Intact LIM 3 and LIM 4 Domains of Paxillin Are Required for the Association to a Novel Polyproline Region (Pro 2) of Protein-Tyrosine Phosphatase-PEST*

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The focal adhesion protein p130Cas was identified as a substrate for the protein-tyrosine phosphatase (PTP)-PEST, and the specificity of this interaction is mediated by a dual mechanism involving a Src homology 3 domain-mediated binding and PTP domain recognition. Recently, paxillin was also demonstrated to interact with PTP-PEST (Shen, Y., Schneider, G., Cloutier, J. F., Veillette, A., and Schaller, M. D. (1998) J. Biol. Chem. 273, 6474–6481). In the present study, we show that amino acids 344–397 of PTP-PEST are sufficient for the binding to paxillin. We demonstrate that a proline-rich segment of PTP-PEST (Pro 2), 355PPEPHVPVPPPSTPSFPEP 374, is essential for this interaction in vivo. Furthermore, mutation of proline residues within the Pro 2 motif reveals that proline 362 is critical for the binding of paxillin. Conversely, using deletion and point mutants of paxillin, LIM 3 and 4 domains were both found to be necessary for binding of PTP-PEST. Finally, using a “substrate trapping” approach, we demonstrate that, unlike p130Cas, paxillin is not a substrate for PTP-PEST. In conclusion, we show that a novel proline-rich motif found in PTP-PEST serves as a ligand for the LIM domains of paxillin. Interestingly, the focal adhesion targeting of paxillin by LIM 3 and 4, which is essential for this interaction, is mediated by a dual mechanism involving a Src homology 3 domain-mediated binding and PTP domain recognition. Protein-tyrosine kinases are involved in the propagation of extracellular signals from a number of trans-membrane receptors. Although some receptors like the epidermal growth factor receptor and platelet-derived growth factor receptors have intrinsic tyrosine kinase activities. Others such as the integrin and T cell receptor must couple intracellular tyrosine kinases to propagate their signals. In both scenarios, protein-tyrosine phosphatases (PTPs)

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† The abbreviations used are: PTP, protein-tyrosine phosphatase; aa, amino acid(s); TCL, total cell lysate; HA, hemagglutinin epitope; p125FAK, focal adhesion kinase; p130Cas, p130 Crk-associated substrate; regulating, in a positive or negative manner, these signaling cascades (1, 2).

PTP-PEST is ubiquitously expressed throughout murine development as well as in the adult animal (3). The enzyme is composed of a N-terminal catalytic domain and a C-terminal tail rich in proline stretches that serve as binding sites for other signaling molecules. PTP-PEST has been proposed to influence mitogenic signaling downstream of the epidermal growth factor receptor due to its direct association with two key adaptor proteins, Shc and Grb2 (4–6). More recently, using substrate trapping approaches (7), the adaptor protein p130Cas was identified as a substrate of PTP-PEST (8). The SH3 domain of p130Cas as well as those of the related proteins Hef1 and Sin/Efs were shown to directly bind to the Pro 1 of PTP-PEST (9, 10). This interaction thus serves as an additional mechanism to confer specificity to PTP-PEST toward its substrates. p130Cas is a focal adhesion-localized protein whose tyrosine phosphorylation is mainly regulated by integrin engagement via the catalytic activity of p125FAK and c-Src (11). Focal adhesions are the sites of cell contact with the extracellular matrix (for review, see Refs. 12 and 13). Proteins found in focal adhesions have either structural or signaling functions. Through the juxtaposition of several enzymes and adaptor proteins such as p125FAK, p130Cas, and Src, the extracellular matrix can connect with the actin-cytoskeleton (12). Studies on PTP-PEST −/− fibroblasts have demonstrated impaired cell migration (14). This is related to the hyperphosphorylation of three focal adhesion-localized proteins, namely p130Cas, p125FAK, and paxillin in the −/− cells. In addition, a higher number of vinculin-containing focal adhesions were observed in the −/− fibroblast, in agreement with increased phosphorylation on tyrosine of the focal adhesion proteins mentioned above. These results indicate a role for PTP-PEST in focal adhesion breakdown and turnover, which are required in events such as cell migration (15). Consistent with these results, it has recently been demonstrated that Rat-1 fibroblasts overexpressing WT PTP-PEST are not efficient for migration in a wound healing assay due to p130Cas hypophosphorylation (16).

Paxillin is a member of a family of adaptor proteins that also includes Hic-5 (17) and leuapaxin (18). Located in focal adhesions, paxillin associates with important cytoskeletal proteins such as talin and vinculin as well as protein-tyrosine kinases found in adhesion plaques such as p125FAK Pyk2, and c-Src (19). In particular, the association of p125FAK with paxillin has been shown to be essential for focal adhesion targeting of

GST, glutathione S-transferase; PCR, polymerase chain reaction; SH, Src homology; PAGE, polyacrylamide gel electrophoresis; IP, immunoprecipitation; WT, wild type; PVDF, polyvinylidene difluoride; TBS-T, Tris-buffered saline with Tween 20.
p125FAK (20). In addition, following integrin engagement, paxillin has been demonstrated to be phosphorylated by p125FAK and c-Src (21, 22). This creates docking sites for the SH2 domain of the Crk proteins (23) and links the integrin activation to signal transduction pathways via the proteins C3G or SOS that are bound to Crk. In addition to its tyrosine phosphorylation, paxillin is also heavily phosphorylated on serine and threonine residues following plating of cells on fibronectin (24). Serine and threonine phosphorylation of paxillin have been implicated in its targeting to focal adhesions and attachment to fibronectin (25).

Structurally, paxillin and the paxillin-like proteins are composed of N-terminal LD motifs and four C-terminal LIM domains. The LD motifs of paxillin (reviewed in Ref. 26) have been shown to be implicated in the paxillin binding to p125FAK and vinculin (19). LD motifs have also been observed in a variety of proteins, where they also act as mediating protein-protein interaction (26). LIM domains are approximately 50 amino acids in length and known to mediate protein-protein associations (for review, see Refs. 27 and 28). The LIM domains have a conserved consensus sequence: (CXCCX(X4–6)XXHXX)XX(CXXC(X16–21)CXXC/D/H) (28). Proteins harboring LIM domains often harbor other domains such as homeodomain, kinase, SH3, and LD domains. One of the most characterized LIM domain mediated interaction involves the association of the LIM 3 of Enigma to the tyrosine-based motif (tyrosine tight turn) of the insulin receptor (29). Similarly, the LIM 2 of Enigma interacts with the Ret receptor tyrosine kinase (29).

The presence of intact LIM 3 and 4 domains of paxillin were required for its association with PTP-PEST. Failure to recognize and interact with specific protein domains. The LIM domains of paxillin, especially LIM 3, are essential for proper focal adhesion targeting. Although the focal adhesion targeting ligand of LIM 3 has not been identified (19), it has recently been shown that LIM 2 and 3 bind protein(s) with serine kinase activity (25).

In a recent report, paxillin was shown to associate directly with the C-terminal tail of PTP-PEST by a still uncharacterized mechanism (30). In the present study, we reveal that a non-classical proline-rich motif of PTP-PEST (Pro 2) is essential for both in vitro and in vivo binding of PTP-PEST to paxillin. More precisely, mutation of proline 362 to alanine completely abolishes this association. The presence of intact LIM 3 and 4 domains of paxillin were required for its association with PTP-PEST. Finally, using mutants of PTP-PEST which have a C231S mutation, we demonstrate that paxillin is not a substrate for PTP-PEST in a substrate trapping assay. Together, these results demonstrate a novel association between LIM domains and a proline-rich motif. We propose that a physiological function for this association could be that, once PTP-PEST is recruited to adhesion plaques, it could regulate the breakdown and turnover of focal adhesions by dephosphorylating and/or binding proteins such as paxillin, p130Cas, She, and Grb2.

**MATERIALS AND METHODS**

**Cell Lines, Transient Transfections, and Pervanadate Treatment—** NIH 3T3, NIH 3T3 clones overexpressing a Src Y527F constitutively active mutant, and HEK 293T cell lines were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (Life Technologies, Inc.). HEK 293T cells were transfected with 5 μg of PTP-PEST plasmid using the calcium phosphate technique as described previously (10). NIH 3T3 cells were pervanadate-treated for 30 min as described previously (10). Antibodies—The monoclonal antibodies against PTP-PEST Pro 1 (P15320) and p130Cas (P7280) were from Transduction Laboratories. The polyclonal antibody specific for avian paxillin was described previously (19). The anti-GST and anti-HA tag antibody 12CA5 were obtained from Santa Cruz Biotechnology. The anti-phosphotyrosine antibodies 4G10, FY20, and FY99 were from Upstate Biotechnologies, Transduction Laboratories, and Santa Cruz Biotechnology, respectively. The polyclonal antibody 1075 against PTP-PEST has been described previously (3). Plasmid Construction—PTP-PEST cDNA in the expression vector pACTAG (HA epitope-tagged) was described previously (3). The Pro 1 (PPKFPFR) and Pro 2 (PEPHVPVPIIPTSPPSAPF) domains were amplified by PCR. Briefly, an oligonucleotide upstream of the Pro 1 (5’-CCGCGTGGACGACTTGTGCTC-3’) designed with a Xhol site was used to amplify the cDNA combination in synthesis with the sense oligonucleotide T7. This PCR product, encoding for the N terminus of PTP-PEST, was gel-purified (QIAEX II, Qiagen) and digested with NotI and XhoI. A second PCR product encoding for the C terminus of PTP-PEST was generated using a sense oligonucleotide site designed downstream of pro 1 (5’-CCACTGGAAGCTGGAATGACTTTC-3’) and an antisense oligonucleotide with a XbaI site (5’-CCCTAGATCATGATGCTTCA-3’). The PCR product, encoding for the C terminus of PTP-PEST, was gel-purified and digested with XhoI and XbaI. To reconstitute the full-length PTP-PEST with the deleted Pro 1 region, the digested PCR products encoding for the PTP-PEST N and C terminus domains were ligated in the NorI and XbaI sites of pACTAG. The Pro 2 region of PTP-PEST was deleted using a similar strategy. The deletions were verified by dideoxy sequencing of the mutated region using Sequenase (Amersham Pharmacia Biotech). Truncations of the GST-PTP-PEST 344–437 (Pro 2) (GST 344–427, 344–417, 344–407, and 344–397) were performed by PCR using 7T primer and PTP-PEST specific oligonucleotide, harboring an EcoRI site. The PCR products were ligated with BamHI and EcoRI and ligated in the BamHI and EcoRI sites of pGEX RC. GST PTP-PEST 344–385 was generated by digestion of the pGEX RC 344–437 vector with KpnI and EcoRI, treatment with mung bean nuclease, followed by religation of the plasmid.

The constructs encoding for the N terminus, C terminus, and LIM 1–4 of avian paxillin fused to GST in the pGEX 2TK vector have been described previously (19). The paxillin GST LIM 1–3 was constructed by subcloning from the GST C terminus pGEX 2TK construct with BamHI and EcoRI lact in the BamHI and EcoRI sites of pGEX RC. Pro 1 region of PTP-PEST was deleted using a similar strategy. The deletions were verified by dideoxy sequencing of the mutated region using Sequenase (Amersham Pharmacia Biotech). Truncations of the GST-PTP-PEST 344–437 (Pro 2) (GST 344–427, 344–417, 344–407, and 344–397) were performed by PCR using 7T primer and PTP-PEST specific oligonucleotide, harboring an EcoRI site. The PCR products were ligated with BamHI and EcoRI and ligated in the BamHI and EcoRI sites of pGEX RC. GST PTP-PEST 344–385 was generated by digestion of the pGEX RC 344–437 vector with KpnI and EcoRI, treatment with mung bean nuclease, followed by religation of the plasmid. Site-directed Mutagenesis—Eight proline residues (highlighted) for Pro 1 and Pro 2 of PTP-PEST (Pro 1 (355PPFR) and Pro 2 (355PPFPFR)) were mutated to alanine residues by PCR. Briefly, mutagenic oligonucleotides were engineered: P355A (5’-GACGCCCAAGAAGCTTGCCCGCCCATC-3’), P360A (5’-CCGAAACCTCACGGGTCGGACCCCATC-3’), P362A (5’-CTCTACCGGTCGCGTATCCTGCAG-3’), P363A (5’-CAGCCGTGGCAGCCATCCTGCAGC-3’), P366A (5’-CTCCATGCGCCATATCCTGCAG-3’), P367A (5’-CTCCATCGCCGATATCCTGCAG-3’), P370A (5’-CTGCGCCATATCCTGCAG-3’), P374A (5’-CTTCCGGCTCCGGCAACCGTGTCAG-3’), and P377A (5’-CTTCCGGCTCCGGCAACCGTGTCAG-3’). The PCR product, encoding for the C terminus of paxillin in pGEX 2TK in the Smal and EcoRI sites of pGEX RC. The paxillin GST C terminus or LIM 3 was constructed by digesting the paxillin GST C terminus construct with XhoI and ScaI, followed by treatment with mung bean nuclease and religation of the plasmid.

**In Vitro Translation—** mRNA was transcribed using T7 RNA polymerase (New England Biolabs). Two μl of the transcription reactions were used to perform in vitro translation in the
presence of \(^{[35]}\)Smethionine and rabbit reticulocyte lysate (Promega). The \textit{in vitro} translated products were tested for binding to 1 \(\mu\)g of GST PTP-PEST 344–397 or GST alone.

\textbf{In Vitro Binding Assay Using GST Fusion Proteins—} NIH 3T3, NIH 3T3 Src Y527F, pervanadate-treated NIH 3T3, and HEK 293T cells were lysed in HNTG buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 1% Triton X-100, and 10% glycerol) supplemented with Complete protease inhibitors (Roche Molecular Biochemicals) and 1 mM Na3VO4, as described previously (10). The lysates were cleared of cellular debris by centrifugation at 16,000 \(\times\) g in a 4°C microcentrifuge. Protein concentrations were determined using the Bradford method (Bio-Rad) using bovine serum albumin as a standard. PTP-PEST, p130\(^{Cas}\), and paxillin GST fusion proteins were expressed by induction for 2 h of exponentially growing bacterial cultures with 1 \(\mu\)g of GST-PEST Pro 5, and GST alone failed to associate with paxillin as seen in Fig. 2B (top panel). Paxillin was detected in every binding assay except with GST alone. Coomassie Blue staining of the GST fusion proteins used for the binding assay (Fig. 2B, bottom panel) was used to demonstrate the integrity of the purified proteins. From these results, the paxillin binding site lies between amino acids 276–437 of PTP-PEST.

The only characterized protein-binding domain within aa 276–437 of PTP-PEST is a proline-rich region (Pro 1, 333PPK-STAFAQ374), which is a 20-amino acid segment rich in proline (20). It was recently reported that PTP-PEST and paxillin associate in chicken embryo fibroblasts and Swiss 3T3 cells (30). In order to define the region of PTP-PEST involved in the binding of paxillin, we have used the non-catalytic C-terminal tail of PTP-PEST in addition to several deletion mutants of PTP-PEST expressed as GST fusion proteins (Fig. 2A). The GST PTP-PEST fusion proteins (aa 276–775, 276–613, 276–567, 276–453, and 276–437 or GST alone) bound to glutathione-Sepharose were incubated with 500 \(\mu\)g of proteins extracted from NIH 3T3 cells. Following a binding incubation period, the beads were washed several times and bound proteins were separated by SDSPAGE. Associated paxillin was detected by Western blotting. As seen in Fig. 2B (top panel), paxillin was detected in every binding assay except with GST alone. Coomassie Blue staining of the GST fusion proteins used for the binding assay (Fig. 2B, bottom panel) was used to demonstrate the integrity of the purified proteins. From these results, the paxillin binding site lies between amino acids 276–437 of PTP-PEST.

**RESULTS**

\textbf{Paxillin Is Associated with PTP-PEST in Various Mouse Tissues—} It was recently reported that PTP-PEST and paxillin associate in chicken embryo fibroblasts and Swiss 3T3 cells (30). To verify that PTP-PEST and paxillin were physically associated in vitro, co-immunoprecipitation experiments were performed in lysates of various mouse tissues. As seen in Fig. 1, paxillin co-precipitated with PTP-PEST in liver, brain, heart, lung, spleen, and thymus lysates. The highest amounts of PTP-PEST-associated paxillin were found in lung, spleen, and liver. No paxillin was detected in PTP-PEST immunoprecipitate from kidney lysate, since PTP-PEST is expressed in low levels in kidney (31). In addition, paxillin was not found in preimmune IP made from liver extracts demonstrating the specificity of the association. When the blot was reprobed with a PTP-PEST antibody, comparable amounts of PTP-PEST were found in the precipitates except in the kidney where no signal was detected (data not shown). These results demonstrate that the PTP-PEST-paxillin association described previously in fibroblasts (30) also occurs \textit{in vivo} in a majority of mouse tissues.

\textbf{Paxillin Associates in Vitro with a Fragment of PTP-PEST Containing a Proline-rich Region—} In order to define the region of PTP-PEST involved in the binding of paxillin, we have used the non-catalytic C-terminal tail of PTP-PEST in addition to several deletion mutants of PTP-PEST expressed as GST fusion proteins (Fig. 2A). The GST PTP-PEST fusion proteins (aa 276–775, 276–613, 276–567, 276–453, and 276–437 or GST alone) bound to glutathione-Sepharose were incubated with 500 \(\mu\)g of proteins extracted from NIH 3T3 cells. Following a binding incubation period, the beads were washed several times and bound proteins were separated by SDS-PAGE. Associated paxillin was detected by Western blotting. As seen in Fig. 2B (top panel), paxillin was detected in every binding assay except with GST alone. Coomassie Blue staining of the GST fusion proteins used for the binding assay (Fig. 2B, bottom panel) was used to demonstrate the integrity of the purified proteins. From these results, the paxillin binding site lies between amino acids 276–437 of PTP-PEST.

The only characterized protein-binding domain within aa 276–437 of PTP-PEST is a proline-rich region (Pro 1, 333PPK-PPP) that acts as binding site for the SH3 domain of p130\(^{Cas}\), Hef1, Sin, and Grb2 (6, 9, 10). Also present in this PTP-PEST fragment is Pro 2 (685PPEHPVPPILTSPPSAFFF), which is a 20-amino acid segment rich in proline residues that has no determined function. In order to delimit the minimal region required for binding to paxillin, a binding assay was thus performed using Pro 1, Pro 2 344–437, Pro 2 344–427, Pro 2 344–417, Pro 2 344–407, Pro 2 344–397, and Pro 2 344–385 of PTP-PEST expressed as GST fusion proteins (Fig. 2A). Paxillin was associated with the Pro 2 344–437, Pro 2 344–427, Pro 2 344–417, Pro 2 344–407, and Pro 2 344–397 PTP-PEST fusion proteins (Fig. 2C, top panel). GST Pro 2 344–385, GST Pro 1, GST Pro 5, and GST alone failed to associate with paxillin as seen in Fig. 2C (top panel). These results suggest that amino acids 344–397 of PTP-PEST are sufficient for its association with paxillin.

\textbf{The Pro 2 Region of PTP-PEST Is Required for Paxillin Binding in Vitro and in Vivo—} The Pro 2 domain of PTP-PEST lies between aa 355 and 374. Since the smallest GST Pro 2 fusion protein used in Fig. 1C encodes for aa 344–397, a PTP-PEST mutant lacking Pro 2 was generated to rigorously demonstrate its role in paxillin binding. A binding assay was per-
formed using two paxillin GST fusion proteins: paxillin N (encoding for the LD motifs) and paxillin C (encoding for the 4 LIM domains). These two proteins in addition to GST p130Cas SH3 and GST alone were purified and incubated with cell extracts of HEK 293T transfected either with WT or ΔPro 2 HA-PTP-PEST as described above. The bound PTP-PEST proteins were monitored by Western blotting using the anti-HA antibody 12CA5. As shown in Fig. 3A (top panel), the GST paxillin C and p130Cas SH3 were bound to WT HA PTP-PEST. The GST paxillin N and GST alone failed to bind WT HA PTP-PEST. Five μg of HA WT PTP-PEST-transfected HEK 293T extract were loaded to demonstrate the proper expression

**Fig. 2. Pro 2 of PTP-PEST is required for paxillin binding in NIH 3T3 cells.** A, schematic representation of the PTP-PEST GST fusion proteins used in the binding assays. Deletion mutants (B) or proline-rich motifs (C) of the PTP-PEST C terminus were incubated with NIH 3T3 cell lysate and bound paxillin was monitored by Western blotting (top panels). A Coomassie Blue-stained gel of the fusion proteins used in the binding assay is shown in the bottom panels of B and C.
of the transfected construct. When the same binding assay was performed using ∆Pro 2 HA PTP-PEST-transfected cell extracts, GST paxillin C failed to bind ∆Pro 2 PTP-PEST (Fig. 3A, top panel). The SH3 domain of p130Cas was still capable of association with the ∆Pro 2 mutant of PTP-PEST since it only requires an intact Pro 1 domain. ∆Pro 2 PTP-PEST was expressed to similar levels to WT as seen in the TCL (5 μg of protein) lanes. To verify the presence and integrity of the GST fusion proteins, the blots were stripped and probed with an anti-GST polyclonal antibody (Fig. 3A, bottom panel). From these results, it is clear that the Pro 2 motif of PTP-PEST is implicated in paxillin binding. In addition, the LIM domains of paxillin rather than the LD motifs are implicated in the association with PTP-PEST.

The importance of the Pro 2 domain of PTP-PEST for paxillin binding was also confirmed in vitro in a co-immunoprecipitation experiment. HEK 293T cells were transfected with: mock (empty pACTAG), HA WT PTP-PEST, HA ∆Pro 1 PTP-PEST, or HA ∆Pro 2 PTP-PEST plasmids. The proper expression of each construct was verified by immunoblotting 5 μg of TCL with the anti-HA antibody 12CA5 (top panel). Paxillin was immunoprecipitated with a monoclonal antibody and the presence of PTP-PEST was assayed by immunoblotting with the anti-HA antibody 12CA5 (middle panel). Equal precipitation of paxillin from each sample was verified by reprobing the blot with a monoclonal antibody against paxillin (bottom panel).

Mutational Analysis of the Pro 2 Region Reveals the Importance of Proline 362 for Paxillin Binding Activity—The Pro 2 domain of PTP-PEST is defined by 20 amino acids, half of which are proline residues (Fig. 4A). Although three PXXP motifs are found in the Pro 2, none of them have the consensus sequence for SH3 binding sites (class 1, RXXPXXP; and class 2, PXXPXR) or WW domains (PPXY) (32). Eight of the proline residues were individually mutated to alanine (Fig. 3A) in the context of the GST Pro 2 344–437 construct (see Fig. 2) in order to get a better understanding of this novel paxillin binding domain. The WT GST Pro 2 as well as the eight proline to alanine mutants were purified from induced bacterial cultures and incubated with NIH 3T3 cell lysates. As seen in Fig. 4B, paxillin was bound to all fusion proteins used except for the P362A mutant. Equal amounts of GST fusion proteins as well as the integrity of the products are shown in Fig. 4C. From these results, we can conclude that the proline residue 362 is critical for binding to paxillin.

The LIM Domains 3 and 4 of Paxillin Are Required for PTP-PEST Binding—Having delimited the region on PTP-
PEST important for binding to paxillin, we were next interested in mapping the segment of paxillin required for its association to PTP-PEST. A GST fusion protein encoding the four LIM domains of paxillin was demonstrated to have the PTP-PEST binding activity (Fig. 2A). A panel of GST fusion proteins of paxillin LIM domains was generated in order to investigate which LIM domain is important for PTP-PEST binding. A schematic representation of these constructs is shown in Fig. 5A. These fusion proteins were purified from induced bacterial cultures and incubated with HA-PTP-PEST expressing 293T cell lysates. Bound PTP-PEST was detected by Western blotting using the anti-HA antibody 12CA5. As seen in Fig. 5B, the paxillin C terminus (paxillin C, LIM 1–4) was bound to PTP-PEST. The C-terminal deletion mutants (LIM 1–3, LIM 1–4ΔLIM3, and LIM 1–2) as well as the LIM domains expressed separately failed to bind PTP-PEST. Interestingly, the LIM 3–4 construct was the only deletion construct retaining PTP-PEST binding activity (Fig. 5B). GST alone used as a negative control was not capable of precipitating PTP-PEST. To demonstrate proper expression of each construct as well as the integrity of the products, a Coomassie Blue-stained gel of the GST fusion proteins used in the binding assay is shown in Fig. 5C.

It is possible that only one of the LIM domains (3 or 4) is interacting with PTP-PEST, but both may be required to have a biologically active conformation. This may be an important concern especially since the fusion proteins have been expressed in bacteria. In order to test this hypothesis, co-immunoprecipitation experiments were performed between PTP-PEST and full-length paxillin WT, ΔLIM3, or ΔLIM4 mutants. HEK 293T cells were co-transfected with HA-PTP-PEST and either pcDNA3 (empty), pcDNA3 WT, ΔLIM3, or ΔLIM4 avian paxillin. Five μg aliquots of each sample was analyzed by Western blotting to verify PTP-PEST expression (Fig. 6C). Aliquots of each lysate were immunoprecipitated with an antibody specific for avian paxillin. The presence of PTP-PEST in the paxillin precipitates was investigated by Western blotting using the anti-HA antibody 12CA5. As seen in Fig. 6A, PTP-PEST was detected after precipitation of WT paxillin but not either of the LIM 3 or 4 deletion mutants. To verify equal precipitation of paxillin in each sample, the blot was stripped and reprobed with a monoclonal antibody against paxillin. As seen in Fig. 6B, equal amounts of paxillin were detected in all samples except in the negative control (pcDNA3, Fig. 6B).

**Intact LIM 3 and LIM 4 Are Required for Binding to PTP-PEST**—In order to confirm that both LIM 3 and LIM 4 domains of paxillin participate in PTP-PEST binding, point mutations were introduced in these domains. Each LIM domain is composed of two zinc fingers stabilized by critical cysteine, histidine, or aspartic acid residues (19). The C467A and the C470A mutants disrupt the first and the second zinc finger of the LIM 3 domain, respectively, and the C467A/C470A is a double mutant. The C523S disrupts the first zinc finger of LIM 4. The WT paxillin and all the mutants were in vitro translated in the presence of [35S]methionine. These products were then incubated with GST PTP-PEST Pro 2 (344–397). As seen in Fig. 7 (top panel), only the WT paxillin was able to interact with the PTP-PEST GST fusion proteins. As a control, GST alone did not interact with WT paxillin (last lane, top panel). In the bottom panel, 15% of the amount of each product used in the binding assays is shown to demonstrate the integrity of the products and also as a loading control. These data clearly demonstrate that the integrity of both LIM 3 and LIM 4 is critical for binding to PTP-PEST.

**Paxillin Is Not a Substrate for PTP-PEST**—Since paxillin is a tyrosine-phosphorylated protein, a substrate trapping approach (7, 8, 10) using C231S mutants of the catalytic domain of PTP-PEST, was used to investigate if paxillin is a physiological substrate for PTP-PEST. The substrate trapping mutants used were GST 1–453 C231S (containing Pro 2) and GST 1–354 C231S (Pro 2 is deleted) PTP-PEST fusion proteins. Protein extracts were prepared from control NIH 3T3, NIH 3T3 stably expressing Src Y527F, and pervanadate-treated NIH 3T3 cells. These samples were incubated with either PTP-PEST C terminus (aa 276–775), PTP-PEST 1–453 WT, PTP-PEST 1–453 C231S, or PTP-PEST 1–354 C231S expressed as GST fusion proteins. Tyrosine-phosphorylated proteins precipitating with PTP-PEST GST fusion proteins were visualized by immuno-
FIG. 5. LIM domains 3 and 4 of paxillin are required for binding to PTP-PEST in vitro. A, schematic representation of a panel of paxillin LIM domains GST fusion proteins used to identify the PTP-PEST binding site. B, the paxillin GST fusion proteins were purified and incubated with HEK 293T cell lysates transiently expressing HA-PTP-PEST. Bound PTP-PEST was detected by immunoblotting with the anti-HA antibody 12CA5 (top panel). The expression as well as the integrity of each fusion protein used in the binding assay was verified by Coomassie Blue staining of a gel containing the resolved proteins.
FIG. 6. LIM domains 3 and 4 of paxillin are required for binding to PTP-PEST in vivo. HEK 293-T cells were transiently co-transfected with HA-PTP-PEST and either pcDNA3, WT, ΔLIM3, or ΔLIM4 avian paxillin. A, 48 h after transfection, paxillin was immunoprecipitated from each lysate with a avian specific polyclonal antibody and the co-precipitation of PTP-PEST was assayed by immunoblotting with the anti-HA antibody 12CA5. B, the blot was reprobed with a monoclonal antibody against paxillin to verify equal precipitation. No paxillin was detected in the empty vector (pcDNA3) sample. C, HA-PTP-PEST expression levels were monitored from each transfection by immunoblotting 5 μg of TCL with the anti-HA antibody 12CA5.

FIG. 7. Intact LIM 3 and LIM 4 of paxillin are required for binding to PTP-PEST. pcDNA3, WT, or mutant paxillin (ΔLIM 3, C467A, C470A, C467/470A, ΔLIM4, and C523S) were in vitro translated in the presence of [35S]methionine. These in vitro translated products were incubated with GST-PTP-PEST Pro 2 (344–397). Following repeated washes of the GST matrix, bound proteins were separated on 10% SDS-PAGE and bound proteins were visualized by a 8-h exposure to film (top panel). GST alone was incubated with WT paxillin and serves as a negative control. In the bottom panel, 15% of the amount of each paxillin products used in the binding assay demonstrate integrity of the products (8-h exposure).

FIG. 8. Paxillin is not a substrate for PTP-PEST. Lysates of NIH 3T3, NIH 3T3 expressing Src Y527F, and NIH 3T3 treated with pervanadate were incubated with four different PTP-PEST GST fusion proteins: C terminus (aa 276–775), 1–453 WT, 1–453 C231S, and 1–354 C231S. A, bound proteins were resolved by SDS-PAGE and tyrosine-phosphorylated proteins were detected by immunoblotting with an anti-phosphotyrosine antibody. The blot was reprobed with anti-paxillin (B) and anti-p130Cas (C) monoclonal antibodies. D, the expression and integrity of each fusion proteins was verified by Coomassie Blue staining of the PVDF blots.

blotting with an anti-phosphotyrosine antibody. As seen in Fig. 8A, in the Src Y527F and pervanadate (PV)-treated cells, a protein of 130 kDa is the major tyrosine-phosphorylated protein bound to the C231S mutants of PTP-PEST. This protein is known to be p130Cas (8, 10) and thus serves as a control to ensure that the trapping experiment worked, as seen by blotting with an anti-p130Cas antibody in Fig. 8C. The 60-kDa band detected in the PTP-PEST C terminus, 1–453 WT, and C231S but not in the 1–354 C231S. This p70 protein was identified as paxillin when the blot was reprobed with an anti-paxillin monoclonal antibody (Fig. 8B). Importantly, no paxillin was detected in the GST PTP-PEST 1–354 C231S lanes, the only construct lacking the Pro 2, thus unambiguously showing that
the PTP-PEST catalytic domain is not interacting directly with tyrosine-phosphorylated paxillin. This result clearly demonstrates that paxillin is not a substrate for the PTP-PEST catalytic domain in a substrate trapping assay. Interestingly, when 3T3 cells are treated with pervanadate, only a small amount of paxillin is detected (overexposure of the blot, data not shown) bound to the PTP-PEST fusion proteins having the Pro 2. This suggest that hyperphosphorylation of paxillin on tyrosine residues could prevent binding to PTP-PEST. The integrity of each fusion protein used in the trapping assay was verified by Coomassie Blue staining of the PVDF blots as seen in Fig. 8D.

DISCUSSION

In a recent report, Shen et al. (30) demonstrated that FAK complexes with proteins having protein-tyrosine phosphatase activity (PTP). One of these PTPs, PTP-PEST, was found in FAK complexes via a direct association with paxillin (30). These findings are significant since they suggest a dynamic regulation of protein-tyrosine phosphorylation in focal adhesions. In support of this hypothesis, fibroblasts lacking FAK (33) or PTP-PEST (14) exhibit migration defects and a higher number of focal adhesions indicating that both tyrosine kinases and phosphatases have an active function in the assembly and disassembly of these adhesive cellular structures. We have analyzed the binding of PTP-PEST to paxillin in order to get a better understanding of the function of PTP-PEST in the regulation of focal adhesion turnover.

The co-precipitation of paxillin and PTP-PEST was reported in chicken embryo and Swiss 3T3 cultured fibroblasts (30). We demonstrate that the association between paxillin and PTP-PEST is physiologically relevant since both proteins co-precipitate from a variety of normal mouse tissues namely liver, brain, heart, lung, spleen, and thymus (Fig. 1). Paxillin was not detected in PTP-PEST precipitate from kidney since PTP-PEST is poorly expressed in kidney as shown previously (31).

In this study, we have identified the domains in PTP-PEST and paxillin that are responsible for their association. Our present data demonstrate that in vitro, a segment of PTP-PEST from aa 344–397 was sufficient for binding paxillin. In vivo, a proline-rich motif on PTP-PEST, Pro 2 (ProPheProProPheProPhePro), is essential for binding to paxillin. In addition, intact LIM 3 and LIM 4 domains of paxillin are required for binding to PTP-PEST. Furthermore, tyrosine-phosphorylated paxillin was not able to complex with a substrate trapping mutant of PTP-PEST (C231S) lacking the Pro 2, demonstrating that it is not a physiological substrate of PTP-PEST in this “substrate trapping” type of assay. The catalytic domain of PTP-PEST is flanked by a C-terminal tail rich in protein binding motifs. In this respect, the SH3 domains of p130Cas, Hef1, Sin/Efs (10, 34), Grb2, v-Src (6), and Csk (31) have been shown to directly associate with proline-rich sequences in PTP-PEST. In addition, the coiled-coil domain of PSTPIP also associates with a non-classical proline-rich domain of PTP-PEST (35) and the PTB domain of Shc was shown to bind a NPLH motif on PTP-PEST (5). The Pro 2 of PTP-PEST contains none of the consensus sequences that can act as ligands for either SH3 or WW domains. This suggests that the Pro 2 of PTP-PEST is a novel protein binding motif that can associate with PTP-PEST domains of at least paxillin. Interestingly, Pro 2 is conserved between human and mouse PTP-PEST but is not present on other members of the PTP-PEST family of enzymes (PEP, PTP-HSCF, and PTPD20). One concern is that the Pro 2 deletion, which prevents paxillin binding, might produce a misfolded PTP-PEST. However, this hypothesis is unlikely since p130Cas can still interact with PTP-PEST ΔPro 2 through an SH3-Pro 1 association (Fig. 3A). In addition, site-directed mutagenesis analysis demonstrated that Proline 362 is critical for binding to paxillin, whereas seven other proline mutants had little if no effect on paxillin binding. To gain a better understanding of the Pro 2, experiments involving more point mutations of residues surrounding Pro-362 and a NMR structure determination of the peptide bound to its paxillin partner are currently under investigation.

Paxillin is an adaptor protein composed entirely of protein binding modules including LD, LIM, SH2 binding sites, and proline-rich domains. Our results indicate that only LIM 3 and LIM 4 are essential for PTP-PEST binding activity. When expressed as GST fusion proteins, neither LIM 3 nor LIM 4 alone was able to negotiate binding with PTP-PEST whereas a construct containing both LIM 3 and LIM 4 domains supports the association. It is possible that LIM 3 and LIM 4 do not fold properly when expressed in bacteria; however, a recent report by Brown et al. (25) indicates that GST LIM 2 and LIM 3 alone associate with serine and threonine kinases, suggesting that they are properly folded domains (25). We have also observed that, in vivo, full-length paxillin lacking either LIM 3 or LIM 4 was unable to bind to PTP-PEST. In addition, we have clearly demonstrated that point mutation that disrupt the zinc fingers in either LIM 3 or LIM 4 of paxillin prevents binding to PTP-PEST. Together, these data point to LIM 3 and LIM 4 as critical domains for paxillin association with PTP-PEST.

LIM domains ligands are extremely varied (27, 28). For example, some LIM domains have been shown to heterodimerize while others bind to structurally distinct protein motifs (27). Among others, it has been previously shown that the LIM 3 of Enigma associates with a tyrosine-based motif (tyrosine tight turn) of the β chain of the insulin receptor (29). Interestingly, the tyrosine tight turn of the insulin receptor (GPLGPLYA) contains a PXXP motif and mutation of the two prolines to alanines abolishes binding to the LIM 3 of Enigma (29). Furthermore, when the LIM 3 of Enigma was used to screen a random peptide library with a fixed tyrosine (position 0), prolines at position −1 and +2 were favored. A consensus sequence for the preferred ligand of the LIM 3 of Enigma was determined: GPhyGPhyHy/Y/FA (Hy=hydrophobic residue). Our data demonstrate that the Pro 2 of PTP-PEST, PILTPSPPSAPF (25), is the binding site for paxillin and that Pro-362 (in bold) is critical for the association. The consensus ligand sequence for the LIM 3 of Enigma was not found in PTP-PEST Pro 2. LIM 3 and LIM 4 of paxillin thus associates with a novel polyproline motif, and adds to the wide variety of LIM domain ligands. The discovery of other ligands for LIM 3 and LIM 4 of paxillin and their comparison to the PTP-PEST Pro 2 will allow the elaboration of a preferred ligand sequence for these LIM domains.

In parallel to this work, PTP-PEST has also been reported to associate with the paxillin homologue Hic-5 (36). The C-terminal LIM domains of Hic-5 is 68% similar to the LIM domains of paxillin. It was shown that the LIM 3 of Hic-5 is the most important for the binding to PTP-PEST but is still not sufficient. Surprisingly, LIM 4 was not critical for the binding of Hic-5 to PTP-PEST. In addition, point mutations in the zinc finger of either LIM 3 or LIM 4 of Hic-5 did not prevent association with PTP-PEST in a co-precipitation experiment. These results differ from the one observed in the association between PTP-PEST and paxillin, and suggest that even though the LIM domains of Hic-5 and paxillin are 68% similar, the mechanism of binding to PTP-PEST is not identical.

PTP-PEST has been shown to be very selective for its physiological substrates (8, 10). The selectivity toward p130Cas can...
be explained by the fact that both a substrate recognition by the PTP domain and a SH3-mediated association occur before dephosphorylation. An important issue that needed to be resolved in order to understand the significance of paxillin-PTP-PEST association was to clarify if paxillin is a substrate for PTP-PEST. Our data clearly demonstrates that tyrosine-phosphorylated paxillin was not bound to a PTP-PEST C231S mutant lacking the Pro2, indicating that paxillin is not directly recognized by the PTP domain. In addition, equal amounts of paxillin were also found in the precipitates of GST PTP-PEST WT or C231S and paxillin was not found to be more tyrosine-phosphorylated in the C231S samples, suggesting an absence of cooperation between the catalytic domain and the Pro2 (Fig. 8C). In support of these findings, Garton and Tonks (16) have demonstrated that overexpression of PTP-PEST in Rat-1 fibroblasts prevents cells from migrating in a wound healing assay. In these cells, p130Cas phosphotyrosine level was greatly reduced whereas paxillin and FAK tyrosine phosphorylation levels were unaltered (16). Thus, these results also indicate that paxillin is not a target for PTP-PEST.

Other PTPs were reported to have remarkable specificity toward substrates including PTP1B (7, 37), T cell-PTP (38), and SHP-1 (39). In contrast, the presence of the PSTPIP binding motif on PTP-HSCF was demonstrated to be essential for a specific tyrosine dephosphorylation of PSTPIP since the PTP domain alone did not dephosphorylate PSTPIP (35). Because we based our conclusions only on a substrate trapping approach, it remains a possibility that paxillin is a weak substrate for PTP-PEST in vivo. It is also possible that the formation of some protein complexes could favor paxillin dephosphorylation by PTP-PEST. In a \textit{in vitro} dephosphorylation assay, GST-PTP-PEST dephosphorylated weakly a paxillin peptide compared with a p130Cas peptide.\textsuperscript{3} The known

\textsuperscript{3} J. F. Côté and M. L. Tremblay, unpublished observations.
promiscuous activity of PTPases in vitro prevents us from basing our substrate identification using such an assay.

If paxillin is not a substrate for PTP-PEST, what is the physiological significance of paxillin-PTP-PEST association? A first clue to answer this question came from findings by Brown et al. (19), indicating that the intracellular localization of paxillin depends on the association of a yet unknown binding protein to the LIM 3 of paxillin. A reasonable assumption is that this LIM 3 ligand must co-localize with paxillin in focal contact sites. PTP-PEST is most likely not the protein responsible for paxillin focal adhesion localization since it is found mainly in the cytoplasm. We have demonstrated in a previous study that PTP-PEST can translocate to the membrane periphery following integrin engagement (14). Hence, we propose a model where PTP-PEST is translocated (14) to focal adhesions and associates with paxillin (Fig. 9A). This would allow the SH3-mediated association with focal adhesion located p130Cas and inhibit its downstream signaling via dephosphorylation of residues of p130Cas critical tyrosine residues. Importantly, the LIM 3 of paxillin would be negotiating to bind with the Pro 2 of p125FAK. With p125FAK, in a similar manner to the LIM 3 of paxillin, the PEST instead of p125 FAK. Together, this cascade would result in p130Cas having also been shown to be critical for proper focal adhesion turnover via its tyrosine phosphatase activity toward p130Cas and via direct binding to critical domains of p130Cas and paxillin required for focal adhesion targeting.

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