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Direct Association of Protein-tyrosine Phosphatase PTP-PEST with Paxillin*

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Tyrosine phosphorylation of focal adhesion-associated proteins may be involved in the regulation of the cytoskeleton and in the control of signals for growth and survival. The focal adhesion kinase (FAK) functions in regulating tyrosine phosphorylation of several of these proteins, including paxillin, tensin, and p130^{cas}. Protein-tyrosine phosphatases, the counterparts of protein-tyrosine kinases, also presumably regulate phosphorylation of these proteins. We have tested the hypothesis that FAK intimately associates with a protein-tyrosine phosphatase. Protein-tyrosine phosphatase activity associated with the recombinant C-terminal domain of FAK *in vitro* and could be coimmunoprecipitated with both FAK and paxillin from lysates of chicken embryo cells. However, the interaction with FAK appeared to be indirect and mediated via paxillin. The protein-tyrosine phosphatase was subsequently identified as protein-tyrosine phosphatase-PEST, a nonreceptor protein-tyrosine phosphatase. The C-terminal noncatalytic domain of protein-tyrosine phosphatase-PEST directly bound to paxillin *in vitro*. The association of both a protein-tyrosine kinase and a protein-tyrosine phosphatase with paxillin suggests that paxillin may play a critical role in the regulation of the phosphotyrosine content of proteins in focal adhesions.

The integrins were originally identified as cell surface receptors for extracellular matrix proteins. They participate in a variety of cellular events, including adhesion, migration, invasion, cytoskeleton organization, and the generation of cytoplasmic signals (1–3). Engagement of the integrins with the extracellular matrix results in the formation of protein complexes termed focal adhesions. In cultured fibroblasts, focal adhesions are specialized structures that link the actin cytoskeleton to extracellular matrix (4, 5). As a communication point between extracellular matrix and the inside of cells, focal adhesions perform both structural and signaling functions. One of the most important events after the engagement of integrins with their ligands is the rapid tyrosine phosphorylation of focal adhesion-associated proteins (2, 3, 6).

Tyrosine phosphorylation of focal adhesion-associated proteins may be controlled by the focal adhesion kinase (FAK),[1] a nonreceptor tyrosine kinase that localizes principally in focal adhesions (2, 3, 6, 7). FAK is a major substrate for tyrosine phosphorylation following integrin-dependent cell adhesion and simultaneously becomes enzymatically activated (8–11). Autophosphorylation of FAK creates a binding site for Src family PTKs, and the assembly of this complex appears to be important for tyrosine phosphorylation of focal adhesion-associated proteins (12–14). p130^{cas} and paxillin are, potentially, downstream components of FAK signaling. They are colocalized with FAK in focal adhesions, associate with FAK, and become tyrosine-phosphorylated in coordination with FAK (9, 15–20). Tyrosine phosphorylation of p130^{cas} and paxillin by FAK and/or Src-like PTKs likely regulates the assembly of signaling complexes in focal adhesions (13, 21–23).

The results of a number of pharmacological experiments suggest that tyrosine phosphorylation may be involved in the regulation of focal adhesion and stress fiber formation (9, 24–27). Other evidence suggests that tyrosine phosphorylation may not contribute to the assembly of focal adhesions. Microinjection of a dominant-negative FAK protein ablated tyrosine phosphorylation in focal adhesions, yet the integrity of the focal adhesions was unaffected, and new focal adhesions could still form (28). Experiments using various strategies to perturb normal FAK signaling suggest that FAK controls cell spreading, the rate of focal adhesion formation, and cell migration (7, 28, 29). Recent evidence also suggests that FAK may function in the transmission of a cell adhesion-dependent signal that is required for cell survival (30, 31).

The phosphorylation of proteins on tyrosine can also be controlled by PTPases (32). The role of PTPases in the regulation of cytoskeletal structure and signaling via focal adhesions is only beginning to be addressed. Several candidate PTPases that may be involved in regulating tyrosine phosphorylation in focal adhesions have been identified; they include LAR, PTP1B, and PTP-PEST. LAR is a transmembrane PTPase expressed on the cell surface as two noncovalently associated subunits derived by cellular processing of a proprotein (33). LAR and an associated protein LIP.1 can be localized to focal adhesions in MCF7 cells (34). PTP1B and PTP-PEST are implicated in focal adhesion signaling because both associate with p130^{cas}, a tyrosine-phosphorylated focal adhesion-associated protein. PTP1B directly associates with the SH3 domain of p130^{cas}, and overexpression of PTP1B leads to decreased tyrosine phosphorylation of p130^{cas} (35). A proline-rich sequence of PTP-PEST

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[1] The abbreviations used are: FAK, focal adhesion kinase; PTK, protein-tyrosine kinase; cas, crk-associated substrate; PTP or PTPase, protein-tyrosine phosphatase; SH, Src homology; CE, chicken embryo; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis.

can also bind the p130^{cas} SH3 domain, but in addition, the catalytic domain can directly interact with tyrosine-phosphorylated p130^{cas}, and PTP-PEST may function to specifically dephosphorylate p130^{cas} (36, 37). *Yersinia pseudotuberculosis*, which is a bacterial pathogen, encodes a PTPase that binds and dephosphorylates both FAK and p130^{cas} upon entry into cells (38). This enzyme is essential for the virulence of a number of *Yersinia* species (39). Thus, three cellular PTPases and one bacterial PTPase have been implicated as potential regulators of tyrosine phosphorylation of focal adhesion proteins.

In this study, we have tested the hypothesis that a PTPase may be intimately associated with FAK. PTPase activity was found to specifically associate with FAK, both *in vitro* and *in vivo*. However, this interaction appeared to be indirect and was in part mediated by the focal adhesion-associated protein paxillin. The PTPase was identified as PTP-PEST and was found to directly bind to paxillin *in vitro* via its noncatalytic C-terminal domain. The finding that paxillin associates with both a PTK and a PTPase suggests novel models for the regulation of tyrosine phosphorylation by the assembly of complexes containing both enzymes.

MATERIALS AND METHODS

Cell Culture Techniques—Chicken embryo (CE) cells were prepared and maintained as described (40). Swiss 3T3 cells were maintained in Dulbecco's modified Eagle's medium-H + 10% calf serum and Cos cells in Dulbecco's modified Eagle's medium-H + 10% fetal bovine serum.

Expression of Exogenous Proteins in CE and Cos Cells—The replication competent avian retroviral vector RCAS A (BH) (41) was used to express wild type FAK, dl853–963, dl965–1012, and C-tagged FAK as described previously (42, 43). For expression of PTP-PEST in Cos cells, the murine cDNA was engineered to encode a C-terminal epitope tag (KT3) using a PCR-based strategy. A 3′ primer that created a *Bam*HI site and destroyed the termination codon was used, and the PCR product was inserted into the plasmid pctag (43) (the *Bam*HI site was engineered so the PTP-PEST and KT3 codons of pctag were in frame). The tagged cDNA was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA). To create a deletion mutant lacking the paxillin binding site, the sequences encoding amino acids 1–300 were amplified by PCR using a primer that created an *Eco*RI site at codon 300 that was in frame with the *Eco*RI site at codon 494 of PTP-PEST. The PCR product was substituted for the sequences encoding amino acids 1–494 of epitope-tagged PTP-PEST, then subcloned into pcDNA3. Cos cells were transiently transfected with the PTP-PEST constructs using LipofectAMINE and the protocol recommended by the manufacturer (Life Technologies, Inc.), and the cells were lysed 48 h later.

In Gel Phosphatase Assay—Glutathione *S*-transferase (GST)-FER (kindly provided by Dr. Shelton Earp, University of North Carolina-Chapel Hill) was used as a PTK source for phosphorylation of the substrate. One mg of poly(Glu,Tyr) was incubated with 10 μg GST-FER in 500 μl of reaction buffer (200 μCi of [γ-^{32}P]ATP, 50 mM imidizole (pH 7.2), 10 mM dithiothreitol, 30 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM vanadate, 0.05% Triton X-100, 0.2 mM cold ATP, and 0.1% β-mercaptoethanol) for 4–6 h at room temperature with constant rotation as described (44). The labeled poly(Glu,Tyr) was purified by trichloroacetic acid precipitation and chromatography through a Sephadex G-50 column. [γ-^{32}P]ATP-labeled poly(Glu,Tyr) (1×10^6 cpm) was incorporated into a 10% SDS-polyacrylamide gel prior to polymerization. After separating the samples by electrophoresis, the SDS was removed, and the gel was incubated in denaturing buffer (6 M guanidine chloride and 0.3% β-mercaptoethanol) and then in renaturing buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.04% Tween 20, and 0.3% β-mercaptoethanol) as described (44). The gel was incubated in reaction buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.04% Tween 20, 0.3% β-mercaptoethanol, and 3 mM dithiothreitol) for 1 h and then in fresh buffer overnight. After drying, the gel was exposed to x-ray film overnight.

Protein Analysis—Polyclonal antiserum directed against part of the noncatalytic portion of PTP-PEST was generated in rabbits using a trpE fusion protein containing amino acids 286–471 (49). A polyclonal antiserum was used to recognize FAK (BC3; a gift of Tom Parsons, University of Virginia), and commercially available monoclonal antibodies were used to recognize paxillin and p130^{cas} (Transduction Laboratories, Lexington, KY). Cells were lysed in ice-cold modified radioimmunoprecipation assay buffer (50 mM Tris-HCl [pH 7.3], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.5% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM orthovanadate) or Triton X-100 buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5% aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1.5 mM orthovanadate). The protein concentration of the lysates was determined by the bicinchoninic acid assay (Pierce). For *in vitro* phosphatase assays, the cells were lysed in the absence of vanadate. For immunoprecipitations, antibodies were incubated with 0.2–1 mg of cell lysate for 1 h on ice. The immunecomplexes were recovered by incubation with protein A-Sepharose beads (Pharmacia Biotech Inc.) or anti-mouse IgG-coupled agarose beads (Sigma) for 1 h at 4 °C. After washing three times in lysis buffer and twice in Tris-buffered saline (50 mM Tris-HCl (pH 7.5), 150 mM NaCl), the immunecomplexes were dissociated by boiling in SDS sample buffer. The samples were analyzed using the in gel PTPase assay (44) or by SDS-PAGE and Western blotting (50). For Western blotting, primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies or protein A and ECL (Amersham Corp.).

Expression of GST Fusion Proteins—GST fusion proteins containing the N- and C-terminal domains of FAK have been described (45). Additional fusion constructs were engineered using PCR to amplify C-terminal domain fragments of FAK, and the PCR products were inserted into pGEX2TK (Pharmacia). GST-H-FAK contains codons 765–1052, GST-B-FAK contains codons 858–1052, and GST-S-FAK contains codons 909–1052. Similarly, fragments of PTP-PEST were amplified by PCR and subcloned into pGEX2TK. Fusion proteins containing residues 1–301, 297–775, 441–775, and 297–494 were engineered. The fusion proteins were expressed by incubation with 0.2 mM isopropyl β-D-thiogalactopyranoside for 4–5 h at 37 °C, the bacteria were lysed by sonication, and the fusion proteins were purified using glutathione agarose.

Binding Experiments Using GST Fusion Proteins—One-half to one mg of cell lysate was precleared with 10 μg of GST bound to glutathione agarose beads for 60 min at 4 °C. The cleared supernatant was then incubated with 0.1–1 μg of GST fusion protein immobilized on glutathione agarose beads for 1 h at 4 °C. The beads were washed three times in lysis buffer and twice in Tris-buffered saline. The bound proteins were released by boiling in SDS sample buffer and analyzed by either Western blotting or the in gel PTPase assay.

Far-Western Blotting—Six to 10 μg of purified GST fusion protein was added to 40 μl of HMK buffer (20 mM Tris (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$) containing 1 mM dithiothreitol, 50 units of protein kinase A (Sigma) and 100 μCi of [γ-^{32}P]ATP (6000 Ci/mmol) (NEN Life Science Products) (46, 47). After 45 min of incubation at room temperature, the reaction was terminated with HMK stop buffer (10 mM sodium phosphate (pH 8.0), 10 mM sodium pyrophosphate, 10 mM EDTA). Radiolabeled GST-PTP-PEST was separated from [γ-^{32}P]ATP by chromatography on a Sephadex G-50 column. As described previously (46, 47), protein samples were subjected to SDS-PAGE, transferred to nitrocellulose, and denatured in buffer containing 6 M guanidine hydrochloride. The samples were renatured by incubation in serial dilutions of denaturation buffer. Filters were washed and then blocked in 5% milk as described (46, 47). Radioactive probe was added (to 6×10^6 cpm/ml) and incubated for 8 h at 4 °C, and then the filter washed three times for 15 min and exposed to film.

RESULTS

Association of PTPase Activity with FAK in Vitro—Overexpression of FAK in CE cells does not induce tyrosine phosphorylation of cellular proteins unless the cells are treated with vanadate, a PTPase inhibitor (22). These results suggest that tyrosine phosphorylation of focal adhesion proteins may be controlled by both FAK and an antagonistic, perhaps intimately associated PTPase. To test whether FAK could bind a PTPase, GST fusion proteins containing the N-terminal and C-terminal noncatalytic domains of FAK were used to pull down FAK-binding proteins from lysates of CE cells. The PTPase activity associated with these fusion proteins was examined using an in gel PTPase assay. The GST-C-terminal domain of FAK associated with four major bands of PTPase activity with molecular masses of 97, 75–80 (a doublet), and 50 kDa (Fig. 1). It is not clear whether these are different PTPases or represent breakdown products of the largest, 97-kDa PTPase. The PTPase activities specifically bound to the C-terminal domain of FAK because GST alone and GST-N-FAK failed to associate with any detectable PTPase activity. A series

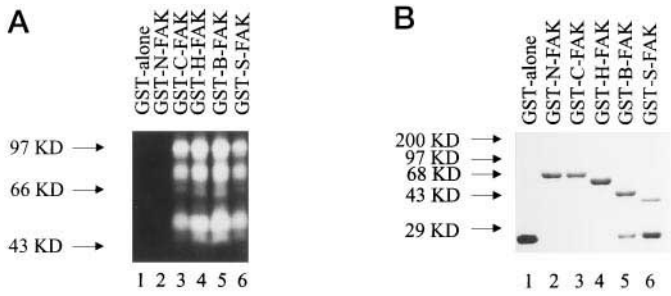

FIG. 1. **The focal adhesion targeting sequence of FAK binds PTPase activity *in vitro*.** *A*, lysates of CE cells were incubated with GST alone (*lane 1*), GST-N-FAK (*lane 2*), GST-C-FAK (*lane 3*), GST-H-FAK (*lane 4*), GST-B-FAK (*lane 5*), or GST-S-FAK (*lane 6*). The PTPases associated with these GST fusion proteins were analyzed using the in gel PTPase assay. *B*, the GST fusion proteins used in *A* were analyzed by SDS-PAGE and Coomassie Blue staining. *Lane 1*, GST alone; *lane 2*, GST-N-FAK; *lane 3*, GST-C-FAK; *lane 4*, GST-H-FAK; *lane 5*, GST-B-FAK; *lane 6*, GST-S-FAK.

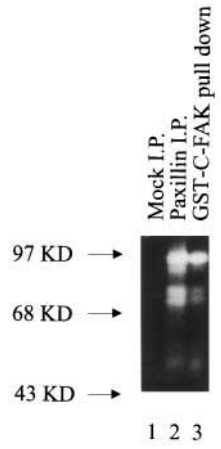

FIG. 2. **PTPase activity coimmunoprecipitates with paxillin.** CE cells were lysed in modified radioimmunoprecipation assay buffer, and the lysates were subjected to immunoprecipitation with secondary antibody alone (*Mock I.P., lane 1*) or an antibody against paxillin (*paxillin I.P., lane 2*). Lysates were also incubated with GST-C-FAK (*lane 3*). The PTPases associated with each immunecomplex and with GST-C-FAK were analyzed using the in gel phosphatase assay.

of GST-fusion proteins containing smaller fragments of the C-terminal domain of FAK was used to further map the FAK sequences required for association with PTPase activity. The GST-S-FAK construct contains only the focal adhesion targeting sequence, a 150-amino acid sequence that has been shown to localize FAK to focal adhesions (42). Each of these constructs associated with PTPase activity *in vitro* (Fig. 1). Analysis of the fusion proteins by SDS-PAGE and Coomassie Blue staining demonstrated that equivalent amounts of each fusion protein were used in the analysis (Fig. 1*B*). These results indicate that FAK can associate with PTPase activity *in vitro* and that the focal adhesion targeting sequence is sufficient for association with the PTPase.

Association of PTPase Activity with Paxillin—The FAK sequences required for association with PTPase activity *in vitro* contain a binding site for paxillin, raising the possibility that FAK associates with a PTPase(s) via its interaction with paxillin. To determine whether paxillin associates with PTPase activity, paxillin immunecomplexes were analyzed using the in gel PTPase assay. No PTPase activity was detected in control immunoprecipitations lacking the primary antibody (Fig. 2). The paxillin immunecomplex, however, exhibited PTPase activity, and the molecular masses of the associated PTPase(s) were 97, 75–80, and 50 kDa (Fig. 2). The pattern of PTPase activities associated with GST-C-FAK was compared with the PTPase pattern coimmunoprecipitated with paxillin and found to be virtually identical (Fig. 2). Thus, paxillin associates with PTPase activities *in vivo*, and the molecular masses of the PTPase(s) are similar to those that associate with the C-terminal domain of FAK *in vitro*.

Paxillin Mediates the Association between PTPase and FAK—To determine whether paxillin was required for the association of recombinant FAK with PTPase activity *in vitro*, a clearing experiment was performed. Paxillin was cleared from cell lysates by repeated immunoprecipitation. After three rounds of immunodepletion using a paxillin monoclonal antibody, the amount of paxillin remaining in the lysate was undetectable by immunoprecipitation and immunoblotting (Fig. 3*C*). After each immunoprecipitation, the lysate was incubated with GST-C-FAK, and the PTPase activity that associated with FAK *in vitro* was measured using the in gel PTPase assay. GST-C-FAK could no longer associate with PTPase activity after paxillin was cleared from the lysate, whereas GST-C-FAK bound PTPase activity in uncleared lysate (Fig. 3*A*). Following four rounds of control immunoprecipitation using anti-mouse IgG, the amount of PTPase that associated with GST-FAK was undiminished (Fig. 3*B*). These results indicate that paxillin is required for the association of FAK with PTPase activity *in vitro*.

To determine whether paxillin mediates the association of FAK with PTPase activity *in vivo*, mutants of FAK were expressed in CE cells, FAK was immunoprecipitated, and the immunecomplexes were analyzed using the in gel PTPase assay. A 97-kDa PTPase was coimmunoprecipitated from CE cells overexpressing FAK (Fig. 4*A*). Under these lysis conditions, little PTPase activity coimmunoprecipitated with endogenous FAK (Fig. 4*A, lane 1*). However, under milder lysis conditions, a 97-kDa PTPase could be coprecipitated with endogenous FAK (Fig. 4*B*). The three FAK mutants analyzed, two deletion mutants and a variant in which the C-terminal 13 residues are replaced with an epitope tag, each failed to coimmunoprecipitate PTPase activity (Fig. 4*A*). Western blotting of the immunecomplexes using BC3 antiserum indicated that similar amounts of each mutant were recovered (Fig. 4*A*). Mutants dl853–963 and dl965–1012 are defective in focal adhesion targeting and paxillin binding (42, 47). Conversely, epitope-tagged FAK is defective for paxillin binding but is correctly localized to focal adhesions. Thus, paxillin binding correlates with ability of FAK to coimmunoprecipitate PTPase activity from cell lysates. These two results support the hypothesis that FAK associates indirectly with PTPase activity and that paxillin may serve as an intermediary binding protein.

The 97-kDa PTPase Is PTP-PEST—To determine whether PTP-PEST was the paxillin-associated PTPase, paxillin was immunoprecipitated from CE cells or Swiss 3T3 cells, and the immunecomplexes were probed with an anti-PTP-PEST antibody in a Western blot (Fig. 5*A*). PTP-PEST was detected in paxillin immunecomplexes formed from each cell type. Control immunecomplexes lacking the primary paxillin monoclonal antibody did not contain PTP-PEST. Interestingly, the molecular mass of PTP-PEST differed between species. Murine PTP-PEST exhibited a molecular mass of 120 kDa, whereas avian PTP-PEST was 97 kDa. Thus, the molecular mass of avian PTP-PEST coincides with that of the major PTPase activity that associates with paxillin. In addition, PTP-PEST was immunoprecipitated from CE cells and Swiss 3T3 cells, and the immunecomplexes were Western blotted for paxillin. Control immunoprecipitations using nonimmune serum contained no paxillin, whereas PTP-PEST immunecomplexes contained detectable paxillin (Fig. 5*B*). To confirm that the PTPase activity

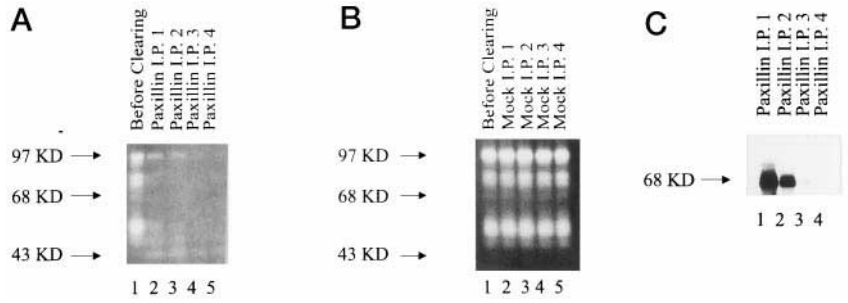

FIG. 3. **Paxillin mediates the FAK/PTPase association.** *A*, CE cell lysates, either before clearing (*lane 1*) or after each of four consecutive paxillin immunoprecipitations (*lanes 2–5*) were incubated with GST-C-FAK, and bound PTPase activity was detected using the in gel PTPase assay. *B*, as in A, except that four consecutive control immunoprecipitations using secondary antibody alone were performed. *C*, the immunecomplexes from each of the four consecutive paxillin immunoprecipitations described in *A* were analyzed by Western blotting for paxillin.

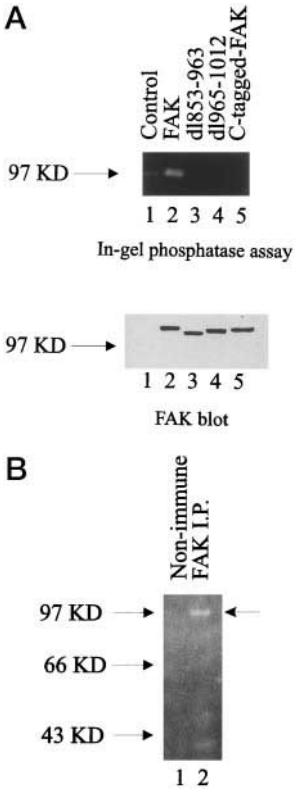

FIG. 4. **C-terminal FAK mutants fail to bind PTPase activity *in vivo*.** *A*, CE cells (*lane 1*) or CE cells expressing wild type FAK (*lane 2*), dl853–963 (*lane 3*), dl965–1012 (*lane 4*), or epitope-tagged FAK (*lane 5*) were lysed in modified radioimmunoprecipation assay buffer and FAK immunoprecipitated using BC3. The immunecomplexes were analyzed using the in gel PTPase assay (*top panel*). Each immunecomplex was also Western blotted using BC3 to verify that equivalent amounts of wild type and mutant FAK were recovered (*bottom panel*). *B*, CE cells were lysed in Triton X-100 lysis buffer. Endogenous FAK was immunoprecipitated using BC3, and the immunecomplex was analyzed using the in gel PTPase assay (*lane 2*). Immunecomplexes formed using nonimmune serum were used as the negative control (*lane 1*).

originally detected by its association with recombinant FAK was indeed PTP-PEST, CE and Swiss 3T3 cell lysates were incubated with GST-C-FAK, and the bound proteins were blotted for PTP-PEST. Indeed, PTP-PEST was found to be associated with GST-C-FAK (Fig. 5*C*). Despite the presence of PTPase activity in FAK immunecomplexes (Fig. 4), PTP-PEST could not be detected in FAK immunecomplexes by Western blotting. This presumably reflects the low stoichiometry of association with FAK. These results identify PTP-PEST as the PTPase that coimmunoprecipitates with paxillin and associates with the C-terminal domain of FAK *in vitro*.

At first glance, comparing the amount of PTP-PEST in paxillin immunecomplexes with the amount immunoprecipitated directly with the PTP-PEST antiserum, PTP-PEST appears to associate with paxillin at a relatively high stoichiometry. Therefore, experiments were performed to more carefully examine the stoichiometry of association. Serial paxillin immunoprecipitations were performed. Most of the paxillin was recovered in the first immunoprecipitation, some was recovered in the second immunoprecipitation, and vanishingly small amounts recovered in subsequent immunoprecipitations (Fig. 6*C*). Coimmunoprecipitating PTP-PEST was recovered predominantly in the first immunoprecipitation, although it was detectable in the second immunoprecipitation as well (Fig. 6*B*). To examine the stoichiometry of association, lysates were Western blotted before and after paxillin was removed by immunoprecipitation. By comparison with serial dilutions of the lysate prior to paxillin immunoprecipitation, it was estimated that up to 50% of the PTP-PEST was removed upon immunoprecipitation of paxillin (Fig. 6*A*). Control immunoprecipitations did not detectably diminish the amount of PTP-PEST in the lysate (Fig. 6*A*). In addition, the amount of PTP-PEST recovered in paxillin immunoprecipitations was compared with the amount of PTP-PEST in the lysate by Western blotting. In Fig. 6*B*, the lysate loading control contains 50 μg of cell lysate (*lane 7*), whereas paxillin was immunoprecipitated from 300 μg of lysate. Thus, if equivalent signals were present in the lanes containing lysate (*lane 7*) and the paxillin immunecomplex (lanes 4–6), 15% of the PTP-PEST had been recovered in the paxillin immunecomplexes. It is apparent that the amount of PTP-PEST recovered in the paxillin immunecomplexes easily exceeded 15% of that found in the lysate. It is therefore concluded that PTP-PEST associates with paxillin with a relatively high stoichiometry (a conservative estimate would be 20–25%).

The Noncatalytic Domain of PTP-PEST Binds Paxillin—To map the paxillin binding site within PTP-PEST, fragments of the PTPase were expressed as GST fusion proteins. The fusion proteins were immobilized on glutathione beads and incubated with CE cell lysates, and the cellular proteins that bound to PTP-PEST were probed for paxillin by Western blotting. The PTP-PEST 1–305 construct (containing the catalytic domain) did not associate with paxillin (Fig. 7), whereas the PTP-PEST 297–775 construct did. Thus, the paxillin binding site resides in the C-terminal noncatalytic domain of PTP-PEST. To further narrow the binding site, two additional constructs were engineered. PTP-PEST 441–775 exhibited no paxillin binding activity, whereas PTP-PEST 297–494 bound paxillin as well as the construct containing the entire C-terminal domain (Fig. 7). Therefore, the paxillin binding site on PTP-PEST resides between residues 297 and 494. To determine whether this region of PTP-PEST also mediated association with paxillin *in vivo*, a deletion mutant lacking residues 300–494 was engineered. Both wild type PTP-PEST and the mutant were epitope-tagged using the KT3 tag and subcloned into pcDNA3. The constructs were transiently transfected into Cos cells. Cells were lysed, and exogenously expressed PTP-PEST was immunoprecipitated with the antibody recognizing the tag. Each immunecomplex was probed for paxillin by Western blotting. Wild type

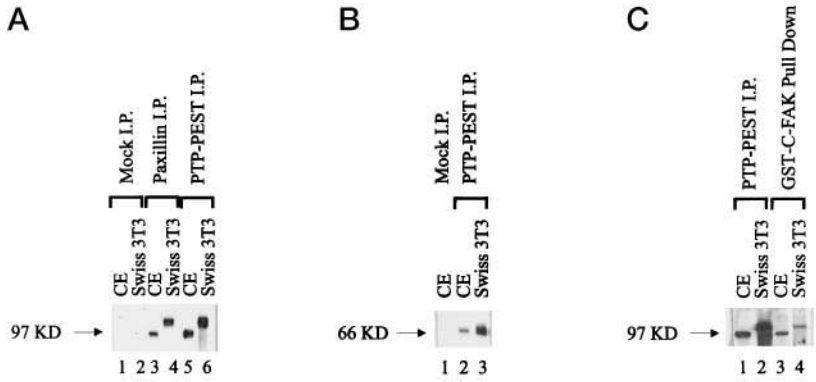

FIG. 5. **Paxillin coimmunoprecipitates with PTP-PEST.** *A*, CE cell lysates (*lanes 1, 3*, and *5*) or Swiss 3T3 cell lysates (*lanes 2, 4*, and *6*) were incubated with a control antibody (*lanes 1* and *2*), a paxillin monoclonal antibody (*lanes 3* and *4*), or the PTP-PEST antiserum (*lanes 5* and *6*). The immunecomplexes were collected and analyzed by Western blotting using the PTP-PEST antiserum. *B*, CE cell lysates (*lanes 1* and *2*) or Swiss 3T3 cell lysates (*lane 3*) were subjected to immunoprecipitation with the PTP-PEST antiserum (*lanes 2* and *3*). The immunecomplexes were blotted with a paxillin monoclonal antibody. A control immunoprecipitation was performed on the CE cell lysate (*lane 1*). *C*, lysates of CE cells (*lanes 1* and *3*) or Swiss 3T3 cells (*lanes 2* and *4*) were either subjected to immunoprecipitation using the PTP-PEST antiserum (*lanes 1* and *2*) or incubated with GST-C-FAK (*lanes 3* and *4*). The immunecomplexes and GST-C-FAK binding proteins were Western blotted with the PTP-PEST antiserum.

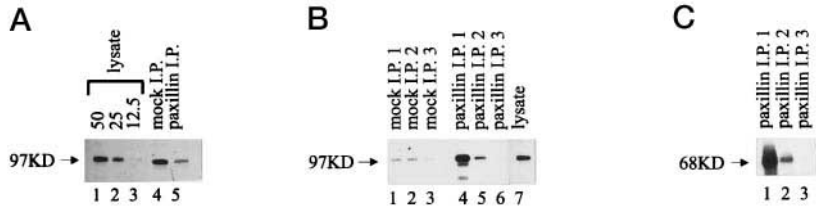

FIG. 6. **Stoichiometry of PTP-PEST association with paxillin.** Three sequential paxillin immunoprecipitations or control immunoprecipitations were performed on 300 μl of CE cell lysate (at a concentration of 1 mg/ml). *A*, 50 μl of the supernatant following the paxillin immunoprecipitations (*lane 5*) or control immunoprecipitations (*lane 4*) was analyzed by Western blotting for PTP-PEST. For comparison, 50 (*lane 1*), 25 (*lane 2*) or 12.5 (*lane 3*) μl of the original lysate was analyzed in parallel. *B*, each of the sequential paxillin immunecomplexes (*lanes 4–6*) and the control immunecomplexes (*lanes 1–3*) was analyzed by Western blotting for PTP-PEST. As a control, 50 μg of lysate was analyzed in parallel (*lane 7*). Because the immunoprecipitations were performed using 300 μg of lysate, the signal intensity of PTP-PEST in the paxillin immunecomplexes equals the signal intensity in the lysate loading control if 15% of the PTP-PEST is recovered in the immunecomplex. *C*, the three sequential paxillin immunecomplexes were probed for paxillin by Western blotting.

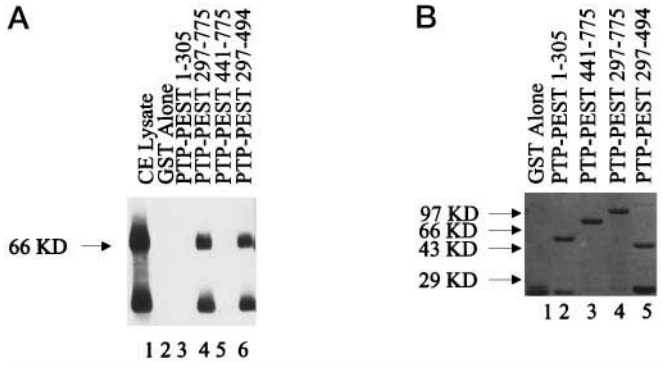

FIG. 7. **Noncatalytic domain of PTP-PEST binds paxillin *in vitro*.** *A*, CE cell lysate was precleared with GST alone and then incubated with GST (*lane 2*) or GST fusion proteins containing the catalytic domain of PTP-PEST (1–305) (*lane 3*), the noncatalytic domain of PTP-PEST (297–775) (*lane 4*), or fragments of the noncatalytic domain of PTP-PEST containing residues 441–775 (*lane 5*) or residues 297–494 (*lane 6*). Bound proteins were eluted with sample buffer and analyzed by Western blotting for paxillin. CE cell lysate was directly loaded in *lane 1* as a control. *B*, the GST fusion proteins utilized in *A* were analyzed by SDS-PAGE and Coomassie Blue staining to verify that equivalent amounts of protein were used in the analysis.

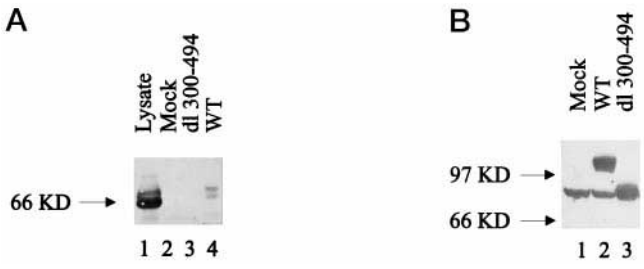

FIG. 8. **Noncatalytic domain of PTP-PEST bind paxillin *in vivo*.** *A*, Cos cells were transiently transfected with pcDNA constructs encoding epitope-tagged PTP-PEST constructs. After 48 h, the cells were lysed and PTP-PEST immunoprecipitated with the monoclonal antibody recognizing the KT3 epitope tag. Immunecomplexes formed from mock-transfected cells (*lane 2*) or cells transfected with dl300–494 (*lane 3*) or with wild type PTP-PEST (*WT, lane 4*) were Western blotted for paxillin. Lysate was loaded in *lane 1* as a control. *B*, 50 μg of lysate from mock-transfected (*lane 1*), wild type PTP-PEST transfected (*WT, lane 2*), or dl300–494-transfected (*lane 3*) cells were Western blotted with monoclonal antibody KT3. The 90-kDa protein recognized by KT3 in the mock and wild type samples is SV40 large T antigen, which is expressed in Cos cells and contains the KT3 epitope. Note, however, that paxillin is not coimmunoprecipitated with large T antigen (*A, lane 2*).

PTP-PEST coimmunoprecipitated paxillin from Cos cells, whereas control immunoprecipitations lacking the primary antibody did not (Fig. 8). Cos cells express SV40 large T antigen, which also contains the KT3 tag. Paxillin was not coimmunoprecipitated with large T antigen, because KT3 immunoprecipitations from untransfected Cos cells did not contain detectable paxillin (Fig. 8). Thus, in transiently transfected Cos cells, the paxillin/PTP-PEST interaction can be reconstituted. The mutant PTP-PEST protein, dl300–494, was successfully expressed in Cos cells but failed to coimmunoprecipitate paxillin (Fig. 8). Therefore, the region of PTP-PEST extending from residue 297 to residue 494 is required for association with paxillin both *in vitro* and *in vivo*.

PTP-PEST Binds Directly with Paxillin—A far-Western blotting (also known as gel overlay) strategy was applied to deter-

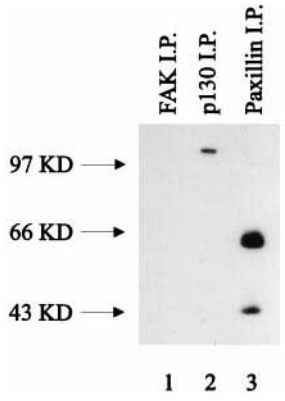

FIG. 9. **Noncatalytic domain of PTP-PEST binds directly to paxillin.** FAK immunecomplexes (*lane 1*), p130^{cas} immunecomplexes (*lane 2*) and paxillin immunecomplexes (*lane 3*) were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with a radiolabeled GST fusion protein containing residues 297–494 from PTP-PEST as a probe.

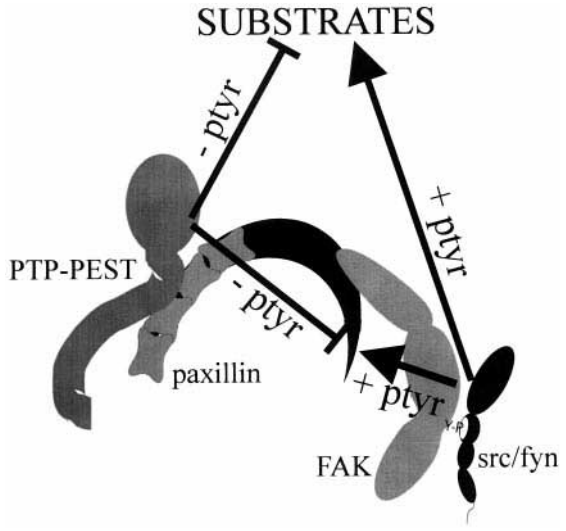

FIG. 10. **Model of PTP-PEST and focal adhesion signaling.** PTP-PEST binds directly to paxillin, and via paxillin, it may indirectly associate with FAK. Through these interactions, PTP-PEST may be directed to dephosphorylate paxillin, FAK, Src, or other focal adhesion-associated substrates and function to regulate signaling at these sites.

mine whether PTP-PEST might directly bind paxillin. Paxillin immunecomplexes were subjected to SDS-PAGE and transferred to nitrocellulose. The GST fusion protein containing PTP-PEST sequences 297–494 was radiolabeled using [γ-^{32}P]ATP and protein kinase A, which phosphorylates a site at the junction of the GST and PTP-PEST polypeptides. This fusion protein was used as the probe for the proteins immobilized on the membrane. The PTP-PEST probe bound to two proteins in the lane containing the paxillin immunecomplex (Fig. 9). The 68-kDa protein is paxillin, and the 45-kDa protein may be a paxillin-related species that can be immunoprecipitated and detected in Western blots using paxillin monoclonal antibodies (65). As controls, p130^{cas} and FAK immunecomplexes were also analyzed. The PTP-PEST probe did not bind any protein in the lane containing the FAK immunecomplex. It did, however, bind to p130^{cas} (Fig. 9). These results demonstrate that a fragment of the noncatalytic domain of PTP-PEST can directly bind to paxillin and p130^{cas} *in vitro*.

DISCUSSION

In this study, we set out to test the hypothesis that FAK intimately associated with a PTPase. Indeed, PTPase activity did associate with FAK, both *in vitro* and *in vivo*. PTPase activity was also detected in paxillin immunecomplexes. Two lines of evidence suggest that the observed association of PTPase activity with FAK was indirect. First, following immunodepletion of paxillin from cell lysates, recombinant FAK was unable to associate with PTPase activity *in vitro*. Second, mutants of FAK that are defective for paxillin binding fail to coimmunoprecipitate PTPase activity. PTP-PEST, a cytoplasmic member of a family of PTPases, was identified as the paxillin binding PTPase. A region of the noncatalytic domain of PTP-PEST contains the paxillin binding site and can directly bind paxillin *in vitro*. Therefore, we favor a model in which PTP-PEST can directly associate with paxillin and the small amount of PTP-PEST associated with FAK does so indirectly, via paxillin (Fig. 10).

PTP-PEST is a widely expressed PTPase belonging to the PEST family of PTPases, which are characterized by proline, glutamic acid, serine, and threonine rich sequences (48, 51). Immunofluorescence and subcellular fractionation studies indicate that PTP-PEST is localized to the cytoplasm of the cell (48). The enzymatic activity of PTP-PEST can be repressed by phosphorylation of serine residue 39 by either protein kinase A or protein kinase C (52). The adaptor molecule Shc binds via its phosphotyrosine binding (PTB) domain to a sequence in the noncatalytic domain of PTP-PEST (NPLH) (53, 54). An abundance of proline rich sequences in the noncatalytic domain of PTP-PEST serve as docking sites for other signaling molecules. Grb2 binds via its SH3 domain to the noncatalytic domain of PTP-PEST, and through this interaction PTP-PEST can associate with tyrosine-phosphorylated EGF receptor (48). The SH3 domain of the Csk PTK can also associate with a proline rich sequence located distally, in the noncatalytic domain of PTP-PEST (49). A distinct proline rich sequence in the noncatalytic domain of PTP-PEST serves as a docking site for the SH3 domain of p130^{cas} (37). Tyrosine-phosphorylated p130^{cas} associates tightly with the catalytic domain of "substrate trapping" mutants of PTP-PEST and can be dephosphorylated by PTP-PEST, suggesting that p130^{cas} may be a specific substrate (36). The SH3 domain mediated interaction between p130^{cas} and PTP-PEST may facilitate dephosphorylation of p130^{cas} *in vivo* (37).

In addition to these associated proteins, we have found that paxillin directly binds to the noncatalytic domain of PTP-PEST. Residues 297–494 of PTP-PEST contain both paxillin and p130^{cas} binding sites because this fragment of PTP-PEST recognizes both proteins on a far-Western blot. A sequence within this region (PPPKPPR) has been identified as the binding site for the SH3 domain of p130^{cas} (37). The paxillin binding site has not been further delineated; thus, the proximity of the paxillin and p130^{cas} binding sites is not known. Likewise, it remains to be determined whether paxillin and p130^{cas} can simultaneously dock to PTP-PEST. It may be noteworthy that paxillin and p130^{cas} can be coimmunoprecipitated (55).[2] Whether this observation is due to a direct interaction or is mediated via an intermediate protein, *e.g.* PTP-PEST or FAK, remains to be established. Coimmunoprecipitation and Western blotting experiments suggest that more than 15% of the cellular PTP-PEST is in complex with paxillin. This relatively high stoichiometry of association between endogenous PTP-PEST and endogenous paxillin presumably reflects the functional importance of the interaction. The finding that PTP-PEST associates with paxillin lends further credence to the hypothesis that PTP-PEST may control signaling by dephosphorylation of focal adhesion-associated proteins.

[2] Y. Shen and M. D. Schaller, unpublished observations.

Presumably, the function of the paxillin/PTP-PEST-containing complex is to regulate the PTPase. Conceivably, paxillin binding could regulate the enzymatic activity of the PTPase, in either a stimulatory or inhibitory manner. A more likely scenario is that paxillin regulates the accessibility of PTP-PEST to its substrates. PTP-PEST may be targeted to focal adhesions, the site of some of its substrates, *e.g.* p130^{cas}, via its association with paxillin. However, immunofluorescence experiments have failed to reveal PTP-PEST in focal adhesions (48).[2] This observation is somewhat surprising, given the relatively high stoichiometry of association of PTP-PEST with paxillin. Perhaps only a fraction of the cellular PTP-PEST resides in focal adhesions or retargeting of PTP-PEST to focal adhesions may only occur under certain conditions, *e.g.* immediately following cell adhesion. Alternatively, paxillin may control the access of PTP-PEST to its substrates by serving as a scaffold to recruit both enzyme and its substrates into a complex.

The discovery that PTP-PEST binds paxillin suggests that paxillin itself or other paxillin-binding partners might also serve as PTP-PEST substrates. FAK is a paxillin-binding protein and therefore could also serve as a PTP-PEST substrate (47, 56). An epitope-tagged FAK variant, which fails to bind paxillin (47) and PTP-PEST, is remarkably normal in the regulation of its tyrosine phosphorylation.[2] Thus, the recruitment of PTP-PEST via paxillin may not be the exclusive mechanism for the regulation of dephosphorylation of FAK. It is particularly intriguing to speculate that PTP-PEST might be able to dephosphorylate only a subset of the phosphorylation sites on FAK or paxillin. One other interesting set of candidate substrates are the Src family PTKs. Src can bind via its SH3 domain to paxillin (57), and both Src and Fyn can bind via their SH2 domains to FAK (12, 58, 59). If PTP-PEST were brought into the proximity of Src (or the FAK/Src complex) via paxillin, the active conformation of Src might be stabilized by dephosphorylation of the negative site of regulation (60). This mechanism of activation is somewhat analogous to the activation of the Src-like PTK, lck, in T cells by the CD45 PTPase (61, 62). Alternatively, PTP-PEST might dephosphorylate tyrosine 416 of Src, which is the major site of autophosphorylation and a positive regulatory site (60). Thus, models evoking PTP-PEST as both a positive and negative regulator of Src can be proposed.

The findings that PTP-PEST dephosphorylates p130^{cas} and associates with paxillin provides clues into how the enzyme might be regulated. Stimuli that induce the dephosphorylation of focal adhesion-associated proteins might enhance the activity of PTP-PEST. Alternatively, stimuli that induce tyrosine phosphorylation of focal adhesion-associated proteins might inhibit the activity of PTP-PEST. In this regard, it may be noteworthy that integrin-dependent cell adhesion induces tyrosine phosphorylation of focal adhesion proteins and stimulates the activity of protein kinase C (63, 64). Because serine phosphorylation of PTP-PEST by either protein kinase C or protein kinase A inhibits its activity (52), integrin-induced serine phosphorylation of PTP-PEST by protein kinase C could contribute to the elevation of phosphotyrosine levels following cell adhesion.

Finally, it is intriguing that multimolecular complexes containing a PTK (FAK), a PTPase (PTP-PEST), and paxillin can be immunoprecipitated from cell lysates. We speculate that paxillin may play a role in coordinating the tyrosine phosphorylation of focal adhesion associated proteins by recruiting PTP-PEST and or FAK to their substrates. Thus, paxillin may play a novel role in regulating tyrosine phosphorylation of cellular proteins in both a spatial and a temporal fashion.

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