

Cooperative Inhibition of T-Cell Antigen Receptor Signaling by a Complex between a Kinase and a Phosphatase

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Summary

Antigen receptor-triggered T-cell activation is mediated by the sequential action of the Src and Syk/Zap-70 families of protein tyrosine kinases (PTKs). Previously, we reported that another PTK termed p50^{csk} was a potent negative regulator of T-cell receptor (TCR) signaling because of its ability to inactivate Src-related kinases. This inhibitory effect required the catalytic activity of Csk, as well as its Src homology (SH)3 and SH2 domains. Subsequent studies uncovered that, via its SH3 domain, p50^{csk} was associated with PEP, a proline-enriched protein tyrosine phosphatase (PTP) of unknown function expressed in hemopoietic cells. Herein, we have attempted to identify the role of the Csk-PEP complex in T lymphocytes. The results of our experiments showed that, like Csk, PEP was a strong repressor of TCR signaling. This property was dependent on the phosphatase activity of PEP, as well as on the sequence mediating its binding to p50^{csk}. Through reconstitution experiments in Cos-1 cells, evidence was obtained that Csk and PEP act synergistically to inhibit protein tyrosine phosphorylation by Src-related kinases, and that this effect requires their association. Finally, experiments with a substrate-trapping mutant of PEP suggested that PEP functions by dephosphorylating and inactivating the PTKs responsible for T-cell activation. In addition to giving novel insights into the mechanisms involved in the negative regulation of T-cell activation, these findings indicate that the association of an inhibitory PTK with a PTP constitutes a more efficient means of inhibiting signal transduction by Src family kinases in vivo.

Key words: T-cell activation • Csk • PEP • protein tyrosine phosphatase • protein tyrosine kinase

Activation of T cells by antigen bound to MHC molecules results in a rapid rise in protein tyrosine phosphorylation. This response predates all subsequent events of T-cell activation, including transcriptional activation, lymphokine release, cytolysis, and proliferation. Whereas the TCR, as well as the associated CD3 and ζ subunits, do not possess intrinsic protein tyrosine kinase (PTK)¹ activity, the CD3 and ζ chains contain within their cytoplasmic domain a signaling sequence termed Immunoreceptor Tyrosine-based Activation Motif (ITAM). This sequence undergoes rapid tyrosine phosphorylation in response to TCR stimulation, thereby triggering a signaling cascade leading to T-cell activation.

Based on analyses of genetically manipulated cells and reconstitution experiments in nonlymphoid cells, evidence

has accumulated that TCR signaling is mediated by the sequential involvement of the Src and Syk/Zap-70 families of PTKs. The Src family kinases Lck and Fyn^T are responsible for the initiation of T-cell activation, as a result of their capacity to phosphorylate the ITAMs of the TCR complex. Subsequently, the diphosphorylated ITAMs of CD3/ ζ bind the tandem Src homology (SH)2 domains of Zap-70 and, in some T-cell subsets, Syk. This association results in tyrosine phosphorylation and activation of Zap-70 and Syk. The Syk/Zap-70 family is involved in the amplification of TCR-induced signals, by causing tyrosine phosphorylation of proteins such as phospholipase C- γ 1, Vav, Slp-76, and LAT.

Src-related kinases possess, from the NH₂ terminus to the COOH terminus: (a) an NH₂-terminal myristylation signal needed for membrane targeting; (b) a unique domain, presumed to mediate interactions with specific regulators; (c) SH3 and SH2 domains allowing associations with proline-rich sequences and tyrosine phosphorylated pro-

¹Abbreviations used in this paper: ITAM, Immunoreceptor Tyrosine-based Activation Motif; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; SH, Src homology.

teins, respectively; (d) a linker domain involved in an intramolecular regulatory mechanism (1–3); (e) a kinase region bearing a typical phosphotransfer motif, as well as an autophosphorylation site involved in positive regulation; and (f) a negative regulatory domain containing the major site of *in vivo* tyrosine phosphorylation. Phosphorylation of the COOH-terminal tyrosine triggers an intramolecular association between the COOH terminus and the SH2 domain, thereby inactivating Src family kinases. Such a phosphorylation is not the result of autophosphorylation, but rather is caused by another PTK termed Csk.

Csk is a 50-kD cytoplasmic PTK expressed in all cell types, albeit in greater amounts in hemopoietic cells (4, 5). It contains NH₂-terminal SH3 and SH2 regions, in addition to a COOH-terminal catalytic domain. Previously, we reported that Csk was a potent negative regulator of TCR signaling (6). Furthermore, Csk was shown recently to control the development of mature $\alpha\beta$ T cells from precursor thymocytes (7). These effects correlated with the ability of Csk to phosphorylate the inhibitory COOH-terminal tyrosine of Lck and FynT (6, 7). In addition, in at least one of the two systems (6), it was dependent on the SH3 and SH2 domains of Csk, implying that the association with other cellular proteins was also critical for the activity of Csk *in vivo* (8). Subsequent studies uncovered that, by way of its SH3 domain, p50^{csk} is associated with PEP (9), an intracellular protein tyrosine phosphatase (PTP) contained in hemopoietic cells (10). Although the biological significance of this association was not elucidated, its high stoichiometry suggested that it may have an important role. As recently reported for other kinase-phosphatase complexes (CaMKIV-PP2A and Erk2-MKP3) (11, 12), it is possible that PEP represses the activity of Csk, either by dephosphorylating the inhibitory tyrosine of Src-like enzymes or by regulating Csk itself. Alternatively, it is conceivable that PEP cooperates with Csk to inhibit intracellular signaling events.

Herein, we have examined the potential role of the Csk-PEP association in signal transduction. We found that, like Csk, PEP is a strong repressor of antigen receptor signaling in T cells. Evidence was obtained that PEP and Csk cooperate to inhibit signaling by Src family kinases, and that this cooperation requires their association.

Materials and Methods

Cells. The previously described antigen-specific mouse T-cell hybridoma BI-141 was maintained in RPMI medium supplemented with 10% fetal bovine serum and antibiotics (13). BI-141 derivatives expressing wild-type Csk, Δ SH3 Csk, or the neomycin phosphotransferase alone (Neo) have been reported previously (5). All BI-141 clones were propagated in the presence of G418 (0.6 mg/ml). Cos-1 cells were grown in α -MEM containing 10% fetal bovine serum and antibiotics.

cDNAs and Antibodies. The cDNAs for wild-type PEP, Δ P1 PEP, wild-type Csk, Δ SH3 Csk, wild-type FynT, kinase-inactive (lysine 295 to arginine 295) Zap-70, and Tac- ζ were reported previously (6, 8, 9, 14, 15). Those coding for the aspartate 195 to

alanine 195 (D195A), cysteine 227 to serine 227 (C227S), and arginine 233 to methionine 233 (R233M) mutants of PEP were created by overlap extension PCR. All mutated cDNAs were sequenced to verify that they did not contain unwanted mutations (data not shown). Antibodies directed against PEP, Csk, PTP-PEST, phosphotyrosine, Zap-70, Tac (mAb 7G7), ζ (mAb H146), and Fyn were described elsewhere (6, 8, 9, 15–17).

Transfections. BI-141 T cells were stably transfected by electroporation (260 V, 960 μ F) and selected in the presence of G418 (0.75 mg/ml). Monoclonal cell lines were derived by limiting dilution. Cells were tested for PEP expression by immunoblotting with anti-PEP antibodies. All clones selected for our studies had unchanged levels of TCR, CD3, CD45, and Thy-1, and remained CD4 negative (data not shown). Cos-1 cells were transiently transfected with Lipofectamine-plus (Bethesda Research Laboratories) according to the protocol provided by the manufacturer, using a fixed total amount of DNA (8 μ g). Cells were harvested 24–48 h after transfection.

Immunoprecipitations and Immunoblots. Cells were washed with phosphate-buffered saline and lysed in TNE buffer (50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 2 mM EDTA) supplemented with 10 μ g/ml each of the protease inhibitors leupeptin, aprotinin, n-tosyl-L-phenylalanine chloromethyl ketone (TPCK), n-p-tosyl-L-lysine chloromethyl ketone (TLCK), and PMSF, as well as the phosphatase inhibitors sodium fluoride (50 mM) and sodium orthovanadate (1 mM). Immunoprecipitations were performed with the appropriate antibodies using equivalent amounts of cellular lysate. Immune complexes were recovered using *Staphylococcus aureus* protein A (Pansorbin; Calbiochem) or protein A-Sepharose (Pharmacia Biotech). After washing three times in TNE buffer containing sodium orthovanadate (1 mM), proteins were eluted in sample buffer, boiled, and subjected to SDS-PAGE. Immunoblots were performed according to a protocol detailed previously (15), using ¹²⁵I-protein A (Amersham Corp.) unless specified.

Lymphokine Secretion Assays. Cells were stimulated in triplicate with the indicated concentrations of anti-TCR V β 8 mAb F23.1 coated on 96-well plastic plates. After a 24-h incubation, supernatants were harvested and frozen at -70° C to destroy any carry-over cells. The presence of secreted lymphokines in the supernatant was determined by measuring incorporation of tritiated thymidine in the IL-2-dependent indicator cell line HT-2.

TCR-mediated Protein Tyrosine Phosphorylation. BI-141 derivatives were activated for the indicated periods of time at 37°C using biotinylated mAb F23.1 (30 μ g/ml) and avidin (38 μ g/ml). They were then lysed in TNE buffer supplemented with protease and phosphatase inhibitors, and equal amounts of total cellular lysate were subjected to immunoprecipitation using the protocol described above.

PTP Assay. PEP variants were expressed by transient transfection in Cos-1 cells as explained earlier. After washing with PBS, they were lysed according to the usual protocol, except that the phosphatase inhibitors were omitted. PEP polypeptides were subsequently recovered by immunoprecipitation from 25 μ g of cellular lysate. After collecting the immune complexes with *S. aureus* protein A, they were washed three times in lysis buffer, followed by two washes in phosphatase assay buffer (25 mM Tris-HCl, pH 7.2, 1 mg/ml BSA, 1 mM dithiothreitol). They were resuspended in phosphatase assay buffer and incubated for the indicated periods of time at 30°C with tyrosine phosphorylated myelin basic protein (5 μ M). Reactions were stopped by the addition of 20% (wt/vol) trichloroacetic acid, followed by incubation on ice. After centrifugation, the release of ³²PO₄ in the

supernatant was measured by scintillation counting. Immunoprecipitates obtained from cells transfected with vector alone were used to measure spontaneous hydrolysis. In our calculations, this amount was subtracted from those of PEP immunoprecipitates. Preparation of tyrosine phosphorylated myelin basic protein was performed using the Protein Tyrosine Phosphatase Assay System (New England Biolabs, Inc.), according to the protocol provided by the manufacturer. All experiments were conducted under linear assay conditions (data not shown).

Substrate Trapping. Transfected Cos-1 cells were prepared as above, except that they were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 200 mM sodium chloride, 5 mM iodoacetamide, and 1 mM benzamidine, supplemented with leupeptin, aprotinin, TPCK, TLCK, PMSF, and sodium fluoride. Subsequently, immunoprecipitations and immunoblots were performed using the standard protocols.

Metabolic Labeling and Peptide Mapping Studies. For $^{32}\text{P}_i$ labeling, cells were incubated for 2 h in phosphate-free DMEM supplemented with 1.0 mCi of $^{32}\text{P}_i$ (carrier free; NEN Research Products) per ml, and 2% dialyzed FCS. Cells were then washed with phosphate-free medium and lysed in TNE buffer containing protease and phosphatase inhibitors, as described above. FynT polypeptides were recovered by immunoprecipitation and resolved in 8% SDS-PAGE. Cyanogen bromide cleavage was performed as described elsewhere (18). Phospholabeled FynT peptides were resolved in 18% SDS-polyacrylamide gels and detected by autoradiography.

Results

Impact of PEP on Antigen Receptor-induced Lymphokine Secretion. To evaluate the role of PEP in T lymphocytes, we opted to use the antigen-specific mouse T-cell line BI-141,

as it has proven very useful in ascertaining the role and regulation of cytoplasmic PTKs in T lymphocytes (6, 8, 15, 19). Cells were transfected by electroporation with a cDNA coding for mouse PEP, and stable monoclonal cell lines were selected after growth in G418-containing medium. Derivatives overexpressing PEP were identified by immunoblotting of anti-PEP immunoprecipitates with anti-PEP antibodies. Representative clones are shown in Fig. 1 A. In comparison with control neomycin-resistant BI-141 cells (Neo; lanes 1–3), these cells (lanes 4–6) contained three to four times greater amounts of a 110-kD polypeptide consistent with PEP.

Next, it was determined whether PEP overexpression resulted in an increase in the abundance of Csk-PEP complexes (Fig. 1, B and C). When anti-Csk immunoprecipitates were probed by immunoblotting with anti-PEP antibodies (Fig. 1 B), we observed that PEP overexpression (lanes 5–8) induced a three- to fourfold increase in the quantity of PEP that was coimmunoprecipitated with Csk. As was the case for control Neo cells (lanes 1–4), ~50–80% of PEP molecules contained in these cells were associated with Csk (lanes 5–8). In a similar way, enforced expression of PEP induced a proportional augmentation in the amount of Csk that could be coimmunoprecipitated with PEP (Fig. 1 C; compare lanes 2 and 6). Whereas ~5% of Csk was associated with PEP in control Neo cells (lane 2), this fraction was elevated to ~15% in PEP-overexpressing cells (lane 6). PEP overexpression did not affect the ability of Csk to associate with PTP-PEST, a PEP-related PTP expressed ubiquitously (compare lanes 3 and 7) (16, 20). Importantly, all cell lines used in our experiments expressed

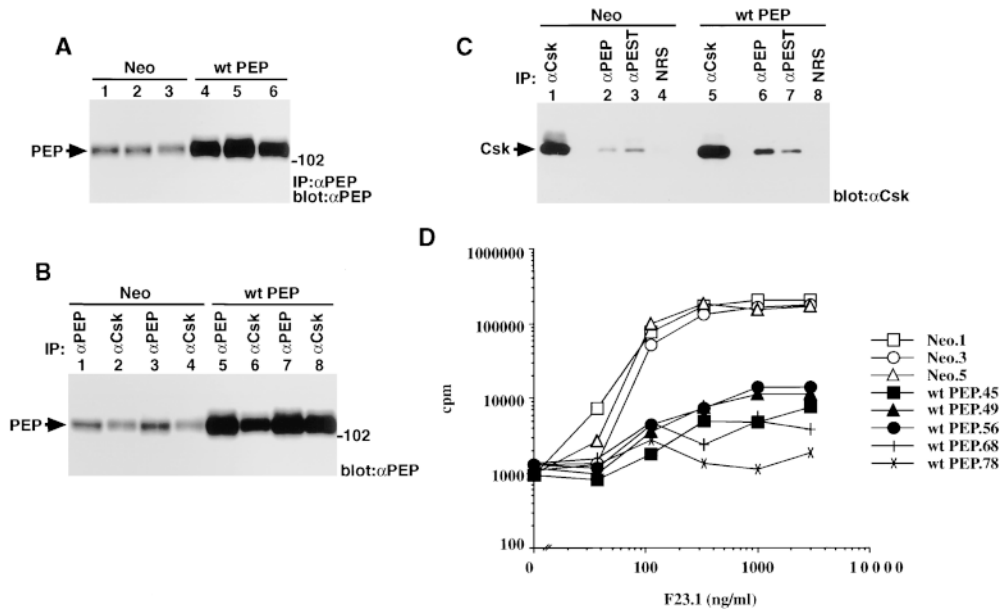


Figure 1. Effect of PEP on TCR-mediated lymphokine secretion. (A) Expression of PEP in BI-141 T cells. The abundance of PEP in representative cell lines was determined by anti-PEP immunoblotting of anti-PEP immunoprecipitates. Lane 1, Neo.1; lane 2, Neo.3; lane 3, Neo.5; lane 4, wt PEP.68; lane 5, wt PEP.78; and lane 6, wt PEP.82. The position of a molecular mass marker is shown on the right, whereas that of PEP is indicated on the left. Exposure: 10 h. (B) Association of PEP with Csk. The association of PEP with Csk was evaluated by immunoblotting of anti-Csk immunoprecipitates with an anti-PEP serum. Lanes 1 and 2, Neo.1; lanes 3 and 4, Neo.3; lanes 5 and 6, wt PEP.68; and lanes 7 and 8, wt PEP.78. The migration of a molecular weight marker is shown on the right;

that of PEP is indicated on the left. Exposure: 18 h. (C) Association of Csk with PEP. The association of Csk with PEP was determined by immunoblotting of anti-PEP immunoprecipitates with anti-Csk antibodies. Lanes 1–4, Neo.5; and lanes 5–8, wt PEP.78. The migration of Csk is indicated on the left. Exposure: 45 s (enhanced chemiluminescence). (D) TCR-mediated lymphokine secretion. Cells were stimulated for 24 h with the indicated concentrations of anti-TCR MAb F23.1 (abscissa) coated on plastic. Lymphokine production was assayed using the IL-2-dependent indicator cell line HT-2. Thymidine incorporation is shown on the ordinate in cpm. All assays were done in triplicate.

unaltered levels of TCR, CD3, and CD45 (data not shown).

The effect of PEP on antigen receptor-mediated lymphokine secretion, a late marker of T-cell activation (21, 22), was first evaluated (Fig. 1 D). Cells were stimulated with increasing concentrations of anti-TCR mAb F23.1 immobilized on plastic, and the release of lymphokines in the supernatant was monitored using a standard bioassay. This analysis revealed that, compared to Neo cells, cells overexpressing PEP had dramatically reduced lymphokine production in response to TCR stimulation. This diminution was observed in all clones tested, and at all concentrations of anti-TCR mAbs.

Structural Requirements for the Inhibitory Effect of PEP on T-Cell Activation. To begin elucidating the mechanism by which PEP affected TCR signaling, the relevance of its phosphatase activity was determined. To this end, catalytically defective PEP mutants in which either cysteine 227 was replaced by serine (C227S), or arginine 233 was substituted by methionine (R233M), were created. As described for other phosphatases (23–25), mutation of these highly conserved residues located in the catalytic domain strongly diminished the ability of PEP to dephosphorylate the substrate myelin basic protein in an immune complex phosphatase assay (Fig. 2 B; data not shown). When introduced in BI-141 cells, the two PEP mutants (Fig. 2 A; lanes 7–11) were expressed in quantities comparable to those of wild-type PEP (lanes 4–6). Furthermore, they were able to asso-

ciate with Csk with a similar stoichiometry (Fig. 2 C; lanes 9–12). However, they were incapable of inhibiting TCR-triggered lymphokine production (Fig. 2 D).

Then we examined the importance of the association of p50^{Csk} with PEP by testing the function in BI-141 cells of a PEP mutant carrying a deletion in the proline-rich region mediating binding to Csk (Δ P1 PEP; Fig. 3 A) (9, 26). While this mutation did not affect the ability of PEP to dephosphorylate the exogenous substrate myelin basic protein in vitro (Fig. 2 B), it significantly reduced (approximately fivefold) its capacity to be coimmunoprecipitated with Csk (Fig. 2 C; lanes 13–16). By opposition to our earlier finding in Cos-1 cells (9, 26), there appeared to be a partial association of Δ P1 PEP with Csk in BI-141 cells. Although the exact basis for this difference is undetermined, it is possible that other structural determinants in PEP (such as the proline-rich domain P2) mediated binding to Csk in the T-cell line. In support of this notion, we reported elsewhere that PEP P2 could also associate with the Csk SH3 domain in vitro, albeit with a greatly reduced apparent affinity in comparison with P1 (26). Alternatively, it is conceivable that the presence of Δ P1 PEP in BI-141 cells augmented by an unknown mechanism the ability of endogenous PEP molecules to associate with Csk. In any case, we observed that Δ P1 PEP failed to inhibit antigen receptor-mediated lymphokine production in BI-141 cells (Fig. 3 B), indicating that the small (approximately twofold) increase in the abundance of Csk-associated PEP in these cells was insuffi-

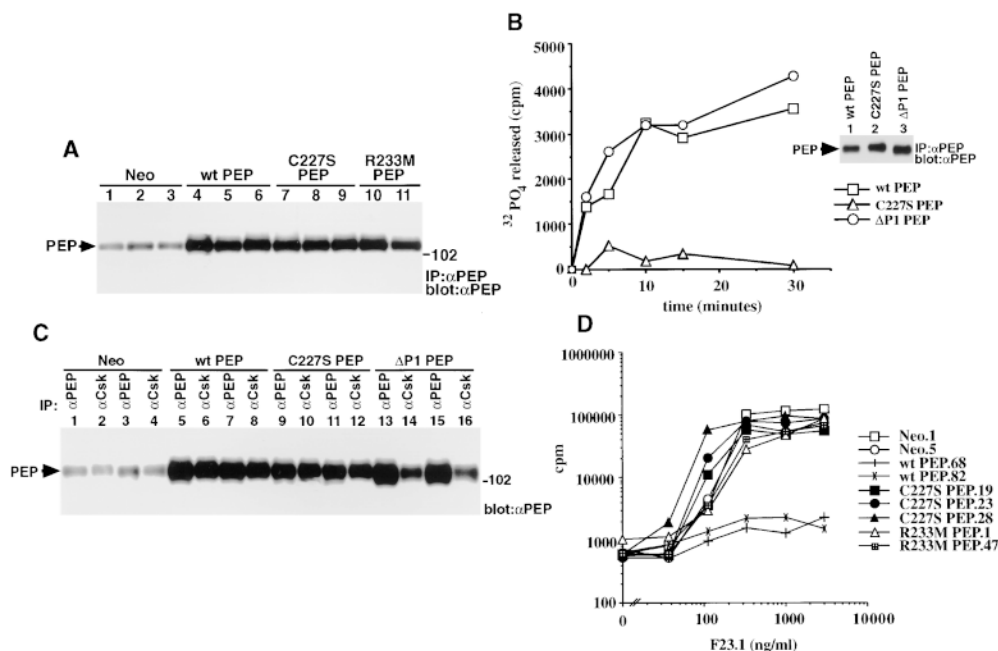


Figure 2. Role of phosphatase activity of PEP. (A) Expression of catalytically defective PEP mutants. The expression levels of PEP in representative cell lines containing C227S PEP or R233M PEP were determined as for Fig. 1 A. Lane 1, Neo.1; lane 2, Neo.3; lane 3, Neo.5; lane 4, wt PEP.68; lane 5, wt PEP.78; lane 6, wt PEP.82; lane 7, C227S PEP.19; lane 8, C227S PEP.23; lane 9, C227S PEP.28; lane 10, R233M PEP.1; and lane 11, R233M PEP.47. The position of a molecular mass marker is shown on the right, whereas that of PEP is indicated on the left. Exposure: 14 h. (B) Tyrosine phosphatase activity of PEP mutants. The catalytic activity of the PEP mutants was measured by immune complex phosphatase assay using radiolabeled myelin basic protein as a substrate. Immune complexes were incubated with myelin basic protein for the indicated periods of time (abscissa). Release of inorganic phosphate was measured by scintillation counting and is shown on the ordinate (cpm). The abundance of the various PEP mutants was also monitored by anti-PEP immunoblotting of parallel anti-PEP immunoprecipitates (inset). The position of PEP is indicated on the left. Exposure: 12 h. (C) Association of PEP mutants with Csk. The ability of the PEP mutants to coimmunoprecipitate with Csk was assayed as outlined for Fig. 1 B. Lanes 1 and 2, Neo.1; lanes 3 and 4, Neo.3; lanes 5 and 6, wt PEP.68; lanes 7 and 8, wt PEP.82; lanes 9 and 10, C227S PEP.19; lanes 11 and 12, C227S PEP.23; lanes 13 and 14, Δ P1 PEP.3; and lanes 15 and 16, Δ P1 PEP.29. The migration of a molecular weight marker is indicated on the right, while that of PEP is shown on the left. Exposure: 16 h. (D) Lymphokine production assay. Assay was carried out as described for Fig. 1 D.

of time (abscissa). Release of inorganic phosphate was measured by scintillation counting and is shown on the ordinate (cpm). The abundance of the various PEP mutants was also monitored by anti-PEP immunoblotting of parallel anti-PEP immunoprecipitates (inset). The position of PEP is indicated on the left. Exposure: 12 h. (C) Association of PEP mutants with Csk. The ability of the PEP mutants to coimmunoprecipitate with Csk was assayed as outlined for Fig. 1 B. Lanes 1 and 2, Neo.1; lanes 3 and 4, Neo.3; lanes 5 and 6, wt PEP.68; lanes 7 and 8, wt PEP.82; lanes 9 and 10, C227S PEP.19; lanes 11 and 12, C227S PEP.23; lanes 13 and 14, Δ P1 PEP.3; and lanes 15 and 16, Δ P1 PEP.29. The migration of a molecular weight marker is indicated on the right, while that of PEP is shown on the left. Exposure: 16 h. (D) Lymphokine production assay. Assay was carried out as described for Fig. 1 D.

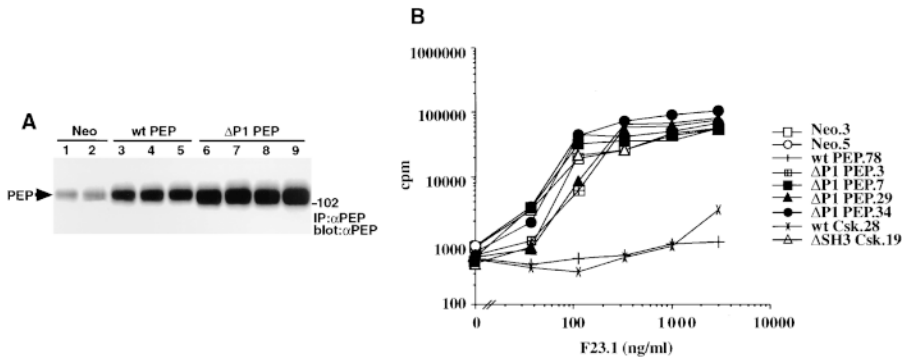


Figure 3. Importance of Csk-binding domain of PEP. (A) Expression of ΔP1 PEP. The abundance of PEP in representative cell lines expressing ΔP1 PEP was evaluated as described in the legend of Fig. 1 A. Lane 1, Neo.1; lane 2, Neo.3; lane 3, wt PEP.68; lane 4, wt PEP.78; lane 5, wt PEP.82; lane 6, ΔP1 PEP.3; lane 7, ΔP1 PEP.7; lane 8, ΔP1 PEP.29; and lane 9, ΔP1 PEP.34. The position of a molecular mass marker is shown on the right, whereas that of PEP is indicated on the left. Exposure: 10 h. (B) Lymphokine production assay. Assay was carried out as described for Fig. 1 D.

cient to inhibit TCR signaling. The defective function of ΔP1 PEP was reminiscent of the inability of an SH3 domain-deleted version of Csk (ΔSH3 Csk) to suppress TCR-mediated lymphokine secretion in BI-141 cells (8; Fig. 3 B). From these data, we deduced that the inhibitory effect of PEP on TCR-mediated lymphokine secretion not only requires its phosphatase activity, but also is correlated with the capacity to associate with Csk.

Impact of PEP on Antigen Receptor-mediated Protein Tyrosine Phosphorylation. The influence of PEP overexpression on TCR-mediated protein tyrosine phosphorylation was investigated next (Fig. 4). BI-141 cells were stimulated for various periods of time at 37°C with biotinylated anti-

TCR mAb F23.1 and avidin. After stimulation, they were lysed in detergent-containing buffer, and tyrosine phosphorylated proteins were immunoprecipitated and detected by antiphosphotyrosine immunoblotting (Fig. 4 A). In contrast to Neo cells (lanes 1–6), cells overexpressing PEP (lanes 7–12) demonstrated a clear reduction in TCR-induced protein tyrosine phosphorylation. Tyrosine phosphorylation of most, if not all, TCR-regulated substrates was diminished approximately fourfold. The impact of PEP on tyrosine phosphorylation of Zap-70 and the ζ subunit of TCR, the two most proximal substrates in TCR signaling (21, 22), was also specifically evaluated. Antiphosphotyrosine immunoblotting of Zap-70 immunoprecipitates

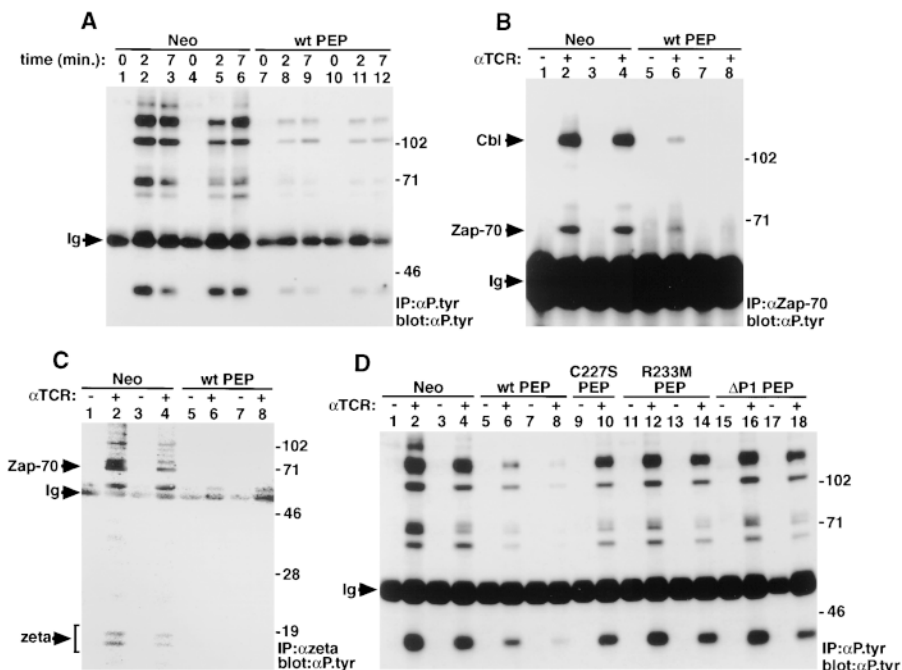


Figure 4. Impact of PEP on TCR-mediated protein tyrosine phosphorylation. (A) Overall protein tyrosine phosphorylation. Cells were stimulated for the indicated times with biotinylated anti-TCR mAb F23.1 and avidin. After cell lysis, tyrosine phosphorylated proteins were immunoprecipitated with antiphosphotyrosine (αP.tyr) antibodies and revealed by subsequent antiphosphotyrosine immunoblotting. Lanes 1–3, Neo.1; lanes 4–6, Neo.3; lanes 7–9, wt PEP.68; and lanes 10–12, wt PEP.78. The migrations of prestained molecular mass markers are indicated on the right, while that of the H chain (Ig) is shown on the left. Exposure: 16 h. (B) Tyrosine phosphorylation of Zap-70. Cells were activated as outlined for A, except that stimulation was for 2 min only. Tyrosine phosphorylation of Zap-70 was monitored by immunoblotting of anti-Zap-70 immunoprecipitates with antiphosphotyrosine antibodies. Lanes 1 and 2, Neo.1; lanes 3 and 4, Neo.3; lanes 5 and 6, wt PEP.68; and lanes 7 and 8, wt PEP.78. The migrations of prestained molecular weight markers are shown on the right; those of c-Cbl, Zap-70, and H chain (Ig) are indicated on the left. Exposure: 24 h.

(C) Tyrosine phosphorylation of ζ. Experiment was as for B, except that the ζ subunit of TCR was immunoprecipitated with anti-ζ mAb H146. Lanes 1 and 2, Neo.1; lanes 3 and 4, Neo.3; lanes 5 and 6, wt PEP.68; and lanes 7 and 8, wt PEP.78. The migrations of prestained molecular mass markers are indicated on the right; those of Zap-70, H chain (Ig), and ζ (zeta) are shown on the left. Exposure: 12 h (using a PhosphorImager). (D) Effect of PEP mutants on TCR-mediated protein tyrosine phosphorylation. Experiment was as outlined for A, except that cells expressing the various PEP mutants were studied. Cells were stimulated for 2 min. Lanes 1 and 2, Neo.1; lanes 3 and 4, Neo.3; lanes 5 and 6, wt PEP.68; lanes 7 and 8, wt PEP.78; lanes 9 and 10, C227S PEP.28; lanes 11 and 12, R233M PEP.1; lanes 13 and 14, R233M PEP.47; lanes 15 and 16, ΔP1 PEP.3; and lanes 17 and 18, ΔP1 PEP.29. The positions of prestained molecular weight markers are shown on the right, whereas that of H chain (Ig) is indicated on the left. Exposure: 16 h.

(Fig. 4 B) showed that PEP (lanes 5–8) markedly reduced the phosphotyrosine content of Zap-70 in TCR-stimulated BI-141 cells. Tyrosine phosphorylation of c-Cbl, a 120-kD adaptor molecule associated with Zap-70 (27, 28), was also lowered. In a similar way, PEP diminished tyrosine phosphorylation of the ITAMs of ζ (Fig. 4 C), the first biochemical change known to occur during T-cell activation (21, 22).

Lastly, the structural requirements for the diminution of TCR-induced protein tyrosine phosphorylation by PEP were ascertained (Fig. 4 D). This analysis showed that, unlike wild-type PEP (lanes 5–8), the two phosphatase-inactive variants of PEP (lanes 9–14), as well as Δ P1 PEP (lanes 15–18), were unable to inhibit TCR-triggered protein tyrosine phosphorylation.

Cooperative Inhibition of Protein Tyrosine Phosphorylation by PEP and Csk Is Enhanced by Their Physical Association. These findings demonstrated that, in a manner analogous to Csk (6), PEP was a negative regulator of antigen receptor signaling in T cells. Together with previously published results (8), they also indicated that the domains mediating the physical association between Csk and PEP were critical for the respective inhibitory impacts of these two molecules. On this basis, it seemed plausible that Csk and PEP needed to be associated with each other in order to inhibit TCR-mediated signal transduction events. To further test this possibility, attempts were made to recreate the influence of Csk and PEP on TCR-mediated tyrosine phosphorylation events in a heterologous system (Fig. 5). The Cos-1 cell system was chosen for these studies, because it has been extensively validated to dissect the proximal events of TCR signaling (17, 29, 30). Cells were transiently transfected with cDNAs coding for a chimeric receptor containing the cytoplasmic portion of ζ (Tac- ζ), the Src family kinase FynT, and Zap-70. A kinase-inactive version of Zap-70 (lysine 295 to arginine 295 mutant) was used in these assays to ensure that baseline protein tyrosine phosphorylation was caused exclusively by FynT.

As described elsewhere (29), expression of wild-type FynT allowed tyrosine phosphorylation of the ζ chimera (Fig. 5 A; first panel, lane 1) and Zap-70 (second panel, lane 1). Neither substrate was phosphorylated in cells lacking FynT (data not shown). In the presence of FynT, Tac- ζ was also detectably associated with tyrosine phosphorylated Zap-70 molecules (first and second panels, lane 1). In cells overexpressing p50^{csk} alone (lane 2), there was a small (<25%) diminution in the phosphotyrosine content of Tac- ζ and Zap-70. This decrease was more marked (30–40%) in cells containing PEP alone (lane 3), although residual phosphorylation of Tac- ζ and Zap-70 was clearly observed. However, in contrast, cells coexpressing Csk and PEP (lane 4) displayed a total abolition of tyrosine phosphorylation of both the chimeric receptor (first panel) and Zap-70 (second panel). Similar results were obtained in cells transfected with the Src-like enzyme Lck rather than FynT (data not shown).

Since this system recapitulated the effects of Csk and PEP on the initial events of TCR signaling, we wanted to

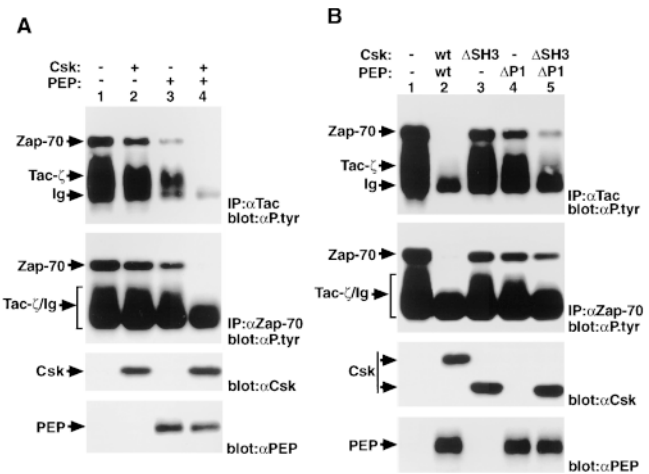


Figure 5. Cooperative inhibition of protein tyrosine phosphorylation by Csk and PEP in Cos-1 cells. (A) Effects of wild-type Csk and PEP on protein tyrosine phosphorylation in Cos-1 cells. Cos-1 cells were transiently transfected with cDNAs coding for Tac- ζ , wild-type FynT, and kinase-inactive Zap-70, in the absence or presence of Csk and/or PEP. Tyrosine phosphorylation of Tac- ζ (top panel) and Zap-70 (second panel) was examined by immunoblotting of either anti-Tac mAb 7G7 or anti-Zap-70 immunoprecipitates with antiphosphotyrosine antibodies. The expression of Csk (third panel), and PEP (bottom panel) was monitored by immunoblotting of total cell lysates with the indicated antisera. Equivalent expression of Tac- ζ , FynT, and Zap-70 in the transfected cell populations was confirmed by immunoblotting of cell lysates with the appropriate antibodies (data not shown). The migrations of Zap-70, Tac- ζ , H chain (Ig), Csk, and PEP are indicated on the left. Exposures: 6 h. (B) Effects of Δ SH3 Csk and Δ P1 PEP on protein tyrosine phosphorylation in Cos-1 cells. Experiment was as in A, except that cDNAs coding for Δ SH3 Csk and Δ P1 PEP were used. Equivalent levels of Tac- ζ , FynT, and Zap-70 were demonstrated by immunoblotting of total cell lysates with the appropriate antibodies (data not shown). The migrations of Zap-70, Tac- ζ , H chain (Ig), Csk, and PEP are indicated on the left. Exposures: 5 h, except for the bottom panel (7 h).

test whether the ability of these enzymes to associate was important for their cooperation. Thus, mutants that were defective in this association (Δ SH3 Csk and Δ P1 PEP; 9) were evaluated (Fig. 5 B). Like wild-type p50^{csk} or PEP alone (Fig. 5 A, lanes 2 and 3), Δ SH3 Csk (Fig. 5 B, lane 3) or Δ P1 PEP (lane 4) alone had a partial effect on tyrosine phosphorylation of Tac- ζ (first panel) and Zap-70 (second panel). However, contrary to wild-type Csk and PEP together (lane 2), coexpression of Δ SH3 Csk and Δ P1 PEP (lane 5) could not eliminate tyrosine phosphorylation of Tac- ζ and Zap-70. Together, these data supported the notion that the optimal ability of PEP and Csk to inhibit protein tyrosine phosphorylation by Src family kinases required their physical association.

Identification of Potential PEP Substrates. The ability of PEP overexpression in BI-141 cells to repress tyrosine phosphorylation of most TCR-regulated substrates, including Zap-70 and ζ , indicated that this phosphatase acted on a proximal step of TCR signaling. However, unfortunately, in light of the complex interplay between Src family kinases, ITAMs, and Zap-70, it was difficult to identify the immediate targets of PEP using the T-cell system. To help identify the PEP substrates, substrate-trapping experiments

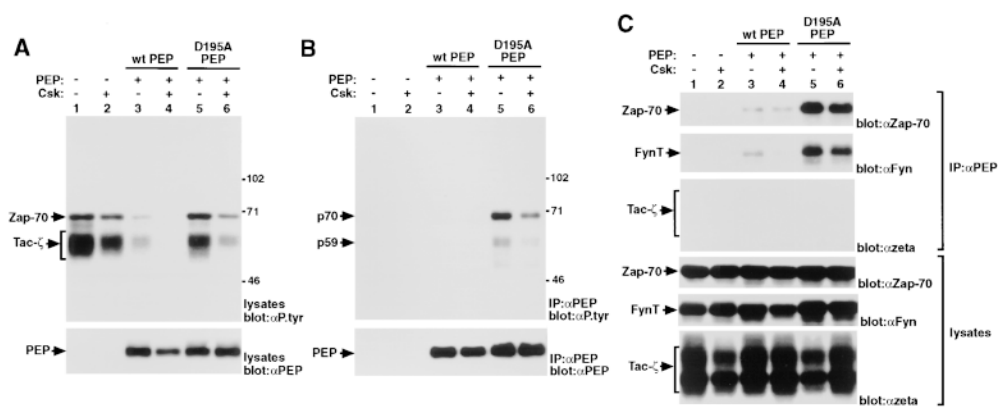


Figure 6. Identification of potential PEP substrates by substrate trapping. (A) Effect of D195A PEP on protein tyrosine phosphorylation in Cos-1 cells. Cos-1 cells were transiently transfected as for Fig. 5 A. Appropriate levels of expression of Tac-ζ, FynT, Zap-70, and Csk were documented by immunoblotting of total cell lysates with the appropriate antibodies (data not shown). The migrations of Zap-70, Tac-ζ and PEP are indicated on the left, whereas those of prestained molecular weight markers are shown on the right. Exposures: 13 h. (B) Association

of tyrosine phosphorylated proteins with D195A PEP. Lysates from the experiment shown in A were immunoprecipitated with anti-PEP antibodies and probed by immunoblotting with either antiphosphotyrosine (αP.tyr; top panel) or anti-PEP antibodies (bottom panel). The positions of the 70- and 59-kD tyrosine phosphorylated proteins are indicated on the left, whereas those of prestained molecular weight markers are shown on the right. Exposures: top panel, 13 h; bottom panel, 7 h. (C) Identification of tyrosine phosphorylated proteins associated with D195A PEP. Experiment was as in A and B, except that PEP immunoprecipitates and total cell lysates were immunoblotted with anti-Zap-70, anti-Fyn, or anti-ζ antibodies. The positions of Zap-70, FynT, and Tac-ζ are shown on the left. Exposures: top three panels, 48 h; bottom three panels, 13 h.

were conducted in the Cos-1 system (Fig. 6). Cells were transfected as described in Fig. 5, except that a PEP variant carrying a replacement of a conserved aspartate in the catalytic domain (aspartate 195 to alanine 195 mutant; D195A PEP) was used. Mutation of the equivalent residue in other PTPs has been shown to abolish their catalytic activity while preserving the ability to associate with substrates (24, 31–33). Such a property has provided a very useful tool in identifying targets of PTPs.

Immunoblotting of total cell lysates with antiphosphotyrosine antibodies showed that, when compared with wild-type PEP (Fig. 6 A, lane 3), D195A PEP (lane 5) was less efficient at repressing protein tyrosine phosphorylation in Cos-1 cells. Nonetheless, it was clearly able to reduce the tyrosine phosphorylation of certain substrates, especially Tac-ζ. Furthermore, the mutant could cooperate with Csk (lane 6) to inhibit protein tyrosine phosphorylation, albeit to a lesser degree than wild-type PEP (lane 4). The ability of D195A PEP to repress protein tyrosine phosphorylation may appear paradoxical, especially since the catalytic activity of this mutant was found to be abolished in *in vitro* phosphatase assays (data not shown). However, these results are consistent with the findings of others (33). Seemingly, substrate-trapping mutants of other PTPs can interfere with protein tyrosine phosphorylation as a result of steric hindrance.

To identify which proteins were the immediate substrates of PEP, PEP molecules were recovered by immunoprecipitation and probed by antiphosphotyrosine immunoblotting (Fig. 6 B, top panel). This experiment revealed that D195A PEP (lanes 5 and 6) associated with a major tyrosine phosphorylated polypeptide of ~70 kD. This association occurred independently of coexpression of Csk (compare lanes 5 and 6). Small quantities of a ~59-kD phosphotyrosine-containing protein were also present in PEP immunoprecipitates. Unlike D195A PEP, wild-type

PEP (lanes 3 and 4) was not associated with any detectable tyrosine phosphorylated protein. This difference was not caused by variations in the levels of expression of the two forms of PEP, as revealed by reprobing of the immunoblot membrane with anti-PEP serum (bottom panel).

To characterize the tyrosine phosphorylated proteins associated with D195A PEP, anti-PEP immunoprecipitates were also probed by immunoblotting with antibodies against either Zap-70, FynT, or Tac-ζ (Fig. 6 C). This analysis revealed that D195A PEP (lanes 5 and 6) formed a stable complex with Zap-70 (first panel) and FynT (second panel). However, it was not detectably associated with Tac-ζ (third panel). It is noteworthy that the ability of D195A PEP to associate with FynT and Zap-70 was not influenced by the presence of Csk (compare lanes 5 and 6), implying that Csk did not mediate these interactions. When compared with D195A PEP, wild-type PEP (lanes 3 and 4) bound much smaller quantities of Zap-70 and FynT, in keeping with the results obtained with substrate-trapping mutants of other phosphatases (31, 32). Thus, the results of these experiments suggested that PEP inhibited TCR signaling by dephosphorylating Zap-70 and FynT, but not ζ.

PEP Induces Dephosphorylation of the Positive Regulatory Site of FynT, Tyrosine 417. Like other Src family kinases, FynT is regulated by phosphorylation of two distinct tyrosine residues (5, 34, 35). Phosphorylation of the major site of autophosphorylation, tyrosine 417, is involved in the activation of FynT. In contrast, phosphate occupancy of the COOH-terminal tyrosine residue, tyrosine 528, which is mediated by Csk, causes an inhibition of the catalytic function of FynT. To assess which of these residues was(were) targeted for dephosphorylation by PEP, peptide mapping studies were conducted (Fig. 7). Cos-1 cells were transfected with cDNAs coding for wild-type FynT, Tac-ζ and kinase-inactive Zap-70 as above, in the absence or presence of wild-type PEP. Subsequently, they were la-

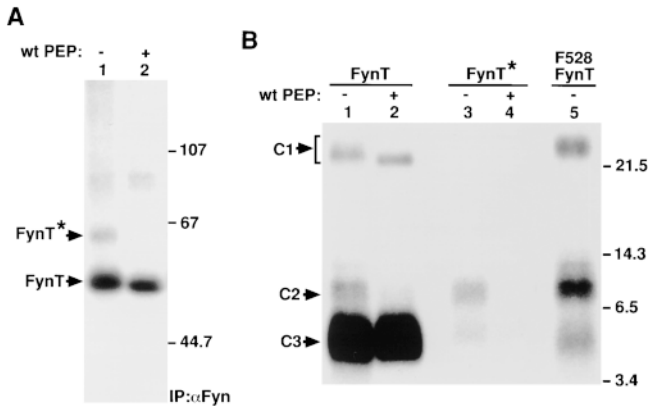


Figure 7. Peptide mapping studies. (A) Immunoprecipitations. Cells were metabolically labeled with $^{32}\text{P}_i$, as outlined in Materials and Methods. After lysis, FynT polypeptides were recovered by immunoprecipitation (IP) and analyzed by SDS-PAGE. The positions of prestained molecular mass markers are shown on the right. Those of the 59- and 65-kD species of FynT (FynT*) are indicated on the left. Exposure: 13 h. (B) Cyanogen bromide cleavage. Radiolabeled FynT was cleaved with cyanogen bromide and the resulting fragments were resolved in 18% SDS-PAGE gels. Phosphorylated products were detected by autoradiography. To allow better identification of the fragment (C2) containing the site of autophosphorylation of FynT, tyrosine 417, a peptide map of radiolabeled F528 FynT from cells treated with pervanadate is shown in lane 5. Under these conditions, the mutant FynT protein is extensively phosphorylated at tyrosine 417. The presence of a phosphorylated C3 fragment in this lane was due to contamination of the immunoprecipitates with endogenous Fyn molecules from Cos-1 cells. The positions of C1 (which contains NH_2 -terminal sites of serine and threonine phosphorylation), C2 (which contains tyrosine 417), and C3 (which bears tyrosine 528) are shown on the left. The migrations of prestained molecular mass markers are indicated on the right. Exposure: Lanes 1, 2, and 5, 3.5 h; lanes 3 and 4, 9 h.

beled metabolically with $^{32}\text{P}_i$, and FynT molecules were isolated by immunoprecipitation (Fig. 7 A). Gel electrophoresis revealed that FynT obtained from cells lacking PEP (lane 1) migrated as a phosphorylated product of 59 kD, in addition to a species of ~ 65 kD (FynT*). The existence of a similar form of FynT, having a retarded electrophoretic mobility, was shown previously to correlate with FynT activation (36). In comparison, FynT polypeptides recovered from cells expressing PEP (lane 2) were resolved as a single phosphorylated product of 59 kD. Little or no FynT* was found in these cells.

To identify more precisely the sites of phosphorylation on these molecules, the phosphorylated products were cleaved with cyanogen bromide and the resulting fragments were resolved in 18% SDS-PAGE gel (Fig. 7 B). Previous studies demonstrated that cleavage of FynT with cyanogen bromide produces three major phosphorylated peptides: C1 (21 kD), which contains NH_2 -terminal sites of serine and threonine phosphorylation; C2 (9.6 kD), which possesses tyrosine 417; and C3 (4.2 kD), which bears tyrosine 528 (37–39). To help identify the C2 fragment, a peptide map of constitutively activated tyrosine 528 to phenylalanine 528 (F528) FynT was performed in parallel (lane 5). This mutant is known to be extensively phosphorylated at tyrosine 417 (38).

We observed that the 59-kD FynT molecules in cells

lacking PEP (Fig. 7 B, lane 1) were phosphorylated within the tyrosine 528-containing C3 peptide and to a lesser, albeit appreciable extent, the tyrosine 417-bearing C2 peptide. Phosphorylation within the NH_2 -terminal C1 fragment was also present. The 65-kD products (lane 3) were more prominently phosphorylated within the tyrosine 417-containing C2 peptide, relative to the C3 fragment. This finding is consistent with the notion that FynT* represented activated FynT molecules. In cells expressing PEP (lane 2), there was no reduction in the extent of phosphorylation of $\text{p}59^{\text{FynT}}$ within the tyrosine 528-bearing C3 peptide. However, phosphorylation at tyrosine 417 was abolished. Furthermore, the existence of FynT* was abrogated (lane 4). Even though the global extent of phosphorylation in the C1 fragment was not altered by the presence of PEP, the electrophoretic mobility of this peptide was noticeably faster (lane 2). The exact basis for this difference is not known. Thus, in combination, these data indicated that PEP caused dephosphorylation of tyrosine 417, but not of tyrosine 528, of FynT.

Discussion

Previously, we found that the ability of Csk to inhibit T-cell activation required the presence of its SH3 domain and that, through this region, Csk interacted at a high stoichiometry with the proline-enriched protein tyrosine phosphatase PEP (8, 9). Herein, we demonstrated that, like Csk, PEP was a strong negative regulator of antigen receptor-mediated protein tyrosine phosphorylation and lymphokine secretion in T cells. This effect required the phosphatase activity of PEP, and correlated with the capacity of PEP to bind to Csk. Reconstitution experiments in Cos-1 cells provided further support for the idea that PEP and Csk cooperate to inhibit TCR signaling, and that this synergism is dependent on their association. Finally, studies using a substrate-trapping mutant of PEP suggested that PEP inhibited TCR signaling by dephosphorylating the Src-related enzymes themselves and at least one of their targets, the protein tyrosine kinase Zap-70.

These observations suggested that the SH3 domain of Csk is needed for its inhibitory function in T cells because it mediates binding to PEP (or a PEP-related phosphatase; see below). Although it is formally possible that this domain also interacts with other proteins contributing to its activity, it should be mentioned that we and others have failed to identify additional ligands for the Csk SH3 region (9 and references therein). Taking into consideration the similar impact of Csk and PEP on TCR signaling, the high stoichiometry and specificity of their association, and the structural requirements for their respective inhibitory effects, it appears likely that the ability of Csk and PEP to inhibit TCR signaling requires their physical association. Through its dual ability to inactivate Src-related enzymes by phosphorylating their COOH-terminal tyrosine and to dephosphorylate critical TCR-regulated substrates, the Csk-PEP complex constitutes a potent mechanism to inac-

tivate T cells. The purpose of this kinase-phosphatase complex is clearly different from that of CaMKIV-PP2A, Erk2-MKP3, and Lck-CD45, in which the associated phosphatase directly regulates the activity of the kinase (11, 12, 40).

We found that enhanced PEP expression in BI-141 T cells caused a diminution in tyrosine phosphorylation of most TCR-regulated substrates, including Zap-70 and the ITAMs of ζ . Given the complex regulatory interactions between Src family kinases, ITAMs, and Zap-70, it is difficult to determine whether PEP acted by dephosphorylating and inactivating the ITAMs, Zap-70, and/or the positive regulatory site of Src family kinases. To help resolve this issue, we attempted to identify the immediate target(s) of PEP using a substrate-trapping variant of PEP (D195A PEP). In transiently transfected Cos-1 cells, this mutant was found to associate with Zap-70 and FynT. A similar association could be seen with Lck (data not shown). By contrast, D195A PEP did not interact with ζ . Hence, these findings suggested that PEP inhibited TCR signaling by regulating Zap-70 and Src family kinases, and that the reduction in ITAM phosphorylation in PEP-overexpressing cells was secondary to inactivation of Src-related enzymes. Of course, we cannot exclude the possibility that PEP also dephosphorylated more downstream targets in the TCR signaling cascade.

It is unlikely that the impact of PEP in BI-141 cells was the nonspecific consequence of overexpression of an irrelevant PTP. Firstly, the biochemical and biological effects of PEP were seen with moderate degrees (three- to fourfold) of overexpression. Secondly, and most importantly, they were not noted in cells containing Δ P1 PEP, a PEP variant that was defective in the ability to associate with Csk, but that remained catalytically active. On this basis, we feel confident that the effects of PEP in these cells were genuine, and that they reflected a physiological role of PEP in TCR signaling. The inability of phosphatase-inactive versions of PEP (C227S and R233M PEP) to behave as dominant-negative mutants and enhance TCR signaling in BI-141 cells may be viewed as a contradiction of this model. However, it should be remembered that \sim 5% of Csk is associated with endogenous PEP molecules. As indicated in Fig. 1 C, there remains a large pool of presumably unoccupied Csk molecules than can accommodate additional PEP polypeptides. Consequently, it is not surprising that expression of the phosphatase-inactive mutants had no effect on TCR signaling, as they would not be expected to displace the endogenous PEP from Csk.

In addition to PEP, other PTPs have been implicated in the control of T-cell activation. The transmembrane PTP CD45 was shown to be a positive regulator of TCR signaling, as a consequence of its ability to dephosphorylate the negative regulatory site of Src-like kinases (41, 42). SHP-1 and SHP-2, two PTPs bearing tandem SH2 domains at their NH₂ termini, have also been linked to this process.

There is evidence that SHP-1 has an inhibitory impact on T-cell activation (43–45), and that it can be activated by tyrosine phosphorylated Zap-70 (45) or, in some T-cell subsets, inhibitory receptors such as KIRs and LAIR-1 (46, 47). In comparison, the function of SHP-2 is less certain. Whereas it has been proposed to be a negative regulator of TCR signaling based on its association with the inhibitory receptor CTLA-4 (48), recent studies with dominant-negative SHP-2 mutants have suggested an opposite role (49). Finally, other phosphatases such as PAC-1 and HePTP have been found to inhibit T-cell activation by acting on more downstream events of TCR signaling, such as MAP kinase activation (50, 51). Thus, in a manner analogous to protein tyrosine kinases, several PTPs appear to be involved in regulating protein tyrosine phosphorylation in T cells. Even though certain PTPs, especially SHP-1, share substrates with PEP, these enzymes are most probably regulated by distinct mechanisms. As a result, they are likely to be involved in different aspects of T-cell inactivation. Additional studies, including determination of the mechanism by which Csk-PEP is recruited to the site of Src family kinase activation, should help to substantiate this notion.

As p50^{Csk} is ubiquitous and PEP is also expressed in hemopoietic cells other than T cells, the Csk-PEP complex may provide a similar function in cell types such as B cells and macrophages. Furthermore, it is noteworthy that Csk also associates via its SH3 domain with PTP-PEST (16), a PEP-related PTP contained in all cell lineages (20). Since the SH3 region of Csk seems to be critical for its function in nonhemopoietic cells (52–54), the inhibitory kinase may also cooperate with PTP-PEST in these cells. In keeping with this notion, it was reported that expression of the catalytic domain of PTP-PEST was able to suppress the lethal effect of Src in yeast cells, seemingly by dephosphorylating its substrates (55). Hence, a complex between Csk and a PTP could have a generalized role in the regulation of Src family kinases *in vivo*.

Because of the central role played by Src family kinases in cell signaling, significant interest has been directed towards understanding their regulation. Earlier studies showed that Csk-mediated phosphorylation of the COOH-terminal tyrosine of Src-like enzymes decreases their catalytic activity, by allowing an intramolecular interaction between the phosphorylated COOH terminus and the SH2 domain (5, 34, 56). However, as revealed by recent structural data (1, 2), this phosphorylation is not sufficient for full inactivation of Src-related enzymes. Other mechanisms, including a documented intramolecular association between their SH3 domain and linker region, are involved in additional inhibition. The data presented in this report raise the possibility of a novel facet in this regulation, by suggesting that a complex between Csk and a PTP is also critical to repress signaling by Src family kinases *in vivo*.

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