Protein-tyrosine Phosphatase PTPD1 Regulates Focal Adhesion Kinase Autophosphorylation and Cell Migration*

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PTPD1 is a cytosolic nonreceptor tyrosine phosphatase and a positive regulator of theSrc-epidermal growth factor transduction pathway. We show that PTPD1 localizes along actin filaments and at adhesion plaques. PTPD1 forms a stable complex via distinct molecular modules with actin, Src tyrosine kinase, and focal adhesion kinase (FAK), a scaffold protein kinase enriched at adhesion plaques. Overexpression of PTPD1 promoted cell scattering and migration, short hairpin RNA-mediated silencing of endogenous PTPD1, or expression of PTPD1 mutants lacking either catalytic activity (PTPD1C1108S) or the FERM domain (PTPD1A1-325) significantly reduced cell motility. PTPD1 and Src catalytic activities were both required for epidermal growth factor-induced FAK autophosphorylation at its active site and for downstream propagation of ERK1/2 signaling. Our findings demonstrate that PTPD1 is a component of a multivalent scaffold complex nucleated by FAK at specific intracellular sites. By modulating Src-FAK signaling at adhesion sites, PTPD1 promotes the cytoskeleton events that induce cell adhesion and migration.

Activation of tyrosine kinase receptor by a given growth factor initiates a highly orchestrated signaling cascade involving reversible phosphorylation/dephosphorylation of key cellular substrates. These signaling cascades control major cellular functions, including growth, differentiation, motility, development, and survival (1, 2). The tyrosine kinase pathway may be regulated at each point of the cascade (3). Protein-tyrosine phosphatases (PTPs) represent key regulatory molecules in kinase cascades. PTPs act both as inhibitors and enhancers of tyrosine kinase signaling. Several PTPs possess scaffold domains that assemble and target multienzymatic signaling complexes to specific intracellular compartments. Co-localization of PTPs with their substrates increases the rate, magnitude, and specificity of PTP-mediated dephosphorylation of a given target molecule, thus efficiently modulating specific cellular functions (4–7).

Motility allows a cell to change reversibly its interaction with other cells and with the extracellular matrix. This requires a coordinated process of assembly and disassembly of cell-to-matrix adhesion receptors and cell-cell interactions as well as a dynamic remodeling of the actin cytoskeleton. Several PTPs have been identified as major regulators of cell motility (8–17).

Protein-tyrosine phosphatase D1 (PTPD1) is a widely expressed cytosolic nonreceptor tyrosine phosphatase (18). PTPD1 cDNA encodes a protein of 1174 amino acids containing an N-terminal sequence homologous to the four-point-one ezrin-radixin-moesin (FERM domain) protein family, which includes PTPH1 and PTPMEG1 (Fig. 1A). The FERM motif is a modular structure present within a family of peripheral membrane proteins that link cytoskeleton to plasma membrane. The catalytic domain (PTP) is positioned at the extreme C terminus of PTPD1. An intervening sequence of about 580 residues without apparent homology to known proteins separates the FERM and the PTP domains. PTPD1 regulates signaling induced by Tec family members of membrane receptors (19) and associates with KIF1C, a tyrosine-phosphorylated kinesin-like protein that controls retrograde transport of vesicles from the Golgi apparatus to the endoplasmic reticulum (20). Expression of PTPD1 is significantly elevated in several human cancers (21).

PTPD1 associates with and activates Src, enhancing growth factor-induced signal transduction. PTPD1-Src complex also associates with AKAP121, a protein kinase A-anchoring protein that attaches to the outer mitochondrial membrane. The PTPD1-Src-AKAP121 complex is required for efficient maintenance of mitochondrial membrane potential and ATP oxidative synthesis. When not in complex with AKAP121, PTPD1 directs EGF-Src signaling to the nucleus, activating ERK1/2 and Elk1-dependent gene transcription (22–24).

In this report, we show that PTPD1 binds to and co-localizes with actin microfilaments and adhesion plaques. By manipulating the expression, localization, and activity of PTPD1, we have uncovered an important role of this phosphatase in the dynamic organization of actin cytoskeleton and in regulation of cell adhesion and motility.
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EXPERIMENTAL PROCEDURES

**Cell Lines**—The human embryonic kidney cell line (HEK293) was cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum in an atmosphere of 5% CO₂. Primary cultures of human fibroblasts were obtained from skin biopsies of healthy subjects and were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in an atmosphere of 5% CO₂. Human breast cancer cells (MCF-7) were obtained from the American Type Culture Collection (Manassas, VA). MCF-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamine (2 mM), insulin (60 ng/ml), cortisone (3.75 ng/ml). Cells were cultured in an atmosphere of 5% CO₂ at 37°C. NIH3T3 cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

**Antibodies**—Antibodies against the following proteins were used: phospho-Tyr-204 ERK1/2, ERK2, actin, and GST (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); hemagglutinin epitope (HA.11) (monoclonal; Covance); phospho-Tyr-397 FAK (BIOSOURCE International); FAK (for immunoblot, from Upstate Biotechnology (clone 2A7)); anti-Src antibody (monoclonal, Oncogene). A polyclonal antibody directed against human PTPD1 was raised as follows. A polypeptide segment spanning residues 751–910 of human PTPD1 was expressed and purified from BL21 was used to immunize rabbits. The specificity of the antibody was tested by Western blot (Fig. 1B) and immunofluorescence (see Fig. 1C). We also used an anti-PTPD1 antibody raised against the peptide sequence gctggtacc (KpnI) TGAGATGTAAAGGTGGCGGGACAG (KpnI) TGATGTCTCTGCCTAAACCCCAGCC; reverse, 5'-acgcgtcga (Sall) GATGAGCCCTGAGCTTTTCAG. PCR products were digested with the indicated restriction enzymes and cloned in the pEGFP-N3 vector (Clontech).

The plasmids were purified using Qiagen tip columns (Qiagen, Chatsworth, CA) and sequenced using the CEQ2000 DNA analysis system and a Beckman automated sequencer. Plasmids encoding for wild type Src and GST-SH2 fusion were kindly provided by Dr A. Migliaccio (II Medical School, Naples). Transfection of HEK293 and MCF-7 cells was performed using FuGENE 6 transfection reagent (Roche Applied Science), according to the manufacturer's instructions. Primary cultures of skin fibroblasts were transiently transfected using Effectorine (Qiagen).

**Immunoprecipitation and Immunoblot Analysis**—Cells were homogenized and sonicated in lysis buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 10 mM EDTA, 0.5% Nonidet P-40) containing aprotinin (5 μg/ml), leupeptin (10 μg/ml), pepstatin (2 μg/ml), 0.5 mM phenylmethanesulfonyl fluoride, 2 mM orthovanadate, 10 mM NaF. The lysates were cleared by centrifugation at 15,000 × g for 15 min. Cell lysates (1 mg) were subjected to immunoprecipitation using the anti-HA (2 μg/ml), anti-Src (4 μg/ml), or anti-FAK (4 μg/ml) antibody overnight at 4°C. Protein G-Sepharose (25 μl/sample) was added to the samples and incubated at 4°C for 60 min. The immunoprecipitates were washed three times with lysis buffer. Samples of cell lysates (100 μg) and the precipitates were resolved by SDS-PAGE, transferred to PROTRAN membrane, and immunoblotted with specific antibodies. Chemiluminescent (ECL) signals were quantified by scanning densitometry (Amersham Biosciences).

**GST Pull-down Experiments**—PTPD1 and its mutants were in vitro synthesized by the TNT quick-coupled transcription/translation system (Promega) in the presence of 5 μCi of [35S]methionine. Translated product was incubated overnight with 10 μl of GST or GST-SH2 polypeptides in buffer containing 20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 10 mM EDTA, 0.5% Nonidet P-40, supplemented with protease inhibitors and 2 mM orthovanadate. The pellets were washed four times in the binding buffer containing 500 mM NaCl and eluted in Laemmli buffer. The eluted samples were resolved in 10% polyacrylamide gels. Gels were fixed with methanol/acetic acid, enhanced with sodium salicylate, dried, and exposed to x-ray film. GST fusions were visualized by immunoblotting one-fifth of the eluted samples with anti-GST antibody.

**Immunofluorescence Analysis**—Cells were rinsed with PBS and fixed in paraformaldehyde for 20 min. After permeabilization with 0.5% Triton X-100 in PBS for 5 min, the cells were incubated with 1× phosphate-buffered saline plus 0.1 mg/ml bovine serum albumin for 60 min at room temperature. Double immunofluorescence was carried out with the following antibodies: TRITC-conjugated phalloidin (Sigma), anti-PTPD1 rabbit polyclonal, and anti-FAK. Fluorescein- or rhodamine-tagged anti-rabbit and anti-mouse IgG (Technogenetics) secondary antibodies were used. Coverslips were analyzed by confocal microscopy.

**Cell Migration, Invasion, and Wound Healing Assays**—Migration was assayed in a standard Transwell kit assay (Costar). 1 × 10⁵ cells were suspended in migration medium and added.
to the upper chamber. At selected time points, the cells were washed with phosphate-buffered saline, fixed in 3.7% formaldehyde for 20 min, and stained with crystal violet. Migrated cells were counted and scored. Cell invasion was tested using the Transwell kit assay as described above, with the modification that the polycarbonate membrane was coated with a thin layer of extracellular matrix (Matrigel; Collaborative Research Inc., Bedford, MA). The matrix layer occludes the membrane pores, blocking noninvasive cells from migrating through. Invasive cells, on the other hand, migrate through the matrix layer and cling to the bottom of the polycarbonate membrane. Cells migrated into the membrane were stained with crystal violet and counted. Wound healing assays were performed as follows. Confluent HEK293 cells stably transfected with either empty vector (CMV), PTPD1, or PTPD1C1108S expression vector were scratched with a pipette tip, and cells migrating into the wound were monitored as a function of time.

**FIGURE 1.** PTPD1 localizes along actin stress fibers. A, schematic representation of the PTPD1 protein. PTP, catalytic domain; AcR, acidic region; FERM, four point one-ezrin-radixin-moesin domain. B, lysates (100 μg) from human embryonic kidney cells were immunoblotted with anti-PTPD1 or preimmune serum. Where indicated, anti-PTPD1 antibody was preincubated with the immunogen. C, human fibroblasts were subjected to immunostaining with anti-PTPD1 antibody (raised against residues 751–910 of human PTPD1) or preimmune serum. Where indicated, the antibody was preincubated with the immunogen. D, human fibroblasts were subjected to double immunostaining with the following antibodies: Texas Red-conjugated phalloidin (a, d, g, and j) and anti-PTPD1 (raised against residues 618–631 of human PTPD1) (b, e, h, and k). The right panel of each set of the images is a merged composite of both signals. Bar, 5 μm.

**FIGURE 2.** PTPD1 localizes at the adhesion sites. A, human fibroblasts were subjected to double immunostaining with the following antibodies: anti-FAK (a) and anti-PTPD1 (b). The cells were subjected to hypoosmotic shock and subsequently immunostained with anti-FAK (d) and anti-PTPD1 (e) antibodies. The right panel (c and f) of each set of images is a merged composite of both signals (red and green). The arrows indicate adhesion plaques. Bar, 5 μm. B, HEK293 cells were serum-deprived overnight, stimulated with EGF for 30 min, and subjected to double immunostaining with anti-PTPD1 (b and e) and anti-FAK (a and d) antibodies. Right panels (c and f) show merge composites of both signals.

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**RESULTS**

**PTPD1 Localizes along Actin Filaments and at Focal Adhesion Sites**—PTPD1 contains a FERM domain located at its extreme NH2 terminus that is thought to mediate interaction with the actin cytoskeleton. To determine if PTPD1 associated with actin in situ, we performed immunocytochemical analysis using polyclonal antibodies directed against human PTPD1. The antibody specifically recognized PTPD1, as shown by immunoblot analysis (Fig. 1B) and immunofluorescence (Fig. 1C). Human fibroblasts were immunostained with anti-PTPD1 antibody, and actin was detected with TRITC-labeled phalloidin. As shown in Fig. 1D (a–c), PTPD1 staining co-localized in part with phalloidin along actin filaments. PTPD1 was also present in membrane extensions and cellular organelles where no actin staining was evident. Serum deprivation for 18 h disassembled actin microfilaments and dispersed phalloidin and PTPD1 signals throughout the cytoplasm (Fig. 1D, d–f).

Platelet-derived growth (PDGF) receptor activation is functionally coupled to the small Rho family GTPase, Rac (26). Activated Rac induces reorganization of the actin cytoskeleton, formation of stress fibers, and a “ring structure” at internal sites of actin reorganization.

**RNA Interference**—Two ~70-bp DNA inserts encoding short hairpin RNAs (shRNAs) targeting human PTPD1 (nucleotides 184–204 and 335–355, ATG +1) were subcloned in the pRNA-H1/hygro vectors (GenScript Corp.). A Blast search confirmed that both sequences specifically recognize human PTPD1. A scrambled sequence subcloned in the same vector was used as control. shRNA vectors were transiently transfected using the Lipofectamine protocol. Cells were harvested at 72 h after transfection, and down-regulation of endogenous PTPD1 was monitored by immunoblot analysis with anti-PTPD1 antibody.

**Hypoosmotic Shock (Lysis Squirting)**—Hypoosmotic shock was performed as previously described (25). Human fibroblasts were grown overnight on coverslips. Cells were then rinsed in Pipes buffer (20 mM Pipes, 100 mM KCl, 5 mM MgCl2, 3 mM EGTA, pH 6.0) and incubated in a 20% Pipes buffer (1:5 dilution of Pipes buffer) for 10 min. This procedure caused osmotic swelling of the cells and destabilization of plasma membranes. Dorsal membranes and most cellular contents were removed with Pipes buffer, pH 7.0. Coverslips were then rapidly fixed with 4% paraformaldehyde in Pipes, pH 7.0, and subjected to immunofluorescence analysis.

**Focal Adhesions**—Focal adhesions are cellular loci where actin filaments concentrate and create adhesive links between cell membrane and extracellular matrix. Scaffold proteins assemble multifunctional platforms at these structures, where distinct signaling pathways are generated and focused (27, 28). Since PTPD1 co-localizes with actin filaments, we asked if PTPD1 might also be concentrated at adhesion plaques. As shown in Fig. 2A, PTPD1 staining partly overlaps with that of FAK (a–c), a major component of adhesion plaques. To prove that PTPD1 physically interacts with these loci, the cells were lysed by hypoosmotic shock. This procedure disperses cellular membrane, organelles, and actin filaments but leaves some adhesion plaques intact, as shown by immunostaining with anti-FAK antibody (Fig. 2A, d). Treated cells retained some PTPD1 staining, which clearly overlapped with FAK (Fig. 2A, e and f).
Since PTPD1 modulates EGF signaling, we asked whether EGF stimulation regulates PTPD1 localization. HEK293 cells express functional EGF receptor that couples growth factor signaling to reorganization of actin cytoskeleton and adhesion plaque formation. HEK293 cells were serum-deprived overnight, exposed for 30 min to EGF, and immunostained with anti-FAK and anti-PTPD1 antibodies. Fig. 2B (a–c) shows that PTPD1 and FAK co-localized at adhesion sites. Moreover, some PTPD1 staining was evident at plasma membrane and at actin-enriched sites (Fig. 2B, d–f).

PTPD1 Interacts with Actin, Src, and FAK—We then demonstrated biochemically that actin forms a complex with PTPD1. Cell lysates prepared from HEK293 cells transiently transfected with empty vector (CMV) or with HA-PTPD1 vector were immunoprecipitated with anti-HA antibody. Fig. 3A shows that anti-HA antibody co-precipitated PTPD1 and actin. Interaction with actin requires the FERM domain; a PTPD1 mutant lacking residues 1–325 (PTPD1Δ1–325) failed to pull-down significant amounts of actin.

Although PTPD1 was demonstrated to associate with Src, the interacting domains of the two proteins were not defined (18). To identify these domains, HEK293 cells were transiently transfected with empty vector (CMV) or HA-PTPD1 vectors and harvested 24 h later. Cell lysates were subjected to immunoprecipitation with anti-Src antibody. As shown in Fig. 3B, PTPD1 and Src form a stable complex in the cell extract. In contrast, the PTPD1Δ1–325 mutant did not associate with Src. PTPD1 activity was not required for Src binding, since the catalytic inactive mutant of PTPD1 (PTPD1C1108S; see below) was efficiently immunoprecipitated by anti-Src antibody (data not shown).

The PTPD1 FERM domain contains two highly conserved SH2 binding motifs (Y158ESQ and Y217GEE) that may mediate interaction with Src (29). We then performed a pull-down assay using GST-SH2 and lysates from HEK293 cells that expressed endogenous PTPD1 (Fig. 3C). Again, PTPD1 was recovered in association with GST-SH2 (Fig. 3C, column 1). We also found that
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PTPD1 phosphorylation and binding to Src SH2 were regulated by EGF. Thus, serum deprivation decreased tyrosine phosphorylation of PTPD1 by 2-fold and its binding to Src SH2 by 55–60% (Fig. 3C, column 2). Phosphorylation and binding were restored by EGF stimulation (Fig. 3C, column 3). Furthermore, we found that EGF-stimulated SH2 interaction to PTPD1 requires the FERM domain (Fig. 3D).

PTPD1 and FAK co-localize at specific subcellular sites, suggesting that both proteins may be part of the same complex in vivo. To demonstrate this association biochemically, lysates from HEK293 cells were subjected to co-immunoprecipitation with anti-FAK and anti-PTPD1 antibodies. As shown in Fig. 4A, endogenous PTPD1 and FAK form a stable complex in vivo. These experiments were replicated using an epitope-tagged PTPD1 transgene. Fig. 4B confirmed the interaction between FAK and exogenous HA-tagged PTPD1. Interestingly, a PTPD1 mutant lacking the FERM domain (PTPD1Δ31–325) still bound FAK. This implies that Src and FAK bind distinct molecular modules on PTPD1.

To identify the PTPD1 domain that interacts with FAK, we performed experiments using in vitro translated, 35S-labeled recombinant proteins. As shown in Fig. 4C, anti-FAK antibody efficiently co-precipitated both PTPD1 and the FERM mutant (PTPD1Δ1–325). However, a PTPD1 mutant lacking residues 1–581 (PTPD1Δ1–581) did not complex with FAK.

Next, we mapped the PTPD1 binding domain on FAK. Two segments of FAK, the FERM domain and the COOH terminus (FRNK) fused to GST polypeptide (30) were used as bait for pull-down experiments with labeled PTPD1. Fig. 4D shows that the FAK FERM domain directly binds to PTPD1 (left). No binding between the FRNK segment and PTPD1 was detected. Furthermore, residues 329–587 on PTPD1 were sufficient to interact with the FAK FERM domain (Fig. 4D, right).

To investigate if the FAK binding domain (residues 325–580) was required to target PTPD1 to adhesion sites, we generated recombinant genes carrying green fluorescent protein fused to the COOH terminus of PTPD1Δ1–325 or PTPD1Δ1–600. Human fibroblasts were transiently transfected with the GFP fusions and subsequently immunostained with anti-FAK antibody. Fig. 4E (a–c) shows that PTPD1Δ1–325-GFP fusion partly co-localized with FAK at the adhesion sites. In contrast, PTPD1 deleted for the FAK binding domain (PTPD1Δ1–600-GFP) failed to co-localize with FAK and instead dispersed throughout the cytoplasm and nucleus (Fig. 4E, d–f). We noticed that full-length PTPD1-GFP formed large intracytoplasmic precipitates, perhaps reflecting drastic conformational changes occurring within the structure of PTPD1 when fused to GFP (data not shown).

PTPD1 Activity Regulates Actin Filament Stability—PTPD1 interacts with and localizes along the actin filaments and adhesion sites. This typical localization pattern led us to investigate whether PTPD1 activity was necessary for the assembly/stability of actin filaments. Fibroblasts were transfected with vectors encoding PTPD1 or the catalytic inactive PTPD1 mutant (PTPD1C1108S). To identify transfected cells, a vector encoding GFP was included in the transfection mixture. 48 h after transfection, cells were fixed and stained with TRITC-conjugated phalloidin. Transfection with wild type PTPD1 (Fig. 5, g–i) did not affect the number of actin filaments or cellular appearance compared with transfection with vector (Fig. 5, d–f). In contrast, expression of PTPD1C1108S drastically reduced the actin filaments (Fig. 5, a–c). These findings, summarized in Fig. 5, bottom, suggest a physical and functional interaction between PTPD1 and actin filaments.

PTPD1 Activity Is Required for FAK Autophosphorylation and Adhesion Plaque Stability—Src is both an upstream regulator and downstream effector of FAK. Integrin engagement or stimulation by growth factor(s) induces FAK autophosphorylation at Tyr-397, creating a high affinity binding site for the Src SH2 domain. Src then phosphorylates FAK at several tyrosine residues (Tyr-576, Tyr-577, Tyr-861, and Tyr-925), enhancing FAK activity (28, 31, 32). As an upstream regulator, Src positively controls FAK autophosphorylation by regulating the cellular response to integrin or EGF. We have shown that PTPD1

FIGURE 5. PTPD1 activity is required for actin filament stability. Vectors encoding for PTPD1Δ1–325 (a–c), PTPD1 (g–i), and GFP (green) were co-transfected at a DNA molar ratio of 3:1 in human fibroblasts. Empty vector (CMV) co-transfected with GFP was used as control (d–f). 24 h after transfection, cells were fixed and stained with TRITC-conjugated phalloidin. The right panel of each set of images is a merged composite of both signals. Bar, 7 μm. The data shown are representative of 45–80 cells scored for each set of experiments.
forms a complex with FAK and Src (see Figs. 2–4). We postulated that PTPD1, by targeting Src to FAK, might regulate biochemical events at adhesion sites. To test this notion, HEK293 cells were stably transfected with HA-PTPD1, HA-PTPD1C1108S, or CMV vector. Transgene expression, monitored by Western blot analysis using anti-HA antibody, was shown to be comparable in the two transfectants. Cells were serum-deprived overnight and harvested at various times after EGF stimulation. As shown in Fig. 6 (A and B), expression of PTPD1 slightly increased FAK autophosphorylation at Tyr-397. However, PTPD1C1108S strongly reduced FAK autophosphorylation in response to EGF.

Next, we asked if Src activity was required for PTPD1 signaling to FAK. As a first approach, we evaluated FAK phosphorylation in EGF-stimulated and transfected cells that were pretreated with PP2, a potent and selective inhibitor of Src family kinases. Fig. 6C shows that PP2 reduced EGF-dependent FAK autophosphorylation in PTPD1-transfected cells.

Second, we postulated that PTPD1C1108S inhibits EGF signaling by sequestering Src in an inactive form. In this model, overexpressed Src would interact with and titrate the mutant PTPD1, restoring EGF signaling. We tested this hypothesis using HEK293 cells that were transiently transfected with PTPD1C1108S vector and/or wild-type Src vector. 48 h after transfection, cells were serum-deprived overnight, stimulated with EGF, and harvested at the indicated times. Immunoblot analysis showed that EGF stimulation enhanced FAK autophosphorylation in HEK293 cells in a time-dependent manner (Fig. 6D, lanes 1–3). EGF simulation of FAK autophosphorylation was stimulated by Src (lanes 4–6). As expected, PTPD1C1108S decreased EGF-induced FAK autophosphorylation (lanes 7–9). In contrast, the kinetics and extent of FAK phosphorylation in cells co-transfected with Src and PTPD1C1108S was equivalent to that of cells transfected with Src alone (lanes 10–12). These data indicate that Src mediates, at least in part, PTPD1 stimula-

Further evidence that PTPD1 is, indeed, a physiological modulator of FAK signaling was obtained by down-regulating PTPD1 expression using RNA interference. We designed DNA vectors that encoded two shRNAs targeting distinct segments
PTPD1 Promotes Cell Motility—Having demonstrated that PTPD1 physically and functionally associates with actin cytoskeleton, we investigated the role of PTPD1 in biological events that require proper actin condensation. The epithelial-mesenchymal transition depends on EGF activation and Src-dependent signaling and is associated with increased cell motility (3, 33–36). Since PTPD1-Src up-regulates the EGF pathway, we suspected that PTPD1 might promote cell motility. HEK293 cells are migratory and express integrin subunits that act as receptors and intracellular transducers of matrix ligands (3). First, we performed a wound-healing assay by scraping a confluent dish with a pipette tip, creating a cell-free space. Migration of the bordering cells into the wound was followed over time. Fig. 8A shows that cells stably expressing PTPD1 migrate significantly faster than controls, closing the gap at around 36 h after wounding. Conversely, expression of PTPD1C1108S dramatically slowed migration. Transgene expression was equivalent in the two clones (Fig. 8A, bottom).

We performed a second migration test using the Transwell apparatus. As shown in Fig. 8B, cell migration was inhibited by PP2, indicating the role of Src in this pathway. Migration was increased by PTPD1 in an Src-dependent fashion. PTPD1 catalytic activity was required; PTPD1C1108S inhibited migration. To ask if PTPD1 enhancement of cell motility necessitated interaction with actin filaments, we assayed cells expressing untransfected cells), 35 focal adhesions in 19 cells (CMV), 28 focal adhesions in 22 cells (PTPD1), 23 focal adhesions in 31 cells (C1108S), or 31 focal adhesions in 24 cells (PTPD1Δ1–325). Fluorescence intensity of individual focal adhesions was calculated using Meta-Morph/MetaFluor Imaging System software (Universal Imaging). Cumulative data are expressed as mean ± S.E. and are relative to control untransfected (GFP-negative) cells that were set as 100.

To establish the role of PTPD1 in vivo, we analyzed adhesion plaques in fibroblasts transiently transfected with either PTPD1, PTPD1C1108S, or PTPD1Δ1–325. Plaques were visualized by immunostaining cells with anti-phospho-Tyr-397 FAK antibody or anti-paxillin, a major adhesion plaque component. To monitor transgene expression, a vector expressing GFP was included in the transfection mixture. Fig. 7A shows that cells transfected with control (A and B) or PTPD1 vector (C and D) immunostain for adhesion plaques in a fashion similar to that of control, untransfected cells. In contrast, transfection with PTPD1C1108S (E and F) and, to a lesser extent, PTPD1Δ1–325 (G and H) significantly reduced the number and the size of adhesion plaques. Quantitative analysis of these experiments is summarized in Fig. 7B. Taken together, these findings lend further support to the role of PTPD1 in regulation of adhesion plaque formation/stability.

PTPD1 activity is required for adhesion plaque stability. A, fibroblasts were transiently co-transfected with GFP and the indicated vectors, immunostained with anti-phosphotyrosine 397 FAK antibody or anti-paxillin, and analyzed by confocal microscopy. Bar, 5 μm. Magnification of the images is shown in the middle and right. B, focal adhesion disassembly was determined from values obtained from 34 focal adhesions in 17 cells (control untransfected cells), 35 focal adhesions in 19 cells (CMV), 28 focal adhesions in 22 cells (PTPD1), 23 focal adhesions in 31 cells (C1108S), or 31 focal adhesions in 24 cells (PTPD1Δ1–325). Fluorescence intensity of individual focal adhesions was calculated using Meta-Morph/MetaFluor Imaging System software (Universal Imaging). Cumulative data are expressed as mean ± S.E. and are relative to control untransfected (GFP-negative) cells that were set as 100.
PTPD1 controls cell motility and invasion. A, wound-healing assay. Confluent HEK293 cells stably transfected with either empty vector (CMV), PTPD1, and PTPD1C1108S expression vector were wound healed with a pipette tip, and migrating cells were filmed at the indicated time from wounding. A representative set of two independent experiments is shown. Bar, 100 μm. B, motility assays on growing HEK293 cells transiently transfected with the following vectors: CMV, PTPD1, PTPD1C1108S, PTPD1Δ1–325, shRNAPTPD1, and shRNAcontrol. 24 h after transfection, cells were plated on a Transwell apparatus. Where indicated, the cells were treated for 6 h before harvesting with the Src inhibitor, PP2. 24 h after plating, migrated cells were fixed, stained, and counted. Similar data were obtained performing motility assay at 12 h from plating (data not shown). C, same as in B, except the motility assay was performed in medium supplemented with 1% serum and EGF (100 ng/ml). Experiments from B were replicated in NIH3T3 mouse fibroblasts (D) and in mammary tumor-derived cells (MCF-7) (E). F, matrix invasion assay on control and PTPD1C1108S-transfected MCF-7 cells. The experiment was performed in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cumulative data are presented as mean ± S.E. of 3–5 independent experiments. Values from control (CMV) cells were set as 100 (arbitrary units). Lower panels (from A–F) show expression analysis of HA-tagged transgenes (arrows) or endogenous PTPD1 (B, upper right) and ERK (B, lower right).

PTPD1Δ1–325, a mutant lacking the FERM domain. This mutant does not bind actin and Src (see Fig. 3A). Fig. 8B shows that PTPD1Δ1–325 significantly reduced cell motility. Finally, shRNA knockdown of PTPD1 inhibited cell motility. Transgene expression, monitored by immunoblotting, was similar in all samples (Fig. 8B; see also Fig. 8, B–E, lower panels).

Since PTPD1 is a positive modulator of EGF signaling, the Transwell experiment was replicated using EGF as stimulus. Fig. 8C shows that PTPD1 increases, whereas PTPD1C1108S decreases, EGF-dependent motility, at the two time points (12 and 24 h after EGF exposure) tested. The effects of PTPD1 and PTPD1C1108S on cell motility were reproduced in NIH3T3 mouse fibroblasts (Fig. 8D), MCF-7 human mammary adenocarcinoma cells (Fig. 8E), and in other cell types, including human bladder cancer and mouse melanoma cells (data not shown).

In cancer cells, motility is functionally coupled to extracellular matrix invasion (3). Therefore, we asked if PTPD1 stimulates invasiveness in MCF-7 cells using an in vitro reconstituted extracellular matrix. In this assay, cells are seeded on a polycarbonate membrane coated with a thin layer of Matrigel, and movement into the Matrigel is monitored over time. As shown in Fig. 8F, PTPD1C1108S significantly reduced tumor cell invasion at the two time points tested.

DISCUSSION

The findings presented here demonstrate for the first time that the protein-tyrosine phosphatase, PTPD1, is a regulatory component of the actin cytoskeleton. PTPD1 partly localizes along the actin filaments and at adhesion plaques. It also forms a complex with actin, Src tyrosine kinase, and FAK. Functional studies disclosed an important role of PTPD1 in the regulation of actin filaments, FAK signaling, and cell motility.

PTPD1 belongs to the FERM-PTP family of tyrosine phosphatases, which is characterized by a four-point-one/ezrin/radixin/moesin domain. The FERM domain is a modular structure that mediates interaction between membrane glycoproteins and actin filaments. We showed that the PTPD1 FERM domain is required for association with actin filaments. Remodeling of the actin cytoskeleton underlies important biological functions, including cell spreading and motility, vesicle transport, growth cone guidance, and cell division. The dynamic assembly and
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disassembly of actin filaments is a complex process that requires the coordinated modulation of key effector molecules (37–41). The typical localization pattern of PTPD1 along the actin filaments reflects its role in regulation of actin cytoskeleton remodeling and cell-to-matrix contact sites. Consistent with this idea, expression of a PTPD1 catalytically inactive mutant in fibroblasts disassembled actin filaments and reduced the number of adhesion plaques.

FAK is a high molecular weight scaffold protein kinase that is localized principally at adhesion sites. It is essential for turnover of focal adhesions and for cell motility. We found that PTPD1 interacts with FAK and regulates its autophosphorylation and signaling. The FAK binding domain (residues 329–587; see Fig. 1) lies COOH-terminal to the PTPD1 FERM domain. The FAK binding domain was necessary and sufficient to bind FAK in vitro and is required to localize PTPD1 at adhesion sites in vivo. Conversely, the PTPD1 binding domain lies within the FERM segment of FAK. PTPD1 residues 323–581 contain a proline-rich motif (P60PPPYPPRPR77) and six PYX motifs (where X represents a nonpolar residue). These motifs may serve as substrates of tyrosine kinases and could be involved in Src homology 3 binding activities (18, 42). Interestingly, the FAK FERM domain associates with proline-rich regions of a variety of signaling proteins, including integrins, growth factor receptors, and Tec family kinase Etk (43–46). Thus, PTPD1 may complex to FAK via proline-rich motifs.

Growth factors or components of extracellular matrix induce FAK phosphorylation at multiple sites and its consequent activation. Activated FAK undergoes autophosphorylation at Tyr-397, thereby creating a docking motif for the Src SH2 domain. FAK-Src complex promotes tyrosine phosphorylation of several substrates, including paxillin, p130Cas, and p190RhoGAP, and activates downstream signaling cascades (47–49). Our findings indicate that PTPD1 activity is required for EGF stimulation of FAK autophosphorylation and signaling. Inhibition of Src abrogated PTPD1 signaling, whereas overexpression of Src bypassed the block to FAK autophosphorylation imposed by PTPD1C1108S.

These findings support a model whereby PTPD1, in response to EGF, activates and recruits Src close to FAK. By sustaining and focusing the EGF pathway, the PTPD1-Src complex may generate a local positive loop between growth factor signaling and FAK. This regulatory feedback might play a significant role in the initial formation of focal adhesions (Fig. 9). Although Src represents an important effector of PTPD1, additional mechanism(s) modulated by this phosphatase may also operate in vivo.

In response to EGF, FAK and Src cooperate to induce cell scattering and migration (28). We find that PTPD1 promotes growth factor-dependent cell motility. Silencing of endogenous PTPD1 or expression of PTPD1 mutants lacking either catalytic activity (PTPD1C1108S) or the FERM domain (PTPD1Δ1–325) significantly reduced cell motility. FAK and Src are implicated in several aspects of tumorigenesis, and their levels and activity directly correlate with the invasive potential of human cancer (3). Given its role in motility, we hypothesized that PTPD1 might be involved in tumor cell invasion. Accordingly, expression of PTPD1 inactive mutant (PTPD1C1108S) in MCF-7 human breast cancer cells inhibited cell migration and extracellular matrix invasion. Finally, PTPD1 levels are significantly elevated in human bladder cancers tested, and the extent of elevation correlates with tumor stage.

In conclusion, our observations reveal an unanticipated role of PTPD1 in actin cytoskeleton remodeling and directly implicate this phosphatase in critical aspects of cell-cell communication, adhesion to extracellular matrix, and cell motility. Deregulation of PTPD1-FAK signaling may represent an early event in cancer progression and metastasis in vivo. The identification of molecular targets and the exact role of PTPD1 in critical aspects of tumor biology represent important goals for future investigation.

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