCl). Coverslips were washed in TBS and then co-stained for PTP-PEST expression with the KT3 mAb followed by FITC anti-mouse (Chemicon) and for actin with Rhodamine-phalloidin (Molecular Probes; Eugene, OR). GFP-transfected cells were stained

for actin. The percentage of spread cells was determined among KT3 positive or GFP positive cells for 5 fields (at least 100 cells total and for at least three separate experiments). Cells co-expressing PTP-PEST and AU-tagged Q61LRac1 were triple stained for PTP-PEST expression with anti-mouse PTP-PEST serum, anti-AU mAb (Babco), and actin. To assess focal adhesion and stress fiber formation, cells were plated on FN coverslips as described above for 1, 3, or 6 hours in SFM. Cells were triple stained for PTP-PEST overexpression, for focal adhesions with an anti-vinculin mAb, 7F9, and for actin with Texas-Red-phalloidin. Secondary antibodies for triple staining were Cascade Blue anti-rabbit (Molecular Probes) and FITC anti-mouse (Chemicon; Temecula, CA). For membrane ruffling in response to growth factors, parental Rat1 fibroblasts or Rat1 cell lines expressing PTP-PEST were seeded onto coverslips in complete growth medium, serum starved for 24 hours, and then stimulated with PDGF-BB (20 ng/ml) for 15 minutes. Coverslips were fixed and stained for actin. Immunofluorescence was performed on a Zeiss Axiophot microscope. Images were obtained using a Hammamatsu cooled CCD digital camera and Metamorph imaging software (Universal Imaging).

Migration assays

The ability of CHOK1 or transfected CHOK1 cells to migrate in a haptotactic assay towards a FN gradient was assessed using a modified Boyden chamber, or transwell insert. Transwell filters (Costar; Cambridge, MA) with a 0.8 μ m pore size were used for all experiments. The bottom surface of the membrane was coated with 10 μ g/ml FN in PBS for 1 hour at 37°C. The membranes were washed and blocked in 2% BSA-PBS. To facilitate quantitation, 24 hours after transfection cells were pre-labeled with a vital nuclear stain, Hoechst 33342 (Molecular Probes) at a concentration of 10 μ g/ml in growth medium for 30 minutes at 37°C prior to preparation for the assay. Cells were then trypsinized, washed and resuspended in SFM as described above. SFM was added to the lower chamber and 10^5 cells were added to the top of the filter. Migration was assayed after 2 hours at 37°C. The number of total nuclei on the lower surface of the membrane were scored for at least 5 fields for each membrane. The number of nuclei (or cells migrated) for control CHOK1 cells was treated as 100% migration. The migration of the transfected cells is expressed as a percentage of CHOK1 cells. Similar results were obtained in at least three separate experiments.

For the effects of PDGF on motility, parental Rat1 fibroblasts or Rat1 cells expressing PTP-PEST were serum starved for 24 hours. Nuclei were labeled as above in SFM. Cells were trypsinized, washed and resuspended in SFM. The bottom membranes of transwell filters were coated with FN (10 μ g/ml in PBS) and SFM with or without PDGF-BB (20 ng/ml) was added to the lower chamber. Cells were seeded in the top chamber and migration was assayed after 2 hours at 37°C. Migration is expressed relative to parental cells in the absence of growth factor and is the average of results obtained in three separate experiments.

Cell lysis and western blots

For the detection of transfected proteins, cells were lysed 24 hours after transfection in modified RIPA buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM PMSF) on ice. For antiphosphotyrosine experiments, 1 mM Na Orthovanadate was added to the lysis buffer. Protein content was determined using Coomassie reagent (Pierce) with BSA as the standard. Lysates were boiled in sample buffer for 5 minutes and 10–20 μ g of lysates were loaded on 10% SDS-PAGE gels (Laemmli, 1970). Proteins were transferred to nitrocellulose membranes (Towbin et al., 1979) and blocked in TBST (50 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20) with 5% non-fat dry milk overnight at 4°C. For PY20 western blots membranes were blocked

in TBST containing 10% cold fish gelatin (Sigma). Primary antibodies were diluted in blocking buffer as follows: anti-GFP, 1:250; anti-KT3, 1:1000; anti-PTP-PEST sera, 1:1000; anti-Rac1, 1:1000; PY20, 1:1000 and incubated for 1 hour at room temperature. Membranes were washed for 1 hour in TBST followed by HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson Labs) at a 1:10,000 dilution. After washing in TBST, proteins were detected by chemiluminescence using the Super Signal reagent (Pierce). Membranes were exposed to Kodak X-Omat AR X-ray film and developed in an automatic processor.

Rac1 activity assay

The glutathione-S-transferase-PAK-binding domain (GST-PBD) fusion protein was grown and purified with glutathione Sepharose beads (Pharmacia) as described (Glaven et al., 1999). For affinity precipitation of Rac1-GTP (active Rac1) from cell lysates, cultures were lysed in either PLB (25 mM Hepes, pH 7.5; 150 mM NaCl, 1% NP-40, 10% glycerol, 10mM MgCl₂, 2 mM EGTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin) or in modified RIPA containing 10 mM MgCl₂, 2 mM EGTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin. 15-20 µg of the GST-PBD protein bound to glutathione beads were added to 0.5 mg of lysate and incubated at 4°C on a rotator for 30 minutes. Precipitates were washed three times in lysis buffer. The bead pellets were boiled in SDS sample buffer for 5 minutes and the entire sample loaded onto a 15% SDS gel. Equal protein aliquots of lysates were included as loading controls. After transfer to nitrocellulose, the amount of Rac1-GTP present was determined by western blotting for Rac1. Densitometry was performed on the western blots using Metamorph imaging software. Rac1 activity is normalized against total Rac1 in the lysate.

Results

PTP-PEST activity and localization are regulated by the ECM

PTP-PEST is a widely expressed protein tyrosine phosphatase that has recently been implicated in cell motility. Both Rat1 fibroblasts that overexpress PTP-PEST (Garton and Tonks, 1999) and fibroblasts isolated from PTP-PEST null mice (Angers-Lousteau et al., 1999a) display inhibited cell motility. These findings suggest that at normal levels of expression, either the activity of PTP-PEST or its localization are tightly regulated. Therefore, we first determined whether PTP-PEST was regulated in response to cell-ECM adhesion. The catalytic activity of PTP-PEST was assayed by immunoprecipitation of PTP-PEST followed by measuring the release of free ³²P from a radiolabeled poly-Glu-Tyr peptide substrate. PTP-PEST activity was compared under serum-free conditions in CHOK1 cells held in suspension (S), plated on a fibronectin (FN) substrate, or plated on a poly-L-lysine (PL) substrate. As shown in Fig. 1A, cell attachment to FN for 45 minutes stimulated PTP-PEST activity 2-3-fold compared with either suspension or poly-L-lysine controls. Western blots of the PTP-PEST immunoprecipitations with an anti-PTP-PEST antibody showed that equal amounts of the phosphatase were present in the activity assays. Similar activation results were obtained with Swiss 3T3 fibroblasts plated under identical conditions (data not shown). A time course of PTP-PEST activity in response to cell attachment and spreading on FN showed that PTP-PEST is activated 1.5-2-fold compared with suspension cultures within 10-15 minutes after plating. PTP-PEST activity persists at an elevated level (2-3 fold) for up to 2 hours (data not shown).

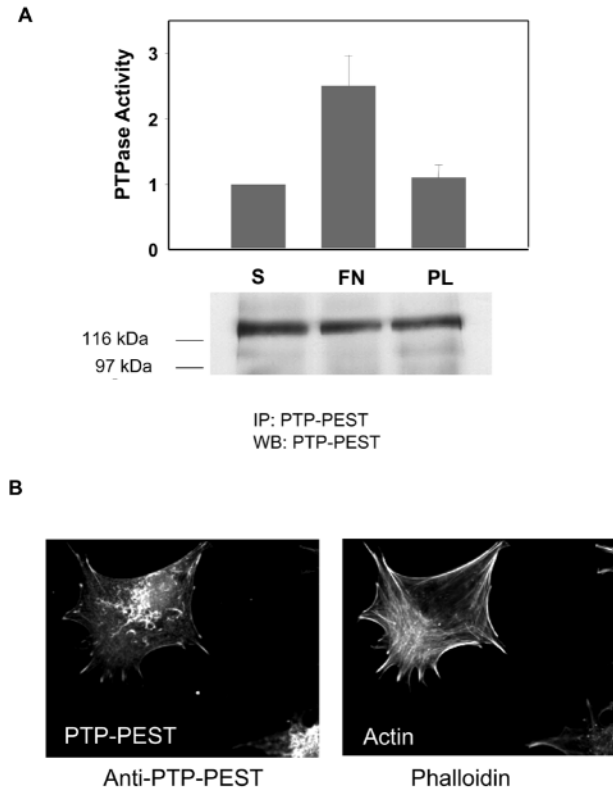


Fig. 1. PTP-PEST is activated by integrins and localizes to the tips of membrane protrusions. (A) CHOK1 fibroblasts were held in suspension (S) or plated on fibronectin-coated (FN, 10 µg/ml) or poly-L-lysine-coated (PL, 10 µg/ml) plates for 1 hour in serum-free medium. PTP-PEST was immunoprecipitated from TX-100 lysates. PTPase activity was determined by incubating the immunoprecipitates with a ³²P-labeled peptide substrate (³²P polyglu-tyr) and measuring the amount of ³²P released. PTP-PEST activity is 2-3-fold higher in cells plated on FN compared with cells in suspension (S) or plated on a non-specific substrate (PL). The results represent three independent experiments. (Lower panel) Immunoprecipitates of PTP-PEST from CHOK1 cells in suspension, plated on FN or PL, show that equal amounts of PTP-PEST were precipitated for the activity assay. (B) Swiss 3T3 fibroblasts were plated on FN-coated coverslips for 20 minutes and then immunostained for PTP-PEST (left) and actin (right). PTP-PEST transiently localizes at the tips of protrusions.

We next determined the localization of endogenous PTP-PEST in spreading fibroblasts. Swiss 3T3 fibroblasts were plated on FN-coated coverslips for various time points from 20 minutes to 2 hours and then stained with an anti-mouse PTP-PEST antiserum. At 20-40 minutes after plating, when cells were still in the process of spreading, PTP-PEST was detected in adhesion sites at the cell periphery at the tips of membrane protrusions (Fig. 1B). In some spreading cells, PTP-PEST also localized in membrane ruffles at the edge of lamellipodia (not shown). This localization was transient. At later times after adhesion and spreading on FN such as 2 hours, PTP-PEST was diffusely distributed and could not be detected in focal adhesions (data not shown). Taken together, these data indicate that PTP-PEST is activated by integrins and localizes to sites where new adhesive contacts are forming at the tips of protrusive structures.

PTP-PEST regulates motility and membrane protrusion via its catalytic activity

Since adhesion to the ECM enhanced PTP-PEST catalytic activity, we addressed whether PTP-PEST effects on motility were dependent on its catalytic or scaffolding function. To accomplish this, we transiently overexpressed either wild-type PTP-PEST (WT), or a catalytically inactive mutant, C231S, both of which had been epitope-tagged, in CHOK1 cells. The integrity of these constructs for phosphatase activity was confirmed by immunoprecipitation and *in vitro* PTPase assays (P.D.L., unpublished). We used a GFP expression vector, pGreen Lantern, as a mock condition. As assessed by immunofluorescence, 40–60% of cells were expressing either GFP, WT PTP-PEST, or PTP-PESTC231S 24 hours following transfection. Western blot analysis with the KT3 epitope tag mAb showed that WT PTP-PEST and PTP-PESTC231S were equally expressed. (Fig. 2A).

We next examined the effect of this overexpression on cell motility using a haptotaxis assay. CHOK1 cells or transfected cells were tested for their ability to migrate towards FN in the absence of serum using a transwell chamber. Relative to control CHOK1 cells, 90–95% of GFP-transfected cells

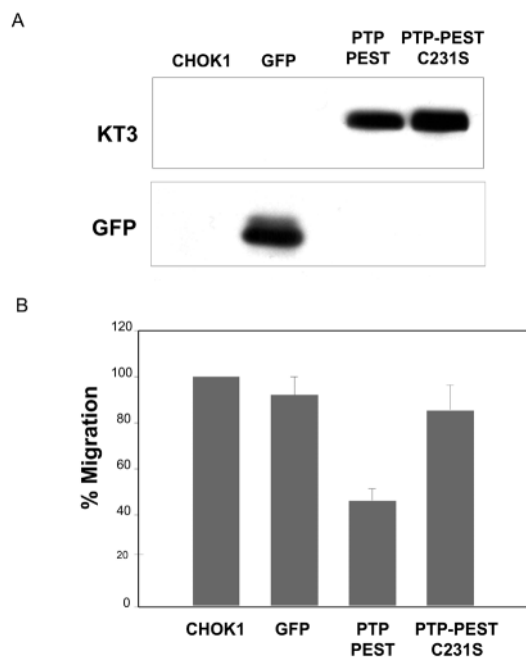


Fig. 2. PTP-PEST effect on motility depends on its catalytic activity. (A) Transient expression of GFP, PTP-PEST or PTP-PESTC231S in CHOK1 cells. Western blots for the KT3 epitope tag (KT3) or for GFP show expression levels of transfected proteins. (B) CHOK1 cells transfected with wild-type PTP-PEST, PTP-PESTC231S, or GFP were analyzed for their ability to migrate towards a FN substrate using a haptotactic migration assay. The lower membrane of a transwell chamber was coated with 10 μ g/ml FN. While expression of a mock gene, GFP, did not affect CHOK1 migration, expression of wild-type PTP-PEST significantly impaired their migration. In contrast, expression of a catalytically inactive mutant, PTP-PESTC231S, did not inhibit CHOK1 migration, indicating that the catalytic function is critical for motility. The number of cells per field for five fields were scored in three separate experiments. Migration of transfected cells is expressed as a percentage of CHOK1 controls.

migrated after 2 hours (Fig. 2B). In contrast, PTP-PEST overexpression led to a 40–50% inhibition of cell migration. Since the transfection efficiency is 40–60%, this indicates that virtually all PTP-PEST-expressing cells are blocked in migration. This finding agrees with that reported by Garton and Tonks (Garton and Tonks, 1999). Expression of the catalytically inactive mutant did not alter cell migration in the transwell assay. Close to 90% of PTP-PESTC231S-transfected cells migrated towards FN, much like control cells expressing GFP. Therefore, impaired cell motility in response to PTP-PEST overexpression is primarily due to its catalytic activity and likely not to a scaffolding function.

An important aspect of motility is membrane ruffling and protrusion of the leading edge. PTP-PEST is implicated in

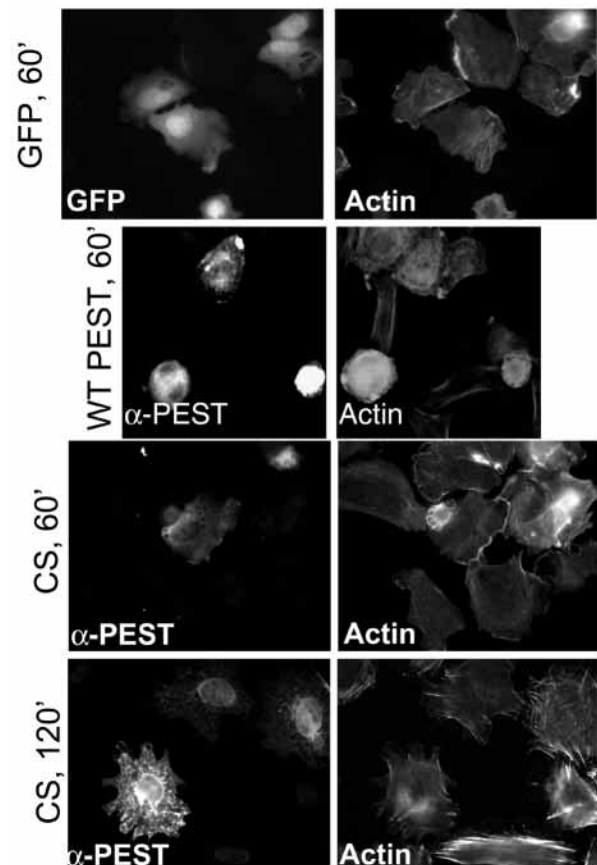


Fig. 3. PTP-PEST regulates the formation of membrane protrusions. CHOK1 cells were transiently transfected with expression plasmids encoding wild-type PTP-PEST, a catalytically inactive mutant PTP-PESTC231S, or a mock vector encoding GFP. 24 hours after transfection, cells were trypsinized and replated onto FN-coated coverslips in serum-free medium for indicated times. PTP-PEST or PTP-PESTC231S-transfected cells were fixed and co-stained for PTP-PEST with the KT3 mAb and for actin with Texas-Red-phalloidin. GFP-transfected cells were co-stained for actin only. MOCK-transfected cells spread on a FN substrate extending prominent lamellipodia and form membrane ruffles. WT PTP-PEST-transfected cells are inhibited in spreading. These cells are able to attach but have a rounded morphology. They do not extend protrusion or form ruffles. Catalytically inactive PTP-PEST(C231S) enhances membrane ruffling at early times of adhesion (30–60 min). At later times after plating (2 hours) these cells extend numerous protrusions in all directions.

controlling cell spreading (Angers-Lousteau et al., 1999a) however it is not known if the catalytic activity of PTP-PEST plays a role. Since cell spreading mimics many of the events that occur at the leading edge, we analyzed the effect of overexpressing WT PTP-PEST or the C231S mutant on CHOK1 cell morphology. Fig. 3 shows that 45-60 minutes after plating CHOK1 cells expressing GFP spread on FN and formed small lamellipodia, with modest actin staining in membrane ruffles. CHOK1 cells that overexpress WT-PTP-PEST, while able to initially attach to a FN substrate, did not efficiently spread. These cells did not extend lamellipodia or form membrane ruffles, unlike untransfected cells in the same field. In addition, phalloidin staining shows actin to be poorly organized in these cells. This impaired cell spreading persisted up to 3 hours after plating (not shown). In contrast, overexpression of the catalytically inactive mutant, C231S, produced an opposite effect on cell morphology. 45-60 minutes after plating, C231S-transfected cells exhibited enhanced membrane ruffling as observed by actin staining. More dramatically however, over a longer time course of 2 or more hours, C231S-expressing cells continued to extend numerous membrane protrusions and lamellipodia. Strikingly, these protrusions lacked a defined polarity and emanated from the cell in all directions. Control cells at this time point ceased protruding and ruffling and in general adopted a triangular, fibroblastic shape. Taken together, these results demonstrate that an elevated level of PTP-PEST interferes with motility by blocking membrane protrusion.

PTP-PEST impairs membrane ruffling and motility in response to growth factors

In addition to integrin-mediated adhesion, membrane ruffling and motility are also regulated by growth factors such as PDGF (Ridley et al., 1992; Anand-Apte et al., 1997). Furthermore, PTP-PEST associates with the adaptor proteins grb2 (Charest et al., 1997) and shc (Habib et al., 1994; Charest et al., 1996) both of which participate in growth factor signaling and motility. Therefore, we next determined if PTP-PEST overexpression altered the migratory response of fibroblasts to PDGF. For these assays, we utilized a stable Rat1 cell line overexpressing PTP-PEST which exhibits decreased motility in a wound assay but has a less severe morphology phenotype (Garton and Tonks, 1999). We first assessed the effect of PTP-PEST overexpression on membrane ruffling. Subconfluent mock-transfected Rat1 cells or cells overexpressing PTP-PEST were serum starved. Membrane ruffling was assayed by staining for actin in the presence or absence of PDGF (20 ng/ml in serum-free medium) for various time points. Within 15 minutes after addition of PDGF, mock-transfected Rat1 cells displayed extensive membrane ruffling and actin reorganization (Fig. 4). PTP-PEST overexpression greatly diminished the ruffling response of Rat 1 fibroblasts to PDGF.

We next assayed the ability of PTP-PEST overexpressing Rat1 cells to migrate in response to PDGF. Parental Rat1 cells and a PTP-PEST clone were compared using a transwell assay in which the lower surface of the filter was coated with FN and the bottom chamber contained serum-free medium in the presence or absence of PDGF (20 ng/ml). In parental Rat1 cells, PDGF stimulated a 3-fold increase in migration relative to untreated cells (Fig. 4). In contrast, PDGF treatment of PTP-

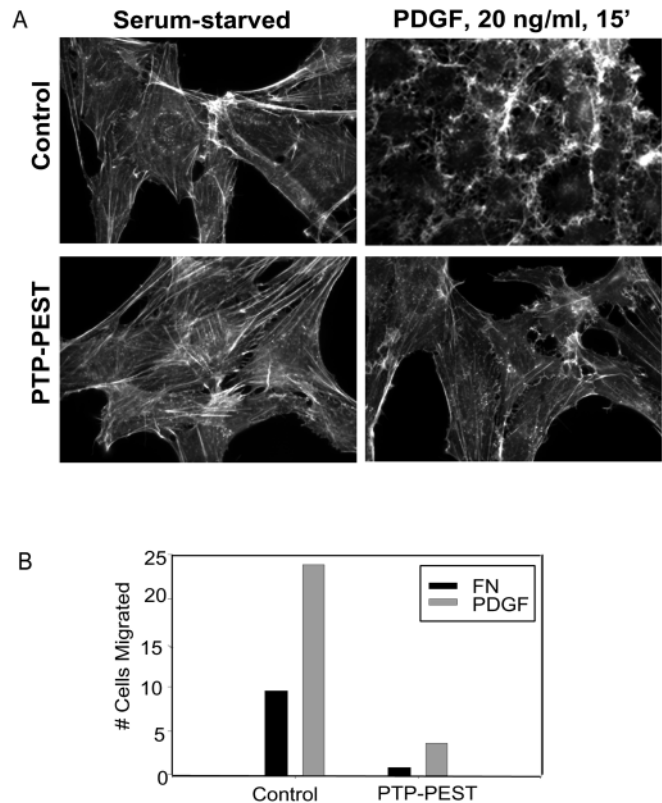


Fig. 4. PTP-PEST impairs membrane ruffling and motility in response to PDGF. (A) Parental Rat-1 fibroblasts or Rat-1 cells stably overexpressing PTP-PEST were serum starved (left panels) and then stimulated with PDGF-BB (20 ng/ml, 15 minutes, right panels) to assess membrane ruffling. Whereas control cells reorganize actin into ruffles, those that express PTP-PEST show less prominent membrane ruffling. (B) Parental Rat-1 fibroblasts or those stably expressing PTP-PEST were added to transwell filters in which the bottom membrane was coated with FN in the presence or absence of PDGF-BB in the lower chamber. While control cells are induced to migrate in the presence of PDGF, PTP-PEST-expressing cells exhibit reduced motility in response to PDGF.

PEST overexpressing cells did not stimulate motility relative to parental cells. A western blot with PTP-PEST antiserum indicated that PDGF stimulation had no effect on the level of PTP-PEST expression in either control Rat1 cells or in a Rat1 PTP-PEST clone (not shown). It should be noted that PTP-PEST overexpression did not perturb all PDGF-induced responses. In Rat1 cells erk1 and erk2 activation in response to PDGF were unaffected by PTP-PEST overexpression (data not shown).

PTP-PEST expression levels modulate activation of Rac1

The alterations in cell morphology, membrane ruffling, and migration resulting from PTP-PEST overexpression suggested that PTP-PEST might act by regulating a rho family GTPase. The rho family of GTPases has been widely demonstrated to control these cellular phenomena (Schmitz et al., 2000; Hall, 1998). In particular, Rac1 has been implicated in controlling cell spreading (Price et al., 1998; Clark et al., 1998), the

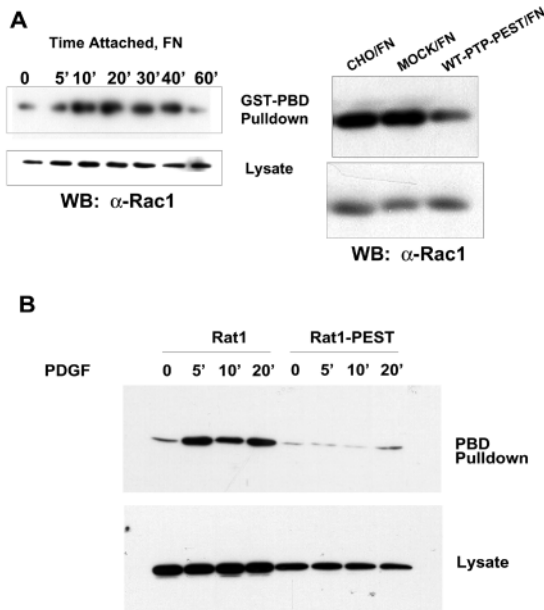


Fig. 5. PTP-PEST overexpression suppresses adhesion- and growth factor-dependent activation of Rac1. Rac1 activity was measured using an affinity precipitation assay in which a GST fusion protein of the Rac1-binding domain of the Rac1 effector PAK3 (GST-PBD), which specifically binds GTP-Rac1 (active), is used to 'pull down' active Rac1 from cell lysates. The amount of GTP-Rac1 isolated by GST-PBD immobilized on glutathione beads was determined by a Rac1 immunoblot. (A) A timecourse of Rac1 activation in response to CHO/K1 attachment and spreading on a FN substrate shows that Rac1 activity is stimulated 10–20 minutes after attachment, remains active for 30–40 minutes and then begins to decrease by 60 minutes. Rac1 activity in the pulldown is normalized against total Rac1 in the lysate. Overexpression of PTP-PEST, but not GFP (MOCK), decreases the level of Rac1 activity in response to cell attachment to FN for 45 minutes. (B) Parental or PTP-PEST-expressing Rat-1 fibroblasts were serum starved and then stimulated with PDGF-BB (50 ng/ml) for the times indicated. PDGF robustly activates Rac1 in parental cells. PTP-PEST-expressing cells have a lower baseline of Rac1 activity and respond poorly to PDGF.

formation of membrane protrusions and membrane ruffling (Ridley et al., 1992), and cell motility (Keely et al., 1997; Nobes and Hall, 1999). Therefore we next determined if PTP-PEST overexpression affected activation of Rac1 either in response to cell attachment to FN or in response to PDGF. Rac1 activity was assayed by affinity precipitation with the Rac1/cdc42 effector-binding domain of PAK3 fused to GST (GST-PBD=p21-binding domain) (Glaven et al., 1999). This fragment of PAK3 specifically binds activated Rac1-GTP and not inactive Rac1-GDP (Burbelo et al., 1995). The amount of Rac1-GTP bound to the beads was determined by a Rac1 western blot.

The timecourse of Rac1 activation in response to cell attachment to FN was first determined in control CHO/K1 cells. When compared to CHO/K1 cells kept in suspension for 1 hour (Fig. 5A), adhesion to FN leads to increased amounts of active Rac1 within 10 minutes. Rac1 activity is elevated 2-fold, as determined by densitometry, up to 40 minutes after attachment to FN. By 60 minutes, Rac1 activity is reduced to a basal level. Equivalent amounts of total Rac1 were present in the lysates

indicating that Rac1 expression is not affected by cell attachment. CHO/K1 cells overexpressing PTP-PEST exhibit a 2-fold decrease in levels of Rac1-GTP 45–50 minutes after attachment, while Rac1 activity was unaffected in GFP-transfected cells. In addition, GFP or PTP-PEST expression had no effect on the amount of total Rac1 expressed. Therefore, PTP-PEST overexpression suppresses integrin-dependent activation of Rac1.

We then examined the effect of PTP-PEST overexpression on PDGF-stimulated activation of Rac1. Control rat1 fibroblasts were serum starved and then stimulated with PDGF (50 ng/ml) for 5, 10, and 20 minutes. Rac1 activity peaked within 5–10 minutes after PDGF stimulation at 2–3-fold over basal levels (Fig. 6B). Rat1 cells overexpressing PTP-PEST showed a lower baseline activity of Rac1 and little if any activation in response to PDGF treatment (Fig. 5B). Therefore, PTP-PEST interferes with Rac1 activation both in response to integrin-mediated adhesion and growth factors.

Fibroblasts which lack PTP-PEST expression show enhanced cell spreading (Angers-Lousteau et al., 1999a). Therefore, we next determined whether PTP-PEST expression was required to regulate Rac1 activity. We examined Rac1 activity in PTP-PEST null fibroblasts (PTP-PEST $-/-$) or in null fibroblasts where PTP-PEST was re-expressed at wild-type levels (PTP-PEST-RE). Cells were plated on FN-coated dishes for times indicated (Fig. 6) in serum-free medium. As with CHO/K1 cells (Fig. 5A), Rac1 activity in control cells (PTP-PEST-RE) peaked within 30 minutes of plating on FN and returned to baseline by one hour. PTP-PEST null fibroblasts showed enhanced and sustained Rac1 activity compared to controls at each timepoint. Thus, PTP-PEST expression is required to downregulate Rac1 activity in response to adhesive stimuli.

Finally, to demonstrate that PTP-PEST utilizes a Rac1-dependent pathway to mediate its effects on morphology and motility, we next determined whether the effects of WT-PTP-PEST overexpression on cell spreading and migration could be counteracted by activated Rac1. WT-PTP-PEST and an activated mutant of Rac1, AU-tagged or GFP-tagged Q61LRac1, were co-expressed in CHO/K1 cells and then analyzed for cell spreading and migration. As shown in Fig. 7A, activated Rac1 ameliorated the ability of PTP-PEST-

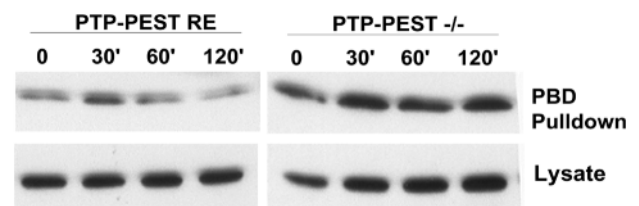


Fig. 6. PTP-PEST is required to regulate Rac1 activity. PTP-PEST null fibroblasts (PTP-PEST $-/-$) or null fibroblasts that re-express PTP-PEST (PTP-PEST RE) were maintained in suspension or plated on FN in serum-free medium for 30, 60 or 120 minutes. Rac1 activity was determined using the GST-PBD pulldown assay. In cells that re-express PTP-PEST (left), Rac1 is transiently activated by adhesion to FN within 30 minutes and then returns to baseline. In PTP-PEST $-/-$ cells (right), Rac1 is activated by adhesion after 30 minutes to a greater degree than in control cells. This elevated activity is sustained throughout the time course.

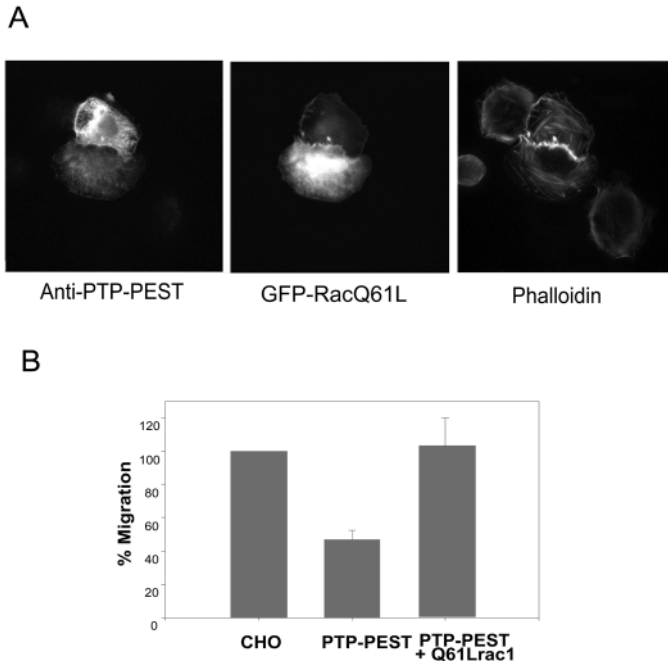


Fig. 7. Activated Rac1 overcomes the phenotypic effects of PTP-PEST overexpression. Wild-type PTP-PEST and GFP-tagged Q61LRac1 were co-expressed in CHOK1 cells and assayed for cell spreading and migration. (A) For cell spreading, co-transfected cells were plated on FN-coated coverslips for 45 minutes in serum-free medium. Cells were fixed and then triple-stained for PTP-PEST overexpression (left), GFP (middle) and actin (right). Compared with PTP-PEST overexpression alone (see Fig. 2C), co-expression of activated Rac1 resulted in cells that were able to spread. (B) Co-expression of activated Rac1 also restored the ability of PTP-PEST-transfected cells to migrate towards FN in a haptotaxis transwell assay.

transfected cells to spread on FN within 45 minutes. The percentage of co-transfected cells able to spread was approximately 80% compared with 15% when PTP-PEST was expressed alone. Actin staining shows that expression of activated Rac1 also restores membrane ruffling. In addition, CHOK1 cells co-expressing WT-PTP-PEST and Q61LRac1 regained the ability to migrate towards FN in a transwell assay (Fig. 7B). These data demonstrate that activated Rac1 can overcome the phenotypic effects of PTP-PEST. Activated forms of RhoA (Q63L) or cdc42 (Q61L) did not rescue the PTP-PEST phenotype (not shown). Thus, PTP-PEST functions to suppress Rac1-dependent responses at the leading edge of migrating cells.

Discussion

In this study, we examined the cellular and molecular mechanisms by which the cytoplasmic tyrosine phosphatase, PTP-PEST, controls cell motility. We found that PTP-PEST is activated in response to integrin-mediated cell adhesion. This catalytic activity is important for events that occur at the leading edge of a migrating cell including membrane protrusion and membrane. We further demonstrate that PTP-PEST overexpression suppresses motile responses to adhesive and growth factor stimuli. Finally, we show that the level of

PTP-PEST expression affects the activity of Rac1. Overexpression of PTP-PEST suppresses Rac1 activity, whereas targeted deletion enhances Rac1 activity. Thus, PTP-PEST controls motile events at the leading edge via modulation of Rac1.

One of the most prominent responses to integrin-mediated cell adhesion is an increase in tyrosine phosphorylation (Clark and Brugge, 1995; Schwartz et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996). Not only are tyrosine kinases such as FAK and Src family members activated, but this is paralleled by a general decrease in PTPase activity (Maher, 1993). The net result is an increase in the tyrosine phosphorylation of several proteins, many of which are concentrated in focal adhesions, sites where integrins are clustered and stress fibers anchored. PTP-PEST associates with and dephosphorylates at least two focal adhesion proteins, p130cas (Garton et al., 1996; Garton et al., 1997; Cote et al., 1998) and paxillin (Shen et al., 1998; Cote et al., 1999; Shen et al., 2000). We anticipated that PTP-PEST activity would be inhibited by integrin-mediated adhesion. Contrary to these expectations, we found that there was a stimulation of PTP-PEST activity during cell attachment to a FN matrix. The mechanism by which integrins activate PTP-PEST is unclear. A negative regulation of PTP-PEST occurs in response to its phosphorylation on serine by PKC or PKA (Garton and Tonks, 1994). Future studies will be aimed at exploring the phosphorylation state of PTP-PEST in response to integrin-mediated adhesion.

In the context where cell adhesion promotes tyrosine phosphorylation, the concomitant activation of a PTPase presents an apparent paradox. However, several PTPases have been implicated in the activation of Src family kinases (Liu et al., 1998; Arregui et al., 1998; Su et al., 1999; Cheng et al., 2001) indicating that these seemingly antagonistic classes of enzymes can act in parallel. PTP-PEST associates with the src inhibitory kinase, csk, although a functional role for this interaction has not been demonstrated (Davidson et al., 1997). In addition, PTP-PEST regulates the activity of c-abl (Cong et al., 2000), and in PTP-PEST null cells, FAK is hyperphosphorylated (Angers-Lousteau et al., 1999a). PTP-PEST activation may be necessary to limit the extent to which its substrates are phosphorylated. For example, enhanced tyrosine phosphorylation of p130cas, a major substrate of PTP-PEST (Garton et al., 1996; Cote et al., 1998), is associated with enhanced motility and invasiveness of pancreatic carcinomas (Klemke et al., 1998). Tyrosine phosphorylation of integrins and other focal adhesion proteins such as talin occurs as a result of v-src transformation (Hirst et al., 1986). Indeed, paxillin, p130cas, and FAK were also identified as hyperphosphorylated proteins in v-src transformed fibroblasts (Kanner et al., 1990). A safeguard mechanism whereby some PTPases, such as PTP-PEST, are activated in response to cell attachment would avert uncontrolled tyrosine kinase signaling which might otherwise result in excessive invasion.

Our findings indicate that the catalytic activity of PTP-PEST plays a significant role in its function during cell migration. Previous findings implicate PTP-PEST as a critical player in motility (Garton and Tonks, 1999; Angers-Lousteau et al., 1999a), however these studies did not distinguish the catalytic versus scaffolding activities of this phosphatase. To explore this question, we compared the consequences of

overexpressing catalytically active and inactive forms of PTP-PEST. We found that overexpression of wild-type PTP-PEST impaired haptotaxis towards a FN matrix. In contrast, overexpression of a catalytically inactive form did not block this type of migration, leading us to conclude that the effect on motility we observe is due to the catalytic activity of PTP-PEST. Interestingly, fibroblasts that are null for PTP-PEST expression display enhanced spreading but are blocked in their migration. Our observations with the C231S mutant only partially mimic the knockout phenotype. A significant difference between our system and the knockout cells is the presence or absence of the C-terminal scaffolding domain. It is conceivable that the inability of PTP-PEST null cells to migrate derives from the absence of the scaffolding domain, which may be either necessary or permissive for motility.

We found that PTP-PEST affects motile events at the leading edge. Specifically, we observed that overexpression of active PTP-PEST impaired cell spreading whereas the catalytically inactive mutant enhanced the number of membrane protrusions. Based on the knockout cells, which display increased spreading (Angers-Lousteau et al., 1999a), this result was not surprising. Using time-lapse video microscopy, we have seen that cells overexpressing wild-type PTP-PEST, while maintaining a round morphology after plating, are not quiescent in their membrane activity (S.K.S., unpublished). These cells extend small protrusions but then immediately retract them suggesting that the protrusions are not being stabilized. In contrast, overexpression of catalytically inactive PTP-PEST results in cells that continue to extend protrusions well past the timepoint where parental cells have ceased this activity. Finally, we found that PTP-PEST overexpression impairs membrane ruffling and actin reorganization in response to growth factors such as PDGF. Taken together, these results strongly support a role for PTP-PEST in regulating events at the leading edge of a migrating cell.

Several intracellular targets of PTP-PEST have been identified which might mediate the phenotypes described above. Besides p130cas, PTP-PEST substrates include paxillin (Shen et al., 2000), c-abl (Cong et al., 2000), PYK2/CAK β (Lyons et al., 2001; Davidson and Veillette, 2001), and PSTPIP, a WASP-binding protein (Spencer et al., 1997; Wu et al., 1998a). The functional relationship between PTP-PEST and these substrates is yet to be determined but each is likely to impinge on some aspect of cell motility. For instance, c-abl participates in membrane ruffling in response to growth factor stimulation (Plattner et al., 1999) and antagonizes migration under some conditions (Kain and Klemke, 2001). PSTPIP, through its association with WASP, can affect actin polymerization (Wu et al., 1998a; Cote et al., 2002). Additionally, paxillin's function in motility depends partly on its state of tyrosine phosphorylation (Yano et al., 2000; Petit et al., 2000).

We considered the possibility that PTP-PEST may act via a member of the Rho family of GTP-binding proteins. The involvement of PTPases in the regulation of Rho family proteins has been preceded by studies showing that Shp-2 acts upstream of RhoA (Schoenwaelder et al., 2000) and PTEN acts upstream of Rac and cdc42 (Liliental et al., 2000). A key protein regulating cell migration, membrane ruffling and spreading is Rac1 (Ridley et al., 1992; Hotchin and Hall, 1995; Anand-Apte et al., 1997; Keely et al., 1997; Price et al., 1998;

Clark et al., 1998). The phenotypic effects of PTP-PEST overexpression, are reminiscent of cells in which Rac1 is inhibited. Therefore, we investigated whether Rac activity was decreased in these cells. Rac1 is transiently activated in response to adhesion to FN at a time when these cells are actively spreading, extending lamellipodia, and ruffling (del Pozo et al., 2000). We found that overexpression of PTP-PEST results in decreased Rac activity in response to integrin-mediated adhesion. In contrast, we found that cells lacking PTP-PEST have enhanced Rac1 activity when allowed to spread on FN. Our observation of decreased Rac activity in response to PDGF stimulation indicates that PTP-PEST acts in both integrin and growth factor pathways leading to Rac. These data suggest that PTP-PEST expression is required to control membrane ruffling and protrusion through negative regulation of Rac1.

How might PTP-PEST act on Rac1? This could occur either directly or indirectly. Rac1 activity is regulated by GEFs and GAPs (Van Aelst and D'Souza-Schorey, 1997). One possibility is that PTP-PEST regulates the activity of a GEF or GAP by regulating its level of tyrosine phosphorylation. Some Rac GEFs, for example, VAV, are regulated by tyrosine phosphorylation (Crespo et al., 1997). Vav, specific to hematopoietic cells, is tyrosine phosphorylated in response to integrin clustering and cell adhesion (Cichowski et al., 1996; Zheng et al., 1996; Gotoh et al., 1997; Yron et al., 1999; Miranti et al., 1998). The more ubiquitously expressed family member, VAV2, is tyrosine phosphorylated in response to growth factors (Schuebel et al., 1998; Pandey et al., 2000; Liu and Burridge, 2000; Moores et al., 2000; Marignani and Carpenter, 2001). VAV or VAV2 stimulate membrane ruffling and motility when overexpressed (Miranti et al., 1998; Liu and Burridge, 2000; Marignani and Carpenter, 2001). Some GEF's are proto-oncogenes that can contribute to anchorage-independent growth (Schwartz et al., 1996) and a highly invasive phenotype (Michiels et al., 1995). It is attractive to speculate that a PTPase such as PTP-PEST may counteract these tumor promoters. We are currently investigating the possibility that PTP-PEST acts on a GEF for Rac1.

Interestingly, a link between the Cas/Crk complex and Rac has also been made. p130cas/crk induced cell motility can be perturbed by dominant negative Rac1 (Klemke et al., 1998). This suggests that p130cas or crk may recruit a protein involved in rac activation. Indeed, Crk interacts with DOCK180 (Kiyokawa et al., 1998a; Dolfi et al., 1998), a protein that has been shown to activate Rac (Kiyokawa et al., 1998b). Although it does not appear to be a Rac guanine nucleotide exchange factor (GEF) itself, DOCK180 binds to Rac and DOCK180 overexpression results in elevated Rac-GTP levels (Kiyokawa et al., 1998b). In future work, it will be interesting to investigate whether PTP-PEST affects the activity of DOCK180 or the activity of a Rac GEF downstream of DOCK180.

Additional pathways leading to Rac regulation downstream of PTP-PEST may involve paxillin. Similar to p130cas, paxillin recruits a Rac activating complex through its indirect association with PIX (Turner et al., 1999), a GEF for Rac and cdc42 (Manser et al., 1998). Alternatively, paxillin may participate in the targeting of active Rac1 to the membrane via an interaction with PKL, an ARF-GAP (Turner et al., 1999; Kondo et al., 2000). ARFs are small GTPases involved in

membrane trafficking which can potentiate the activity of Rho family proteins (Radhakrishna et al., 1999; Norman et al., 1998). Specifically, ARF6 affects Rac1-dependent membrane protrusion and actin reorganization (Radhakrishna et al., 1999). The region of paxillin that binds PKL has recently been shown to be important for Rac1 activation and membrane protrusion (West et al., 2001).

The data we have presented show that PTP-PEST plays a significant role in controlling cell migration. PTP-PEST is activated by integrin engagement and it can target to sites of membrane protrusion where it antagonizes membrane extension and membrane ruffling. PTP-PEST suppresses Rac1 to mediate these effects, thereby destabilizing the leading edge. Tyrosine phosphorylation events in response to integrin-mediated cell adhesion control multiple cell behaviors including migration. The misregulation of these tyrosine phosphorylation events is associated with tumorigenesis. Activation of PTP-PEST in response to adhesion may provide a mechanism to control the level of tyrosine kinase signaling from integrin receptors thereby maintaining cells in a normal state.

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