Inhibitory Tyrosine Protein Kinase \( p50^{\text{csk}} \) Is Associated with Protein-tyrosine Phosphatase PTP-PEST in Hemopoietic and Non-hemopoietic Cells\(^*\)

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\( p50^{\text{csk}} \) is a cytosolic tyrosine protein kinase expressed in all cell types. Accumulating data show that it inhibits multiple cellular processes, as a consequence of its ability to repress the enzymatic activity of Src family tyrosine protein kinases. We previously demonstrated that, via its Src homology 3 (SH3) domain, Csk is tightly bound to PEP, a protein-tyrosine phosphatase (PTP) exclusively expressed in hemopoietic cells. In this report, we have tested the possibility that Csk also interacts with PTP-PEST, a ubiquitous PTP sharing structural homology with PEP. Our studies revealed that Csk was associated with PTP-PEST in a variety of cell types, including non-hemopoietic cells. This interaction involved the SH3 region of \( p50^{\text{csk}} \) and a proline-rich region (PPPLPERTPESFLADM) outside the catalytic region of PTP-PEST. Even though both PTP-PEST and PEP were associated with Csk, significant differences were noted between these two PTPs. PTP-PEST, but not PEP, was also complexed with Shc, an adaptor molecule implicated in the Ras pathway. Moreover, PTP-PEST and PEP were found to accumulate primarily in distinct intracellular compartments in cell fractionation studies. In combination, these findings indicated that, like PEP, PTP-PEST is probably involved in Csk-mediated functions in mammalian cells. Moreover, they suggested that the roles of Csk-PTP-PEST and Csk-PEP are likely to be different.

\( p50^{\text{csk}} \) is a 50-kDa cytoplasmic tyrosine protein kinase (TPK)\(^1\) expressed ubiquitously (Ref. 1 and reviewed in Ref. 2). It contains, from the amino terminus to the carboxy terminus, a Src homology (SH) 3 region, an SH2 domain, and a catalytic domain. Significant interest in Csk stems from its unique ability to phosphorylate the inhibitory carboxyl-terminal tyrosine of Src family TPKs. This phosphorylation provokes an intracellular association between the carboxyl terminus and the SH2 domain of Src-related enzymes, thereby repressing their enzymatic activity (reviewed in Ref. 3; see Refs. 4 and 5).

As a corollary to the widespread involvement of Src-related TPKs in cell signaling, \( p50^{\text{csk}} \) has the ability to inhibit several cellular responses. We previously showed that it is a potent negative regulator of antigen receptor-mediated T-cell activation, by inactivating the Src family kinases \( p56^{\text{ck}} \) and \( p59^{\text{ckT}} \) (6). In addition, Csk can suppress the Na\(^+\)/H\(^+\) antiporter in kidney epithelial cells (7), endothelin-1-mediated responses in vascular cells (8), and G-protein-mediated activation of mitogen-activated protein kinase in PC-12 rat pheochromocytoma cells (9). Finally, analyses of mice engineered to lack \( p50^{\text{csk}} \) as a result of homologous recombination in embryonic stem cells have illustrated its requirement for normal central nervous system development and embryonic viability (10, 11).

Little is known of the regulation of \( p50^{\text{csk}} \). As it is primarily localized in the cytoplasm, it is postulated to be regulated by recruitment to cellular membranes, where Src-related kinases are residing. In keeping with this idea, FcYRIIA-mediated activation of human erythroleukemia cells was suggested to provoke an increase in the extent of membrane association of Csk (12). Moreover, we reported that constitutive membrane targeting of Csk, via addition of a myristoylation signal from Src or farnesyl/palmitoylation sequences from Ras, enhanced its ability to inhibit antigen receptor-mediated signals in T-cells (6). Although the exact mechanism by which Csk is physiologically recruited to cellular membranes remains to be elucidated, studies in fibroblasts have indicated that its SH3 and SH2 sequences are crucial for colocalization with activated Src molecules at focal adhesions (13). It is presumed that the SH3 and SH2 domains allow the association of Csk with docking proteins located in focal adhesions.

Because constitutive membrane targeting failed to rescue the ability of SH3 or SH2 domain-deletion mutants of Csk to repress T-cell activation (14), these two regions are also likely to provide additional functions that are crucial for the biological activity of Csk. Presumably, these modules also interact with effectors and/or regulators of \( p50^{\text{csk}} \). While these proteins are mostly unidentified, the SH2 domain of Csk was found to bind tyrosine-phosphorylated focal adhesion proteins such as paxillin and \( p125^{\text{ck}2} \) in fibroblasts (15). It can also associate with the docking molecules IRS-1 (16) and \( p62^{\text{ck}2} \) (previously termed GAP-associated p62) (17). Furthermore, we and others (14, 18) reported that it recognizes tyrosine-phosphorylated proteins from activated T-cells.

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\(^{1}\) The abbreviations used are: TPK, tyrosine protein kinase; SH, Src homology; PTP, protein-tyrosine phosphatase; GST, glutathione S-transferase; mAb, monoclonal antibody; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.
the same PTP family. Similarities, PTP-PEST and PEP are postulated to belong to the motif PPPLPERT, which is present both in PEP (PEP P1 and P2, less than 10% sequence identity). One notable exception is the sequence conservation in this domain is otherwise limited to the carboxyl terminus of PTP-PEST and PEP are nearly identical. The conserved carboxyl-terminal tail is represented by a hatched box. The proline-rich sequences P1 and P2 in PEP and PTP-PEST are listed at the bottom.

Recently, we identified the first known ligand for the Csk SH3 domain (19). Using the yeast two-hybrid system, we observed that Csk could interact with PEP, a non-receptor protein-tyrosine phosphatase (PTP) exclusively expressed in hematopoietic cells (Fig. 1) (20, 21). We found that 20–50% of PEP molecules was associated with p56 

\[ \text{PTP-PEST-related protein-tyrosine phosphatases, Schematic representations of the primary structure of PEP, PTP-PEST, and PTP-HSCF. The positions of the amino-terminal phosphatase domain and the carboxyl-terminal non-catalytic domain are indicated. The locations of the proline-rich regions P1 and P2 in PEP and PTP-PEST, as well as of the Shc-binding sequence NPLH in PTP-PEST, are also highlighted. The conserved carboxyl-terminal tail is represented by a hatched box. The proline-rich sequences P1 and P2 in PEP and PTP-PEST are listed at the bottom.} \]

\[ \text{FIG. 1. PEP/PTP-PEST-related protein-tyrosine phosphatases. Schematic representations of the primary structure of PEP, PTP-PEST, and PTP-HSCF. The positions of the amino-terminal phosphatase domain and the carboxyl-terminal non-catalytic domain are indicated. The locations of the proline-rich regions P1 and P2 in PEP and PTP-PEST, as well as of the Shc-binding sequence NPLH in PTP-PEST, are also highlighted. The conserved carboxyl-terminal tail is represented by a hatched box. The proline-rich sequences P1 and P2 in PEP and PTP-PEST are listed at the bottom.} \]

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\[ \text{PTP-PEST is a ubiquitously expressed PTP that bears structural homology with PEP (Fig. 1) (22, 23). It contains an amino-terminal phosphatase domain, sharing ~60% sequence identity with PEP. Additionally, the last 20 amino acids at the carboxyl terminus of PTP-PEST and PEP are nearly identical. Both PTPs also possess a long non-catalytic carboxyl-terminal region, which contains several PEST-like sequences. However, the sequence conservation in this domain is otherwise limited (less than 10% sequence identity). One notable exception is the motif PPPLPERT, which is present both in PEP (PEP P1 and P2) and in PTP-PEST (PEST P2) (Fig. 1). On the basis of these similarities, PTP-PEST and PEP are postulated to belong to the same PTP family. The association of PEP with Csk is likely to be physiologically relevant. However, it is noteworthy that PEP is not expressed in non-hematopoietic cells, where Csk has important roles. Hence, it is plausible that a PEP-related PTP existing in non-hematopoietic cells is also capable of interacting with p56. In this paper, we have examined the possibility that Csk interacts with PTP-PEST. We found that PTP-PEST could be co-immunoprecipitated with p56 in a variety of cell types, including hematopoietic and non-hematopoietic cells. This association involved the SH3 domain of Csk and the P2 sequence in PTP-PEST. Despite their shared ability to bind Csk, only PTP-PEST was also associated with the Shc adaptor molecule. Moreover, PTP-PEST was found to be primarily located in the cytosolic fraction, whereas PEP mainly accumulated in the particulate fraction of a T-cell line.} \]

\[ \text{MATERIALS AND METHODS} \]

\[ \text{Tissues and Cell Lines—Tissues were obtained from 3 to 6 week-old Balb/c mice. B1-141 is an antigen-specific mouse T-cell line (24, 25) and was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. COS-1 cells were propagated in α-minimal essential medium containing 10% fetal bovine serum and antibiotics. Antibodies—Polyclonal rabbit anti-PTP-PEST antibodies were produced using a trpE fusion containing part of the non-catalytic portion of PTP-PEST (amino acids 286–471) as immunogen. These antibodies did not cross-react with PEP. Rabbit antisera reacting with PEP were reported previously (19). They did not recognize PTP-PEST. Antibodies against Csk, Chk, Fyn, Chk, PEP, or PTP-PEST were used for immunoprecipitation. Anti-TrpE monoclonal antibodies (mAbs) were purchased from Oncogene Science (Cambridge, MA). Mouse anti-Myc mAb 9E10 and anti-hemagglutinin (HA) mAb 12CA5 were previously described.} \]

\[ \text{cDNAs—A mouse ptp-pest cDNA was generated by PCR, using RNA from NIH 3T3 cells as template. A myc epitope-tagged version of this cDNA, in which the sequences MASMEQKLISEEDLNGSSS was added to the amino terminus of PTP-PEST, was created by overlap extension PCR. A variant lacking the nucleotides coding for the sequence PPPLERT in PEST P2 (Fig. 1) was also produced by PCR. All cDNAs were fully sequenced to ensure that no unwanted mutations had been introduced during PCR (data not shown). cDNAs coding for the HA-tagged Csk mutants and Myc-tagged PEP were reported previously (19). Immunoprecipitations and Immunoblots—Cells or tissues were lysed in 1 × TNE buffer (50 mM Tris, pH 8.0, 1% Nonidet P-40, 2 mM EDTA, pH 8.0) supplemented with 10 µg per ml each of the protease inhibitors leupeptin, aprotnin, N-tosyl-l-phenylalanine chloromethyl ketone, N-tosyl-l-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride, as well as the phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (1 mM). Proteins were recovered by immunoprecipitation using the indicated antibodies. Immune complexes were collected with either Protein A-Sepharose (Pharmacia Biotech Inc., Baie d’Urfé, Québec) or formalin-fixed Staphylococcus aureus Protein A (Pansorbin; Calbiochem-Novabiochem Int., San Diego), coupled, if indicated, to rabbit anti-mouse IgG. Immunoprecipitates were washed three times in TNE buffer containing 1 mM sodium orthovanadate. Proteins were subsequently eluted in sample buffer, boiled, electrophoresed in 8% SDS-polyacrylamide gel electrophoresis (PAGE) gels, and transferred onto Immobilon membranes (Millipore, Mississauga, Ontario, Canada) for immunoblotting. Immunoblots were performed according to a previously described protocol (32), using either 125I-Protein A (Amersham Canada, Oakville, Ontario, Canada), 125I-goat anti-mouse IgG (ICN Pharmaceuticals Canada Ltd., Montréal, Québec, Canada), or enhanced chemiluminescence reagents (Amersham Canada, Oakville, Ontario, Canada). Immunoreactive products were detected by autoradiography. All quantitations were done with a PhosphorImager (BAS 2000; Fuji), using 125I-Protein A- or 125I-goat anti-mouse IgG-labeled immunoblots.} \]

\[ \text{Bacterial Fusion Proteins and In Vitro Binding Assays—To identify the protein-rich motif of PTP-PEST involved in binding to the SH3 region of Csk, the necessary DNA fragments from ptp-pest were amplified by PCR and cloned in-frame in the PATH vector. The resulting TrpE fusion proteins encompassed amino acids 324–348 (IDSEKDSSPPKPRTRSRCLVEGDA, PEST P1) or 689–698 (DADVSESSPPKPRTRSRCLVEGDA, PEST P2) of PTP-PEST. All plasmids were verified by sequencing (data not shown). TrpE fusion proteins containing the P1 or P2 region of PEP were described elsewhere (19). Plasmids encoding GST fusion proteins were reported previously (19). Production and purification of GST fusion proteins were as described elsewhere (33). In vitro binding assays were conducted as detailed in an earlier publication (19).} \]

\[ \text{Transient Transfections—Transient transfections in Cos-1 cells were performed as described previously (19).} \]

\[ \text{2 D. Davidson, J.-F. Cloutier, A. Gregoireff, and A. Veillette, unpublished data.} \]
Cell Fractionation Studies—For cell fractionation, BL-141 T-cells were incubated for 15 min in hypotonic buffer (10 mM Tris, pH 7.4, 2 mM EDTA pH 8.0) supplemented with the protease and phosphatase inhibitors outlined above. Then, membranes were mechanically broken with a Dounce homogenizer. In all cases, staining with trypan blue confirmed that over 95% of cells had been lysed (data not shown). After adjusting the homogenates to 0.15 m NaCl, nuclei and large membrane sheets (P1) were recovered by two successive centrifugations at 450 × g for 5 min. Supernatants were then separated into soluble (S100) and particulate (P100) fractions by ultracentrifugation at 100,000 × g for 30 min. The various fractions were solubilized in boiling sample buffer, and lysates corresponding to equivalent cell numbers were subjected to immunoblotting. To avoid overloading of the gels, however, lysates from 5.5 times lower cell numbers were used for the S100 fraction. This factor was taken into consideration in the subsequent assessment of relative protein distribution. The validity of the cell fractionation was confirmed by studying the distribution of the z chain of the T-cell antigen receptor complex and lamin B. These two polypeptides are expected to localize primarily to the P100 and P1 fractions, respectively.

RESULTS

p50csk Is Associated with PTP-PEST in Various Cell Types—To examine whether Csk interacts with PTP-PEST, the patterns of expression of these two enzymes were first compared. Equivalent amounts of cell lysates from various mouse tissues were resolved by SDS-PAGE and immunoblotted with antisera directed against either PTP-PEST (Fig. 2, top panel) or Csk (middle panel). In addition, parallel lysates were immunoblotted with anti-PEP antibodies (bottom panel). Importantly, the anti-PTP-PEST (hereafter named anti-PEST) serum did not cross-react with PEP, and vice versa. The experiment showed that the PTP-PEST protein, which migrated at ~120 kDa in these gels, was present in all the tissues tested (top panel), with the exception of kidney (lane 4). Greater quantities of PTP-PEST were detected in the lymphoid organs thymus (lane 1) and spleen (lane 2), as well as in liver (lane 5). In an analogous manner, p50csk was most abundant in thymus (middle panel, lane 1) and spleen (lane 2). Lower amounts were noted in the other tissues (lanes 3–6; data not shown). By contrast, PEP, which exhibited an apparent molecular mass of ~110 kDa, was readily detected only in thymus (bottom panel, lane 1). Small amounts of PEP could also be seen in spleen and kidney in longer exposures (data not shown).

To determine whether p50csk was physically associated with PTP-PEST, lysates from these various tissues were immunoprecipitated with antibodies directed against the carboxy-terminal portion of p50csk. Following several washes, the immunoprecipitates were resolved by SDS-PAGE gels and immunoblotted with anti-PEST antibodies (Fig. 3A). This study revealed that significant quantities of PTP-PEST were present in p50csk immunoprecipitates from thymus (lane 1), spleen (lane 2), and liver (lane 5). Small quantities of PTP-PEST were also associated with Csk in brain (lane 3), although their detection required longer autoradiographic exposures (data not shown). No Csk-PTP-PEST complexes were detected in kidney (lane 4) and heart (lane 6). In general, the amounts of Csk-PTP-PEST detected in these various tissues were proportional to the combined levels of expression of Csk and PTP-PEST (see Fig. 2). PTP-PEST was also co-immunoprecipitated with p50csk in various cell lines, including fibroblasts, T-cells, and B-cells (data not shown). As the anti-PEST antibodies used precipitated over 90% of PTP-PEST from cell lysates, we could estimate the total amounts of PTP-PEST present in these tissues and cell lines (see Fig. 3B for an example). On this basis, we calculated that 5–10% of PTP-PEST molecules were co-immunoprecipitated with Csk (data not shown).

To verify the specificity of this co-immunoprecipitation, thymocyte lysates were immunoprecipitated with antibodies directed against other signaling molecules, and the presence of PTP-PEST in these immunoprecipitates was assessed by anti-PEST immunoblotting (Fig. 3B). Although PTP-PEST was eas-

**Fig. 2. Expression of PTP-PEST, p50csk, and PEP in normal mouse tissues.** The levels of expression of PTP-PEST, Csk, and PEP in various mouse tissues were examined by immunoblotting equivalent amounts of total cell lysates with the appropriate antibodies. Immunoreactive products were detected by enhanced chemiluminescence. The positions of PTP-PEST (PEST), Csk, and PEP are indicated on the left. Exposures: top panel, 15 s; middle panel, 15 s; bottom panel, 3 min.

**Fig. 3. Association of PTP-PEST with signaling molecules in normal mouse tissues.** A, co-immunoprecipitation of PTP-PEST with p50csk in various mouse tissues. The ability of PTP-PEST to co-immunoprecipitate with Csk was ascertained by immunoblotting of anti-Csk immunoprecipitates with anti-PEST antibodies. Immunoreactive products were detected by enhanced chemiluminescence. The identity of the 70–75-kDa polypeptides observed in lanes 3–5 is not known. The positions of PTP-PEST (PEST) and heavy chain of immunoglobulin (Ig) are indicated on the left, whereas those of prestained molecular mass markers are shown on the right. Exposure: 30 s. B, association of PTP-PEST with signaling molecules in thymocytes. Thymocyte lysates were immunoprecipitated with the indicated antibodies, and the presence of PTP-PEST in these immunoprecipitates was determined by immunoblotting with anti-PEST antibodies. Immunoreactive products were detected by enhanced chemiluminescence. Equivalent amounts of cellular proteins (1 mg) were used in all immunoprecipitations. The positions of PTP-PEST (PEST) and heavy chain of immunoglobulin (Ig) are indicated of the left, whereas those of prestained molecular mass markers are shown on the right. Exposure: 15 s.
Csk was present in anti-Fyn immunoprecipitates (immunoprecipitates generated with normal rabbit serum (PTP-PEST—To determine the structural basis for the interaction between Csk and PTP-PEST, attempts were made to reconstitute this association in a heterologous mammalian cell system. Cos-1 cells were transiently transfected with cDNAs encoding various HA-tagged versions of Csk (Csk-HA), in the presence or absence of cDNAs coding for a Myc-tagged version of PTP-PEST (Myc-PEST). Sixty hours after transfection, HA-tagged Csk polypeptides were recovered by immunoprecipitation with anti-HA mAb 12CA5, and their association with Myc-PEST was ascertained by immunoblotting with anti-Myc antibodies. The abundance of Myc-PEST and Csk-HA was verified by immunoblotting of total cell lysates with anti-Myc (middle panel) or anti-HA antibodies (bottom panel).

In cells expressing wild-type Csk-HA (lane 7), we observed that significant amounts of Myc-PEST were recovered in anti-HA immunoprecipitates. In comparison, no Myc-tagged protein was detected when these immunoprecipitates were generated from cells lacking Myc-PEST (lane 2) or Csk-HA (lane 6). Myc-PEST was also associated with the SH2 domain-deleted (lane 9) and kinase-inactive (lane 10) versions of HA-tagged Csk. However, it failed to co-immunoprecipitate with the SH3 domain-deleted variant of Csk-HA (lane 8). These differences were not caused by variations in the levels of expression of the transfected cDNAs, as reflected by the immunoblots of cell lysates with anti-Myc (middle panel) and anti-HA (bottom panel) antibodies.

The Proline-rich Sequence P2, but Not P1, of PTP-PEST Mediates the Association with p50<sup>csk</sup>—Since the SH3 region of Csk was needed for the association with PTP-PEST, special attention was given to proline-rich sequences in PTP-PEST that could mediate this interaction. As mentioned above, PEP interacts with Csk via its proline-rich region P1, located outside the phosphatase domain (Fig. 1) (19). Another proline-rich sequence in PEP, P2, is inefficient at mediating binding to p50<sup>csk</sup>. Although the non-catalytic domain of PTP-PEST is generally divergent from that of PEP (22, 23), its second proline-rich region (P2) also exhibits homology with the two PEP sequences.

To determine whether these PTP-PEST sequences were able to bind the Csk SH3 domain, they were individually expressed as TrpE fusion proteins in bacteria. Their ability to associate with a GST fusion protein encompassing the SH3 region of Csk was evaluated in in vitro binding assays, as detailed under...
**Materials and Methods** (Fig. 6A). As is the case for the P1 region of PEP (top panel, lane 2), the P2 sequence of PTP-PEST (lane 4) was able to associate with the Csk SH3 domain in vitro. In contrast, PEST P1 (lane 5) and TrpE alone (lane 1) did not bind the Csk SH3 domain. In agreement with our previous report (19), PEP P2 (lane 3) was also inefficient at binding the Csk SH3 domain, although longer exposures of this immunoblot revealed that a small amount of binding did occur (data not shown). Immunoblotting of bacterial lysates with anti-PEST antibodies documented that all fusion proteins were expressed comparably (bottom panel).

To gain a better idea of the relative affinities of PEST P2 and PEP P1 for the Csk SH3 domain, serial dilutions of bacterial lysates were used in the binding assays (Fig. 6B). The expression levels of the TrpE fusions in bacteria were verified by immunoblotting of total cellular lysates with anti-TrpE antibodies and 125I-labeled goat anti-mouse IgG. The migration of the TrpE-PEST variant lacking the sequence PPPLPERT in the P2 region (PTP-PESTΔP2) was created by PCR. A myc-tagged version of this cDNA was transiently transfected in Cos-1 cells, and the ability of Myc-PESTΔP2 to associate with Csk was assessed as outlined for Fig. 5. Unlike wild-type Myc-PEST (Fig. 7, top panel, lane 5) and Myc-PEP (lane 7), the Myc-PESTΔP2 mutant (lane 6) was unable to bind Csk. This ineptitude was not caused by a defect in expression of the mutant protein, as it could be easily detected by immunoblotting of total cell lysates with anti-Myc antibodies (middle panel).

**Differences in Intracellular Distribution of PTP-PEST and PEP**—Previous studies have shown that PTP-PEST and PEP were localized to the cytoplasm in transiently transfected Cos-1 cells (19, 23). To address further the intracellular distribution of these two PTPs, cell fractionation studies were performed, using an antigen-specific T-cell line that expresses PTP-PEST and PEP (BI-141) (Fig. 8). After incubation in hypotonic buffer, BI-141 cells were mechanically broken in a Dounce homogenizer, and various cellular fractions were isolated by differential centrifugation. In this manner, three different fractions were generated as follows: the cytosolic fraction (S100), which possesses the cytoplasmic content; the particulate fraction (P100), which encompasses cellular membranes; and the "nuclear" fraction (P1), which contains the nucleus, large sheets of membranes, and cytoskeletal elements.

The abundance of PTP-PEST and PEP in these fractions was determined by immunoblotting of lysates with anti-PEST and anti-PEP antibodies, respectively (Fig. 8A, top panels). To verify the adequacy of the cell fractionation, the distribution of

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**Fig. 6.** Binding of proline-rich sequences of PTP-PEST to the Csk SH3 domain in vitro. A, binding of P2, but not P1, of PTP-PEST to the Csk SH3 domain. The ability of TrpE fusion proteins encompassing proline-rich sequences from PTP-PEST or PEP to bind GST-Csk SH3 domains was assessed using in vitro binding assays (top panel). Binding of the TrpE fusions was revealed by immunoblotting with anti-TrpE antibodies and 125I-labeled goat anti-mouse IgG. The expression levels of the TrpE fusions in bacteria were verified by immunoblotting of total bacterial lysates with the same antibodies (bottom panel). The migrations of TrpE-PEP P1 and TrpE-PEST P2 are indicated on the right, whereas those of a prestained molecular mass marker are shown on the left. Exposures: top panel, 5.5 h and bottom panel, 2 h. B, titrations. As in A, except that various quantities of bacterial lysates were used in the binding assays. Lanes 1, 5, and 6, 50 μg; lanes 2 and 7, 5 μg; lanes 3 and 8, 0.5 μg; and lanes 4 and 9, 0.05 μg. The positions of TrpE-PEP P1 and TrpE-PEST P2 are indicated on the right, whereas that of a prestained molecular mass marker is shown on the right. Exposure: 4 h. C, binding of various SH3 domains to the P2 region of PTP-PEST. As in A, except that GST fusion proteins bearing various SH3 domains were used. Equivalent amounts of GST fusion proteins were used in these assays, as determined by staining SDS-PAGE gels of parallel samples with Coomassie Blue (data not shown). The position of TrpE-PEST P2 is indicated on the left, whereas that of a prestained molecular mass marker is shown on the right. Exposure: 5 h.
Csk, lamin B, and the \( \zeta \) chain of the T-cell receptor complex was also evaluated (lower panels). After correcting for the fact that lysates from 5.5 times lower cell numbers were used for the S100 fraction, it was estimated that \( \approx 80\% \) of PTP-PEST was present in the S100 fraction (a quantitation of these data is represented in Fig. 8). In comparison, \( \approx 60\% \) of PEP was detected in the P100 fraction. It should be noted that the \( 120\text{-kDa} \) product recognized by the anti-PEP serum in the S100 fraction was not PEP. This polypeptide was nonspecifically recognized by the anti-PEP antibody.2 As expected, \( \approx 80\% \) of the Csk polypeptides accumulated in S100. Moreover, \( 90\% \) of the nuclear protein lamin B was in the P1 fraction, whereas \( \zeta \) was equally distributed in P100 and P1 and was largely excluded from S100. These last results confirmed that the cell fractionation procedure was adequate.

**DISCUSSION**

In an earlier report (19), we showed that the inhibitory TPK

p50<sup>csk</sup> is associated with the PEP phosphatase in hemopoietic cells, via an SH3 domain-dependent mechanism. This observation suggested that PEP may be involved in the regulation of Csk-mediated functions in cells of hemopoietic lineages. It is noteworthy, however, that PEP is not expressed in non-hemopoietic cells, in which Csk has important functions. Nevertheless, PTP-PEST, a PTP having structural homology to PEP, is known to accumulate in most cells, including non-hematological cell types (22, 23; this report). On this basis, we examined whether PTP-PEST is associated with p50<sup>csk</sup>. Through co-immunoprecipitation experiments, we found that 5–10% of PTP-PEST was physically associated with Csk in a variety of normal mouse tissues, including thymus, spleen, and liver. Conversely, approximately 5% of p50<sup>csk</sup> molecules was complexed to PTP-PEST. The Csk-PTP-PEST association was also documented in cell lines, including fibroblasts, myeloid cells, T-cells, and B-cells (this report; data not shown). Hence, these results demonstrated that Csk is also bound to PTP-PEST, both in hemopoietic and in non-hemopoietic cells.

Reconstitution experiments in Cos-1 cells revealed that the interaction between Csk and PTP-PEST involved the SH3 domain of Csk and a proline-rich sequence located outside the catalytic domain of PTP-PEST. The ability of these two domains to interact directly was demonstrated by \textit{in vitro} binding assays, using bacterially produced fusion proteins. As pointed out earlier, the PTP-PEST motif responsible for Csk binding (PPPLPERTPESPFLADM, PEST P2) shares significant homology with the Csk-binding sequence in PEP (PPPLPERTPESPFLIVEE, PEP P1) (19). However, the affinity of PEST P2

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**Fig. 7.** The P2 region of PTP-PEST is required for binding to p50<sup>csk</sup> in Cos-1 cells. As in Fig. 5, except that various Myc-PEST polypeptides were tested for their ability to bind wild-type Csk-HA (top panel). The expression levels of Myc-tagged PEP to associate with Csk-HA was also tested in parallel. The expression levels of Myc-tagged PTP-PEST and PEP (middle panel) and Csk-HA (bottom panel) were verified by immunoblotting of total cell lysates with the appropriate antibodies. Immunoreactive products were detected with \(^{125}\text{I}\)-labeled goat anti-mouse IgG. The positions of Myc-PEST, Myc-PEP, Csk-HA, and heavy chain of immunoglobulin (Ig) are shown on the left, and those of pre-stained molecular mass markers are indicated on the right. Exposures: top panel, 6 h; middle panel, 8.5 h; bottom panel, 8.5 h.

**Fig. 8.** \textit{Cellular fractionation studies.} A, the relative cellular distribution of PTP-PEST and PEP in the antigen-specific T-cell line BI-141 was ascertained by cell fractionation studies, as detailed under “Materials and Methods.” To validate the cell fractionation procedure, the distribution of Csk, lamin B, and the \( \zeta \) chain of the T-cell receptor complex was also studied in parallel. Immunoreactive products were detected with \(^{125}\text{I}\)-protein A. The positions of PTP-PEST (PEST), PEP, Csk, lamin B, and \( \zeta \) are indicated on the left. Exposures: 16 h. B, quantitation of the data presented in A. Data were quantitated using a PhosphorImager.
for the Csk SH3 region appeared to be ~10 times lower than that of PEP P1, at least in vitro. Combined with our earlier finding that a related region in PEP, PEP P2 (PPPLPLESTES-FFLAD), was inefficient at associating with the Csk SH3 region in vitro and in vivo (19), this finding suggested that the sequences surrounding the prolinc-rich core PPPLPERT are crucial for high affinity binding to the Csk SH3 domain. In support of this notion, we recently showed that residues localized immediately carboxyl to PPPLPERT are necessary for high affinity binding to the Csk SH3 region.3

Whereas PTP-PEST and PEP share the capacity to associate with Csk, these two enzymes exhibit several noticeable differences. First, PTP-PEST is expressed ubiquitously, whereas PEP is restricted to hemopoietic cells (19–23; this report). Second, PTP-PEST, but not PEP, is also able to associate with Shc via an NPLH sequence in PTP-PEST and the phosphotyrosine-binding domain of Shc (Fig. 1) (34, 35). Third, cell fractionation studies indicated that PTP-PEST mostly accumulated in the cytosolic (S100) fraction, whereas PEP was principally located in the particulate (P100) fraction. Taken together, these data suggest that the functions of the Csk-PTP-PEST and Csk-PEP complexes may not be interchangeable. PTP-PEST may provide a more generalized function common to all cell types, whereas PEP may mediate a specialized function in hemopoietic cells. Possibly, the two Csk-associated PTPs act on different sets of cellular substrates, or have different catalytic efficiencies toward similar sets of substrates. It is also plausible that PTP-PEST and PEP dephosphorylate the same substrates but in response to different stimuli. The singular ability of PTP-PEST to bind Shc raises the possibility that Shc recruits PTP-PEST (and presumably the associated Csk) to affect various signaling pathways. This could be achieved via the ability of the SH2 domain and/or tyrosine phosphorylation sites of Shc to associate with other cellular proteins such as the z chain of the T-cell receptor complex (40) or Grb2 (41). Finally, it is conceivable that PTP-PEST and PEP act on their targets in distinct cellular locales, as suggested by our cell fractionation studies. Obviously, future studies are needed to test these diverse possibilities.

Although the determination of whether PTP-HSCF (36–39; Fig. 1) truly belongs to the PEP/PTP-PEST family must await comparison of the genomic structures of these three PTPs, its shared ability of PTP-PEST and PEP to bind Csk, differences in the recognition function of PTP-PEST and Csk-PEP may not be interchangeable. PTP-PEST may provide a more generalized function common to all cell types, whereas PEP may mediate a specialized function in hemopoietic cells. It is likely that PTP-PEST and Csk-PEP act on their targets in distinct cellular locales, as suggested by our cell fractionation studies. Obviously, future studies are needed to test these diverse possibilities.

As is the case for the association between Csk and PEP, the physiological role of the Csk-PTP-PEST interaction remains to be elucidated. It is conceivable that the phosphorylation state of Csk is regulated by PTP-PEST or vice versa. Alternatively, the binding to PTP-PEST may restrict the extent of intracellular tyrosine protein phosphorylation induced by Csk. To date, however, we have been unable to obtain experimental evidence supporting any of these possibilities.4 A more likely scenario seems to be that PTP-PEST cooperates with p50SH to inhibit intracellular tyrosine protein phosphorylation. In keeping with this idea, Superti-Furga et al. (42) showed that a fragment of PTP-PEST containing the catalytic domain was able to antagonize the lethal impact of activated Src in the fusion yeast Schizosaccharomyces pombe. Although not formally demonstrated, the authors proposed that this effect was due at least in part to dephosphorylation of the positive regulatory site (tyrosine 416) of Src. Perhaps, PTP-PEST also acted by dephosphorylating the substrates of activated p60c-src. Clearly, future experiments should be aimed at further testing this model.

Even though possible targets of PTP-PEST have been identified using yeast cells, little is known of its physiological substrates in mammalian cells. However, using a substrate trapping approach, Garton et al. (43) recently found that the focal adhesion protein p130Cas was a target for PTP-PEST in a variety of non-hematological cell lines. As Csk carries multiple sites of tyrosine phosphorylation, in addition to an SH3 domain and several prolinc-rich regions (44), it triggers several protein-protein interactions at focal adhesions. Dephosphorylation of Cas by PTP-PEST may prevent its ability to recruit signaling molecules and thus ultimately affect theorganization of focal adhesions. It is interesting that, through its SH2 domain, Csk also binds focal adhesion proteins such as paxillin and FAK (15). This association is presumed to facilitate the ability of Csk to regulate Src family kinases at focal adhesions (13). Taking into consideration the individual properties of Csk and PTP-PEST, it is provocative to speculate that their physical association may represent an exquisite mechanism to inhibit tyrosine protein phosphorylation at focal adhesions, at least in non-hemopoietic cells.

In summary, our results showed that p50SH is associated with the non-receptor phosphatase PTP-PEST in hemopoietic and non-hemopoietic cells. Structure-function analyses and in vitro binding studies demonstrated that this interaction is mediated by the SH3 domain of p50SH and by a prolinc-rich region (P2) outside the catalytic domain of PTP-PEST. Despite the shared ability of PTP-PEST and PEP to bind Csk, differences were observed between these two PTPs. Hence, it is likely that Csk-PTP-PEST and Csk-PEP have distinct functions in mammalian cells.

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Interaction between Csk and PTP-PEST

23462

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