The Noncatalytic Domain of Protein-tyrosine Phosphatase-PEST Targets Paxillin for Dephosphorylation in vivo*

Yu Shen‡, Patrick Lyons‡, Marion Cooley‡, Dominique Davidson§, André Veillette§§, Ravi Salgia‖, James D. Griffin¶, and Michael D. Schaller****

From the ‡Department of Cell Biology & Anatomy and the §§Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599, the §McGill Cancer Centre, McGill University, Montreal, Quebec H3G 1Y6, Canada, and the Division of Hematologic Malignancies, Dana Farber Cancer Institute, Boston, Massachusetts 02115

The noncatalytic domain of protein-tyrosine phosphatase (PTP)-PEST contains a binding site for the focal adhesion-associated protein paxillin. This binding site has been narrowed to a 52-residue sequence that is composed of two nonoverlapping, weak paxillin binding sites. The PTP-PEST binding site on paxillin has been mapped to the two carboxyl-terminal LIM (lin11, isl-1, and mec-3) domains. Transient expression of PTP-PEST reduced tyrosine phosphorylation of p130cas, as anticipated. A PTP-PEST mutant defective for binding p130cas does not cause a reduction in its tyrosine phosphorylation in vivo. Expression of PTP-PEST also caused a reduction of phosphotyrosine on paxillin. Expression of PTP-PEST with deletions in the paxillin-binding site did not associate with paxillin in vivo and failed to cause a reduction in the phosphotyrosine content of paxillin. These results demonstrate that paxillin can serve as a PTP-PEST substrate in vivo and support the model that a noncatalytic domain interaction recruits paxillin to PTP-PEST to facilitate its dephosphorylation.

A major mechanism of signal transduction utilized by the integrins is the activation of PTKs1 (1). One prominent PTK in this signaling pathway is FAK. FAK has been implicated in the regulation of a number of important biological functions, including cell spreading, cell motility, cell survival, and cell growth (2–8). One feature of FAK that is essential for signaling is its autophosphorylation and association with Src family PTKs. Several focal adhesion-associated, FAK-binding proteins also become tyrosine-phosphorylated in response to activation of integrin signaling pathways including paxillin and p130cas. Tyrosine phosphorylation of paxillin may function in controlling the rate of cell spreading (9). Tyrosine phosphorylation of p130cas has been implicated in controlling cell motility (10, 11). The interactions of paxillin and p130cas with SH2 domain-containing signaling molecules is regulated by tyrosine phosphorylation, and assembly of the p130cas/ERK complex appears to be important in regulating cell motility (11).

Although tyrosine phosphorylation of focal adhesion-associated proteins has been intensively investigated, the dephosphorylation of these proteins has not been extensively studied until recently. PTPs function to dephosphorylate tyrosine-phosphorylated substrates and a number of candidate PTPs that may regulate signaling through focal adhesion-associated proteins have recently been identified. In MCF7 cells, a fraction of the transmembrane PTP leukocyte common antigen-related protein was localized in focal adhesions, although there is no evidence implicating leukocyte common antigen-related protein in the regulation of tyrosine phosphorylation of focal adhesion-associated proteins (12). There is biochemical evidence suggesting that two focal adhesion-associated proteins, FAK and p130cas, are substrates for PTEN/MMAC1 (phosphatase and tensin homolog deleted on chromosome 10/mutated in multiple advanced cancers 1) (13, 14). Further, perturbation of PTEN expression levels alters the ability of cells to spread and migrate, biological functions that FAK and p130cas are believed to control (13). Similarly, PTP1B has been implicated in regulating the phosphotyrosine content of FAK, paxillin, and p130cas in controlling the rate of cell spreading and migration (15–17). In addition, PTP1B can associate with p130cas via an SH3 domain mediated interaction and may sometimes localize to focal adhesions (16, 17). The SH2 domain containing PTP, SHP2, can be coimmunoprecipitated with FAK (18). In fibroblasts lacking functional SHP2, the phosphotyrosine content of FAK and paxillin is perturbed and the cells exhibit retarded spreading on fibronectin and reduced motility (18, 19).

One other candidate PTP for regulation of focal adhesion-associated proteins is PTP-PEST. This PTP was first implicated in this function when p130cas was identified as a PTP-PEST substrate using a substrate trapping approach (20). In addition, there are binding sites in the noncatalytic domain of PTP-PEST for both the SH3 domain of p130cas and for paxillin (21, 22). Overexpression of PTP-PEST in Rat 1 fibroblasts reduces tyrosine phosphorylation of p130cas, but not tyrosine phosphorylation of FAK or paxillin, and retards cell motility (23). Conversely, PTP-PEST−/− fibroblasts exhibit elevated levels of tyrosine phosphorylation of p130cas (24). These cells also exhibit dramatic elevation in the phosphotyrosine content of both FAK and paxillin (24). The PTP-PEST−/− cells spread more rapidly than wild type cells but show reduced motility (24).

In this report, we further characterize the interaction of PTP-PEST with paxillin. The PTP-PEST binding site on paxillin is localized to the two carboxyl-terminal LIM domains. The paxillin-binding site on PTP-PEST is shown to reside close to,
but is independent of, the p130Cas-binding site. Although paxillin does not associate with substrate trapping constructs of PTP-PEST, expression of PTP-PEST in 293 cells results in a reduction in the level of tyrosine phosphorylation of endogenous paxillin. Using mutants of PTP-PEST, we demonstrate that the interaction of p130Cas and paxillin with the noncatalytic domain of PTP-PEST is required for their dephosphorylation in vivo.

MATERIALS AND METHODS

Cells—Chicken embryo (CE) cells were prepared and cultured as described previously (25). 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. COS cells in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transfected using LipofectAMINE (Life Technologies Inc.) following the manufacturer's recommended protocol.

Molecular Biology—For expression as GST fusion proteins, fragments of PTP-PEST were amplified by PCR using the PTP-PEST cDNA as template. Primers were designed to create a 5' BamHI site and a 3' EcoRI site, and the fragments were inserted between the BamHI and EcoRI sites of the multiple cloning site of pGEX-2TK (Amersham Pharmacia Biotech), in-frame with the GST coding sequence. For expression in yeast, the 2-hybrid system, the fragment of PTP-PEST extending from codon 297 to codon 485 was amplified by PCR using primers that created this fragment was subcloned into the pGBT9 vector (CLONTECH, Palo Alto CA) and pGAD424 (CLONTECH) in-frame with the Gal 4 sequences. Various fragments of paxillin were amplified by PCR and subcloned in-frame into pGGBT9 or pGAD424. The amino-terminal domain of paxillin (residues 3–332) was subcloned into the pGBT9 vector because expression of this fragment of paxillin in the pGBT9 vector results in autoactivation of the reporter gene. The carboxy-terminal domain of paxillin (codons 320–559) was subcloned into pGGBT9. Smaller fragments of paxillin, including LIM domains 1, 2, and 3 (codons 320–502), LIM domains 2, 3, and 4 (codons 376–559), LIM domains 1 and 2 (codons 320–441), LIM domains 2 and 3 (codons 376–502), LIM domains 3 and 4 (codons 436–559), LIM domain 1 (codons 320–381), LIM domain 2 (codons 376–441), LIM domain 3 (codons 436–502), and LIM domain 4 (codons 495–559) were inserted in-frame into pGAD424. Constructs containing the LIM 4 domain terminate at the stop codon of paxillin. The 3' primers used to amplify each of the other fragments created an in-frame stop codon. For expression in mammalian cells, epitope-tagged derivatives of PTP-PEST were engineered into pcDNA 3 as described previously (22). The point mutant P337A was created by site-directed mutagenesis using the Altered Sites kit (Promega, Madison WI). Mutant dl367–400 was created by PCR amplification of a DNA fragment encoding residues 99–367 using a primer creating a 3' KpnI site and the fragments were inserted between the EcoRI and KpnI sites of the multiple cloning site of pGEX-2TK. Sections of the lacZ reporter was tested using a colorimetric assay (Fig. 1 A). When the amino-terminal half of paxillin was expressed alone as a GAL4 DNA binding domain fusion protein, it activated the reporter gene. Therefore reciprocal constructs were engineered to determine whether the amino-terminal domain of paxillin interacted with PTP-PEST. Residues 297–485 of PTP-PEST were expressed as a GAL4 DNA binding domain fusion protein using the pGBT9 vector. A fragment of PTP-PEST containing the paxillin-binding site (residues 297–485) was expressed in COS cells as GST fusion proteins and purified as described (28). Unless otherwise indicated, 1–2 μg of fusion protein (immobilized to glutathione beads) was incubated with 100–200 μg of lysate for 1 h. The beads were washed as described for immune complexes above, and bound paxillin was detected by Western blotting.

RESULTS

PTP-PEST Binds to the LIM Domains of Paxillin—The yeast two-hybrid system was used to map the PTP-PEST binding site on paxillin. The carboxy-terminal half of paxillin was expressed as a GAL4 DNA binding domain fusion protein using the pGBT9 vector. A fragment of PTP-PEST containing the paxillin-binding site (residues 297–485) was expressed as a GAL4 activation domain fusion protein using the pGAD424 vector. Coexpression of these two proteins induced expression of the lacZ reporter as assessed using a colorimetric assay (Fig. 1A). When the amino-terminal half of paxillin was expressed alone as a GAL4 DNA fusion protein, it activated the reporter gene. Therefore reciprocal constructs were engineered to determine whether the amino-terminal domain of paxillin interacted with PTP-PEST. Residues 297–485 of PTP-PEST were expressed as a GAL4 DNA binding domain fusion protein and the amino-terminal domain of paxillin was expressed as a GAL4 activation domain fusion protein. Coexpression of these two proteins failed to activate expression of the reporter gene (Fig. 1A). Despite their inability to interact, both of these fusion proteins were functional in yeast. The amino-terminal domain of paxillin interacted with a GAL4 DNA binding domain fusion protein containing the paxillin-binding site of PAK (Fig. 1A). The GAL4 DNA binding domain/PTP-PEST fusion protein interacted with several GAL4 activation domain/paxillin LIM domain fusion proteins (see below). Thus in the yeast two-hybrid system PTP-PEST binds to the carboxy-terminal, LIM domain containing half of paxillin.

To validate the findings from the yeast two-hybrid system, fragments of paxillin were transiently expressed in COS cells and tested for PTP-PEST binding activity. The amino- and carboxy-terminal halves of paxillin were subcloned into pcDNA3 for transient expression. Each was engineered to contain the KT3 epitope tag at the carboxyl terminus. Both of these constructs were efficiently expressed in COS cells as determined by Western blotting whole cell lysates using the KT3 monoclonal antibody (Fig. 2, lanes 1 and 2). To examine PTP-PEST binding, cell lysates were incubated with a GST fusion protein containing PTP-PEST residues 297–494, which
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was immobilized on glutathione-agarose beads. The beads were collected and washed, and associated fragments of paxillin were detected by Western blotting with KT3. The carboxyl-terminal half of paxillin was found associated with the GST/PTP-PEST fusion protein (Fig. 2, lane 3). Although the amino-terminal half of paxillin was expressed in the COS cell lysates, it failed to interact with the GST/PTP-PEST fusion protein (Fig. 2, lane 4). These results are in accord with the yeast two-hybrid data and demonstrate that the PTP-PEST binding region is within the carboxyl-terminal half of paxillin.

To further refine the PTP-PEST binding site within paxillin, a series of deletion mutants was engineered and analyzed in the yeast two-hybrid system for interaction with PTP-PEST. Originally, the paxillin/PTP-PEST interaction was reconstituted in yeast by expressing the four paxillin LIM domains as the binding site for one of the LIM domains of enigma (29). It was previously demonstrated that a recombinant protein containing PTP-PEST codons 297–494 could directly bind to both p130cas and paxillin (22). The p130cas binding site in PTP-PEST has been identified as the proline-rich region as p130cas, two GST fusion proteins containing PTP-PEST residues 276–400 were utilized. One contained wild type PTP-PEST sequences and the other contained a point mutation, proline 337 to alanine, which has been shown to abrogate association with the p130cas SH3 domain (21). Each fusion protein was immobilized on glutathione beads, incu-

FIG. 1. Yeast 2-hybrid analysis of paxillin/PTP-PEST interaction. A, yeast containing pGBT9/paxillin carboxyl terminus (Cterm) + pGAD424/PTP-PEST, pGBT9/PTP-PEST + pGAD424/paxillin amino terminus (Nterm), or pGBT9/FAK Cterm + pGAD424/paxillin Nterm were grown on nitrocellulose on minimal medium lacking leucine and tryptophan. The resulting colonies were analyzed using the blue/white assay. B, yeast containing pGBT9/paxillin Cterm + pGAD424/PTP-PEST, pGBT9/PTP-PEST + pGAD424/paxillin LM 123, or pGBT9/PTP-PEST + pGAD424/paxillin LM 234 were analyzed as in A. C, yeast containing pGBT9/PTP-PEST and either pGAD424/paxillin LM 12, pGAD424/paxillin LM 23, pGAD424/paxillin LM 34, pGAD424/paxillin LM 33, or pGAD424/paxillin LM 44 were analyzed as in A.

FIG. 2. The LIM domains of paxillin bind PTP-PEST in vitro. Cells transfected with constructs designed to express either the epitope-tagged amino-terminal half of paxillin (lanes 2 and 4) or the tagged carboxyl-terminal half of paxillin (lanes 1 and 3) were analyzed. Expression was verified by Western blotting whole cell lysates with KT3 (lanes 1 and 2). Lysates were incubated with GST/PTP-PEST, and bound fragments of paxillin were detected by Western blotting with KT3 (lanes 3 and 4).

domains 2, 3, and 4 did interact with PTP-PEST in the two-hybrid system (Fig. 1B). Next, fusion proteins containing pairs of LIM domains from paxillin were analyzed. Whereas a protein containing LIM domains 3 and 4 from paxillin interacted with PTP-PEST, fusion proteins containing either LIM domains 1 and 2 or LIM domains 2 and 3 failed to interact (Fig. 1C). Additional constructs containing each of the individual LIM domains of paxillin fused to the GAL 4 activation domain were tested but all failed to interact with PTP-PEST in the two-hybrid system. The failure of individual LIM domains to interact with PTP-PEST could indicate that a functional binding site is composed of sequences from both LIM 3 and LIM 4. In contrast, the stability of the structure or the strength of the interaction of individual LIM domains may simply be compromised. To attempt to strengthen the potential interaction between individual LIM domains and PTP-PEST, multiple copies of individual LIM domains were engineered in-frame with the Gal 4 activation domain. However, tandem copies of LIM domain 3 (called LIM 33) or LIM domain 4 (called LIM 44) both failed to interact with PTP-PEST in the yeast 2-hybrid system (Fig. 1C). The results of this analysis demonstrate that the PTP-PEST binding site on paxillin lies within LIM domains 3 and 4.

PTP-PEST Contains Separate p130cas and Paxillin Binding Sites—It was previously demonstrated that a recombinant fusion protein containing PTP-PEST codons 297–494 could directly bind to both p130cas and paxillin (22). The p130cas binding site in PTP-PEST has been identified as the proline-rich sequence extending from amino acid 333 to 338 (21). The recognition binding sites for several LIM domains have been identified, and a proline containing sequence has been implicated as the binding site for one of the LIM domains of enigma (29). To exclude the possibility that paxillin binds to the same proline rich region as p130cas, two GST fusion proteins containing PTP-PEST residues 276–400 were utilized. One contained wild type PTP-PEST sequences and the other contained a point mutation, proline 337 to alanine, which has been shown to abrogate association with the p130cas SH3 domain (21). Each fusion protein was immobilized on glutathione beads, incu-
Removal of residues from either the amino or carboxyl termini of this fragment dramatically reduced, but did not totally abrogate, paxillin-binding activity. This observation could be explained if further deletion destabilized a single binding site. Alternatively, this observation could indicate that there are two weaker paxillin-binding sites within this region of PTP-PEST, and both binding sites are required for full binding activity. This idea is consistent with the observations that GST/PTP-PEST 277–368 and GST/PTP-PEST 371–492 both exhibit very weak but detectable paxillin binding activity. This hypothesis was further tested by more extensively analyzing the binding activity of two nonoverlapping constructs, GST/PTP-PEST 277–368 and GST/PTP-PEST 371–492. Although both fusion proteins bound very weakly to paxillin under the standard conditions of interaction, significant binding activity was detected if 10-fold more fusion protein was used in the binding reaction (Fig. 5C, top panel). Importantly, under these conditions, GST alone did not detectably bind paxillin (Fig. 5C, top panel, lane 2). Therefore, the interaction is apparently still specific under these conditions. These results suggest that there are two sites of PTP-PEST that can weakly interact with paxillin. One site lies between amino acids 338 and 368 and the other resides between residues 371 and 390. Both weak binding sites are required for the efficient association of paxillin with PTP-PEST.

Generation of a PTP-PEST Polyclonal Antiserum—To further characterize PTP-PEST, a polyclonal antiserum recognizing a fragment of the noncatalytic domain was generated. The carboxyl-terminal 304 residues of PTP-PEST were expressed as a GST fusion protein and purified using glutathione-agarose beads. The fusion protein was used as an antigen to raise polyclonal antisera in rabbits. The antiserum was characterized using lysates of 293 cells and 293 cells expressing exogenous PTP-PEST. In a Western blot of whole cell lysate, the antiserum detected proteins of 105 (a doublet), 70, 65, 45, and 35 kDa (Fig. 6A, lane 3). Preimmune serum recognized a major species of 45 kDa and several lower molecular mass species (Fig. 6A, lanes 1 and 2). Therefore these proteins were not specifically recognized by the antiserum in Western blots. The 105-kDa doublet was endogenous PTP-PEST and was recognized by another PTP-PEST antiserum (22). In addition to these bands, lysates from 293 cells expressing exogenous murine PTP-PEST exhibited a major 120-kDa species (Fig. 6A, lane 4). Further, the intensity of the 65-kDa band was elevated. This 65-kDa species was a PTP-PEST breakdown product because lysates of 293 cells expressing deletion variants of PTP-PEST exhibit corresponding increases in the electrophoretic mobility of this species (Fig. 7A). The antiserum was further characterized by immunoprecipitation analysis. The antiserum immunoprecipitated the 105-kDa endogenous PTP-PEST protein from 293 cell lysates and both the endogenous and exogenous proteins from lysates of 293 cells transiently expressing PTP-PEST (Fig. 6B, lanes 7 and 8). The 65- and 70-kDa proteins were weakly detected in these immune complexes. Preimmune serum failed to precipitate these proteins (Fig. 6B, lanes 5 and 6). Thus, this antiserum recognized both endogenous and exogenous PTP-PEST by both immunoprecipitation and Western blotting. The antiserum also recognized an unknown 65/70-kDa doublet by Western blotting, although these species were not efficiently immunoprecipitated by the antiserum.

Expression and Characterization of PTP-PEST Mutants—A previously described deletion mutant of PTP-PEST lacking amino acids 297–493 is defective for paxillin binding (22). Presumably, this mutant is also defective for p130CAS binding because the p130CAS SH3 binding site has also been deleted.
Two additional mutants were engineered to separate the p130cas and paxillin binding defects. First, a point mutation destroying the p130 cas binding site (P337A) was created (21). Second, an additional deletion mutant lacking residues 367–400 was engineered. Epitope-tagged versions of these mutants were subcloned into pcDNA 3.1 for expression in mammalian cells. Wild type PTP-PEST, the P337A point mutant, dl 297–493, and dl 367–400 were transiently expressed in 293 cells. Expression of each construct was verified by Western blotting using a polyclonal PTP-PEST antiserum. Comparable levels of expression were achieved for each protein (Fig. 7A). Each mutant was tested for its ability to associate with endogenous p130cas and paxillin by coimmunoprecipitation and Western blotting. Exogenous PTP-PEST was immunoprecipitated using the KT3 monoclonal antibody and the immune complexes were Western blotted for paxillin. Wild type PTP-PEST and the P337A mutant coimmunoprecipitated paxillin (Fig. 7B, lanes 2 and 5), whereas dl 297–493 and dl 367–400 each exhibited a dramatic reduction in their ability to associate with paxillin (Fig. 7B, lanes 3 and 4). Equal amounts of the wild type and mutant PTP-PEST constructs are recovered by immunoprecipitation with KT3. These results are consistent with the in vitro binding data and verify that the proline-rich p130 cas-binding site is not required for paxillin binding. The association of p130cas with PTP-PEST was examined by immunoprecipitating PTP-PEST with a polyclonal antiserum and Western blotting for p130cas. There was detectable p130 cas found in PTP-PEST immune complexes from lysates of control transfected cells.
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Fig. 6. Characterization of PTP-PEST antiserum. A, 25 µg of lysates from 293 cells (lanes 1 and 3) or 293 cells expressing exogenous PTP-PEST (lanes 2 and 4) was Western blotted using preimmune serum (lanes 1 and 2) or the PTP-PEST polyclonal antiserum (lanes 3 and 4). The position of the molecular mass markers are indicated on the left. B, 300 µg of lysate from 293 cells (lanes 1, 3, 5, and 7) or 293 cells expressing exogenous PTP-PEST (lanes 2, 4, 6, and 8) was incubated with preimmune serum (lanes 1, 2, 5, and 6) or the PTP-PEST polyclonal antiserum (lanes 3, 4, 7, and 8). The immune complexes were Western blotted with preimmune serum (lanes 1–4) or the polyclonal PTP-PEST antiserum (lanes 5–8). The position of the molecular mass markers are indicated on the left. The strongly reactive bands from 40–60 kDa and below 29 kDa are the immunoprecipitating antibodies that were detected with the anti-rabbit secondary antibody used in these experiments.

Fig. 7. Expression and characterization of PTP-PEST mutants. 293 cells were transfected with empty pcDNA (lanes 1) or pcDNA containing wild type PTP-PEST (lanes 2), dl 367–400 (lanes 3), dl 297–493 (lanes 4), or P337A (lanes 5). Twenty-five µg of cell lysate was analyzed by Western blotting using a PTP-PEST polyclonal antiserum (A). B, exogenous PTP-PEST was immunoprecipitated with KT3 and associated paxillin was detected by Western blotting. C, PTP-PEST was immunoprecipitated with a polyclonal antiserum and Western blotted with a p130<sup>cas</sup> monoclonal antibody. (Fig. 7C, lane 1). This reflects the coimmunoprecipitation of p130<sup>cas</sup> with endogenous, wild type PTP-PEST. The amount of p130<sup>cas</sup> recovered by coimmunoprecipitation of lysates of PTP-PEST expressing cells was increased relative to the amount seen from control cells. Both wild type PTP-PEST and dl 367–400 associated with p130<sup>cas</sup> because elevated levels of p130<sup>cas</sup> were detected in PTP-PEST immune complexes (Fig. 7C, lanes 2 and 3). In contrast, the amount of p130<sup>cas</sup> detected in PTP-PEST immune complexes from lysates of dl 297–493 expressing cells was equivalent to the amount of p130<sup>cas</sup> immunoprecipitated from control transfected cells, demonstrating that dl 297–493 failed to associate with p130<sup>cas</sup> (Fig. 7C, lane 4). Equivalent amounts of each PTP-PEST construct were recovered by immunoprecipitation using the polyclonal antiserum. Thus, the P337A and dl 367–400 mutants of PTP-PEST demonstrate the dissociation of p130<sup>cas</sup> binding activity from paxillin binding activity in vivo and provide the tools to assess the role of p130<sup>cas</sup> and paxillin binding in the biochemical and biological functions of PTP-PEST.

Biochemical Consequences of PTP-PEST Expression—PTP-PEST was expressed transiently in 293 cells, and the levels of cellular phosphotyrosine were examined by Western blotting whole cell lysates. There was no detectable difference in the profile of phosphotyrosine containing proteins from control cell lysates and from lysates of PTP-PEST expressing cells (Fig. 8A). Thus, expression of PTP-PEST did not result in the indiscriminate dephosphorylation of cellular proteins. Because p130<sup>cas</sup> has been implicated as a substrate for PTP-PEST, its tyrosine phosphorylation was examined by immunoprecipitation and Western blotting. There was detectable phosphotyrosine on p130<sup>cas</sup> in control 293 cells (Fig. 8C, top panel, lane 1). Expression of PTP-PEST reduced the level of phosphotyrosine on p130<sup>cas</sup> (Fig. 8C, top panel, lane 2). After stripping and reprobing the blot with a p130<sup>cas</sup> antibody, it was evident that equal amounts of protein were present in each lane (Fig. 8C, middle panel). Thus, expression of PTP-PEST results in dephosphorylation of p130<sup>cas</sup>. Paxillin is also a candidate substrate for PTP-PEST because it is an associated phosphotyrosine containing protein. Its level of tyrosine phosphorylation was also examined by immunoprecipitation and Western blotting. Transient expression of PTP-PEST resulted in a reduction of the phosphotyrosine content of paxillin (Fig. 8B, top panel, lanes 1 and 2). Stripping and reprobing the membrane with a paxillin antibody demonstrated that equal amounts of protein were recovered (Fig. 8B, middle panel). These results suggest that paxillin may also serve as a PTP-PEST substrate in vivo.

To determine the importance of the noncatalytic domain of PTP-PEST in targeting these substrates for dephosphorylation, mutants were analyzed. Two mutants that fail to bind p130<sup>cas</sup>, P337A and dl 297–493, were examined. The amount of phosphotyrosine on p130<sup>cas</sup> in cells transiently transfected...
These results demonstrate that the binding site for the SH3 domain with expression of wild type paxillin (Fig. 8A). The phosphotyrosine content of paxillin similar to that seen in control cells (Fig. 8B). Paxillin was immunoprecipitated, and its phosphotyrosine content was then examined by Western blotting (top panel). The blot was stripped and reprobed with a paxillin monoclonal antibody (middle panel). C, p130cas was immunoprecipitated, and its phosphotyrosine content was examined by Western blotting (top panel). The blot was stripped and reprobed with a p130cas monoclonal antibody (middle panel).

with P337A or dl 297–493 was comparable to phosphotyrosine levels in control cells (Fig. 8C, top panel, lanes 4 and 5). In contrast, dl 370–400, which retains p130cas binding activity, reduced tyrosine phosphorylation of p130cas to the same extent as wild type PTP-PEST (Fig. 8C, top panel, lanes 2 and 3). Two mutants defective for paxillin binding, dl 297–493 and dl 367–400, were also analyzed. The phosphotyrosine content of paxillin from lysates of cells expressing either of these mutants was the same as the level seen in control cells (Fig. 8B, top panel, lanes 2 and 3). P337A expression resulted in a reduction of the phosphotyrosine content of paxillin similar to that seen with expression of wild type paxillin (Fig. 8B, top panel, lane 5). These results demonstrate that the binding site for the SH3 domain of p130cas is required for PTP-PEST-induced dephosphorylation of p130cas in vivo and that the paxillin-binding site of PTP-PEST is necessary for PTP-PEST-induced dephosphorylation of paxillin.

**DISCUSSION**

The data presented in this report demonstrate that the two carboxyl-terminal LIM domains of paxillin contain a binding site for PTP-PEST. The paxillin-binding site on PTP-PEST has been mapped to a 52-residue region near the catalytic domain, and the evidence suggests that this binding site is composed of two binding sites of much weaker activity. PTP-PEST mutants that fail to associate with either paxillin or p130cas or both have been engineered and characterized. Expression of PTP-PEST in 293 cells results in dephosphorylation of p130cas and paxillin. The ability of mutants of PTP-PEST to dephosphorylate p130cas and paxillin correlates with their ability to associate with these proteins, demonstrating that interactions with these substrates via the noncatalytic domain is required for their dephosphorylation in vivo.

Paxillin and its related proteins, hic-5 and leupaxin, contain four carboxyl-terminal LIM domains and an amino-terminal domain that contains four LD motifs and multiple tyrosine residues that may serve as sites of phosphorylation (30–33). Paxillin and hic-5 are localized to focal adhesions via their LIM domains, and the primary targeting sequence in paxillin has been further localized to the LIM 3 domain (34, 35). LIM domains have been implicated in mediating protein-protein interactions (29, 36). Presumably, the interaction of an LIM domain-binding partner tethers paxillin and hic-5 in focal adhesions. PTP-PEST is the first identified binding partner of paxillin that interacts with its LIM domains.

Recently, PTP-PEST was isolated as a hic-5-interacting protein using the hic-5 LIM domains as bait in a yeast 2-hybrid screen (37). The hic-5 binding site of PTP-PEST was localized to residues 344–427, a result that is consistent with the results of our experiments mapping the paxillin-binding site of PTP-PEST. A recent report has also described mapping studies of the paxillin/PTP-PEST interaction (38). The paxillin-binding site in this report was narrowed down to PTP-PEST residues 344–397 using GST fusion proteins containing fragments of PTP-PEST. A smaller fusion protein extending from residues 344–385 failed to bind paxillin. This result is similar to our observations that carboxyl-terminal truncation to amino acid 368 dramatically impairs the paxillin binding activity of PTP-PEST. Cote *et al.* (38) further implicate proline residue 362 of PTP-PEST in paxillin binding. Mutation of this residue to alanine eliminated paxillin-binding (38). It was therefore concluded that proline-rich region 2 (residues 355–374) of PTP-PEST is the paxillin docking site. However, this region alone appears insufficient for paxillin binding because a GST fusion protein containing residues 344–385, and thus containing proline-rich region 2, does not associate with paxillin. These observations are consistent with our finding that paxillin binds weakly to two nonoverlapping fragments of PTP-PEST, one of which contains proline-rich region 2 (and the critical residue proline 362) and the other of which lies to the carboxyl-terminal side of proline-rich region 2.

The site of PTP-PEST binding on hic-5 has also been mapped and does not precisely correspond to the site of interaction we have identified on paxillin (37). The binding site on paxillin has been narrowed to LIM domains 3 and 4, which is in agreement with the results of Cote *et al.* (38). The PTP-PEST binding site on hic-5 has been further localized to LIM 3 (37). Two lines of evidence that the hic-5 LIM 3 domain interacts with PTP-PEST have been presented. First, two overlapping fragments of hic-5 interact with PTP-PEST and the region of overlap is the LIM 3 domain (37). Second, mutation of two residues critical for metal coordination in the LIM 3 domain, which disrupts the structure of LIM domains and undoubtedly has dire consequences for the structural integrity of the protein, ablates PTP-PEST binding activity in vivo (37). There is no additional direct evidence that LIM 3 interacts with PTP-PEST because the individual LIM domain does not associate with PTP-PEST (37), a result that is in accord with our findings with paxillin. In contrast to the published hic-5 data, we have failed to detect an interaction between PTP-PEST and paxillin constructs containing LIM domains 1, 2, and 3 or LIM domains 2 and 3. It is unlikely that paxillin and hic-5 interact with PTP-PEST via different sequences given the conserved nature of the paxillin and hic-5 sequences. Most likely, these different results reflect subtle differences in the constructs or the methods of assessing interaction. Because perturbation of the structure of LIM domain 3 of hic-5 does not disrupt PTP-PEST binding in vivo, additional sequences may be required for the interaction (37). Based upon our analysis of the PTP-PEST/paxillin interaction, we speculate that these additional sequences may reside...
PTP-PEST is the first identified protein that interacts with the LIM domains of paxillin/hic-5. Although PTP-PEST binds near the focal adhesion targeting sequence of paxillin/hic-5, it is unlikely to function in directing these proteins to focal adhesions. First, based upon the recoveries of paxillin in PTP-PEST immune complexes and PTP-PEST in paxillin immune complexes, there appears to be much more paxillin than PTP-PEST in the cell (22). Second, a hic-5 mutant that disrupts the LIM 3 domain is still able to bind PTP-PEST, although the hic-5 mutant fails to target to focal adhesions (37). Third, hic-5 has been expressed in PTP-PEST fibroblasts and correctly targets fibroblasts exhibit elevated levels of tyrosine phosphorylation of paxillin, which is the expected consequence of removing the enzyme that functions to dephosphorylate paxillin (24). Although further experimentation will be required to rigorously determine whether paxillin is a PTP-PEST substrate, our data and the data from the PTP-PEST fibroblasts clearly support the candidacy of paxillin as a substrate.

p130cas has emerged as the prototypical substrate for PTP-PEST. It can be substrate-trapped by catalytically defective PTP-PEST mutants, and its phosphotyrosine content is reduced upon overexpression of PTP-PEST and is elevated in PTP-PEST fibroblasts (20, 23, 24, 40). p130cas also associates with the noncatalytic domain of PTP-PEST via an SH3 domain mediated interaction and this interaction is required for the dephosphorylation of p130cas by recombinant PTP-PEST in cell lysates (21). We have extended this observation and demonstrated that the interaction between p130cas and the noncatalytic domain of PTP-PEST is necessary for its dephosphorylation in vivo. Thus, despite the high affinity interaction between the catalytic domain of PTP-PEST and tyrosine-phosphorylated p130cas, it is the interaction with the noncatalytic domain of PTP-PEST that directs p130cas dephosphorylation. We suggest that paxillin is a second class of PTP-PEST substrate. Although it does not exhibit high affinity binding to the catalytic domain of PTP-PEST in vitro, tyrosine phosphorylation of paxillin can be regulated by PTP-PEST in vivo. As with p130cas, interactions with the noncatalytic domain of PTP-PEST direct the dephosphorylation of paxillin in vivo. Thus, the recurring theme is that the noncatalytic domain of PTP-PEST contains binding sites that recruit tyrosine-phosphorylated substrates for dephosphorylation.

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The Noncatalytic Domain of Protein-tyrosine Phosphatase-PEST Targets Paxillin for Dephosphorylation in Vivo
Yu Shen, Patrick Lyons, Marion Cooley, Dominique Davidson, André Veillette, Ravi Salgia, James D. Griffin and Michael D. Schaller

doi: 10.1074/jbc.275.2.1405

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