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Inhibition of the Catalytic Activity of Cell Adhesion Kinase β by Protein-tyrosine Phosphatase-PEST-mediated Dephosphorylation*

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Protein-tyrosine phosphatase (PTP)-PEST is a cytoplasmic tyrosine phosphatase that can bind and dephosphorylate the focal adhesion-associated proteins p130^{CAS} and paxillin. Focal adhesion kinase (FAK) and cell adhesion kinase β (CAKβ)/PYK2/CADTK/RAFTK are protein-tyrosine kinases that can colocalize with, bind to, and induce tyrosine phosphorylation of p130^{CAS} and paxillin. Thus, we considered the possibility that these kinases might be substrates for PTP-PEST. Using a combination of substrate-trapping assays and overexpression of PTP-PEST in mammalian cells, CAKβ was found to be a substrate for PTP-PEST. Both the major autophosphorylation site of CAKβ (Tyr^{402}) and activation loop tyrosine residues, Tyr^{579} and Tyr^{580}, were targeted for dephosphorylation by PTP-PEST. Dephosphorylation of CAKβ by PTP-PEST dramatically inhibited CAKβ kinase activity. In contrast, FAK was a poor substrate for PTP-PEST, and treatment with PTP-PEST had no effect on FAK kinase activity. Tyrosine phosphorylation of paxillin, which is greatly enhanced by CAKβ overexpression, was dramatically reduced upon coexpression of PTP-PEST. Finally, endogenous PTP-PEST and endogenous CAKβ were found to localize to similar cellular compartments in epithelial and smooth muscle cells. These results suggest that CAKβ is a substrate of PTP-PEST and that FAK is a poor PTP-PEST substrate. Further, PTP-PEST can negatively regulate CAKβ signaling by inhibiting the catalytic activity of the kinase.

Tyrosine phosphorylation is a post-translational modification that is essential for many signal transduction cascades, including growth factor receptor and focal adhesion signaling. Cellular phosphotyrosyl levels are kept in homeostasis by the competing action of two classes of enzymes, protein-tyrosine kinases (PTKs)[1] and protein-tyrosine phosphatases (PTPs). There is a vast literature describing the role of PTKs in the regulation of signal transduction. As more PTPs are being discovered, it has become apparent that these proteins, like PTKs, play an integral role in regulating cellular signaling pathways.

PTP-PEST is one of a number of cytoplasmic phosphatases characterized by the presence of several PEST-rich regions (1–4), motifs that have been found in many proteins that are rapidly degraded in the cell (5). PTP-PEST, however, appears to be quite stable (6). Structurally, it is composed of an N-terminal catalytic domain and a C-terminal tail containing five proline-rich regions, several of which constitute consensus Src homology 3 binding sites. Indeed, PTP-PEST binds the adaptor protein Grb2 and the tyrosine kinase Csk in an Src homology 3-dependent manner (7, 8). PTP-PEST also binds the adaptor protein Shc via a phosphotyrosine binding domain-mediated interaction. However, this interaction occurs in a non-phosphotyrosine-dependent manner through an NPLH sequence in the C-terminal tail of PTP-PEST (9). Although PTP-PEST has been shown to associate with these signaling molecules, its role in regulation of these signaling pathways remains to be fully elucidated. Grb2 binding was reported to bring PTP-PEST into association with activated epidermal growth factor receptor; however, the epidermal growth factor receptor does not appear to be a substrate (7). An alternative hypothesis is that PTP-PEST may instead regulate epidermal growth factor receptor signaling by dephosphorylating receptor substrates. Csk was recently shown to cooperate with the tyrosine phosphatase PEP, a structural homologue of PTP-PEST, in inhibiting T-cell antigen receptor signaling (10). PEP could dephosphorylate and inactivate PTKs responsible for T-cell activation but only when associated with Csk. A role for PTP-PEST in Csk signaling events has yet to be established. The Shc proteins are adaptor molecules most closely linked with activation of the Ras pathway (11, 12). Interestingly, Shc is phosphorylated on tyrosine itself (11). PTP-PEST, however, has not been implicated in regulation of the Ras signaling pathway, and Shc has not been identified as a substrate. In addition to binding these signaling molecules, the C-terminal domain of PTP-PEST has docking sites for two known substrates. p130^{CAS} binds PTP-PEST in an Src homology 3-dependent manner (13), and paxillin binds through its two C-terminal LIM domains to two nonoverlapping stretches of a 52-amino acid sequence in PTP-PEST (14, 15). Both p130^{CAS} and paxillin are substrates for PTP-PEST, and their association with the C-terminal domain of PTP-PEST is required for dephosphorylation of these substrates *in vivo* (13, 15, 16).

Paxillin and p130^{CAS} localize to focal adhesions and undergo rapid tyrosine phosphorylation upon cell adhesion (17). Paxillin and p130^{CAS} also become tyrosine-phosphorylated in response to various physiological stimulants including bombesin, platelet-derived growth factor, nerve growth factor, and angiotensin II (18, 19). Both proteins act as adaptor molecules in integrin signaling. Paxillin binds to several focal adhesion-associated proteins including vinculin, FAK, and c-Src (19). Integrin-mediated tyrosine phosphorylation of paxillin creates binding sites for the SH2 domains of Crk and Csk (20, 19). Tyrosine

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[1] The abbreviations used are: PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; CAS, Crk-associated substrate; HEK, human embryonic kidney; FAK, focal adhesion kinase; CAKβ, cell adhesion kinase β; SH2, Src homology 2; LIM, *lin11*, *isl-1*, and *mec-3*; GST, glutathione *S*-transferase.

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phosphorylation of paxillin has been implicated in the control of biological events such as cell spreading (21), cell adhesion (22), and cell motility (23, 24). Tyrosine phosphorylation of $p130^{CAS}$ also creates docking sites for the SH2 domain of Crk (25). The $p130^{CAS}$-Crk complex has been proposed to regulate cell migration (26), and $p130^{CAS}$ is thought to act as a mediator for FAK-promoted cell migration (27). PTP-PEST has been implicated in the regulation of cell motility (28, 29) and may do so by targeting paxillin and/or $p130^{CAS}$ for dephosphorylation.

Paxillin and $p130^{CAS}$ are potential substrates for several candidate tyrosine kinases including FAK and CAKβ. Paxillin and $p130^{CAS}$ can directly bind to FAK and CAKβ and become tyrosine-phosphorylated in response to stimuli that activate these PTKs (17). FAK and CAKβ are structurally related cytoplasmic tyrosine kinases composed of a central catalytic domain flanked by N- and C-terminal noncatalytic domains (30). Although structurally similar and able to associate with many of the same proteins, it is unclear whether these kinases share similar functions. While FAK has been most strongly implicated as a major component of integrin-mediated signaling pathways (17), there have been conflicting reports as to whether CAKβ is regulated by integrin mediated cell adhesion (31–33). Unlike FAK, CAKβ is regulated by stimuli that induce changes in intracellular Ca^{2+} (30). Like other PTKs, tyrosine phosphorylation of FAK and CAKβ plays an important role in regulating signaling. FAK and CAKβ share four conserved sites of tyrosine phosphorylation. They are the Src-family SH2 domain binding site at Tyr^{397} in FAK and Tyr^{402} in CAKβ, the Grb2-SH2 domain binding site at Tyr^{925} in FAK and Tyr^{881} in CAKβ, and two regulatory sites in the activation loop of the kinase domains: $Tyr^{576/577}$ in FAK and $Tyr^{579/580}$ in CAKβ (30, 34).

FAK and CAKβ are particularly good candidate PTP-PEST substrates, since these kinases both bind paxillin and $p130^{CAS}$ (35), two previously identified substrates (15, 16). Two lines of evidence suggest that FAK may be a substrate. First, PTP-PEST activity was coimmunoprecipitated with FAK (36), although this association is indirect and appears to be mediated by paxillin. Second, $PTP\text{-}PEST^{-/-}$ cells exhibited not only increased paxillin and $p130^{CAS}$ phosphotyrosine but also increased tyrosine phosphorylation of FAK (29). These results suggest that FAK may be a substrate of PTP-PEST and that PTP-PEST may regulate signaling both at the level of the kinase and the substrate. On the other hand, PTP-PEST may not play a major role in FAK dephosphorylation, since the two proteins fail to colocalize intracellularly.[2] Alternatively, PTP-PEST may regulate CAKβ signaling. In this report, we explore the hypothesis that FAK and CAKβ may be PTP-PEST substrates. We identify CAKβ as a specific substrate of PTP-PEST using a substrate-trapping approach and by demonstrating dephosphorylation of CAKβ by PTP-PEST *in vivo*. In contrast, PTP-PEST bound very weakly to FAK in a substrate-trapping assay and was a weaker substrate for dephosphorylation than CAKβ. We determined that PTP-PEST could target the major autophosphorylation site of CAKβ as well as one or both tyrosines in the activation loop of the catalytic domain. We demonstrate that PTP-PEST is able to inhibit CAKβ kinase activity both *in vitro* and *in vivo*. Finally, endogenous PTP-PEST and endogenous CAKβ were shown to reside in similar cellular compartments within GN4 and A7r5 cells. These results suggest that PTP-PEST can inhibit CAKβ signaling by impairing the activity of the kinase and by dephosphorylating downstream substrates.

EXPERIMENTAL PROCEDURES

Cells—HEK 293 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal bovine serum and A7r5 vascular smooth muscle cells in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. GN4 cells were obtained from Dr. H. Shelton Earp and were maintained in Richter's minimal essential medium supplemented with 10% fetal bovine serum. Cells were transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's recommended protocol.

Molecular Biology—The epitope-tagged (KT3) wild type, dl367–400, dl297–493, and P337A PTP-PEST constructs have been described previously (15, 36). To construct pcDNA 3.1-FAK, the FAK cDNA was excised from pBS-FAK (37) using *Xba*I and *Sal*I, which cut in the multiple cloning site flanking the FAK insert. The FAK-containing fragment was inserted between the *Nhe*I and *Xho*I sites of pcDNA3.1. Wild type and CAKβ YF mutants were kindly provided by Drs. Xiong Li and H. Shelton Earp (University of North Carolina) and have been described previously (34). For expression as a glutathione *S*-transferase (GST) fusion protein, a fragment of the PTP-PEST cDNA encoding the catalytic domain (amino acids 1–301) was amplified by polymerase chain reaction and subcloned into pGEX2TK (Amersham Pharmacia Biotech) in frame with the GST coding sequence. The C231S PTP-PEST substrate-trapping mutant ($GST/PTP\text{-}PEST^{C231S}$) was generously provided by Dr. André Veillette (McGill University). This mutant encodes amino acids 1–301 with cysteine 231 mutated to serine. $GST\text{-}paxillin^{N\text{-}C3}$ was generated by amplifying a fragment of the paxillin cDNA (encoding amino acids 1–312) by polymerase chain reaction using primers that created a *Bam*HI site at the 5′-end and an *Eco*RI site at the 3′-end. The fragment was subcloned into pGEX2TK in frame with the GST coding sequence. The fusion proteins were expressed by incubation with 0.3 mM isopropyl β-D-thiogalactopyranoside for 5 h at 30 °C, the bacteria were lysed by sonication, and the fusion proteins were purified using glutathione-agarose beads (Sigma) (38).

Protein Analysis—Cells were lysed in modified radioimmune precipitation assay buffer (36), and protein concentrations were determined using the BCA assay (Pierce). Immunoprecipitations were performed using ~500 μg of cell lysate and 10 μl of polyclonal antisera or 2 μg of purified antibody. For CAKβ immunoprecipitations, a polyclonal antiserum was used (described below). Paxillin was immunoprecipitated using a polyclonal antiserum described previously (39), and FAK was immunoprecipitated using the BC4 polyclonal antiserum (37), a gift of Dr. Thomas Parsons (University of Virginia). The immune complexes were recovered by incubation with protein A-Sepharose beads (Amersham Pharmacia Biotech) for 1–2 h at 4 °C. Immune complexes were washed twice with lysis buffer and twice with Tris-buffered saline (10 mM Tris-HCl (pH 7.5), 150 mM NaCl). The samples were boiled in Laemmli sample buffer (40) and analyzed by SDS-PAGE and Western blotting (41). For Western blotting, monoclonal antibodies recognizing CAKβ, paxillin, and the RC20 phosphotyrosine antibody were purchased from BD Transduction Laboratories (Lexington, KY). The FAK 2A7 monoclonal antibody was kindly provided by Dr. Thomas Parsons (University of Virginia). A rabbit polyclonal antiserum recognizing the noncatalytic domain of PTP-PEST has been described previously (15). CAKβ phosphorylation site-specific antibodies (PY402, PY579, and PY579/580) were obtained from BIOSOURCE International (Camarillo, CA). These affinity-purified (using both negative and positive affinity purification methods) rabbit polyclonal antibodies have been shown to be highly selective for the targeted phosphorylation site by analyzing site-directed (YF) mutants at the phosphorylation site of interest (see Fig. 5). For RC20 phosphotyrosine immunoblots, membranes were blocked in Tris-buffered saline (10 mM Tris-HCl (pH 7.5), 150 mM NaCl), supplemented with 0.1% Tween 20 (Sigma). For CAKβ phosphospecific antibodies, immunoblots were blocked in Tris-buffered saline, supplemented with 0.1% Tween 20 (Sigma) and 2% fish gelatin (Sigma). Immunoblots were incubated with 0.5 μg/ml phosphospecific antibody for 1 h at room temperature, washed, and incubated with horseradish peroxidase-conjugated protein A (Amersham Pharmacia Biotech). Enhanced chemiluminescence (Amersham Pharmacia Biotech) was used for detection of proteins.

Generation of CAKβ Polyclonal Antiserum—The rat CAKβ cDNA was used to generate a GST fusion protein containing amino acids 226–306. These residues were amplified by polymerase chain reaction and subcloned into pGEX2TK in-frame with the GST coding sequence. This fusion protein was gel-purified and used as an antigen to prepare polyvalent rabbit antiserum as described previously (37). For primary immunizations, 1 mg of fusion protein in phosphate-buffered saline was emulsified with complete Freund's adjuvant and injected subcutane-

[2] P. D. Lyons, J. M. Dunty, E. M. Schaefer, and M. D. Schaller, unpublished observations.

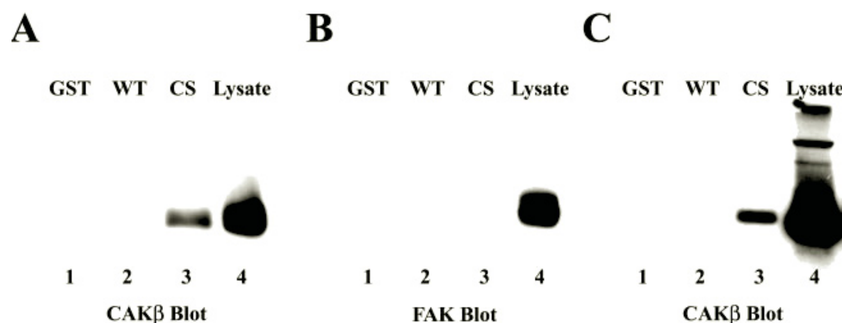

FIG. 1. **PTP-PEST substrate traps CAKβ.** *A*, HEK 293 cells were transfected with wild type CAKβ and treated with pervanadate. 500 μg of cell lysate was first precleared with 10 μg of GST and then incubated with 5 μg of either GST (*lane 1*), GST/PTP-PEST^{WT} (*WT*; *lane 2*), or GST/PTP-PEST^{C231S} (*CS*; *lane 3*). Bound CAKβ was detected by Western blotting with a CAKβ monoclonal antibody. *B*, HEK 293 cells were transfected with wild type FAK and treated as in *A*. Bound FAK was detected by Western blotting. *C*, A7r5 rat smooth muscle cells were treated with pervanadate and lysed. 500 μg of lysate was analyzed as in *A* and *B*, and bound CAKβ was detected by Western blotting. For each blot, 25 μg of cell lysate was included as a positive control (*A–C*, *lanes 4*).

ously into female New Zealand White rabbits. For all boosts, 500 μg of antigen in phosphate-buffered saline was emulsified with Freund's incomplete adjuvant and injected subcutaneously. Serum was prepared by centrifugation of cellular and clotted material from whole blood.

Substrate-trapping Assay—Cells were treated with pervanadate and lysed as described previously (16). Briefly, confluent cells were treated with 50 mM pervanadate for 30 min and lysed in radioimmune precipitation assay buffer supplemented with 5 mM iodoacetic acid for 30 min at 4 °C. After lysis, 10 mM dithiothreitol was added to inactivate any unreacted iodoacetic acid. Pervanadate-treated cell lysates (500 μg) were precleared with 10 μg of GST bound to glutathione-agarose beads at 4 °C for 1 h. The cleared supernatants were then incubated with 5 μg of either GST alone, GST/PTP-PEST^{WT}, or GST/PTP-PEST^{C231S} fusion protein immobilized on glutathione-agarose beads at 4 °C for 1 h. The beads were washed as described for immune complexes above, and bound protein was detected by Western blotting.

In Vitro Kinase Assay—CAKβ and FAK were immunoprecipitated using polyclonal antiserum as described above. Immune complexes were washed twice in lysis buffer and twice in kinase reaction buffer (50 mM Tris-HCl (pH 7.4), 5 mM $MnCl_2$, 5 mM $MgCl_2$) and then resuspended in 20 μl of kinase reaction buffer supplemented with 20 μM cold ATP, 10 μCi of [γ-^{32}P]ATP (6000 Ci/mol; PerkinElmer Life Sciences), and 5 μg of GST-paxillin^{N-C3} as an exogenous substrate. The reaction was stopped by the addition of Laemmli sample buffer after incubation at room temperature for 10 min. Samples were resolved by SDS-PAGE and analyzed by autoradiography. In some experiments, pervanadate-treated lysates (500–1000 μg) were incubated with GST/PTP-PEST^{WT} (1–4 μg) immobilized on glutathione beads for up to 20 min at room temperature or 1 h on ice. The phosphatase was removed by centrifugation, vanadate was added to the sample, and CAKβ or FAK was immunoprecipitated. The immune complexes were analyzed in the *in vitro* kinase assay or by Western blotting.

Immunofluorescence—Cells were fixed in 3.7% formaldehyde and permeabilized with 0.5% Triton X-100 as previously described (42). Commercially available monoclonal antibodies directed against PTP-PEST (Exalpha Biologicals Inc., Boston MA) and CAKβ (BD Transduction Laboratories) were used. A secondary rabbit anti-mouse bridging antibody and tertiary donkey anti-rabbit antibody conjugated to fluorescein (Jackson Immunoresearch Laboratories Inc., West Grove PA) were used to detect the primary antibody. The cells were visualized using a Leitz Orthoplan microscope, and images were collected with a Hamamatsu CCD camera and MetaMorph imaging software (Universal Imaging Corp., West Chester, PA).

RESULTS

PTP-PEST Substrate Traps CAKβ—The hypothesis that FAK and CAKβ are PTP-PEST substrates was initially tested using an *in vitro* substrate-trapping assay. FAK and CAKβ were overexpressed in HEK 293 cells, and the cells were treated with pervanadate to increase the cellular pool of tyrosine-phosphorylated proteins. Cell lysates were prepared and incubated with GST fusion proteins containing the catalytic domains of either wild type PTP-PEST (GST/PTP-PEST^{WT}) or the catalytically inactive, substrate-trapping mutant, GST/PTP-PEST^{C231S}. As shown in Fig. 1*A*, CAKβ bound to GST/PTP-PEST^{C231S} (*lane 3*) but was unable to bind GST/PTP-PEST^{WT} or GST alone (*lanes 1* and *2*). Upon overexposure of the blot, however, a very small amount of CAKβ was observed to bind to GST/PTP-PEST^{WT} (data not shown), implying that there may be a weak interaction between CAKβ and an unidentified site in the PTP-PEST catalytic domain. CAKβ from lysates of cells not stimulated with pervanadate failed to bind to GST/PTP-PEST^{C231S} (data not shown; see Fig. 4*A*). These two results suggest that the observed phosphotyrosine dependent interaction of CAKβ with GST/PTP-PEST^{C231S} was due to substrate binding to the active site of the phosphatase. Whereas CAKβ was readily detectable in complex with GST/PTP-PEST^{C231S} (Fig. 1*A*, *lane 3*), no detectable FAK was bound (Fig. 1*B*, *lane 3*). FAK could very weakly bind to the GST/PTP-PEST^{C231S} fusion protein as seen upon overexposure of the Western blot (data not shown). Note that the signal intensities of the loading controls for the FAK blot (Fig. 1*B*, *lane 4*) and CAKβ blot (Fig. 1*A*, *lane 4*) are similar. These data suggest that CAKβ is a preferred substrate for PTP-PEST in comparison with FAK. In order to examine the ability of PTP-PEST to substrate trap endogenous CAKβ, the A7r5 vascular smooth muscle cell line was employed. Cells were treated with pervanadate, and lysates were prepared. CAKβ from these lysates bound to GST/PTP-PEST^{C231S} but was unable to bind GST/PTP-PEST^{WT} or GST alone (Fig. 1*C*, *lanes 1–3*). CAKβ from cells not treated with pervanadate also failed to bind to any of the fusion proteins (data not shown). These results suggest that CAKβ was being trapped by the catalytically inactive mutant, GST/PTP-PEST^{C231S}, and is likely a substrate for PTP-PEST.

PTP-PEST Dephosphorylates CAKβ in Vivo—Next, the ability of PTP-PEST to dephosphorylate CAKβ *in vivo* was examined. CAKβ was expressed either alone or with PTP-PEST in HEK 293 cells. The cells were then lysed, and CAKβ was immunoprecipitated and Western blotted for phosphotyrosine. As shown in Fig. 2*A*, CAKβ was phosphorylated on tyrosine when expressed alone (*top panel*, *lane 1*) but was dramatically dephosphorylated upon PTP-PEST coexpression (*top panel*, *lane 2*). The differences in phosphotyrosine were not due to differential recovery of CAKβ as shown by stripping and reprobing the phosphotyrosine Western blot with a CAKβ monoclonal antibody (Fig. 2*A*, *middle panel*). A Western blot of whole cell lysates revealed expression of PTP-PEST in the transfected cells (Fig. 2*A*, *bottom panel*, *lane 2*). To determine if CAKβ dephosphorylation was specific, whole cell lysates were Western blotted for phosphotyrosine. Except for a band corresponding to CAKβ, the phosphotyrosine content of cellular proteins in CAKβ expressers and cells expressing both CAKβ and PTP-

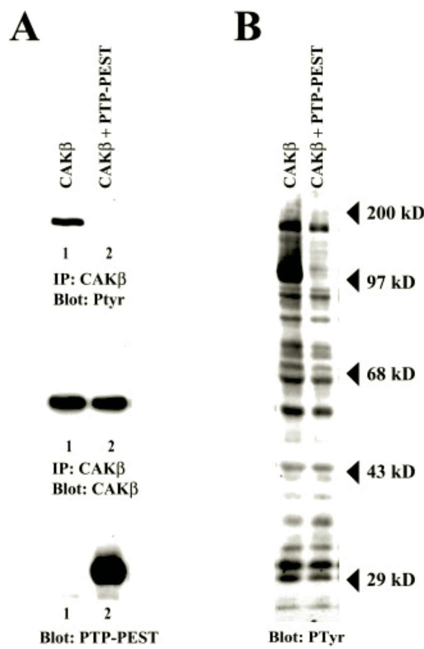

FIG. 2. **PTP-PEST dephosphorylates CAKβ.** *A*, CAKβ was expressed in HEK 293 cells either alone (*lane 1*) or with wild type PTP-PEST (*lane 2*). CAKβ was immunoprecipitated (*IP*), and its phosphotyrosine (*Ptyr*) content was examined by Western blotting (*Blot*; *top panel*). The blot was stripped and reprobed with a CAKβ monoclonal antibody (*middle panel*). PTP-PEST expression is shown in the Western blot in the *bottom panel*. *B*, 25 μg of cell lysate was analyzed by Western blotting using a phosphotyrosine antibody. The positions of molecular weight markers are indicated at the *right*.

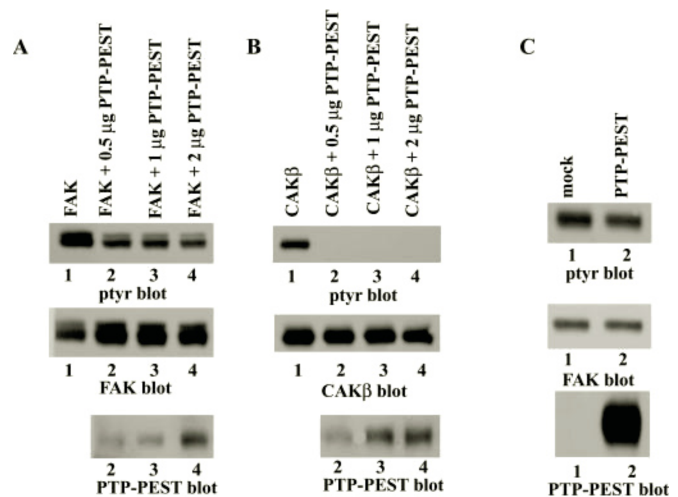

FIG. 3. **Comparison of FAK and CAKβ dephosphorylation *in vivo*.** *A*, FAK was expressed alone (*lane 1*) or cotransfected with 0.5 μg (*lane 2*), 1 μg (*lane 3*), or 2 μg of pcDNA/PTP-PEST (*lane 4*). Cells were lysed, and FAK was immunoprecipitated. The immune complexes were Western blotted with an antibody against phosphotyrosine (*ptyr*; *top panel*) or FAK (*middle panel*). Cell lysates were Western blotted using an antibody against PTP-PEST to verify expression (*bottom panel*). *B*, CAKβ was expressed alone (*lane 1*) or cotransfected with 0.5 μg (*lane 2*), 1 μg (*lane 3*), or 2 μg of pcDNA/PTP-PEST (*lane 4*). Cells were lysed, and CAKβ was immunoprecipitated. The immune complexes were Western blotted with an antibody against phosphotyrosine (*top panel*) or CAKβ (*middle panel*). Cell lysates were Western blotted using an antibody against PTP-PEST to verify expression (*bottom panel*). *C*, HEK 293 cells transfected with empty vector (*lane 1*) or pcDNA/PTP-PEST (*lane 2*) were lysed, and endogenous FAK was immunoprecipitated. The immune complexes were blotted for phosphotyrosine (*top panel*) or FAK (*middle panel*). Lysates were blotted for PTP-PEST to verify expression (*bottom panel*).

PEST was similar (Fig. 2*B*, *lanes 1* and *2*). A reduction in the phosphotyrosine content of a few cellular proteins was observed, including a 68-kDa protein that is probably paxillin. Another CAKβ and PTP-PEST substrate is $p130^{CAS}$, which also becomes dephosphorylated upon expression of PTP-PEST in HEK 293 cells (15). Two other CAKβ-associated substrates are the Nir proteins and PAP/KIAA0400 (43, 44). Nir2, the most broadly expressed Nir family member, is ~140 kDa, and PAP is 112 kDa. Although there are reduced phosphotyrosine levels in this range of molecular masses in cells coexpressing CAKβ and PTP-PEST, tyrosine phosphorylation of endogenous proteins is difficult to discern, since bands in this region of the gel are obscured by the exogenously expressed CAKβ. Further experiments will be required to verify that additional CAKβ substrates become dephosphorylated in PTP-PEST-expressing cells. These results demonstrate that PTP-PEST selectively dephosphorylates cellular proteins when expressed in HEK 293 cells, suggesting that CAKβ is a substrate of PTP-PEST *in vivo*.

To further explore the specificity of CAKβ dephosphorylation, the ability of PTP-PEST to dephosphorylate FAK *in vivo* was also examined. FAK or CAKβ were expressed in HEK 293 cells either alone or with different amounts of PTP-PEST, and their phosphotyrosine content was examined by immunoprecipitation and Western blotting. As seen in Fig. 3*A*, coexpression of PTP-PEST with FAK resulted in a reduction in tyrosine phosphorylation of FAK. However, even in the presence of relatively high levels of PTP-PEST, very substantial levels of tyrosine-phosphorylated FAK remain (Fig. 3*A*, *lane 4*). In contrast, coexpression of even low levels of PTP-PEST with CAKβ virtually abolished tyrosine phosphorylation of CAKβ (Fig. 3*B*, *lane 2*). To further explore the regulation of tyrosine phosphorylation of FAK by PTP-PEST, the level of phosphotyrosine present on endogenous FAK was examined. Expression of PTP-PEST in HEK 293 induced a very modest decrease in the phosphotyrosine content of endogenous FAK (Fig. 3*C*). The expression of endogenous CAKβ in HEK 293 cells was virtually undetectable, precluding a similar analysis for CAKβ (data not shown). These results are consistent with the hypothesis that CAKβ is a specific substrate for PTP-PEST, whereas FAK is a much weaker substrate.

PTP-PEST Targets the Major Autophosphorylation Site and Activation Loop of CAKβ—In order to determine what site(s) of tyrosine phosphorylation of CAKβ is targeted by PTP-PEST, site-specific CAKβ mutants in which tyrosines were replaced with phenylalanine were used (34). These mutants (Y402F, Y579F/Y580F, Y881F) and wild type CAKβ were expressed in HEK 293 cells. The cells were then either left unstimulated or treated with pervanadate, lysed, and subjected to the substrate-trapping assay. As expected, $GST/PTP\text{-}PEST^{C231S}$ was able to trap wild type CAKβ from pervanadate-treated cells, while $GST/PTP\text{-}PEST^{WT}$ could not (Fig. 4*A*, compare *lanes 2* and *4*). Further, neither $GST/PTP\text{-}PEST^{WT}$ nor $GST/PTP\text{-}PEST^{C231S}$ bound to CAKβ from cells that were not treated with pervanadate. $GST/PTP\text{-}PEST^{C231S}$ also trapped Y881F to a similar extent as wild type CAKβ (Fig. 4*A*, compare *lanes 4* and *16*). The Y402F mutant was also trapped by $GST/PTP\text{-}PEST^{C231S}$, although binding was reduced relative to wild type or Y881F CAKβ (Fig. 4*A*, compare *lane 8* with *lanes 4* and *16*). However, the Y579F/Y580F mutant was only very weakly trapped by $GST/PTP\text{-}PEST^{C231S}$ (Fig. 4*A*, *lane 12*), and this was only apparent upon overexposure of the Western blot (data not shown). Similar levels of each CAKβ variant were detected in cell lysates (Fig. 4*B*); thus, these results are not due to differential expression. These results suggest that the activation loop tyrosines are specifically targeted for dephosphorylation by CAKβ.

One trivial explanation why the Y579F/Y580F mutant was poorly trapped is that its phosphotyrosine content is lower than

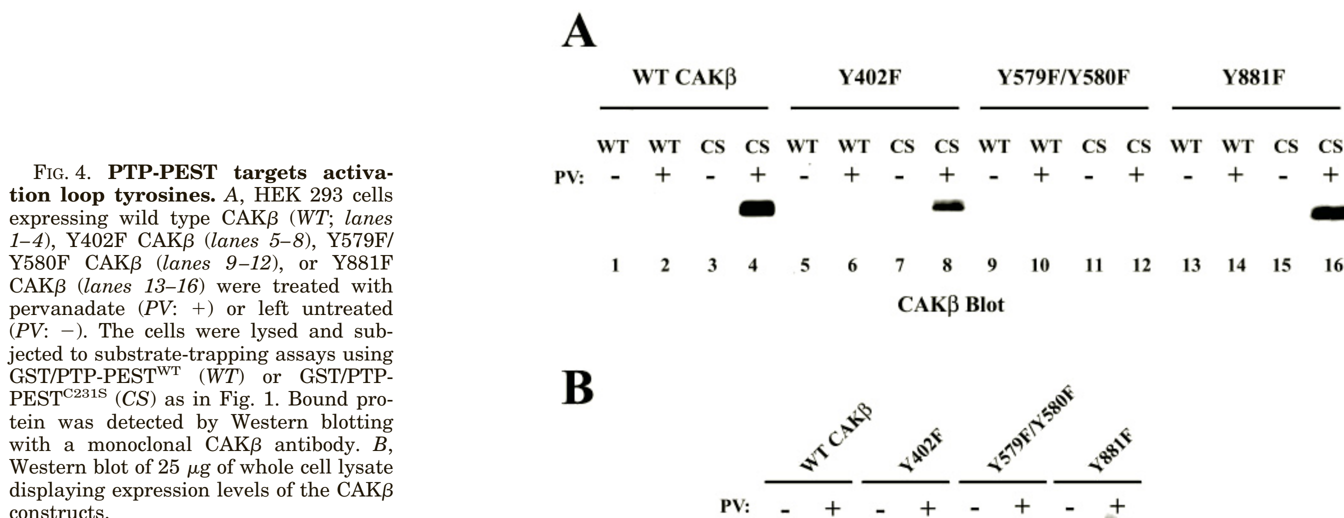

FIG. 4. **PTP-PEST targets activation loop tyrosines.** *A*, HEK 293 cells expressing wild type CAKβ (*WT*; *lanes 1–4*), Y402F CAKβ (*lanes 5–8*), Y579F/Y580F CAKβ (*lanes 9–12*), or Y881F CAKβ (*lanes 13–16*) were treated with pervanadate (*PV*: +) or left untreated (*PV*: −). The cells were lysed and subjected to substrate-trapping assays using GST/PTP-PEST^{WT} (*WT*) or GST/PTP-PEST^{C231S} (*CS*) as in Fig. 1. Bound protein was detected by Western blotting with a monoclonal CAKβ antibody. *B*, Western blot of 25 μg of whole cell lysate displaying expression levels of the CAKβ constructs.

that of the wild type protein or the other CAKβ mutants from pervanadate-treated cells. To examine this possibility, CAKβ was immunoprecipitated from pervanadate-treated lysates and Western blotted for phosphotyrosine. Whereas similar levels of phosphotyrosine were seen in wild type and Y881F (data not shown), Y402F exhibited a slight decrease in its phosphotyrosine content relative to wild type, and Y579F/Y580F had dramatically reduced levels of phosphotyrosine (Fig. 5*A*). A CAKβ Western blot shows that equal amounts of each variant were immunoprecipitated (Fig. 5*D*); thus, the reduced signal on Y579F/Y580F is really due to lower levels of phosphotyrosine. This result could simply reflect the fact that Y579F/Y580F is missing two sites of phosphorylation and thus is expected to be hypophosphorylated. Alternatively, since Tyr^{579} and Tyr^{580} are important for CAKβ kinase activity, it was possible that other sites of tyrosine phosphorylation, especially the major autophosphorylation site at Tyr^{402}, were hypophosphorylated in the Y579F/Y580F mutant. In order to test this hypothesis, phosphospecific antibodies recognizing distinct CAKβ phosphotyrosine residues were employed. CAKβ immune complexes prepared from lysates of pervanadate-treated HEK 293 cells were analyzed by Western blotting. An antibody specific for phosphorylated Tyr^{402} (PY402) was used to examine phosphorylation of this site. Western blotting with PY402 revealed strong reactivity with wild type CAKβ (Fig. 5*B*, *lane 2*). This antibody was specific, since it did not react with the Y402F mutant of CAKβ (Fig. 5*B*, *lane 3*). The Y579F/Y580F mutant did contain dramatically reduced levels of phosphorylation on Tyr^{402} as compared with wild type CAKβ (Fig. 5*B*, compare *lane 4* with *lane 2*). An antibody specific for CAKβ that is dually phosphorylated on Tyr^{579} and Tyr^{580} (PY579/580) recognized wild type CAKβ but very poorly recognized the Y579F/Y580F mutant (Fig. 5*C*, *lanes 2* and *4*); thus, this antibody is also specific. The PY579/580 antibody recognized the Y402F mutant to a lesser extent than wild type CAKβ (Fig. 5*C*, *lanes 2* and *3*), suggesting that Y402F exhibits reduced phosphorylation of the activation loop tyrosine residues. The results of these experiments underscore the complex regulation of CAKβ by tyrosine phosphorylation. The activation loop tyrosine residues must be phosphorylated for maximal catalytic activity of CAKβ, and thus the Y579F/Y580F mutant exhibits reduced tyrosine phosphorylation of the autophosphorylation site (Tyr^{402}). Conversely, the

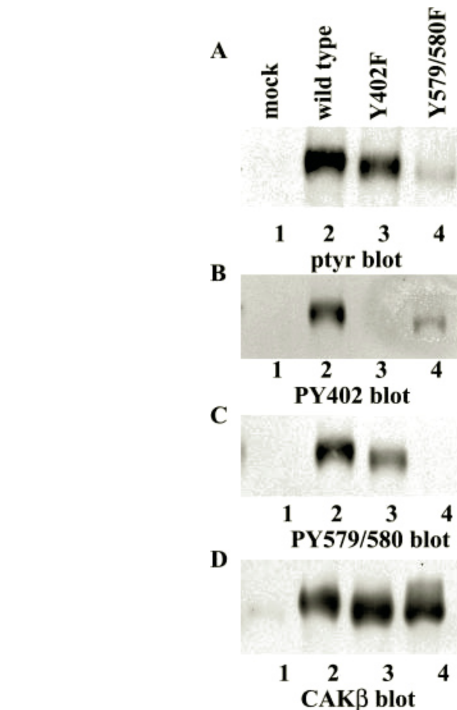

FIG. 5. **Analysis of tyrosine phosphorylation using CAKβ phosphospecific antibodies.** HEK 293 cells were transfected with either empty vector (*lane 1*), wild type CAKβ (*lane 2*), Y402F CAKβ (*lane 3*), or Y579F/Y580F CAKβ (*lane 4*). Cells were treated with pervanadate and lysed. CAKβ was immunoprecipitated and blotted with a phosphotyrosine antibody (*A*), a phosphospecific antibody recognizing Tyr(P)^{402} (*B*), a phosphospecific antibody recognizing Tyr(P)$^{570/580}$ (*C*), or CAKβ (*D*).

association of Src family PTKs with the autophosphorylation site of CAKβ is required for maximal phosphorylation of the activation loop tyrosine residues, and thus the Y402F mutant exhibits reduced tyrosine phosphorylation at these sites.

Dephosphorylation of Tyr^{402} and Tyr$^{579/580}$ by PTP-PEST in Vitro—In order to confirm the dephosphorylation of specific tyrosine residues on CAKβ by PTP-PEST, an *in vitro* phosphatase assay was employed. Lysates of pervanadate-treated HEK 293 cells expressing wild type CAKβ were incubated with GST/

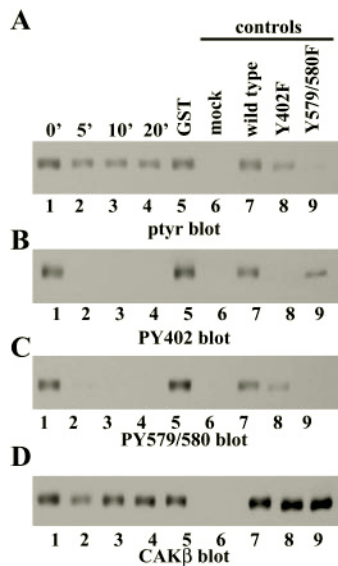

FIG. 6. **Dephosphorylation of Tyr^{402} and Tyr$^{579/580}$ by PTP-PEST *in vitro*.** 1 mg of lysate from pervanadate-treated HEK 293 cells expressing CAKβ was incubated with 4 μg of GST/PTP-PEST^{WT} for 5 min (*lane 2*), 10 min (*lane 3*), or 20 min at room temperature (*lane 4*). As controls, lysate was unexposed to phosphatase (*lane 1*) or incubated with GST for 20 min at room temperature (*lane 5*). CAKβ was immunoprecipitated from 300-μg aliquots, and the immune complexes were Western blotted with an anti-phosphotyrosine antibody (*A*), the phosphospecific PY402 antibody (*B*), the phosphospecific PY579/580 antibody (*C*), or a CAKβ antibody (*D*). As controls for antibody specificity, CAKβ immune complexes from mock-transfected HEK 293 cells (*lane 6*) or cells expressing wild type CAKβ (*lane 7*), Y402F (*lane 8*), or Y579/580F (*lane 9*) were included.

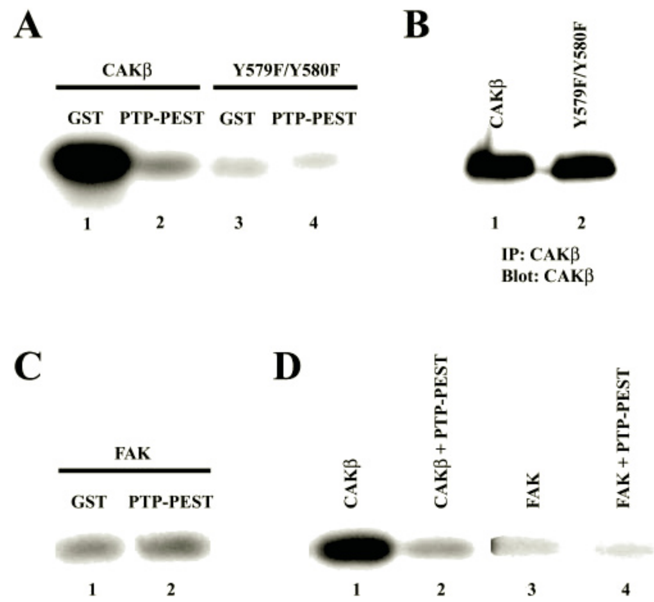

FIG. 7. **PTP-PEST inhibits CAKβ kinase activity.** *A*, 300 μg of pervanadate-treated lysate of HEK 293 cells expressing either wild type CAKβ (*lanes 1* and *2*) or Y579F/Y580F CAKβ (*lanes 3* and *4*) were incubated with either 2 μg of GST alone (*lanes 1* and *3*) or GST/PTP-PEST^{WT} (*lanes 2* and *4*). CAKβ was immunoprecipitated and subjected to an *in vitro* kinase assay using GST-paxillin^{N-C3} as a substrate. The reaction was terminated and analyzed by SDS-PAGE and autoradiography. The region of the autoradiograph containing the recombinant paxillin is shown. *B*, CAKβ was immunoprecipitated as in *A*. Immune complexes were subjected to SDS-PAGE and Western blotting with a CAKβ monoclonal antibody. *C*, HEK 293 cell lysates expressing wild type FAK were incubated with GST or GST/PTP-PEST^{WT}, and FAK was immunoprecipitated and analyzed in an *in vitro* kinase assay as in *A*. *D*, HEK 293 cells were transfected with either wild type CAKβ (*lane 1*), FAK (*lane 3*), CAKβ and wild type PTP-PEST (*lane 2*), or FAK and wild type PTP-PEST (*lane 4*). CAKβ (*lanes 1* and *2*) and FAK (*lanes 3* and *4*) were immunoprecipitated and subjected to kinase assays as in *A* and *C*.

PTP-PEST^{WT} for various times at room temperature or on ice for 1 h. CAKβ was immunoprecipitated from the lysates and analyzed for phosphotyrosine by Western blotting. Incubation with the phosphatase for 5 min at room temperature resulted in a reduction in the phosphotyrosine content of CAKβ, although significant levels of phosphotyrosine remained (Fig. 6*A*, *lane 2*). Incubation for up to 20 min at room temperature did not result in further dephosphorylation of CAKβ (Fig. 6*A*, *lane 4*), and CAKβ was not dephosphorylated when incubated for 20 min with GST alone (Fig. 6*A*, *lane 5*). In contrast to the result using a phosphotyrosine antibody (Fig. 6*A*), the results using phosphospecific antibodies demonstrated very dramatic reductions in the levels of phosphorylation of Tyr^{402} and Tyr$^{579/580}$ (Fig. 6, *B* and *C*, *lanes 2* and *3*). A CAKβ Western blot verified that similar amounts of protein were recovered in the immune complexes (Fig. 6*D*). Since little tyrosine phosphorylation of CAKβ was detected with the phosphospecific antibodies, the phosphotyrosine seen in Fig. 6*A* is probably due to phosphorylation of other tyrosine residues. FAK can be phosphorylated on at least six tyrosine residues (45, 46), and similarly CAKβ is anticipated to have other sites of tyrosine phosphorylation. These results demonstrate that the PTP-PEST can target Tyr^{402} and Tyr$^{579/580}$ for dephosphorylation *in vitro* yet spares other tyrosine residues within CAKβ from dephosphorylation.

PTP-PEST Inhibits CAKβ Kinase Activity—Phosphorylation of Tyr^{579} and Tyr^{580} in the activation loop of CAKβ enhances its kinase activity (34). To test the possibility that PTP-PEST could function to negatively regulate CAKβ kinase activity, HEK 293 cells overexpressing either wild type CAKβ or the Y579F/Y580F mutant were treated with pervanadate. Lysates were then incubated with either GST alone or GST/PTP-PEST^{WT} to catalyze dephosphorylation of CAKβ *in vitro*. CAKβ was then immunoprecipitated and subjected to an *in vitro* kinase assay using GST-paxillin^{N-C3} as an exogenous substrate. CAKβ very effectively phosphorylates this substrate *in vitro* (Fig. 7*A*, *lane 1*). As shown in Fig. 7*A*, CAKβ kinase activity was dramatically reduced in lysates incubated with GST/PTP-PEST^{WT} compared with lysates incubated with GST alone (compare *lanes 1* and *2*). Further, phosphorylation of the activation loop tyrosines was important for optimal catalytic activity of CAKβ, since Y579F/Y580F exhibited dramatically reduced catalytic activity relative to wild type CAKβ (Fig. 7*A*, *lane 3*). Western blotting for CAKβ revealed that equal amounts of wild type and Y579F/Y580F were present in the immune complexes (Fig. 7*B*, *lanes 1* and *2*). If PTP-PEST is negatively regulating CAKβ kinase activity by dephosphorylation of activation loop tyrosines, then the phosphatase should have no effect on the Y579F/Y580F CAKβ mutant. This was indeed the case, since Y579F/Y580F had the same level of kinase activity regardless of whether the lysate was incubated with GST or GST/PTP-PEST^{WT} (Fig. 7*A*, compare *lanes 3* and *4*). Interestingly, when wild type CAKβ was incubated in the presence of GST/PTP-PEST^{WT}, its kinase activity was reduced to almost the level of activity exhibited by the Y579F/Y580F mutant (Fig. 7*A*, compare *lanes 2* and *3*). This result supports the hypothesis that PTP-PEST is targeting activation loop tyrosines and that dephosphorylation of CAKβ by PTP-PEST impairs catalytic activity of the kinase. Unlike CAKβ, PTP-PEST had little or no effect on the kinase activity of FAK. Similar catalytic activities were observed following incubation with GST alone and with GST/PTP-PEST^{WT} (Fig. 7*C*, compare *lanes 1* and *2*). This finding demonstrates the substrate specificity of PTP-PEST, since it inhibited the catalytic activity of CAKβ *in vitro* but had no effect on the highly related tyrosine kinase, FAK.

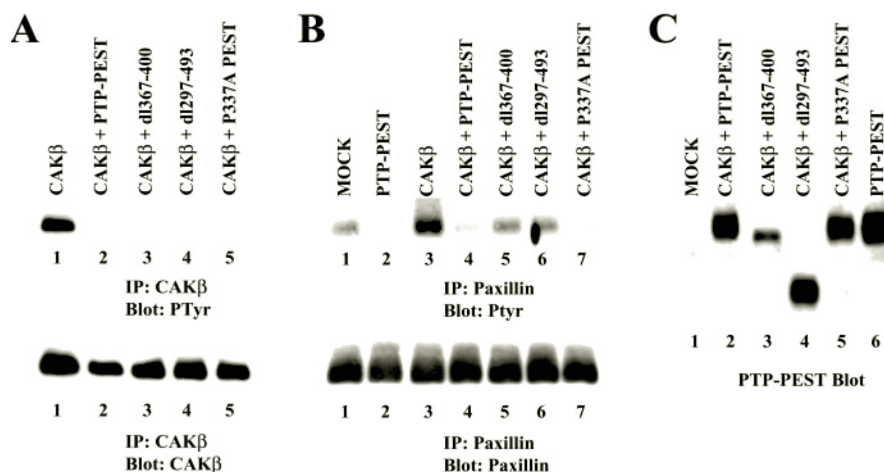

FIG. 8. **PTP-PEST negatively regulates CAKβ-dependent tyrosine phosphorylation *in vivo*.** *A*, CAKβ was expressed in HEK 293 cells either alone (*lane 1*) or with wild type PTP-PEST (*lane 2*), Δ367–400 (*lane 3*), Δ297–493 (*lane 4*), or P337A PTP-PEST (*lane 5*). CAKβ was immunoprecipitated, and its phosphotyrosine content was examined by Western blotting (*top panel*). The blot was stripped and reprobed with a CAKβ monoclonal antibody (*bottom panel*). *B*, paxillin was immunoprecipitated from HEK 293 cell lysate (*lane 1*) or from HEK 293 cell lysates expressing PTP-PEST (*lane 2*), CAKβ (*lane 3*), CAKβ and PTP-PEST (*lane 4*), CAKβ and Δ367–400 (*lane 5*), CAKβ and Δ297–493 (*lane 6*), or CAKβ and P337A PTP-PEST (*lane 7*). The immune complexes were analyzed by Western blotting for phosphotyrosine (*top panel*). The blot was stripped and reprobed with a paxillin monoclonal antibody (*bottom panel*). *C*, 25 μg of whole cell lysates were examined for PTP-PEST expression by Western blotting with a polyclonal antiserum.

PTP-PEST was also able to inhibit the kinase activity of CAKβ *in vivo* when the two were coexpressed in HEK 293 cells. The catalytic activity of CAKβ was measured by immunoprecipitating the kinase from cell lysates and incubating the immune complex in an *in vitro* kinase assay. The catalytic activity of CAKβ immunoprecipitated from cells coexpressing PTP-PEST was dramatically reduced relative to the catalytic activity of CAKβ when expressed alone (Fig. 7*D*, compare *lanes 1* and *2*). In contrast, the catalytic activity of FAK immunoprecipitated from cells coexpressing PTP-PEST was the same as the activity of FAK expressed alone (Fig. 7*D*, compare *lanes 3* and *4*). Thus, PTP-PEST was unable to inhibit FAK kinase activity *in vivo*. These data support the substrate trapping data and suggest that PTP-PEST prefers CAKβ to FAK as a substrate. Furthermore, they suggest that PTP-PEST can directly regulate the catalytic activity of CAKβ by dephosphorylation of activation loop tyrosine residues.

PTP-PEST Negatively Regulates CAKβ-dependent Tyrosine Phosphorylation in Vivo—PTP-PEST is able to bind and dephosphorylate paxillin and p130^{CAS} (13, 15). Since paxillin and p130^{CAS} are also binding partners of CAKβ, it seemed plausible that either of these proteins might serve as a scaffold to bring PTP-PEST into the proximity of CAKβ to allow dephosphorylation to occur. In order to address this, CAKβ was expressed in HEK 293 cells either alone or in combination with wild type PTP-PEST or deletion mutants of PTP-PEST (Δ367–400 and Δ297–493), shown previously to be unable to bind paxillin (15), or a PTP-PEST mutant (P337A PTP-PEST) that is unable to bind p130^{CAS} (13). CAKβ was immunoprecipitated from cell lysates and Western blotted for phosphotyrosine. As already shown in Figs. 2*A* and 3*B*, coexpression with wild type PTP-PEST induced a complete loss of phosphotyrosine on CAKβ (Fig. 8*A*, *lane 2*, *top panel*). Both deletion mutants and the P337A mutant were able to induce CAKβ dephosphorylation to the same extent as wild type PTP-PEST (Fig. 8*A*, *lanes 3–5*, *top panel*). The blot was stripped and reprobed with a CAKβ monoclonal antibody to show that equal levels of protein were immunoprecipitated (Fig. 8*A*, *bottom panel*). This result suggests that the association of PTP-PEST with neither paxillin nor p130^{CAS} is necessary for dephosphorylation of CAKβ by PTP-PEST.

Expression of CAKβ in chick embryo fibroblasts results in enhanced phosphorylation of paxillin (47). Similarly, paxillin becomes hyperphosphorylated when CAKβ is overexpressed in HEK 293 cells (Fig. 8*B*, compare *lanes 1* and *3*). Coexpression of PTP-PEST with CAKβ results in a reduction in paxillin phosphotyrosine levels (Fig. 8*B*, compare *lanes 3* and *4*). This could be the result of PTP-PEST inhibiting phosphorylation of paxillin by negatively regulating CAKβ kinase activity. Alternatively, PTP-PEST could also be dephosphorylating paxillin itself, since paxillin was shown to be a substrate for PTP-PEST (15). The PTP-PEST deletion mutants deficient in paxillin binding were used to address this question. Paxillin binding is essential for the direct dephosphorylation of paxillin by PTP-PEST (15). As shown in Fig. 8*A*, paxillin binding is completely dispensable for dephosphorylation of CAKβ by PTP-PEST. Either Δ367–400 or Δ297–493 PTP-PEST mutants were coexpressed with CAKβ in HEK 293 cells. Paxillin was then immunoprecipitated, and its phosphotyrosine content was examined by Western blotting. Both Δ367–400 and Δ297–493 mutants were able to reduce paxillin phosphotyrosine levels compared with cells expressing CAKβ alone (Fig. 8*B*, compare *lanes 5* and *6* with *lane 3*). Since both mutants fail to directly dephosphorylate paxillin (15), this result implies that PTP-PEST is indirectly reducing tyrosine phosphorylation of paxillin by inhibiting CAKβ. However, both deletion mutants were unable to reduce paxillin phosphotyrosine levels to the same extent as wild type PTP-PEST (Fig. 8*B*, compare *lanes 5* and *6* with *lane 4*). Whole cell lysates were Western blotted with the polyclonal PTP-PEST antiserum to demonstrate that differences in levels of tyrosine phosphorylation of paxillin were not due to differences in expression levels of wild type PTP-PEST and the various mutants (Fig. 8*C*). These results suggest that PTP-PEST also acts to directly dephosphorylate paxillin. Therefore, PTP-PEST is able to negatively regulate a signal sent by CAKβ *in vivo* by targeting both the kinase and its substrate for dephosphorylation.

Subcellular Localization of Endogenous PTP-PEST and CAKβ—FAK is unlikely to be a major target for PTP-PEST *in vivo*, since FAK is localized in focal adhesions and PTP-PEST is found in the cytoplasm (37, 48). To determine if PTP-PEST and CAKβ reside in the same cellular compartment, their subcellular localization was examined by immunofluorescence in the GN4 and A7r5 cell lines, which express both of these proteins

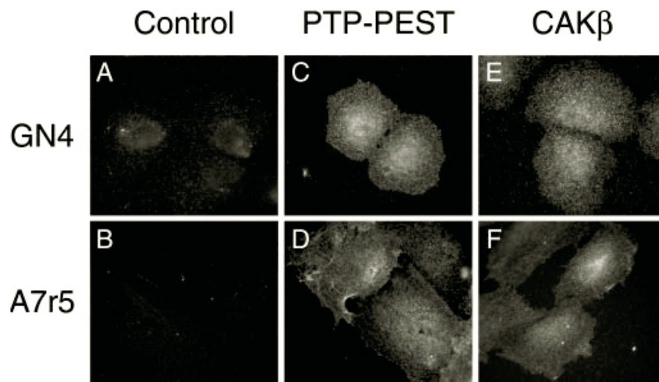

FIG. 9. **Localization of endogenous PTP-PEST and CAKβ in GN4 and A7r5 cells.** GN4 cells (*A*, *C*, and *E*) and A7r5 cells (*B*, *D*, and *F*) were fixed, permeabilized, and incubated with a PTP-PEST monoclonal antibody (*C* and *D*) or a CAKβ monoclonal antibody (*E* and *F*). The primary antibodies were detected with secondary rabbit anti-mouse and tertiary donkey anti-rabbit conjugated to fluorescein. The primary antibody was omitted in control slides (*A* and *B*). The GN4 cells were observed with a × 63 objective, and the A7r5 cells were observed with a × 40 objective.

endogenously. In both cell types, immunofluorescent staining for both PTP-PEST and CAKβ was relatively nondescript. Each protein was located within the cytoplasm of the cells, and the staining appeared particulate (Fig. 9). As a control, the primary antibodies were omitted, resulting in very dim background fluorescence (Fig. 9, *A* and *B*). This result confirms previous findings that PTP-PEST and CAKβ are localized in the cytoplasm of cells (47–49) and supports the contention that CAKβ may be a physiological substrate for PTP-PEST, since both are found in a similar cellular compartment.

DISCUSSION

In this study, we have demonstrated the direct regulation of the PTK CAKβ by PTP-PEST. CAKβ is substrate-trapped by a catalytically inactive PTP-PEST mutant and is dephosphorylated *in vivo* when coexpressed with PTP-PEST. PTP-PEST targets tyrosine residues 579 and 580 in the activation loop of CAKβ for dephosphorylation. As a result, PTP-PEST decreases CAKβ kinase activity both *in vitro* and *in vivo*. This inhibition of CAKβ kinase activity results in the reduced ability of CAKβ to phosphorylate paxillin *in vivo*. In addition, PTP-PEST targets tyrosine residue 402 for dephosphorylation. Loss of phosphorylation at this site will prevent Src family kinases from binding to CAKβ, which in turn will result in reduced tyrosine phosphorylation of CAKβ and other downstream substrates in the pathway. Perhaps as interesting, the results also demonstrate that PTPs can exhibit very strong specificity for substrates, since PTP-PEST appears to strongly prefer CAKβ as a substrate to its highly homologous relative, FAK.

There are several observations regarding tyrosine phosphorylation of CAKβ that merit further discussion. First, when PTP-PEST is coexpressed with CAKβ, a complete loss of phosphotyrosine on CAKβ is observed (Fig. 2*A*, *top panel*). Since PTP-PEST appears to target Tyr^{402}, Tyr^{579}, Tyr^{580}, the complete loss of phosphotyrosine on CAKβ is intriguing. Perhaps these three sites are the only residues phosphorylated when CAKβ is exogenously expressed in cells. If other tyrosine residues are phosphorylated upon CAKβ expression, how are these sites dephosphorylated upon PTP-PEST overexpression? One possible explanation is that by targeting the activation loop, PTP-PEST turns off CAKβ kinase activity, and by directly dephosphorylating Tyr^{402} the Src family kinase binding site is removed. As a result, Src can no longer bind CAKβ and transphosphorylate other tyrosine residues, such as Tyr^{881}, on CAKβ. Second, the observed pattern of tyrosine phosphorylation of CAKβ mutants following pervanadate treatment differs from the published pattern of phosphorylation from untreated cells (34). When expressed in HEK 293(T) cells, the Y402F mutant has been reported to be virtually devoid of phosphotyrosine, and the Y579F/Y580F mutant exhibits levels of phosphotyrosine dramatically lower than wild type CAKβ. In fact, we find similar results in experiments in which the cells are not pervanadate-treated (data not shown). These findings suggest that tyrosine 402 is the major site of tyrosine phosphorylation and/or a prerequisite site of phosphorylation for the phosphorylation of additional tyrosine residues. The observation that pervanadate treatment induces tyrosine phosphorylation of the Y402F mutant suggests that the SH2 domain-mediated association of Src with CAKβ may not be absolutely required for phosphorylation of additional tyrosine residues in CAKβ. This result suggests that there is a second mechanism for tyrosine phosphorylation of CAKβ, although this is probably a minor mechanism of phosphorylation, since it only becomes evident following inhibition of PTPs with pervanadate. However, the SH2-mediated interaction between Src and CAKβ may be necessary for optimal phosphorylation of the activation loop, since Y402F exhibits lower levels of phosphotyrosine on residues 579/580 than wild type CAKβ, even from pervanadate-treated cells (34) (Fig. 5*B*).

PTP-PEST is not the only phosphatase reported to associate with CAKβ. It was recently reported that SHP-1 binds constitutively to CAKβ (50). The data suggest that SHP-1 is involved in dephosphorylation of Tyr^{402}, the major autophosphorylation and Src-SH2 binding site. Unlike PTP-PEST, SHP-1 had no effect on CAKβ kinase activity. SHP-2, another SH2 domain-containing protein-tyrosine phosphatase, was also recently reported to constitutively associate with CAKβ (51). SHP-2 binding to CAKβ was shown to be independent of SHP-2 SH2 domains. Further, CAKβ was dephosphorylated *in vivo* upon expression of a SHP-2 mutant with enhanced phosphatase activity that lacks its two N-terminal SH2 domains. Additional studies are required to establish which site(s) are targeted by SHP-2 and whether SHP-2 can regulate CAKβ kinase activity. One intriguing possibility is that SHP-1 and SHP-2 function cooperatively with PTP-PEST in down-regulating CAKβ signaling. According to this model, PTP-PEST may be responsible for turning off CAKβ kinase activity by dephosphorylation of activation loop residues, and SHP-1 or SHP-2 may be responsible for inhibiting the assembly of the Src-CAKβ complex by dephosphorylating the Src SH2 domain binding site.

Surprisingly, PTP-PEST substrate trapped CAKβ much better than FAK. Small amounts of FAK could sometimes be found in association with the PTP-PEST substrate-trapping mutant, but it was always much less in comparison with CAKβ.[2] This does not rule out the possibility that FAK may be a substrate for PTP-PEST. For example, paxillin was unable to be substrate-trapped but nevertheless appears to be a substrate for PTP-PEST, since it directly binds PTP-PEST and becomes dephosphorylated *in vivo* upon overexpression of wild type PTP-PEST (15). Despite the failure of PTP-PEST to efficiently substrate-trap FAK, there is some evidence suggesting that PTP-PEST could regulate FAK dephosphorylation. First, PTP-PEST activity was coimmunoprecipitated with FAK from lysates of chicken embryo fibroblasts (36). Second, FAK was hyperphosphorylated in fibroblasts where PTP-PEST was removed by gene targeting (29). However, PTP-PEST and FAK have never been colocalized in cells. PTP-PEST is localized diffusely throughout the cytoplasm or at the membrane periphery upon cell adhesion to fibronectin (29), whereas FAK is predominately localized in focal adhesions (37). Clearly, further experimentation is required to define the role of PTP-

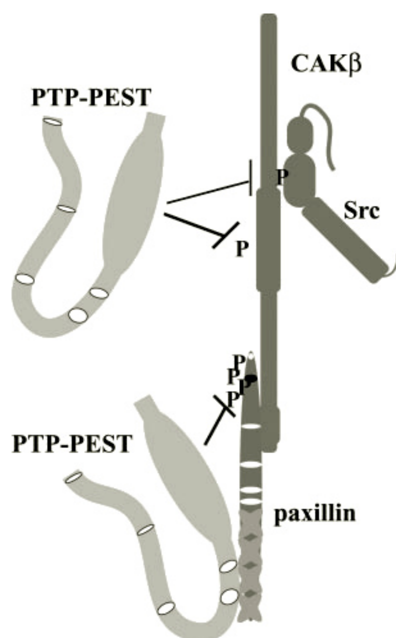

FIG. 10. **Model of PTP-PEST regulation of CAKβ/paxillin signaling.** PTP-PEST can bind directly to and dephosphorylate paxillin. In addition, PTP-PEST can dephosphorylate activation loop tyrosine residues on CAKβ, inhibiting kinase activity, and the major autophosphorylation site of CAKβ, inhibiting association with Src family kinases. Therefore, PTP-PEST can target multiple components of this signaling pathway for dephosphorylation.

PEST in dephosphorylating FAK. Interestingly, FAK has recently been shown to associate with several other tyrosine phosphatases including PTEN/MMAC1 (52, 53), PTP1B (54–56), and SHP-2 (57, 58). It is possible that one or more of these phosphatases is responsible for regulating FAK dephosphorylation.

The observation that CAKβ can be substrate-trapped by PTP-PEST better than FAK raises the interesting question of substrate specificity, since CAKβ and FAK are highly homologous, sharing 45% overall sequence identity and 60% identity in the catalytic domain. As stated previously, they share several conserved tyrosine residues, including two tyrosines in the catalytic domain. Why then does PTP-PEST so strongly prefer CAKβ? One reason may be a slight difference in amino acid sequence surrounding the adjacent tyrosine residues. In the activation loop, the CAKβ sequence is ^{575}EDEDpYpYKAS^{583} (where pY represents phosphotyrosine), while the equivalent FAK sequence is ^{572}EDSTpYpYKAS^{580}. It may be that the difference in the sequences immediately upstream of the phosphorylated tyrosine residues is the reason that PTP-PEST prefers CAKβ as a substrate. This is probably the case, since several reports have suggested that aspartic acid and glutamic acid within the first 5 residues N-terminal to the phosphorylated tyrosine residues increases the binding affinity of several phosphatases for their substrates and increase catalysis of the substrate (59–61). In addition, the crystal structure of another cytoplasmic tyrosine phosphatase, PTP1B, complexed with a high affinity substrate has been determined (62). The crystal structure suggests that substrate specificity is conferred by the interaction of basic residues on the surface of the phosphatase with acidic residues of the peptide that lie to the N-terminal side of the phosphorylated tyrosine residue.

Paxillin is phosphorylated upon overexpression of CAKβ in HEK 293 cells (Fig. 8) and is presumably a downstream effector of CAKβ signaling. Both paxillin and CAKβ are substrates for PTP-PEST, suggesting a model whereby PTP-PEST regulates this pathway at two distinct points (Fig. 10). First, PTP-PEST is able to directly bind and dephosphorylate paxillin. Second, PTP-PEST dephosphorylates activation loop tyrosine residues on CAKβ, rendering the kinase catalytically inactive, and further targets the major autophosphorylation and Src kinase binding site of CAKβ. Inhibition of the kinase activity of CAKβ and blocking recruitment of Src kinases, in turn, prevents the CAKβ-Src kinase complex from phosphorylating its downstream substrate, paxillin. Perhaps regulation at these two distinct points is required for complete inactivation of this signal. Alternatively, there may be additional, independent mechanisms regulating targeting of these two PTP-PEST substrates for dephosphorylation. In this case, CAKβ signaling could be inhibited by dephosphorylation of the kinase. Alternatively, one branch of the CAKβ signaling cascade could be blocked by dephosphorylation of paxillin, allowing other CAKβ-dependent signals to be propagated. This targeting of distinct members of this signaling pathway under specific conditions might increase the versatility of signals transmitted.

The discovery that PTP-PEST can regulate the catalytic activity of CAKβ and phosphorylation of CAKβ substrates provides important new insight into signaling via this pathway. The efficient transmission of downstream signals might require the regulation of both the PTK and the PTP, which could involve changes in the catalytic activity of these enzymes and/or alterations in protein-protein interactions. There is clearly considerable work to be done to unravel the complex interplay between these two enzymes and determine how their activities might be coordinated to allow the propagation of signals.

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