Protein-tyrosine phosphatase (PTP)-PEST is a cytoplasmic tyrosine phosphatase that can bind and dephosphorylate the focal adhesion-associated proteins p130CAS and paxillin. Focal adhesion kinase (FAK) and cell adhesion kinase β (p130CAS)/PYK2/CADTK/RAFTK are protein-tyrosine kinases that can colocalize with, bind to, and induce tyrosine phosphorylation of p130CAS 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 auto-phosphorylation site of CAKβ (Tyr⁴⁰⁸⁹) and activation loop tyrosine residues, Tyr⁴⁷⁸ and Tyr⁵⁵⁶, 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) 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 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. p130CAS 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 p130CAS 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 p130CAS localize to focal adhesions and undergo rapid tyrosine phosphorylation upon cell adhesion (17). Paxillin and p130CAS also become tyrosine-phosphorylated in response to various physiological stimuli 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
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 p130CAS also creates docking sites for the SH2 domain of Crk (25). The p130CAS-Crk complex has been proposed to regulate cell migration (26), and p130CAS 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 p130CAS for dephosphorylation.

Paxillin and p130CAS are potential substrates for several candidate tyrosine kinases including FAK and CAKβ. Paxillin and p130CAS 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 Ca2+. (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 Tyr957 in FAK and Tyr102 in CAKβ, the Grb2-SH2 domain binding site at Tyr295 in FAK and Tyr385 in CAKβ, and two regulatory sites in the activation loop of the kinase domains: Tyr576/77 in FAK and Tyr579/80 in CAKβ (30, 34).

FAK and CAKβ are particularly good candidate PTP-PEST substrates, since these kinases both bind paxillin and p130CAS (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-PEST is able to inhibit FAK-promoted cell migration (27) 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 demonstrated 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.

**Inhibition of CAKβ Catalytic Activity by PTP-PEST**

**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 XhoI and SacI, which cut in the multiple cloning site flanking the FAK insert. The FAK-containing fragment was ligated into the 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 pGEX2T (Amersham Pharmacia Biotech) in frame with the GST coding sequence. The C231S PTP-PEST substrate-trapping mutant (GST/PTP-PESTd231S) was generously provided by Dr. André Veillette (McGill University). This mutant encodes amino acids 1–301 with cysteine 231 mutated to serine. GST-paxillin in34–226 was generated by amplifying a fragment of the paxillin cDNA (encoding amino acids 1–312) by polymerase chain reaction using primers that created a BamHI site at the 5′-end and an EcoRI site at the 3′-end. This fragment was inserted into pGEX2T to create GST-paxillin in34–226. The GST coding sequence. The fusion proteins were expressed by incubation with 0.3 mM isopropyl β-D-thiogalactoside for 5 h at 30 °C, the bacteria were lysed in a bacterial cell lysate in 30°C, the suspension and washed pH 7.5, supplemented with 10% fetal bovine serum. GN4 cells were maintained in Rich Eagle’s 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. Cells were transfected using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s recommended protocol.

**Protein Analysis—**Cells were lysed in modified radioimmunoprecipitation assay buffer (36), and protein concentrations were determined using Bradford dye 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) in 1–2 °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 (CA). The AK2 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, membranes 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.

**Inactivation 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 pGEX2T in-frame with the GST coding sequence. This fusion protein was gel-purified and used as an antigen to prepare polyclonal 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-
Inhibition of CAKβ Catalytic Activity by PTP-PEST

**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-PESTWT) or the catalytically inactive, substrate-trapping mutant, GST/PTP-PESTC231S. As shown in Fig. 1A, CAKβ bound to GST/PTP-PESTC231S (lane 3) but was unable to bind GST/PTP-PESTWT 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-PESTWT (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-PESTC231S (data not shown; see Fig. 4A). These two results suggest that the observed phosphotyrosine dependent interaction of CAKβ with GST/PTP-PESTC231S was due to substrate binding to the active site of the phosphatase. 

**Cell Line Selection**—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-PESTWT (Fig. 1A, lane 3), but was unable to bind GST/PTP-PESTC231S or GST alone (lanes 1 and 2). To determine if CAKβ was being trapped by the catalytically inactive mutant, GST/PTP-PESTC231S, 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. 2A, 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. A Western blot of whole cell lysates revealed expression of PTP-PEST in the transfected cells (Fig. 2A, 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-
PEST was similar (Fig. 2B, 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 p130CAS, which is a substrate of 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-PEST(C231S) was able to trap wild type CAKβ from pervanadate-treated cells, while GST/PTP-PESTWT could not (Fig. 4A, compare lanes 2 and 4). Further, neither GST/PTP-PESTWT nor GST/PTP-PEST(C231S) bound to CAKβ from cells that were not treated with pervanadate. GST/PTP-PESTWT also trapped Y881F to a similar extent as wild type CAKβ (Fig. 4A, compare lanes 4 and 16). The Y402F mutant was also trapped by GST/PTP-PESTWT, although binding was reduced relative to wild type or Y881F CAKβ (Fig. 4A, compare lane 8 with lanes 4 and 16). However, the Y579F/Y580F mutant was only very weakly trapped by GST/PTP-PESTWT (Fig. 4A, 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. 4B); 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...
Inhibition of CAKβ Catalytic Activity by PTP-PEST

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. 5A). A CAKβ Western blot shows that equal amounts of each variant were immunoprecipitated (Fig 5D); 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 Tyr579 and Tyr580 are important for CAKβ kinase activity, it was possible that other sites of tyrosine phosphorylation, especially the major autophosphorylation site at Tyr402, 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 Tyr402 (PY402) was used to examine phosphorylation of this site. Western blotting with PY402 revealed strong reactivity with wild type CAKβ (Fig. 5B, lane 2). This antibody was specific, since it did not react with the Y402F mutant of CAKβ (Fig. 5B, lane 3). The Y579F/Y580F mutant did contain dramatically reduced levels of phosphorylation on Tyr402 as compared with wild type CAKβ (Fig. 5B, compare lane 4 with lane 2). An antibody specific for CAKβ that is dually phosphorylated on Tyr579 and Tyr580 (PY579/580) recognized wild type CAKβ but very poorly recognized the Y579F/Y580F mutant (Fig. 5C, 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. 5C, 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 (Tyr402). Conversely, the

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.

Deglycosylation of Tyr402 and Tyr579/580 by PTP-PEST in Vitro—In order to confirm the deglycosylation 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/
Inhibition of CAKβ Catalytic Activity by PTP-PEST

PTP-PEST\textsuperscript{WT} for various times at room temperature or on ice for 1 h. CAKβ was immunoprecipitated from the lysates and analyzed for phosphoryrosine by Western blotting. Incubation with the phosphatase for 5 min at room temperature resulted in a reduction in the phosphoryrosine content of CAKβ, although significant levels of phosphoryrosine remained (Fig. 6A, lane 2). Incubation for up to 20 min at room temperature did not result in further dephosphorylation of CAKβ (Fig. 6A, lane 4), and CAKβ was not dephosphorylated when incubated for 20 min with GST alone (Fig. 6A, lane 5). In contrast to the result using a phosphospecific antibody (Fig. 6A), the results using phosphospecific antibodies demonstrated very dramatic reductions in the levels of phosphoryrosion of Tyr\textsuperscript{402} and Tyr\textsuperscript{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. 6D). Since little tyrosine phosphorylation of CAKβ was detected with the phosphospecific antibodies, the phosphospecific view in Fig. 6A is probably due to phosphoryrosion of other tyrosines residues. FAK can be phosphoryrosed on at least six tyrosines and that dephosphoryrosion of CAKβ is anticipated to have other sites of tyrosine phosphoryrosion. These results demonstrate that the PTP-PEST can target Tyr\textsuperscript{402} and Tyr\textsuperscript{579/580} for dephosphoryrosion in vitro yet spares other tyrosine residues within CAKβ from dephosphoryrosion.

PTP-PEST inhibits CAKβ Kinase Activity—Phosphoryrosion of Tyr\textsuperscript{402} and Tyr\textsuperscript{579/580} 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\textsuperscript{WT} to catalyze dephosphoryrosion of CAKβ in vitro. CAKβ was then immunoprecipitated and subjected to an in vitro kinase assay using GST-paxillin\textsuperscript{N-C3} as an exogenous substrate. CAKβ very effectively phosphorylates this substrate in vitro (Fig. 7A, lane 1). As shown in Fig. 7A, CAKβ kinase activity was dramatically reduced in lysates incubated with GST/PTP-PEST\textsuperscript{WT} compared with lysates incubated with GST alone (compare lanes 1 and 2). Further, phosphoryrosion 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. 7A, lane 3). Western blotting for CAKβ revealed that equal amounts of wild type and Y579F/Y580F were present in the immune complexes (Fig. 7B, lanes 1 and 2). If PTP-PEST is negatively regulating CAKβ kinase activity by dephosphoryrosion 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 lystate was incubated with GST or GST/PTP-PEST\textsuperscript{WT} (Fig. 7A, compare lanes 3 and 4). Interestingly, when wild type CAKβ was incubated in the presence of GST/PTP-PEST\textsuperscript{WT}, its kinase activity was reduced to almost the level of activity exhibited by the Y579F/Y580F mutant (Fig. 7A, compare lanes 2 and 3). This result supports the hypothesis that PTP-PEST is targeting activation loop tyrosines and that dephosphoryrosion 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\textsuperscript{WT} (Fig. 7C, 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.
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. 7D, 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. 7D, 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 to and dephosphorylate paxillin and p130Cas (13, 15). Since paxillin and p130Cas 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 dephosphorylate paxillin (47). Similarly, paxillin becomes hyperphosphorylated when CAKβ is overexpressed in HEK 293 cells (Fig. 8B, compare lanes 1 and 3). Coexpression of PTP-PEST with CAKβ results in a reduction in paxillin phosphotyrosine levels (Fig. 8B, 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. 8A, 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. 8B, 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. 8B, 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. 8C). 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.
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 of CAKβ is substrate-trapped but nevertheless appears to be a substrate for 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. 2A, top panel). Since PTP-PEST appears to target Tyr402, Tyr579, Tyr580, 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 Tyr402 the Src family kinase binding site is removed. As a result, Src can no longer bind CAKβ and transphosphorylate other tyrosine residues, such as Tyr881, 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. 5B).

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 Tyr402, the major autophosphorylation domain of CAKβ. Surprisingly, PTP-PEST substrate trapped CAKβ kinase activity by dephosphorylation of Tyr402, the major autophosphorylation site dephosphorylation of Tyr402, the major autophosphorylation domain of CAKβ. 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. 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-
Inhibition of CAKβ Catalytic Activity by PTP-PEST

PEST in dephosphorylating FAK. Interestingly, FAK has recently been shown to associate with several other tyrosine phosphatases including PTPN1/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 575EDEDpYpYKAS583 (where pY represents phosphotyrosine), while the equivalent FAK sequence is 572EDSTpYpYKAS580. 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 HEK293 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.

Acknowledgments—We thank Drs. Tom Parsons, Xiong Li, Shelley Earp, and André Veillette for providing reagents and Dr. Jeff Thomas for the GST-paxillin-N-C3 construct. We thank Dr. Ben Peng for generous permission to use the microscope. We also thank Shelley Earp for critical reading of the manuscript. We are particularly indebted to Veronica Gabarra-Niecko for comments and assistance during the course of this work and during preparation of the manuscript.

REFERENCES
1. Yang, Q., Co, D., Sommercorn, J., and Tonks, N. K. (1993) J. Biol. Chem. 268, 6622–6628; Correction (1993) J. Biol. Chem. 268, 17650
2. Matthews, R. J., Bowne, D. B., Flores, E., and Thomas, M. L. (1992) Mol. Cell. Biol. 12, 2386–2405
3. Matthews, R. J., Bowne, D. B., Flores, E., and Thomas, M. L. (1992) Mol. Cell. Biol. 12, 2386–2405
4. Huxley, J. D., and Schaller, M. D. (2000) J. Biol. Chem. 275, 1085–1096
5. Shaw, N., and Hashimoto, S., and Sabe, H. (2000) J. Biol. Chem. 275, 1085–1096
6. Shewan, S., and Latt, A. J., and Schaller, M. D. (2000) J. Biol. Chem. 275, 1085–1096
7. Shaw, N., and Hashimoto, S., and Sabe, H. (2000) J. Biol. Chem. 275, 1085–1096
8. Shaw, N., and Hashimoto, S., and Sabe, H. (2000) J. Biol. Chem. 275, 1085–1096
9. Shaw, N., and Hashimoto, S., and Sabe, H. (2000) J. Biol. Chem. 275, 1085–1096
10. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
11. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
12. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
13. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
14. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
15. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
16. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
17. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
18. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
19. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
20. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
21. Cloutier, J. F., and Veillette, A. (1999) J. Biol. Chem. 274, 20550–20560
Inhibition of CAKβ Catalytic Activity by PTP-PEST

25. Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Nishida, J., Yazaki, Y., and Hirai, H. (1994) J. Biol. Chem. 269, 32740–32746
26. Kleinke, R. L., Leng, J., Molander, E., Brooks, P. C., Vuori, K., and Chretien, D. A. (1998) J. Cell Biol. 140, 961–972
27. Carty, L. A., Han, D. C., Polet, T. R., Hanks, S. K., and Guan, J. L. (1998) J. Cell Biol. 140, 211–221
28. Gorton, A. J. and Tonks, N. K. (1999) J. Cell Biol. 140, 961–972
29. Cary, L. A., Han, D. C., Polte, T. R., Hanks, S. K., and Guan, J. L. (1998) J. Cell Biol. 140, 211–221
30. Garton, A. J., and Tonks, N. K. (1999) J. Biol. Chem. 274, 3811–3818
31. Angers-Loustau, A., Cote, J. F., Charest, A., Dowbenko, D., Spencer, S., Lasky, L. A., and Tremblay, M. L. (1999) J. Cell Biol. 144, 1019–1031
32. Li, J., Avraham, H., Rogers, R. A., Raja, S., and Avraham, S. (1996) Blood 88, 417–428
33. Sasaki, H., Nagura, K., Ishino, M., Tobioka, H., Kotani, K., and Sasaki, T. (1995) J. Biol. Chem. 270, 21206–21219
34. Li, X., Dy, R. C., Cance, W. G., Graves, L. M., and Earp, H. S. (1999) J. Biol. Chem. 274, 8917–8924
35. Schlaepfer, D. D., and Hunter, T. (1998) Trends Cell Biol. 8, 151–157
36. Shen, Y., Schneider, G., Cloutier, J. F., Veillette, A., and Schaller, M. D. (1998) J. Biol. Chem. 273, 6474–6481
37. Schaller, M. D., Borgman, C. A., Cobb, B. S., Vines, R. R., Reynolds, A. B., and Parsons, J. T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5192–5196
38. Smith, D. B., and Johnson, K. S. (1988) Gene (Amst.) 67, 31–40
39. Thomas, J. W., Cooley, M. A., Broome, J. M., Salgia, R., Griffin, J. D., Lombardo, C. R., and Schaller, M. D. (1999) J. Biol. Chem. 274, 36684–36692
40. Laemmli, U. K. (1970) Nature 227, 680–685
41. Kamber, C. B., Reynolds, A. B., Vines, R. R., and Parsons, J. T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3328–3332
42. Cooley, M. A., Broome, J. M., Ohnemach, C., Romer, L. H., and Schaller, M. D. (2000) Mol. Biol. Cell 11, 3247–3263
43. Andreev, J., Simon, J. P., Sabatini, D. D., Kam, J., Polowman, G., Randazzo, P. A., and Schlessinger, J. (1999) Mol. Cell. Biol. 19, 2338–2350
44. Lev, S., Hernandez, J., Martinez, R., Chen, A., Ploewman, G., and Schlesinger, J. (1999) Mol. Cell. Biol. 19, 2278–2288
45. Calab, M. B., Zhang, X., Polte, T. R., and Hanks, S. K. (1996) Biochem. Biophys. Res. Commun. 228, 662–668
46. Calab, M. B., Polte, T. R., and Hanks, S. K. (1995) Mol. Cell. Biol. 15, 954–963
47. Schaller, M. D., and Sasaki, T. (1999) J. Biol. Chem. 274, 25319–25325
48. Charest, A., Wagner, J., Shen, S. H., and Tremblay, M. L. (1995) Biochem. J. 308, 425–432
49. Zheng, C., Xing, Z., Bian, Z. C., Guo, C., Akbay, A., Warner, L., and Guan, J. L. (1998) J. Biol. Chem. 273, 2384–2389
50. Kumar, S., Avraham, S., Bharti, A., Goyal, J., Pandey, P., and Kharbanda, S. (1999) J. Biol. Chem. 274, 30657–30663
51. Tang, H., Zhao, Z. J., Landon, E. J., and Inagami, T. (2000) J. Biol. Chem. 275, 8389–8396
52. Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K. M. (1998) Science 280, 1614–1617
53. Tamura, M., Gu, J., Danen, E. H., Takino, T., Miyamoto, S., and Yamada, K. M. (1999) J. Biol. Chem. 274, 20693–20703
54. Liu, F., Sells, M. A., and Chernoff, J. (1998) Curr. Biol. 8, 173–176
55. Liu, F., Hill, D. E., and Chernoff, J. (1996) J. Biol. Chem. 271, 31290–31295
56. Arregui, C. O., Balasamo, J., and Lilien, J. (1998) J. Cell Biol. 143, 861–873
57. Yu, D. H., Qu, C. K., Henegarau, O., Lu, X., and Feng, G. S. (1998) J. Biol. Chem. 273, 21125–21131
58. Oh, E. S., Gu, H., Saxton, T. M., Timms, J. F., Hausdorff, S., Frevert, E. U., Kahn, B. B., Pawson, T., Neel, B. G., and Thomas, S. M. (1999) Mol. Cell. Biol. 19, 3205–3215
59. Zhang, Z. Y., Thieme-Setter, A. M., MacLean, D., McNamara, D. J., Dobrusin, E. M., Sawyer, T. K., and Dixon, J. E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4446–4450
60. Zhang, Z. Y., MacLean, D., McNamara, D. J., Sawyer, T. K., and Dixon, J. E. (1994) Biochemistry 33, 2285–2290
61. Hippen, K. L., Jakes, S., Richards, J., Jena, B. P., Beck, B. L., Tabatabai, L. B., and Ingebritsen, T. S. (1993) Biochemistry 32, 12405–12412
62. Jia, Z., Barford, D., Plint, A. J., and Tonks, N. K. (1995) Science 268, 1754–1758