Integrin-induced Tyrosine Phosphorylation of Protein-tyrosine Phosphatase-α Is Required for Cytoskeletal Reorganization and Cell Migration*

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Protein-tyrosine phosphatase-α (PTPα) activates Src family kinases (SFKs) to promote the integrin-stimulated early autophosphorylation of focal adhesion kinase (FAK). We report here that integrin stimulation induces tyrosine phosphorylation of PTPα. PTPα was dephosphorylated upon fibroblast detachment from the substratum and rephosphorylated when cells were plated on the integrin ligand fibronectin. PTPα phosphorylation occurred at Tyr789 and required SFKs (Src or Fyn/Yes), FAK, and an intact cytoskeleton. It also required active PTPα or constitutively active Src. These observations indicate that PTPα activates SFKs and that the subsequently activated SFK-FAK tyrosine kinase complex in turn phosphorylates PTPα. Reintroduction of wild-type PTPα or unphosphorylatable PTPα(Y789F) (but not inactive PTPα) into PTPα-null fibroblasts restored defective integrin-induced SFK activation, FAK phosphorylation, and paxillin phosphorylation. PTPα(Y789F) and inactive PTPα could not rescue delayed actin stress fiber assembly and focal adhesion formation or defective cell migration. This study distinguishes two roles of PTPα in integrin signaling: an early role as an activator of SFKs and FAK with no requirement for PTPα phosphorylation and a later downstream role in cytoskeleton-associated events for which PTPα phosphorylation at Tyr789 is essential.

Engagement of the receptor integrins by extracellular matrix ligands determines multiple cellular responses, notably those required for the complex process of cell movement. Many of these responses are mediated through early, tyrosine phosphorylation-based activation of the central signaling molecule focal adhesion kinase (FAK), and require the participation of Src family kinases (SFKs) (1, 2). Integrin-stimulated phosphorylation of FAK and the ensuing formation of the SFK-FAK tyrosine kinase complex result in further phosphorylation and maximal activation of FAK, phosphorylation of FAK at sites that enable proteins such as Grb2 to associate with FAK, and phosphorylation of other substrates such as Cas and paxillin to promote further protein-protein associations and signaling events. Phospho-FAK ultimately serves as a scaffold and coordinating center that regulates focal adhesion formation and disassembly, actin stress fiber and cytoskeletal organization, and dynamic alterations in cell shape.

The precise molecular events linking integrins to the initial and essential phosphorylation of FAK at Tyr397 are still not well defined. Phosphorylation of FAK at this site can be accomplished by autophosphorylation (3), but maximal Tyr397 phosphorylation requires SFKs that participate in an autocatalytic loop by phosphorylating FAK at Tyr576 and Tyr577 to enhance FAK activity and thus promote full autophosphorylation (4–6). The essential role of SFKs in FAK activation is demonstrated by the attenuated FAK autophosphorylation in response to integrin engagement in triple knockout fibroblasts lacking the SFKs Src, Fyn, and Yes (7) or in cells treated with the SFK inhibitor PP2 (8).

SFK activity is constrained by intramolecular interactions involving binding of the SFK SH2 domain to the C-terminal phosphotyrosyl residue (in Src, Tyr527) and binding of the SFK SH3 domain to a region between the SH3 and kinase domains (9–11). In this state, the regulatory tyrosine residue in the activation loop (in Src, Tyr416) is hypophosphorylated. Events that disrupt these interactions lead to conformational changes and the ensuing dephosphorylation of Tyr527, autophosphorylation of Tyr416, and kinase activation. Several mechanisms of integrin-dependent SFK activation have been described, in accord with the physical and functional requirement for SFKs in efficient FAK Tyr397 phosphorylation. FAK itself has been proposed to participate in Src activation, where early low level FAK Tyr397 autophosphorylation enables Src SH2 domain binding and the consequent disruption of Src intramolecular inhibitory constraints to promote Src activity (3). A direct linkage between β integrins and Src (mediated through the Src SH3 domain) may likewise destabilize Src intramolecular inhibitory interactions to permit Src autophosphorylation and activation upon integrin clustering (12, 13). Other SFKs associate with other integrin β-subunits, raising the possibility that activation of these kinases may occur in specific yet similar fashions. Another sequence of events involves the receptor protein-tyrosine phosphatase-α (PTPα), a physiological activator of SFKs (14, 15). Integrin-stimulated FAK Tyr397 phosphorylation is impaired in PTPα-null fibroblasts, demonstrating that PTPα functions as an integrin-proximal upstream regulator of FAK (16). Inhibition of SFKs abrogates FAK Tyr397 phosphorylation (8), and SFK activity is reduced in PTPα−/− fibroblasts (14, 15), suggesting that PTPα dephosphorylates and activates SFKs, which then phosphorylate and activate FAK (16). Src activation through association with FAK or β integrins is accompanied by hypophosphorylation of the inhibitory C-terminal Tyr527 residue of Src (13, 17), a target site for dephosphorylation by PTPα (18, 19).
Thus, FAK- or integrin-mediated SFK activation and PTPα-catalyzed SFK activation may be interconnected rather than exclusive mechanisms of SFK activation in integrin signaling.

How integrin engagement functionally alters PTPα to enable or to regulate subsequent signaling events is unknown. We report here that fibronectin-induced integrin stimulation results in the increased tyrosine phosphorylation of PTPα at Tyr789. Our investigation of the role of this in integrin signaling demonstrates that it is not required for PTPα-mediated activation of SFKs or for efficient FAK Tyr979 phosphorylation. However, it is necessary for actin stress fiber assembly and focal adhesion formation involved in cytoskeletal reorganization and for cell migration. These findings identify and distinguish two roles for PTPα in integrin signaling: an early upstream role in promoting FAK autophosphorylation and SFK activation that is independent of PTPα tyrosine phosphorylation and a second role in focal adhesion formation and cytoskeletal alterations requiring PTPα tyrosine phosphorylation that is likely mediated by the active SFK-FAK complex.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture—**PTPα+/+, (wild type), PTPα−/−, and Src−/− mouse embryonic fibroblasts were derived from the appropriate mouse embryos and spontaneously immortalized. These cell lines were used at passages 30–40 for the experiments described here. SYF (Src−/−/Fyn−/−/Yes−/−), Src+/- (Src+/−/Fyn−/−/Yes−/−), FAK+/-, and FAK−/− mouse embryonic fibroblast cell lines were obtained from American Type Tissue Collection. The cells were all grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, penicillin, and streptomycin. Prior to stimulation by fibronectin (FN), cells were starved in Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum for 18 h. In one experiment, cells were pretreated with 3 μM cytochalasin D (Sigma) for 20 min before trypsinization. The same amount of cytochalasin D was added during suspension and plating of cells on FN-coated dishes in serum-free medium.

**Expression Plasmids—**The mammalian expression vectors pXJ41-PTPα-neo and pXJ41-PTPα(C433S/C723S)-neo have been described previously (18, 20). The pXJ41-PTPα(Y789F)-neo plasmid was created by site-directed mutation of the appropriate PTPα-encoding nucleotides in the plasmid pGEX-KG-PTPα (21), and the PTPα mutant was excised and cloned into pXJ41-neo. Chicken c-src cDNA with a Y527F mutation was excised from the pLNCX vector (a gift from J. S. Brugge) and cloned into pXJ41-neo. The PTPα−/− cells were transfected with these plasmids using Lipofectamine™ reagent (Invitrogen).

**Antibodies and Immunological Detection Reagents—**Rabbit anti-PTPα antisera 2205 has been described previously (22). Antibodies to phosphoysosine (PY20), FAK, paxillin, and Fyn used for immunoblotting were purchased from BD Transduction Laboratories. Phosphorylation site-specific antibodies to FAK Tyr979 and Tyr397 and Src Tyr418 and Tyr416 were from BIOSOURCE. Anti-dephospho-Src antibody (SRC2) and anti-Fyn antibody used for immunoprecipitation were purchased from Santa Cruz Biotechnology, Inc. Anti-v-Src antibody (Ab-1) was from Oncogene Research Products. Anti-vinulin antibody was purchased from Sigma. Alexa Fluor 488-conjugated phallolidin (F-actin staining) and Alexa Fluor 594-conjugated anti-mouse IgG were from Molecular Probes.

**Anti-PTPα Phospho-Tyr789 Antibody—**Anti-PTPα phospho-Tyr789 antibody was custom-made by 21st Century Biochemicals (Marlboro, MA). Rabbits were immunized with the phosphoysosine peptide CYIDAF5DPY789ANFK (sequence confirmed by MS Check™) conjugated to keyhole limpet hemocyanin. To ensure specificity, the sera obtained from immunized animals were subjected to multiple rounds of immunodepletion by passage through an affinity column of immobilized non-phosphopeptide antigen. This was followed by affinity purification using a column with the phosphopeptide antigen as ligand.

**Plating on Extracellular Matrix—**FN (10 μg/ml; Chemicon International, Inc.) and poly-l-lysine (20 μg/ml Sigma) were diluted in phosphate-buffered saline. Diluted FN and poly-l-lysine were added to tissue culture dishes and incubated overnight at 4 °C. Before use, the dishes were washed twice with serum-free Dulbecco’s modified Eagle’s medium and incubated at 37 °C for 1 h. Cells were serum-starved overnight and detached with 0.05% trypsin and 0.35 mM EDTA (Invtrogen). The trypsin action was stopped with 0.5 mg/ml soybean trypsin inhibitor in Dulbecco’s modified Eagle’s medium containing 0.5% bovine serum albumin, and the cells were then washed twice with serum-free medium. After centrifugation, the cells were resuspended in serum-free medium containing 0.1% bovine serum albumin and maintained at 37 °C for 1 h. Suspended cells were plated onto extracellular matrix-coated plates (105 cells/ml) and incubated at 37 °C for various times.

**Cell Migration Assay—**Haptotactic migration assays with FN were carried out as described previously (16). Cells (PTPα+/+, PTPα−/−, and PTPα−/− infected with recombinant adenoviruses expressing different forms of PTPα) were serum-starved overnight prior to use in these assays, and cells (1 × 106/ml) were resuspended in 0.1 ml of serum-free Dulbecco’s modified Eagle’s medium with 0.5% BSA and added to each upper chamber. After 2 h of incubation at 37 °C, the cells were washed, fixed, stained, and counted.

**Cell Lysis, Immunoprecipitation, and Immunoblots—**Cells were lysed in modified radioimmune precipitation assay buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and processed for immunoprecipitation and immunoblotting experiments as described (16).

**Immunofluorescence—**Cells grown on coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.02% Triton X-100 in phosphate-buffered saline for 10 min at room temperature. After blocking with 5% bovine serum albumin in phosphate-buffered saline for 20 min, the cells were incubated with anti-vinulin antibody (1:250 dilution) for 2 h. This was followed by incubation with Alexa Fluor 594-conjugated anti-mouse IgG (1:200 dilution) and Alexa Fluor 488-conjugated phallolidin (1:250 dilution) for F-actin staining. Coverslips were mounted in VECTASHIELD mounting medium (Vector Laboratories) and viewed using an Axioplan 2 fluorescence microscope (Carl Zeiss MicroImaging, Inc.). Images were captured by a Micro-Color CCD digital camera (CRI Inc.) and processed using SmartCapture VP software (Digital Scientific, Ltd.).

**PTPα Adenoviral Expression System—**The AdEasy vector system (Qbiogene, Inc.) was used for PTPα expression in mouse fibroblasts. PTPα and PTPα(C433S/C723S) DNAs with PAC site mutations have been described (16). PTPα(Y789F) with a PAC site mutation was generated using the QuikChange site-directed mutagenesis kit (Stratagene).

The mutant forward primer sequence was 5′-GAT GCA TTC TCA GAT TTC GCC AAC AAG TAA GCC GCG-3′, and the mutant reverse primer sequence was 5′-GCC TTA CTT GAA GAT GGC AAA ATC TGA GAA TGC ATC-3′. The absence of other mutations introduced by PCR was confirmed by sequencing. The cDNAs encoding wild-type PTPα, PTPα(C433S/C723S), and PTPα(Y789F) were cloned into the Sall and NotI sites of the pShuttle-CMV vector. The three resulting plasmids were linearized with Pmel and cotransfected with pAdEasy-1 into Escherichia coli strain B5183 to generate the infectious viral DNA plasmid containing the desired forms of PTPα by homologous recom-
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Catalytically Inactive PTPα or PTPα(Y789F) Is Not Phosphorylated upon Integrin Stimulation—Tyr789 in the C-terminal region of PTPα has been reported to be the major site of PTPα tyrosine phosphorylation (23, 24). We investigated whether this is the site phosphorylated upon integrin stimulation. In addition, because PTPα can dephosphorylate and activate Src and Fyn (18–20), we determined whether the catalytic activity of PTPα is required for its phosphorylation. PTPα−−/− fibroblasts were transiently transfected with plasmids expressing wild-type PTPα, catalytically inactive PTPα lacking the two active-site cysteine residues of each catalytic domain (PTPα(C433S/C723S)), or PTPα with Tyr789 substituted with Phe (PTPα(Y789F)). In adherent growing cells, heterologously expressed wild-type PTPα was tyrosine-phosphorylated as observed in normal fibroblasts, whereas PTPα(Y789F) and catalytically inactive PTPα were not detectably phosphorylated (Fig. 2A). The lack of phosphorylation of catalytically inactive PTPα suggested that PTPα might be required to activate a SFK to induce subsequent PTPα phosphorylation. In accord with this, coexpression of inactive PTPα and constitutively active Src(Y527F) in PTPα−−/− cells caused a pronounced tyrosine phosphorylation of PTPα(C433S/C723S) in adherent cells and in cells plated on FN (Fig. 2B). In contrast, coexpression of PTPα(Y789F) and active Src(Y527F) did not induce phosphorylation of PTPα(Y789F) in cells adhering to the dish or plated on FN (Fig. 2C), suggesting that the negligible phosphorylation of this mutant form of PTPα in the presence of active Src is due to the lack of a major site of integrin-stimulated SFK-mediated phosphorylation at Tyr789.

To verify that Tyr789 was indeed the PTPα residue phosphorylated upon integrin stimulation, we used an antibody raised against a phosphotyrosyl peptide comprising the C-terminal 12 amino acids of PTPα. Wild-type PTPα, PTPα(Y789F), and PTPα(C433S/C723S) were expressed in PTPα-null fibroblasts by adenovirus-mediated infection. Lysates prepared from adherent cells were probed with anti-PTPα phospho-Tyr789 antibody. Consistent with the results obtained by probing with anti-phosphotyrosine antibody, only wild-type PTPα was recognized by anti-PTPα phospho-Tyr789 antibody (Fig. 2D). In other experiments, uninfected wild-type or PTPα-null fibroblasts or PTPα-null fibroblasts expressing wild-type PTPα or PTPα(Y789F) were placed in suspension and then plated on FN for 15 min. Probing cell lysates with anti-PTPα phospho-Tyr789 antibody detected signals corresponding to FN-stimulated increases in PTPα tyrosine phosphorylation in wild-type fibroblasts and in PTPα-null cells re-expressing wild-type PTPα (Fig. 2E). No signal was detected in lysates of uninfected PTPα-null cells or cells re-expressing PTPα(Y789F) (Fig. 2E). These results indicate that the antibody specifically recognizes PTPα phosphorylated at Tyr789 and that this residue is phosphorylated upon integrin stimulation.

RESULTS

Integrin-stimulated Tyrosine Phosphorylation of PTPα—PTPα was tyrosine-phosphorylated in adherent fibroblast cell cultures (Fig. 1A, lanes 1 and 5). To determine whether PTPα phosphorylation status was regulated by cell adhesion and integrin signaling, fibroblasts were placed in suspension for 1 h and then plated on dishes coated with the integrin ligand FN. Detachment of cells from the substratum induced the partial dephosphorylation of PTPα (to 57 ± 4% of that of PTPα in fully adherent and spread cells) (Fig. 1, A, lanes 2 and 6; and B). Plating on FN induced phosphorylation of PTPα to 71 ± 4% of the level in growing adherent cells after 5 min on FN, further increasing to 93 ± 7% of the original level after 30 min on FN (Fig. 1, A, lanes 3 and 4; and B). In contrast, plating the suspended cells on poly-L-lysine-coated dishes for 30 min did not induce significant repophosphorylation of PTPα (68 ± 7%) (Fig. 1, A, lanes 5–7; and B). Under the above conditions, the dynamic tyrosine phosphorylation of PTPα correlated with that of cellular proteins (Fig. 1A), with the major phosphoprotein that was detected in the lysates comigrating with FAK (data not shown).

Determination of phosphotyrosine content of PTPα quantitated for phosphotyrosine content of PTPα by probing for phosphotyrosine and PTPα (upper two panels) in adherent fibroblast cell cultures (Fig. 1). Cells were cultured for 24 h prior to further manipulation.

FIGURE 1. FN-induced tyrosine phosphorylation of PTPα. A, PTPα was immunoprecipitated from lysates of mouse embryonic fibroblasts grown on plastic dishes (Ad), placed in suspension (B), and then replated on FN-coated dishes for 5 (FN5) or 30 (FN30) min or on poly-L-lysine-coated dishes for 30 min (PLL30). Immunoprecipitates (IP) were probed for phosphotyrosine and PTPα in upper two panels. Cell lysates were also probed for phosphotyrosine and actin (lower two panels). B, immunoblot; ECM, extracellular matrix. Autoradiographs from three independent experiments as described for A were quantitated for phosphotyrosine content of PTPα relative to the amount of PTPα protein. The graph shows the mean ± S.D. for phosphotyrosyl PTPα, with that in adherent cells taken as 100%.

Recombinants were selected by kanamycin, and positive colonies were retransformed into E. coli strain DH5α to preserve the correct recombinants. These plasmids were cleaved by PciI, purified using a PCR purification kit (Stratagene), and transfected into QB1-293A cells using Lipofectamine reagent. Viral particles were harvested from the cells by freeze/thaw cycles and purified by continuous CsCl gradient centrifugation. Virus titers were determined by TCID50. To infect fibroblasts, ~10,000 viral particles/cell were used in a minimal volume of medium that completely covered the cells. Following incubation at 37 °C for 90 min, the medium was topped up to the normal level, and the cells were cultured for 24 h prior to further manipulation.

RESULTS

Integrin-stimulated Tyrosine Phosphorylation of PTPα—PTPα was tyrosine-phosphorylated in adherent fibroblast cell cultures (Fig. 1A, lanes 1 and 5). To determine whether PTPα phosphorylation status was regulated by cell adhesion and integrin signaling, fibroblasts were placed in suspension for 1 h and then plated on dishes coated with the integrin ligand FN. Detachment of cells from the substratum induced the partial dephosphorylation of PTPα (to 57 ± 4% of that of PTPα in fully adherent and spread cells) (Fig. 1, A, lanes 2 and 6; and B). Plating on FN induced phosphorylation of PTPα to 71 ± 4% of the level in growing adherent cells after 5 min on FN, further increasing to 93 ± 7% of the original level after 30 min on FN (Fig. 1, A, lanes 3 and 4; and B). In contrast, plating the suspended cells on poly-L-lysine-coated dishes for 30 min did not induce significant repophosphorylation of PTPα (68 ± 7%) (Fig. 1, A, lanes 5–7; and B). Under the above conditions, the dynamic tyrosine phosphorylation of PTPα correlated with that of cellular proteins (Fig. 1A), with the major phosphoprotein that was detected in the lysates comigrating with FAK (data not shown).

SFKs Are Required for Integrin-stimulated PTPα Phosphorylation—The possibility that PTPα phosphorylation is mediated by SFKs was further investigated. As observed in wild-type fibroblasts, PTPα was dephosphorylated when embryonic fibroblasts lacking Src were placed in suspension and was reprophosphorylated when the cells were plated on FN (Fig. 3A, left panels), indicating that Src itself is not essential for integrin-regulated PTPα phosphorylation. However, in adherent embryonic fibroblasts lacking the three SFKs Src, Yes, and Fyn (SYF cells), PTPα phosphorylation was greatly reduced. Detachment from the substratum and replating on FN did not alter PTPα phosphorylation (Fig. 3A, middle panels). In cells with Src but lacking Fyn and Yes expression, PTPα was phosphorylated in adherent cells, dephosphorylated when the cells were placed in suspension, and reprophosphorylated when the cells were plated on FN (Fig. 3A, right panels). Similar effects on PTPα tyrosine phosphorylation were
FIGURE 2. Catalytically active PTPα or Src is required for phosphorylation of PTPα at Tyr789. A, wild-type (+/+ ) or PTPα-null (−/− ) fibroblasts that were left untransfected or were transiently transfected with plasmids expressing wild-type PTPα (wt, mutant PTPα/Y789F), or inactive PTPα(C433S/C723S) (dm) were grown on plastic dishes. IB, immunoblot. B, PTPα-null fibroblasts transiently expressing inactive PTPα(C433S/C723S) alone (+dm) or with constitutively active Src(YS27F) (src-Y527F) were grown on plastic dishes (Ad, placed in suspension (0), and then replated on FN-coated dishes for 30 min (FN30). C, PTPα-null fibroblasts transiently expressing inactive PTPα (Y789F) (α-Y789F) or wild-type PTPα (α-wt) alone or with constitutively active Src(YS27F) (src-Y527F) were grown on plastic dishes, placed in suspension, and then replated on FN-coated dishes for 30 min. PTPα immunoprecipitates (IP) were prepared from the cell lysates in A–C and probed for phosphotyrosine (upper panel) or PTPα (lower panel). D, PTPα-null fibroblasts were left uninfected or were infected with adenovirus (Ad) expressing wild-type PTPα, PTPα(Y789F), or PTPα(C433S/C723S). Cell lysates were probed with antibodies to PTPα phospho-Tyr789 (P-Y789; upper panel) and PTPα (lower panel). E, wild-type and PTPα-null fibroblasts were left uninfected, or the PTPα-null fibroblasts were infected with adenovirus expressing wild-type PTPα or PTPα(Y789F). After 24 h, the cells were placed in suspension (0) and plated on FN for 15 min (FN15) before harvesting. Lysates were probed with antibodies to PTPα phospho-Tyr789 (upper panel) and PTPα (lower panel).

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FIGURE 3. SFKs are required for FN-induced PTPα tyrosine phosphorylation. Mouse embryonic fibroblasts deficient in Src (Src−/−); deficient in Src, Yes, and Fyn (SYF); or deficient in Fyn and Yes (Src+/−) were grown on plastic dishes in the presence of serum (Ad) and then serum-starved overnight and placed in suspension (0) prior to replating on FN-coated dishes for 5 (FN5) or 30 (FN30) min. A, PTPα immunoprecipitates (IP) were prepared from cell lysates were probed for phosphotyrosine (upper panels) or PTPα (lower panels). B, lysates prepared from cells in suspension or plated on FN for 30 min were probed with antibodies to PTPα phospho-Tyr789 (P-Y789; upper panels) and PTPα (lower panels). C, expression of PTPα, Src, Fyn, Yes, and actin in wild-type (WT), PTPα−/−, Src+/−, and SYF fibroblasts was determined by immunoblotting (IB) of cell lysates.

FIGURE 4. FN-induced tyrosine phosphorylation of PTPα requires an intact actin cytoskeleton. Mouse embryonic fibroblasts growing on plastic dishes (Ad) were treated with or without cytochalasin D for 20 min prior to lysis. Other adherent cells were placed in suspension (0) with or without cytochalasin D, and some of the latter cells were then replated on FN-coated dishes for 30 min (FN30) in the absence or presence of cytochalasin D. PTPα was immunoprecipitated (IP) from the lysates and probed for phosphotyrosine and PTPα (upper panels). FAK was immunoprecipitated from the lysates and probed for phospho-Tyr789 and FAK (lower panels). IB, immunoblot.

Observe when lysates prepared from suspended or FN-stimulated cells of these three lines were probed with anti-PTPα phospho-Tyr789 antibody (Fig. 3B). The relative levels of expression of PTPα, Src, Fyn, and Yes in these fibroblasts are shown in Fig. 3C. The above results indicate that one or more SFKs, either Fyn/Yes or Src, are essential for integrin-stimulated phosphorylation of PTPα at Tyr789.

PTPα Phosphorylation Requires an Intact Actin Cytoskeleton and FAK—In addition to SFKs, we tested other requirements for integrin-stimulated PTPα phosphorylation. Integrin activation induces actin polymerization, and the effects of disrupting the actin skeleton by treatment with cytochalasin D were examined. PTPα is phosphorylated in adherent cells, and this was not affected by cytochalasin D (Fig. 4, lanes 1 and 2). The dephosphorylation of PTPα induced by cell detachment was also unaltered by cytochalasin D (Fig. 4, lanes 3 and 4). However, FN-induced tyrosine phosphorylation of PTPα was blocked in the
presence of cytochalasin D (Fig. 4, lanes 5 and 6), as we observed with phosphorylation of FAK at Tyr797 (lanes 5 and 6) and as has been reported for FAK (25). This indicates that an intact actin cytoskeleton is required for integrin-mediated PTPα phosphorylation. Because multiple integrin-stimulated phosphorylation events are mediated by the SFK-FAK complex, we also tested whether FAK is required for PTPα phosphorylation. Adherent FAK-null fibroblasts contained a greatly reduced level of phospho-PTPα compared with wild-type fibroblasts (Fig. 5, lanes 1 and 5), and PTPα phosphorylation remained low when the FAK−/− cells were suspended and replated on FN (compare lanes 5–8 with lanes 2–4). The very low level of PTPα phosphorylation in the FAK−/− cells under all conditions was similar to that observed in the cells lacking Src, Fyn, and Yes (Fig. 2A). These findings demonstrate that FAK (and possibly the SFK-FAK tyrosine kinase complex) is necessary for PTPα phosphorylation and suggest that PTPα phosphorylation may be a consequence of rather than a prerequisite for SFK-FAK activation.

**PTPα Tyr789 Phosphorylation Is Not Required for Src/Fyn Activation or for FAK or Paxillin Phosphorylation—PTPα functions at an early upstream point in integrin signaling as an SFK activator, as it is required for efficient integrin-stimulated SFK-mediated phosphorylation of FAK at Tyr797 (16).** In another process, mitosis, PTPα also activates Src, and phospho-Tyr789 of PTPα was reported to be essential for the ability of PTPα to do so (26). To determine whether PTPα Tyr789 phosphorylation is required for integrin-stimulated SFK activation, FAK Tyr797 phosphorylation, and the ensuing tyrosine phosphorylation of other proteins such as paxillin, we determined whether these impaired events in PTPα−/− cells could be rescued by re-expression of wild-type PTPα, inactive PTPα (PTPa[C433S/C723S]), or PTPα(Y789F). PTPα expression and tyrosine phosphorylation were confirmed following adenovirus-mediated introduction of these forms of PTPα into PTPα−/− cells (Fig. 6A). As expected, only wild-type PTPα was tyrosine-phosphorylated in adherent cells. Integrin-stimulated Src, Fyn, and paxillin tyrosine phosphorylation was then determined in these cells as compared with parental PTPα−/− cells and wild-type fibroblasts after suspension and replating on FN for 30 min. Three different phosphorylation site-specific antibodies were used to examine SFK phosphorylation status. Src phosphorylation was determined by immunoprecipitating Src with the SRC2 antibody, which recognizes Src dephosphorylated at Tyr527, or with anti-Src antibody, which recognizes Src irrespective of its phosphorylation status, and the relative amounts of isolated Src were compared.

**PTPα Tyr789 Phosphorylation Is Required for Integrin-stimulated Cell Spreading—PTPα−/− cells exhibit delayed spreading on FN, accompanied by impaired assembly of actin stress fibers and focal adhesions.** Expression of PTPα in the cells rescued these defects (Fig. 7) (16). We investigated whether the catalytic activity of PTPα and its phosphorylation at Tyr789 are required for PTPα-mediated restoration of these integrin-stimulated processes. Uninfected wild-type or PTPα−/− fibroblasts or PTPα−/− cells infected with adenovirus expressing PTPα, PTPα[C433S/C723S], or PTPα(Y789F) were plated on FN-coated dishes; and 15 and 30 min after plating, the cells were fixed and immunostained to visualize actin and vinculin. After 15 min on FN (Fig. 7A), it was apparent that cells with reintroduced PTPα had spread on the substratum and possessed a clearly visible ring-like enrichment of F-actin and vinculin around the cell periphery, very similar to wild-type cells. In contrast, PTPα−/− cells expressing PTPα[C433S/C723S] exhibited compacted F-actin and vinculin staining consistent with impaired spreading that was comparable with the uninfected PTPα−/− cells. After 15 min, the PTPα−/− cells expressing PTPα(Y789F) had spread poorly (data not shown) or to some extent (Fig. 7A) on FN, and the cells that had spread slightly showed some peripheral localization of F-actin and vinculin, although this was greatly reduced compared with wild-type cells or with PTPα−/− cells re-expressing PTPα. After 30 min on FN (Fig. 7B), wild-type cells or PTPα−/− cells re-expressing PTPα were well spread with a thick peripheral actin ring and clearly defined actin compared with wild-type cells (Fig. 6B). Immunoprecipitation of Fyn, followed by probing with anti-Src phospho-Tyr527 antibody, which recognizes the homologous C-terminal phospho-Tyr528 site of Fyn, showed elevated phosphorylation (2.5-fold) of this site in cells lacking PTPα−/− (Fig. 6B). The reintroduction of PTPα into PTPα−/− fibroblasts resulted in increased dephosphorylation of Src at Tyr416, increased phosphorylation of Src at Tyr416, and reduced phosphorylation of Fyn at Tyr528 to levels approaching those detected in wild-type fibroblasts (Fig. 6B). On the other hand, the reintroduction of catalytically inactive PTPα into PTPα−/− fibroblasts had no effect on Src or Fyn phosphorylation status and was unable to rescue these SFK phosphorylation defects (Fig. 6B). However, expression of PTPα(Y789F) in PTPα−/− cells resulted in altered Src and Fyn tyrosine phosphorylation to levels observed in wild-type fibroblasts and equivalent to those effected by expression of wild-type PTPα (Fig. 6A). Thus, PTPα catalytic activity (but not Tyr789 phosphorylation) is required for PTPα to regulate SFK phosphorylation under conditions of integrin stimulation. Furthermore, expression of PTPα or PTPα(Y789F) induces SFK phosphorylation events (Src Tyr272 and Fyn Tyr528 dephosphorylation and Src Tyr416 phosphorylation) that correlate with enhanced SFK activity.

**SFK activity is required for integrin-stimulated FAK Tyr797 phosphorylation and FAK activation (4, 6–8).** Expression of wild-type PTPα or PTPα(Y789F) in PTPα−/− cells restored integrin-stimulated phosphorylation of FAK at Tyr797 to a level comparable with that in wild-type fibroblasts, whereas in cells expressing PTPα[C433S/C723S], FAK Tyr797 phosphorylation remained at a low level comparable with that observed in uninfected parental PTPα−/− cells (Fig. 6C). Similar rescue of FAK Tyr776 and paxillin phosphorylation, events mediated by the SFK-FAK kinase complex (4, 5, 27–29), were observed upon expression of wild-type PTPα and PTPα(Y789F), but not PTPα[C433S/C723S] (Fig. 6C). Together, these results demonstrate that integrin-induced phosphorylation of PTPα at Tyr789 is not required for these early signaling events. Clearly, PTPα-catalyzed dephosphorylation is required to restore integrin-stimulated SFK, FAK, and paxillin tyrosine phosphorylation, as inactive PTPα cannot compensate for the absence of active PTPα.

**PTPα Tyr789 Phosphorylation Is Required for Integrin-stimulated Cell Spreading**—PTPα−/− cells exhibit delayed spreading on FN, accompanied by impaired assembly of actin stress fibers and focal adhesions. Expression of PTPα in the cells rescued these defects (Fig. 7) (16). We investigated whether the catalytic activity of PTPα and its phosphorylation at Tyr789 are required for PTPα-mediated restoration of these integrin-stimulated processes. Uninfected wild-type or PTPα−/− fibroblasts or PTPα−/− cells infected with adenovirus expressing PTPα, PTPα[C433S/C723S], or PTPα(Y789F) were plated on FN-coated dishes; and 15 and 30 min after plating, the cells were fixed and immunostained to visualize actin and vinculin. After 15 min on FN (Fig. 7A), it was apparent that cells with reintroduced PTPα had spread on the substratum and possessed a clearly visible ring-like enrichment of F-actin and vinculin around the cell periphery, very similar to wild-type cells. In contrast, PTPα−/− cells expressing PTPα[C433S/C723S] exhibited compacted F-actin and vinculin staining consistent with impaired spreading that was comparable with the uninfected PTPα−/− cells. After 15 min, the PTPα−/− cells expressing PTPα(Y789F) had spread poorly (data not shown) or to some extent (Fig. 7A) on FN, and the cells that had spread slightly showed some peripheral localization of F-actin and vinculin, although this was greatly reduced compared with wild-type cells or with PTPα−/− cells re-expressing PTPα. After 30 min on FN (Fig. 7B), wild-type cells or PTPα−/− cells re-expressing PTPα were well spread with a thick peripheral actin ring and clearly defined actin

![FIGURE 5. Defective FN-induced PTPα tyrosine phosphorylation in FAK-null fibroblasts. Mouse embryonic fibroblasts lacking p53 (FAK−/−) or lacking FAK and p53 (FAK−/−) were grown on plastic dishes (Ad), placed in suspension (D), and then replated on FN-coated dishes for 5 (FN5) or 30 (FN30) min. PTPα was immunoprecipitated from the lysates and probed for phosphotyrosine (upper panel) and PTPα (lower panel). ECM, extracellular matrix; IB, immunoblot.](https://example.com/fig5)
stress fibers, well defined membrane protrusions containing actin stress fibers, and numerous vinculin-containing focal adhesions. In contrast, uninfected PTPα−/− cells and PTPα-null fibroblasts expressing PTPα(C433S/C723S) or PTPα(Y789F) had spread more than was observed at 15 min, but were still less well spread than cells with PTPα. The parental PTPα−/− cells and those with PTPα(C433S/C723S) or PTPα(Y789F) had formed some membrane ruffles and small lamellipodia, but still exhibited retarded development of actin stress fibers and focal adhesion formation. The close resemblance of the cells lacking PTPα to those with reintroduced PTPα(C433S/C723S) or PTPα(Y789F) and the clear differences between these cells and wild-type and PTPα−/− fibroblasts re-expressing PTPα indicate that the phosphatase activity of PTPα and its phosphorylation at Tyr789 are necessary for optimal integrin-stimulated spreading, cytoskeletal rearrangement, and focal adhesion formation.

PTPα Tyr789 Phosphorylation Is Required for Integrin-stimulated Cell Migration—The above cell spreading and accompanying morphological changes are integrin-stimulated processes that are required for cell movement. Defects in these processes in PTPα−/− cells resulted in reduced cell migration to FN in a haptotaxis assay (67 ± 4% of wild-type cells migrated) that could be rescued by re-expression of PTPα (90 ± 9% of wild-type cells migrated), but not inactive PTPα (67 ± 3% of wild-
type cells migrated) (Fig. 8). In accord with the inability of introduced PTPα(1Y789F) to restore cell spreading, actin stress fiber assembly, and focal adhesion formation in PTPα−/− cells, this unphosphorylatable mutant PTPα was likewise not able to rescue (67 ± 1% of wild-type cells migrated) the migration defect in PTPα−/− cells (Fig. 8).

DISCUSSION

We have demonstrated that PTPα undergoes regulated tyrosine phosphorylation in response to integrin stimulation. PTPα is phosphorylated in adherent cells, dephosphorylated when cells are placed in suspension, and rephosphorylated when cells are plated on fibronectin. This dynamic phosphorylation occurs at Tyr789 in the C-terminal region of PTPα and requires SFKs, either Src or Fyn/Yes. PTPα dephosphorylates and activates SFKs (14, 15) and acts upstream of SFKs in early integrin signaling (16). Catalytically inactive PTPα is only minimally tyrosine-phosphorylated unless constitutively active Src is present (Fig. 2, A and B), and inactive PTPα cannot mediate FN-stimulated SFK C-terminal tyrosine dephosphorylation and the ensuing Tyr416 autophosphorylation required for SFK activation (Fig. 6). Together, these findings indicate that PTPα-catalyzed SFK activation is required for the SFK-catalyzed phosphorylation of PTPα at Tyr789 that occurs following integrin stimulation. Furthermore, because FAK is required for PTPα phosphorylation (Fig. 5), it is likely that PTPα phosphorylation occurs following formation of the SFK-FAK complex and is indeed effected by this complex. In accord with this, an intact cytoskeleton is required for integrin-stimulated PTPα tyrosine phosphorylation (Fig. 4), as it is for FAK Tyr397 phosphorylation, maximal FAK phosphorylation and likely activation, and full c-Src activation (30, 31).

Consistent with PTPα tyrosine phosphorylation occurring subsequent to PTPα-mediated SFK activation is our finding that PTPα Tyr789 phosphorylation is not required for SFK activation. Indeed, PTPα(1Y789F) was as efficient as wild-type PTPα in rescuing defective Src and Fyn dephosphorylation/phosphorylation in FN-stimulated PTPα−/− fibroblasts and, as a measure of SFK and SFK-FAK activity, in rescuing FAK Tyr397 and Tyr576 and paxillin phosphorylation. This is in contrast to previous reports that phosphorylation of PTPα at Tyr789 is essential for its ability to dephosphorylate and activate Src. In one study, PTPα(1Y789F) expressed in NIH 3T3 cells was found to be unable to dephosphorylate Src at Tyr527, with a consequent lack of Src activation that correlated with an inability of the PTPα mutant to transform the cells, all actions that were effec.
Two Roles of PTPα in Integrin Signaling

by expression of wild-type PTPα (26). In another study of the same cells, PTPα(Y789F) was also found, in contrast to wild-type PTPα, not to be able to catalyze dephosphorylation of Src at Tyr527 and mitotic activation of Src (32). A displacement model of PTPα-mediated Src activation was proposed in which the Src SH2 domain binds to phospho-Tyr789 of PTPα, freeing the phospho-Tyr527 site of Src and enhancing the proximity of PTPα and Src to permit dephosphorylation of Src at Tyr527 by PTPα (26, 32). In this way, phospho-Tyr789 of PTPα was suggested to be essential for PTPα-Src interaction and conformational changes that allow Src Tyr527 dephosphorylation and consequent Src activation. Our results clearly demonstrate that PTPα Tyr789 phosphorylation is not required for integrin-stimulated SFK activation leading to FAK activation, although catalytically active PTPα is an essential upstream participant in this signaling pathway. We propose that integrin engagement provides conditions that replace the PTPα phospho-Tyr789-dependent physical interactions of PTPα and Src postulated in the mitotic displacement model. In our model (Fig. 9), integrin-PTPα interaction(s) provide the enhanced physical proximity of PTPα to integrin-bound SFKs analogous to that occurring through direct PTPα-SFK binding in the displacement model. Indeed, PTPα associates with α-integrins following their binding to ligand (33). In addition, the τ-integrin (or other integrin) interaction with the Src SH3 domain (12, 13) or the initial FAK phospho-Tyr979 interaction with the Src SH2 domain (3, 17) could provide a perturbation of Src intramolecular interactions to induce conformational changes resulting in the release of the Src phospho-Tyr527 tail so that it is accessible for dephosphorylation. In support of these events playing roles in Src activation, both have been demonstrated to be accompanied by Src Tyr527 dephosphorylation (13, 17). In this way, third molecule participants (i.e. integrin subunits, FAK) that engage SFK SH2 or SH3 domains and PTPα can function as scaffolds to promote PTPα-catalyzed SFK dephosphorylation and activation through a PTPα tyrosine phosphorylation-independent mechanism.

Although PTPα Tyr789 phosphorylation is not required for SFK activation, FAK Tyr979 phosphorylation and activation, or tyrosine phosphorylation of the SFK-FAK complex substrate paxillin, it is required for other downstream events in integrin signaling. Cell spreading on FN and the accompanying assembly of actin stress fibers and formation of focal adhesions are impaired in PTPα−/− fibroblasts expressing the unphosphorylatable PTPα(Y789F) mutant, whereas expression of wild-type PTPα rescues these defects. As would be expected as a consequence of such defects, the PTPα(Y789F)-expressing cells, like the parental PTPα-null cells, have a reduced ability to migrate toward an FN stimulus. These observations demonstrate that tyrosine phosphorylation of PTPα is required for a distinct second function of PTPα that is operative at a later point in integrin signaling to regulate cytoskeletal rearrangement and focal adhesion formation (Fig. 9). This indicates that PTPα acts not only upstream but also downstream of SFKs because PTPα phosphorylation is catalyzed by these kinases.

The Rho GTPases Rho, Rac, and Cdc42 play key roles in regulating the actin cytoskeleton in cell migration (34–36). Integrin stimulation activates Rac and Cdc42 to induce actin polymerization and the formation of lamellipodia and filopodia, respectively. It activates Rho to promote myosin contractility, which creates tension and the ensuing assembly of actin stress fibers. All these GTPases play roles in the integrin-mediated formation of focal adhesion complexes. Given that cell spreading, actin stress fiber and lamellipodial assembly, and focal adhesion formation are impaired in PTPα-null cells reconstituted with

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**FIGURE 8.** Delayed migration of PTPα−/− cells is not rescued by inactive or unphosphorylatable PTPα mutants. Wild-type (+/+) and PTPα-null (−/−) fibroblasts and PTPα-null fibroblasts infected with adenovirus (Adv) expressing wild-type PTPα (wt), inactive PTPα(C433S/C723S) (dm), or PTPα(Y789F) were analyzed for migration toward FN as described under “Experimental Procedures.” Each bar represents the mean ± S.D. of three independent experiments, with each experiment comprising three wells/cell type. The number of migrating wild-type cells was taken as 100%, and other values were calculated relative to this. There was a significant difference toward FN as described under “Experimental Procedures.” Each bar represents the mean ± S.D. of three independent experiments, with each experiment comprising three wells/cell type. The number of migrating wild-type cells was taken as 100%, and other values were calculated relative to this. There was a significant difference between the migration of wild-type cells (n = 5) and PTPα-null cells (n = 5; p < 0.0001) or PTPα-null cells reconstituted with PTPα(C433S/C723S) (n = 3; p < 0.03) or PTPα(Y789F) (n = 5; p < 0.001), but not between wild-type cells and PTPα-null cells re-expressing wild-type PTPα (n = 5; p > 0.1). Conversely, there were significant differences between the migration of PTPα-null cells and these cells re-expressing wild-type PTPα (p < 0.001), but not between PTPα-null cells and these cells re-expressing PTPα(C433S/C723S) (p > 0.8) or PTPα(Y789F) (p < 0.002).

**FIGURE 9.** Schematic model of two PTPα actions in integrin signaling. a, prior to integrin engagement, SFKs are in an inactive conformation. b, upon integrin binding to FN, SFKs associate through their SH2 domain with β-integrin and/or through their SH2 domain with phospho-Tyr789 of FAK, increasing the accessibility of the SFK C-terminal Tyr residue. PTPα associates with α-integrin and dephosphorylates SFKs (Step 1), resulting in full SFK activation. c, FAK-associated SFKs then phosphorylate FAK at Tyr397 and Tyr416 to activate FAK and promote further FAK Tyr979 autophosphorylation, phosphorylate other sites on FAK (Tyr527) and other proteins such as paxillin (not shown), and phosphorylate PTPα at Tyr789. d, the SFK-FAK multiphosphoprotein (not shown) signaling complex initiates downstream signaling events, as does phospho-PTPα (Step 2), which include cytoskeletal rearrangement and focal adhesion formation.
PTPα(Y789F), we propose that PTPα phosphorylation plays a role either in the upstream activation of one or more of these GTPases or as an effector in signaling events downstream of Rho, Rac, and/or Cdc42. Phospho-Tyr\(^{789}\) of PTPα is a binding site for the adaptor molecule Grb2 (23, 24). It is unusual that Grb2 associated with constitutively phosphorylated PTPα is not complexed with its common binding partner, the guanine nucleotide exchange factor for Ras GTPase, Sos (23, 24). Intact Tyr\(^{789}\) in PTPα is required for heterologously expressed PTPα to localize to focal adhesions of NIH 3T3 cells, although the putative focal adhesion protein-binding partner regulating this localization has not been identified (37). It remains to be determined whether Grb2 mediates novel or Sos-linked PTPα-protein interactions that signal from integrins to the cytoskeleton. Alternatively, integrin activation could induce a reduction in the affinity of PTPα phospho-Tyr\(^{789}\) for Grb2 and a resulting interaction with another phosphotyrosyl-binding protein (as with Src in the displacement model) that mediates cytoskeleton/focal adhesion-specific effects of PTPα.

This study has distinguished two actions of PTPα in integrin signaling. PTPα plays an early upstream role as an activator of the SFKs Src and Fyn, enabling SFK-mediated FAK phosphorylation and full FAK autophosphorylation at Tyr\(^{925}\), together with SFK-FAK-catalyzed tyrosine phosphorylation of other proteins such as paxillin and PTPα itself. These early events depend upon the catalytic activity of PTPα and its ability to dephosphorylate the C-terminal regulatory residues of Src and Fyn. We have demonstrated that phosphorylation of PTPα at Tyr\(^{925}\) is a result of, but in contrast to, its essential role in Src activation in mitosis, not required for SFK activation in these integrin signaling events. However, the ensuing phosphorylation of PTPα is required for the timely execution of a second sequence of events leading to integrin-induced cell spreading, cytoskeletal rearrangement, focal adhesion formation, and cell migration.

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