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PTP-PEST: a protein tyrosine phosphatase regulated by serine phosphorylation

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The protein tyrosine phosphatase PTP-PEST is an 88 kDa cytosolic enzyme which is ubiquitously expressed in mammalian tissues. We have expressed PTP-PEST using recombinant baculovirus, and purified the protein essentially to homogeneity in order to investigate phosphorylation as a potential mechanism of regulation of the enzyme. PTP-PEST is phosphorylated *in vitro* by both cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC) at two major sites, which we have identified as Ser39 and Ser435. PTP-PEST is also phosphorylated on both Ser39 and Ser435 following treatment of intact HeLa cells with TPA, forskolin or isobutyl methyl xanthine (IBMX). Phosphorylation of Ser39 *in vitro* decreases the activity of PTP-PEST by reducing its affinity for substrate. In addition, PTP-PEST immunoprecipitated from TPA-treated cells displayed significantly lower PTP activity than enzyme obtained from untreated cells. Our results suggest that both PKC and PKA are capable of phosphorylating, and therefore inhibiting, PTP-PEST *in vivo*, offering a mechanism whereby signal transduction pathways acting through either PKA or PKC may directly influence cellular processes involving reversible tyrosine phosphorylation.

Key words: PEST hypothesis/phosphorylation/protein kinase/protein tyrosine phosphatase

Introduction

Protein tyrosine phosphorylation is an essential feature of the regulation of a large number of diverse cellular events involved in growth, division and differentiation (Fantl *et al.*, 1993). The opposing actions of protein tyrosine kinases (PTKs) and phosphatases (PTPs) determine the net phosphorylation state of phosphotyrosine-containing proteins within the cell. This suggests equally important roles for both groups of enzymes in the control of cellular events involving reversible tyrosine phosphorylation.

The PTPs are a stucturally diverse family of enzymes which includes both transmembrane, receptor-like and non-transmembrane, cytoplasmic forms (Charbonneau and Tonks, 1992). They have in common a highly conserved catalytic domain but differ in their non-catalytic segments; this structural diversity presumably reflects differences in the regulation and function of each enzyme. Since these enzymes frequently have specific activities one to three orders of magnitude greater than those of the PTKs (Tonks *et al.*, 1988), it has been suggested that PTP activity must be strictly controlled within the cell in order to allow the tyrosine phosphorylation of proteins that is necessary for cell signalling by PTKs. Many of the PTPs described to date appear to be targeted to particular subcellular locations. This offers a powerful means of controlling PTP activity by limiting the range of substrates available to each enzyme. Examples include PTP1B, which is targeted to the endoplasmic reticulum via its C-terminus (Frangioni *et al.*, 1992), SH-PTP2, which is localized in membrane complexes by binding to specific phosphotyrosine-containing sequences in growth factor receptors via its Src homology 2 (SH2) domains (Feng *et al.*, 1993; Lechleider *et al.*, 1993), and PTPH1, which contains sequences similar to those found in a number of cytoskeleton-associated proteins (Yang and Tonks, 1991). There are also a relatively small number of PTPs which are predicted to be cytosolic; unlike the targeted PTPs, these enzymes would be expected to be less restricted in their available substrates, and are therefore likely to be subject to particularly stringent control mechanisms.

PTP-PEST is an 88 kDa cytosolic human PTP recently cloned in our laboratory (Yang *et al.*, 1993a,b) and by others (Takekawa *et al.*, 1992). The mRNA is widely expressed in cell lines (Yang *et al.*, 1993a), and the murine homologue appears to be ubiquitous in mouse tissues (Yi *et al.*, 1991). Expression of PTP-PEST mRNA has recently been found to be dramatically increased in the cortex of the embryonic rat brain, compared with the levels found in the adult brain, suggesting a role for PTP-PEST in early neuronal development (Sahin and Hockfield, 1993). PEP, a PTP whose expression is restricted to haematopoietic cells, shares a similar arrangement of structural motifs with PTP-PEST (Matthews *et al.*, 1992). Both have a 50–60 amino acid residue N-terminal segment, followed by the 240 amino acid residue PTP catalytic domain, and a C-terminal segment of ~500 amino acids which contains PEST sequences and is highly enriched in proline, glutamic/aspartic acid and serine/threonine residues. PEST sequences have been identified in a large number of proteins with diverse functions, and it has been suggested that these proteins share a common regulatory mechanism involving their rapid destruction (Rogers *et al.*, 1986). Recently, a role for PEST sequence-directed rapid turnover in the regulation of the *Saccharomyces cerevisiae* G1 cyclin Cln3 was suggested by the demonstration that deletion of these sequences in Cln3 generates a protein with increased stability, whose functional impairment results in a small cell size (wee) phenotype (Nash *et al.*, 1988; Tyers *et al.*, 1992). However, it remains to be established whether all PEST sequence-containing proteins, including PTP-PEST, display a short half-life in the cell. It is also likely that there are additional regulatory

mechanisms to modulate the activities of many of these proteins.

Several PTPs, including PTP1B (Flint *et al.*, 1993), cdc25 (Moreno *et al.*, 1990; Strausfield *et al.*, 1994), CD45 (Ostergaard and Trowbridge, 1991; Valentine *et al.*, 1991; Autero *et al.*, 1994), and both SH2 domain-containing PTPs (Yeung *et al.*, 1992; Feng *et al.*, 1993) are known to be phosphoproteins *in vivo*, suggesting the potential for regulation of activity by reversible phosphorylation. We have investigated the possibility that the activity of PTP-PEST may be regulated by reversible phosphorylation. The protein was found to be phosphorylated *in vitro* by both cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC) at two major sites, which we have identified as Ser39 and Ser435. Phosphorylation at Ser39 was inhibitory due to a decrease in the affinity of PTP-PEST for its substrate. This site is also phosphorylated in intact HeLa cells in response to TPA, forskolin or isobutyl methyl xanthine (IBMX), coincident with an inhibition of PTP activity. These data demonstrate that the activity of PTP-PEST *in vivo* may be regulated, at least in part, by the phosphorylation state of the protein at Ser39.

Results

Phosphorylation of PTP-PEST by PKA and PKC in vitro

PTP-PEST was expressed in Sf9 cells using recombinant baculovirus in both full-length (FL) and truncated (CD) forms. The truncated form, comprising residues 1–305, includes the entire PTP catalytic domain while excluding the C-terminal segment of the protein that contains PEST sequences. The proteins were purified essentially to homogeneity (Figure 1) and then tested for their ability to be phosphorylated by protein kinases *in vitro*. Both forms of PTP-PEST were phosphorylated by PKA and PKC, the stoichiometry of phosphorylation being greater with PKA than PKC (Figure 1). Phosphoamino acid analysis revealed that both protein kinases phosphorylated exclusively seryl residues (data not shown). Following phosphorylation of FL PTP-PEST by PKA in the presence of [γ-^{32}P]ATP, the protein was digested with trypsin and the resultant phosphopeptides separated by two-dimensional electrophoresis–chromatography. The label was found primarily in two major phosphopeptides, although some minor species were also observed (Figure 2a). Similar analysis of FL PTP-PEST phosphorylated by PKC showed that

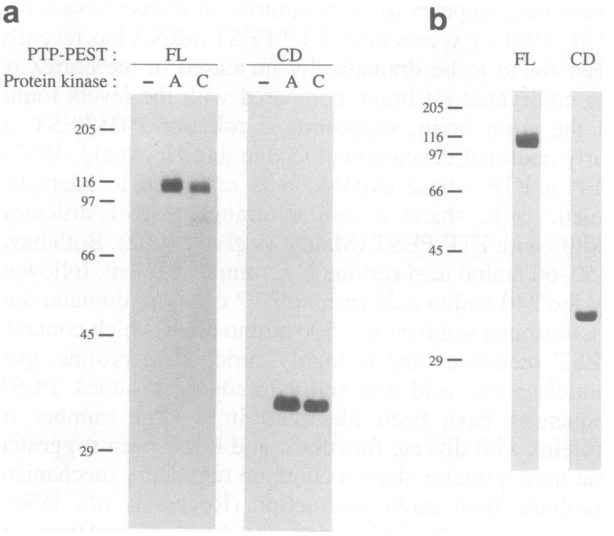

Fig. 1. Phosphorylation of PTP-PEST *in vitro*. PTP-PEST was expressed in Sf9 cells in both full-length (FL) and truncated (CD) forms, and purified as described in Materials and methods. (**a**) Phosphorylation of 1 μg aliquots of FL PTP-PEST, or 2 μg aliquots of CD PTP-PEST by PKA and PKC using [γ-^{32}P]ATP was performed as described in Materials and methods. The extent of phosphorylation was assessed following SDS–PAGE and autoradiography. Control incubations were also carried out in the absence of protein kinase as indicated. (**b**) 2 μg aliquots of the purified proteins were analysed by SDS–PAGE followed by staining with Coomassie blue. Molecular weight markers were from Sigma.

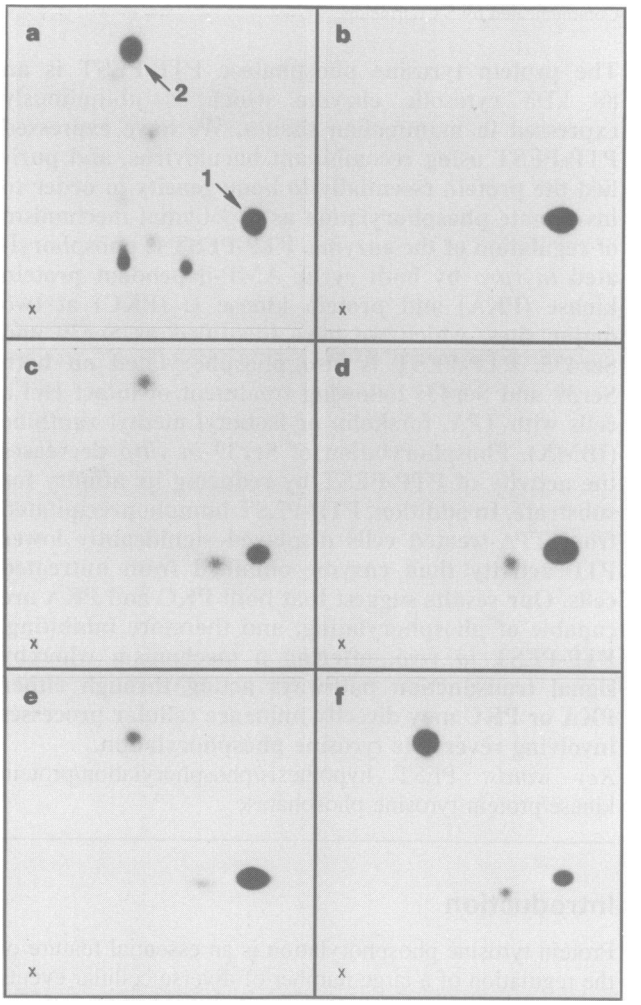

Fig. 2. Phosphopeptide mapping of PTP-PEST following phosphorylation *in vitro*. Gel slices containing ^{32}P-phosphorylated PTP-PEST were incubated with trypsin and the resultant phosphopeptides analysed by two-dimensional phosphopeptide mapping followed by autoradiography. Electrophoresis at pH 1.9 was performed horizontally, with the anode on the left; chromatography was in the vertical direction. Shown are maps resulting from analysis of phosphopeptides generated from FL PTP-PEST phosphorylated by PKA (**a**) and PKC (**c**), or from CD PTP-PEST phosphorylated by PKA (**b**) and PKC (**d**). In panel a, 1 and 2 denote the two major phosphopeptides in PKA phosphorylated FL PTP-PEST, referred to in the text as sites 1 and 2 respectively. These two phosphopeptides were purified by HPLC (see Figure 5), individually mixed with phosphopeptides from PKC-phosphorylated FL PTP-PEST, and subsequently analysed by phosphopeptide mapping [panels **e** (site 1) and **f** (site 2)]. The sample origin is at the bottom left corner, and is marked with an x.

both of the major PKA phosphopeptides (and one additional minor phosphopeptide) were present (Figure 2c, e and f) This indicates that PKA and PKC phosphorylate the same two major sites in PTP-PEST. Phosphorylation of CD PTP-PEST by PKA occurred exclusively at one of these sites, termed site 1 (Figure 2b); PKC also phosphorylated CD PTP-PEST predominantly at site 1, as well as at the minor site seen in FL PTP-PEST (Figure 2d). These results demonstrate that phosphorylation site 1 is within the N-terminal 305 amino acids of PTP-PEST, while the second major site of phosphorylation by both PKA and PKC (site 2) is C-terminal to amino acid 305.

Phosphorylation of PTP-PEST inhibits enzyme activity

The effect on enzyme activity of phosphorylation of both FL PTP-PEST and CD PTP-PEST by PKA *in vitro* was assessed using tyrosine phosphorylated RCM-lysozyme as substrate. Parallel incubations were carried out in which the enzyme was phosphorylated by PKA in the presence of [γ-^{32}P]ATP, to analyse the time course of phosphorylation, or in the presence of unlabelled ATP, from which aliquots were removed for assay. Both forms of PTP-PEST were inhibited over a 20 min time course of incubation with PKA in the presence of ATP (Figure 3c and d), while PTP activity was essentially unaffected in parallel incubations performed in the absence of ATP. The time course of phosphorylation of PTP-PEST by PKA (Figure 3a and b) was found to correlate closely with that of enzyme inhibition. The maximum observed inhibition of PTP-PEST following phosphorylation by PKA was 69%, and in all comparable experiments CD and FL PTP-PEST were found to be inhibited to a similar extent. Since CD PTP-PEST is phosphorylated exclusively at site 1 by PKA, the inhibition of PTP-PEST by PKA results from phosphorylation at this site, while phosphorylation of site 2 has no apparent effect on enzyme activity.

The stoichiometry of phosphorylation of FL PTP-PEST

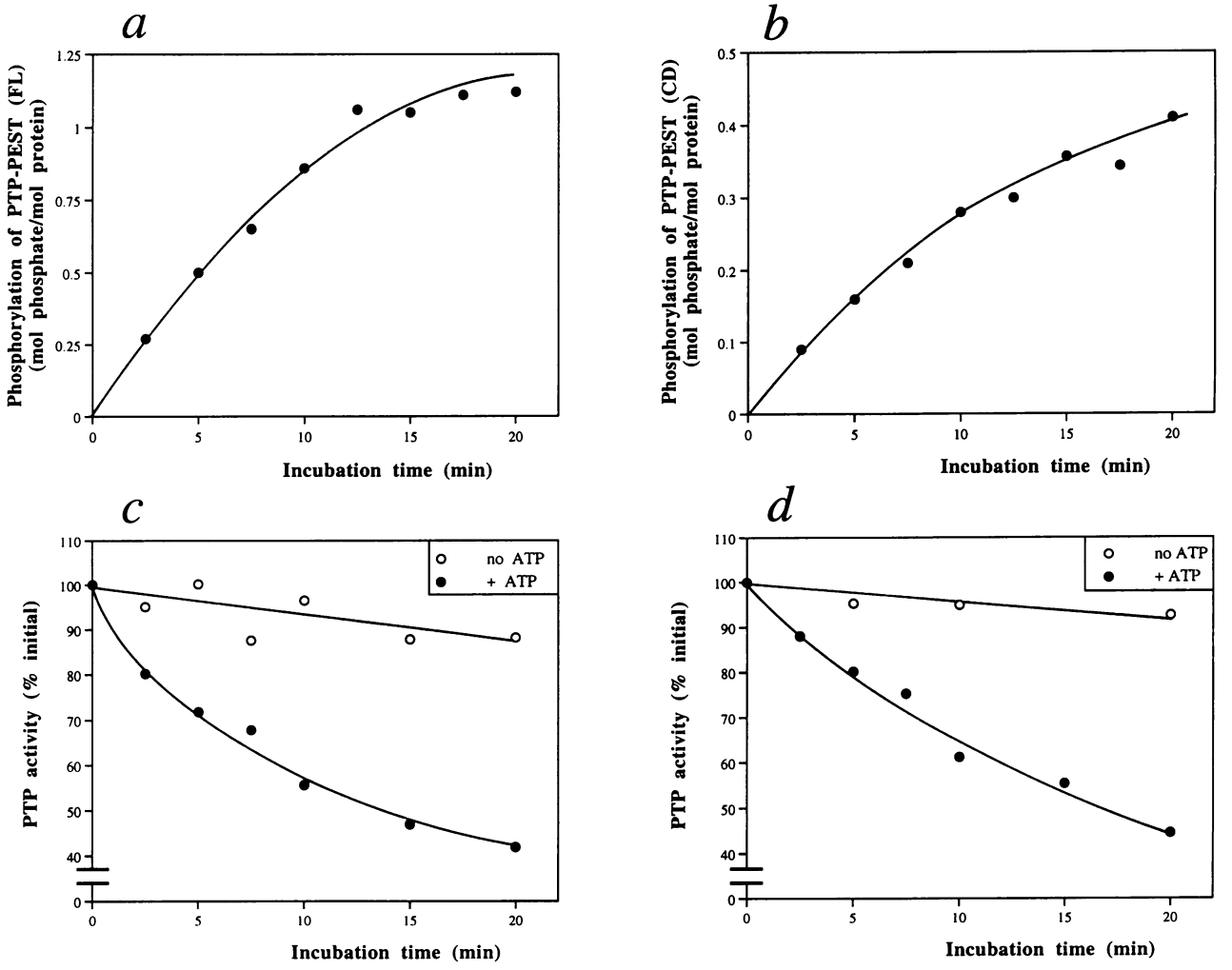

Fig. 3. Phosphorylation and inhibition of PTP-PEST by PKA. FL (**a**) and CD (**b**) PTP-PEST were phosphorylated by PKA using [γ-^{32}P]ATP as described in Materials and methods. Aliquots of the reaction were removed at the indicated time points into an equal volume of SDS−PAGE sample buffer. The extent of phosphorylation of PTP-PEST was then estimated following SDS−PAGE by counting the Cerenkov radiation in the resultant gel slice. The effect of phosphorylation on the PTP activity of FL (**c**) and CD (**d**) PTP-PEST was assessed following incubation under identical conditions except that non-radioactive ATP was used (●). The reaction was terminated, and the enzyme activity determined using 0.1 μM tyrosine phosphorylated RCM-lysozyme as the substrate, as described in Materials and methods. Control incubations were also carried out in which ATP was omitted (○). The results shown are calculated from the mean activity of assays performed in triplicate, and are expressed as a percentage of the initial enzyme activity from the incubation in which ATP was omitted.

by PKA reached a maximum of ~1.2 mol phosphate/mol protein, while CD PTP-PEST was phosphorylated up to a stoichiometry of 0.5 mol phosphate/mol protein. This difference in stoichiometry largely reflects the presence of additional phosphorylation sites in FL PTP-PEST, while the reason for the observed apparent sub-stoichiometric phosphorylation is not clear. Increasing the temperature, the time of phosphorylation, the amount of kinase, or the ATP concentration did not produce a significant increase in stoichiometry. In addition, PTP-PEST is not significantly phosphorylated at either of the two major sites during its production in Sf9 cells, as judged by peptide mapping of ^{32}P-phosphorylated PTP-PEST obtained from labelled recombinant baculovirus-infected Sf9 cells (data not shown).

The inhibition of PTP-PEST observed following phosphorylation by PKA was found to be more pronounced at low (submicromolar) substrate concentrations than at higher concentrations. Assays of enzyme activity of maximally phosphorylated FL PTP-PEST over a range of substrate concentrations revealed that the apparent K_m was increased by ~65%, from 1.9 μM to 3.1 μM, while the V_{max} was essentially unchanged (Figure 4). The major effect of phosphorylation of PTP-PEST by PKA is therefore to reduce the affinity of the enzyme for its substrate. Purified CD PTP-PEST (which has a similar V_{max} but a lower K_m (~0.35 μM) than FL PTP-PEST), was similarly affected in its kinetic parameters by phosphorylation (data not shown), consistent with this effect being due entirely to phosphorylation of site 1.

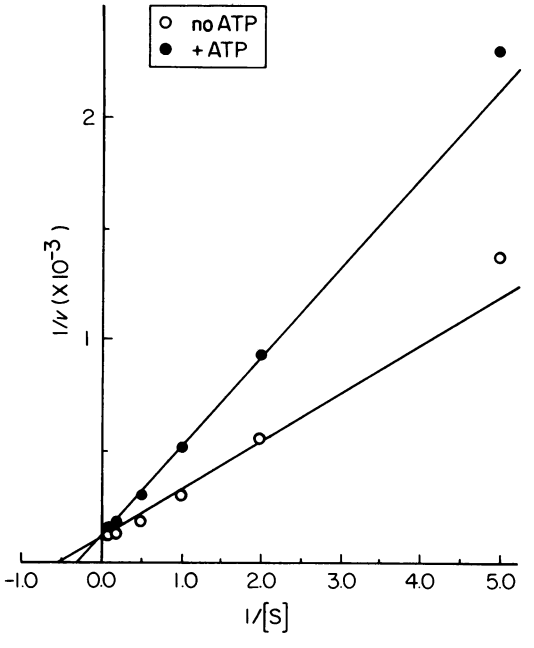

Fig. 4. Lineweaver–Burk analysis of PKA-phosphorylated/non-phosphorylated PTP-PEST. FL PTP-PEST was incubated with PKA for 20 min in the presence (●) or absence (○) of ATP, and the reaction was terminated as described in Materials and methods. The enzyme was then diluted appropriately in PTP assay buffer prior to assaying against tyrosine-phosphorylated RCM-lysozyme at the concentrations shown. The results illustrated are the means from triplicate assays, and are plotted as the reciprocal of the enzyme activity (U/mg) versus the reciprocal of the substrate concentration (μM).

Identification of the major sites of phosphorylation of PTP-PEST by PKA and PKC

In order to identify the two major PKA and PKC phosphorylation sites, FL PTP-PEST was maximally phosphorylated by PKA in the presence of [γ-^{32}P]ATP. The protein was then digested with trypsin and the resultant phosphopeptides separated by reversed phase HPLC on a C_8 column. One of the major phosphopeptides (corresponding to phosphorylation site 1) eluted during the aqueous wash prior to application of the elution gradient, indicating that this phosphopeptide was extremely hydrophilic. The second major phosphopeptide (phosphorylation site 2) eluted at ~28% acetonitrile (Figure 5a). Each

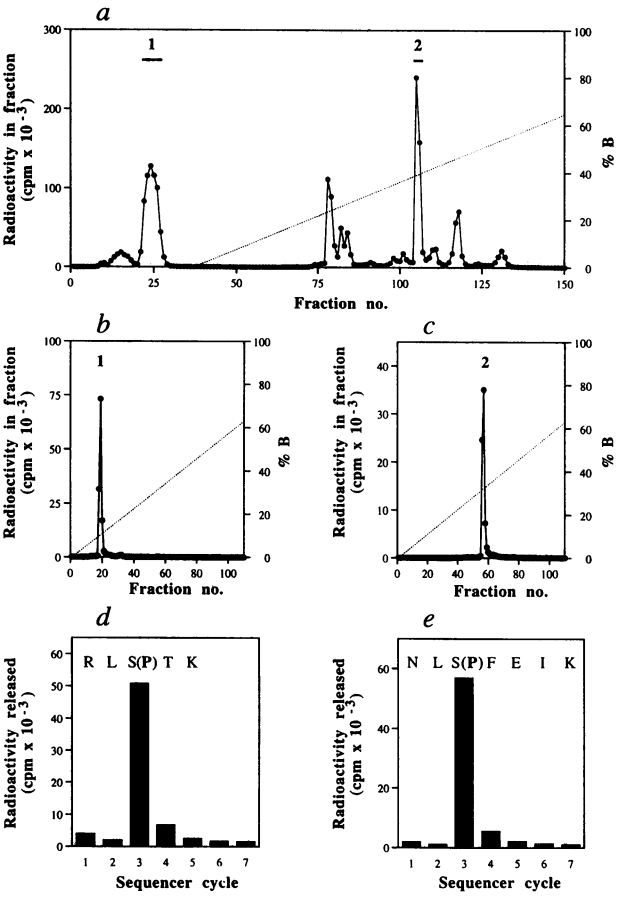

Fig. 5. Phosphopeptide purification and identification of PTP-PEST phosphorylation sites. Following ^{32}P-phosphorylation of FL PTP-PEST by PKA, purification by SDS–PAGE, and trypsin digestion, the resultant phosphopeptides were purified by reversed phase HPLC on a C_8 column (**a**). The sample was applied in an aqueous solution of 0.1% (v/v) TFA, and the column was then washed with the same buffer. Peptides were then eluted with acetonitrile using the gradient shown (buffer B contains 0.1% TFA in a mixture of 70% acetonitrile, 30% water). The radioactivity in each fraction was determined by Cerenkov counting, and the indicated fractions containing phosphopeptides 1 and 2 were pooled for further purification. Phosphopeptides 1 (**b**) and 2 (**c**) were then applied separately to a C_{18} HPLC column and purified using conditions similar to those in (a). An aliquot of each partially purified phosphopeptide (from a) was covalently coupled to a sequencer sample filter and subjected to protein sequencer analysis. The ^{32}P-radioactivity released at each cycle from phosphopeptide 1 (**d**) and 2 (**e**) is plotted. The purified phosphopeptides, resulting from the second round of HPLC purification (b and c) were subjected to direct protein sequencing. The amino acid sequence of phosphopeptides 1 (d) and 2 (e), deduced from this analysis (see text for details), is shown in single-letter code.

phosphopeptide was then purified further by reversed phase HPLC using a C_{18} column, the phosphopeptides eluting at 7% acetonitrile (site 1) and 22% acetonitrile (site 2) (Figure 5b and c). The positions of the phosphorylated serine residues in phosphopeptides 1 and 2 were then determined by sequencer analysis following covalent coupling of a small aliquot of each purified phosphopeptide to the sample filter. In each case, significant radioactivity was released from the coupled peptide only at cycle 3, demonstrating that the phosphorylated serine residue was at position 3 in both phosphopeptides (Figure 5d and e).

The amino acid sequence surrounding phosphorylation site 2 was determined directly by protein sequencing of the phosphopeptide. The sequence obtained was (N)L(S)FEI (assignments in parentheses were not definitive), which corresponds to amino acid residues 433–438 in PTP-PEST. Ser435 in this sequence therefore corresponds to phosphorylation site 2. The sequence obtained from analysis of phosphopeptide 1 was (R)L(S)X(K) (X denotes no assignment at cycle 4). Inspection of the amino acid sequence of PTP-PEST suggested that phosphorylation site 1 probably corresponds to Ser39 in the peptide RLSTK. Presumably, the relatively poor sequence data obtained for this phosphopeptide resulted from its extreme hydrophilic nature, which had also prevented the binding of the phosphopeptide to the C_8 HPLC column (Figure 5a).

In order to establish Ser39 as phosphorylation site 1, the synthetic peptide LRRLSTK, based on the amino acid sequence of residues 35–41 of PTP-PEST, was made and used as a substrate for PKA in a phosphorylation reaction *in vitro*. The peptide was phosphorylated by PKA only on serine (data not shown), and following trypsin digestion co-migrated, on two-dimensional electrophoresis–chromatography maps, with phosphopeptide 1 from FL PTP-PEST phosphorylated *in vitro* by PKA (Figure 6). Phosphopeptide 1 therefore has the sequence RLSpTK (Sp refers to the phosphorylated serine residue) corresponding to amino acids 37–41 of PTP-PEST. The absence of tryptic phosphopeptide LSpTK, resulting from complete tryptic digestion of the phosphorylated synthetic peptide, presumably reflects the relative resistance of the Arg–Leu peptide bond in this phosphopeptide to trypsin digestion. This is likely to be due to the adjacent phosphoserine residue, which has been shown to affect trypsin digestion of other phosphorylated proteins in a similar manner (Wettenhall and Morgan, 1984).

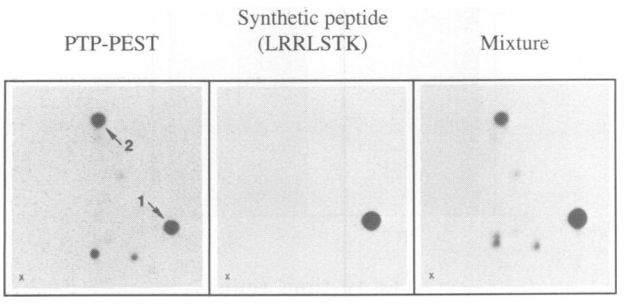

Fig. 6. Confirmation of Ser39 of PTP-PEST as a major site of PKA phosphorylation *in vitro*. [^{32}P]phosphopeptides were obtained from FL PTP-PEST following PKA phosphorylation and analysed by phosphopeptide mapping (as described in the legend to Figure 2) either alone (left panel), or together with PKA phosphorylated, trypsin digested synthetic peptide LRRLSTK (right panel). The middle panel shows a map of phosphorylated, digested peptide alone. In the left panel, 1 and 2 denote phosphopeptides 1 and 2.

Phosphorylation of Ser39 and Ser435 in vivo

The phosphorylation state of PTP-PEST *in vivo* was investigated following ^{32}P-labelling of HeLa cells and incubation in the absence or presence of agents expected to activate either PKA (forskolin or IBMX) or PKC (TPA). PTP-PEST was then immunoprecipitated from lysates of labelled cells, and the precipitated protein analysed by SDS–PAGE and autoradiography. In samples from untreated cells, grown in the presence of serum, PTP-PEST was detectable as a ^{32}P-labelled protein, and the extent of labelling was significantly increased in response to treatment with TPA, forskolin or IBMX (Figure 7). The sites in PTP-PEST phosphorylated *in vivo* were analysed by two-dimensional peptide mapping. The pattern obtained from cells grown in the presence of serum was found to contain one major, and several minor, phosphopeptides (Figure 8a). The major phosphopeptide co-migrated with phosphopeptide 2 derived from PTP-PEST phosphorylated *in vitro* by PKA (Figure 8b), indicating that Ser435 is phosphorylated under these conditions. In contrast, phosphopeptide 1 was absent in these samples. Following treatment of HeLa cells with TPA for 15 min, the pattern of phosphopeptides obtained from PTP-PEST included two major phosphopeptides, which co-migrated with phosphopeptides 1 and 2 from PTP-PEST phosphorylated *in vitro* by PKC or PKA (Figure 8c and d). A similar pattern was obtained from cells treated with forskolin or IBMX (data not shown).

Phosphoamino acid analysis was performed on phospho-

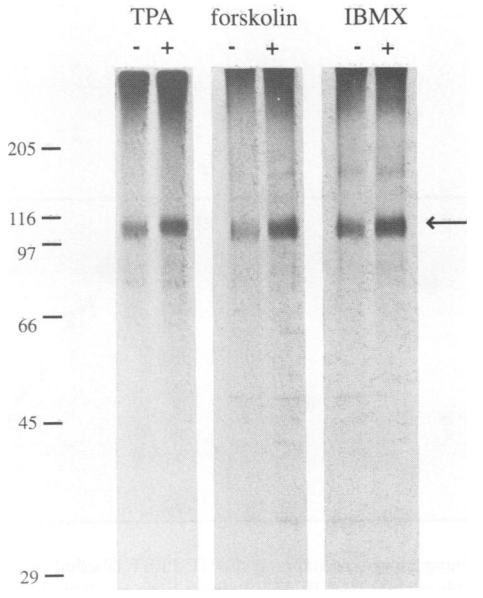

Fig. 7. Phosphorylation of PTP-PEST *in vivo*. HeLa cells were incubated in the presence of [^{32}P]inorganic phosphate, and then treated with the indicated agents prior to cell lysis and immunoprecipitation of the labelled PTP-PEST as described in Materials and methods. An equivalent amount of cell lysate (containing 1.2 mg total protein) was used in each immunoprecipitation. The immunoprecipitated proteins were analysed by SDS–PAGE followed by autoradiography. The position of the PTP-PEST band is indicated by an arrow; molecular weight markers were from Sigma.

peptide 1 of PTP-PEST from stimulated HeLa cells following its extraction from the TLC plate. This established that serine but not threonine residues were phosphorylated *in vivo* (data not shown), demonstrating that Ser39 in PTP-PEST is a major *in vivo* phosphorylation site, while Thr40 is not significantly phosphorylated under these conditions. These results indicate that both PKC and PKA are capable of phosphorylating the inhibitory phosphorylation site (Ser39) on PTP-PEST in intact HeLa cells.

The effect of phosphorylation of PTP-PEST *in vivo* in response to TPA was investigated following immunoprecipitation of the protein from HeLa cell lysates. The activity of the immunoprecipitated protein, assayed against 0.1 μM tyrosine phosphorylated RCML, was reduced by up to 35% in TPA-treated samples relative to samples from unstimulated cells (Figure 9); presumably reflecting the increased phosphorylation of Ser39 in PTP-PEST following TPA stimulation. This result suggests that the activity of PTP-PEST can potentially be controlled *in vivo* by phosphorylation of Ser39 by PKC or PKA.

Discussion

PTP-PEST is a human PTP which has no obvious transmembrane or other targeting sequence and is therefore predicted to be cytosolic (Yang *et al.*, 1993a,b). Following overexpression of the protein either in mammalian cells by calcium phosphate-mediated cDNA transfection, or in Sf9 cells using recombinant baculovirus, the majority (>95%) of the PTP-PEST protein is found in the soluble fraction (unpublished data), suggesting that the endogenous protein is also likely to be cytosolic. The specific activity of the enzyme, assessed following expression in Sf9 cells and purification of the protein, is ~35 000 U/mg protein (unpublished data). This specific activity is considerably higher than that previously reported for PTP-PEST expressed in bacteria as a glutathione S-transferase fusion protein (Yang *et al.*, 1993a), which is likely to reflect the greater stability and solubility of the enzyme expressed in Sf9 cells, compared with bacterially expressed enzyme. The specific activity of PTP-PEST observed in the current work is comparable with that of purified PTP-1B (Tonks *et al.*, 1988), while being several-fold higher than the specific activities of the PTKs. Taken together, these considerations suggest that PTP-PEST may exert a considerable negative influence on the tyrosine phosphorylation state of any intracellular protein which is accessible to the cytoplasm. This further predicts the existence of mechanisms to modulate the activity of the enzyme. We have investigated reversible phosphorylation of PTP-PEST as a potential regulatory mechanism and have identified Ser39 as a major inhibitory phosphorylation site on PTP-PEST *in vivo*, while Ser435 is also phosphorylated in intact cells, but has no apparent effect on enzyme activity.

Both Ser39 and Ser435 were found to be major sites of phosphorylation *in vitro* by the protein kinases PKA

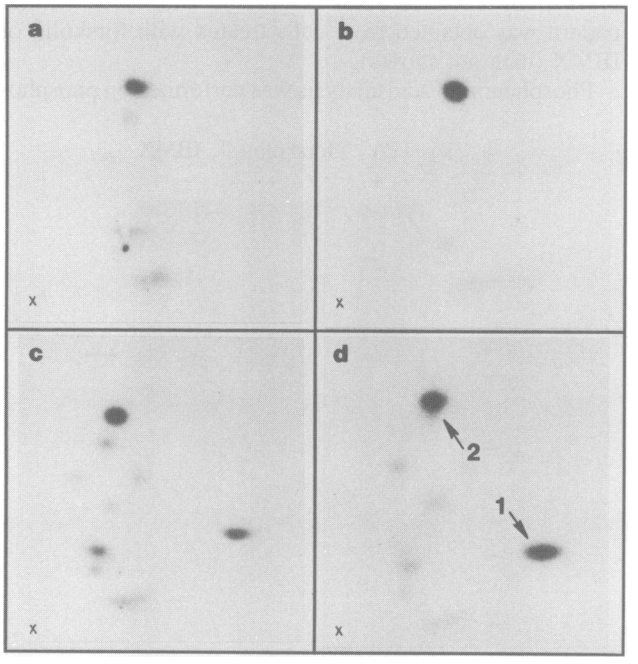

Fig. 8. Phosphopeptide mapping of PTP-PEST labelled *in vivo*. ^{32}P-phosphorylated PTP-PEST, obtained from labelled HeLa cells by immunoprecipitation and SDS−PAGE as in Figure 7, was digested with trypsin and analysed by two-dimensional phosphopeptide mapping. Maps resulting from analysis of PTP-PEST phosphopeptides obtained from untreated cells, grown in the presence of serum (**a**), and from cells treated with TPA (**c**) are shown. In panel **b**, PTP-PEST phosphopeptides from untreated cells were mixed with purified phosphopeptide 2 obtained as in Figure 5. In addition, purified phosphopeptides 1 and 2 were both mixed with PTP-PEST phosphopeptides from cells stimulated with TPA (**d**). The positions of phosphopeptides 1 and 2 are indicated in panel d.

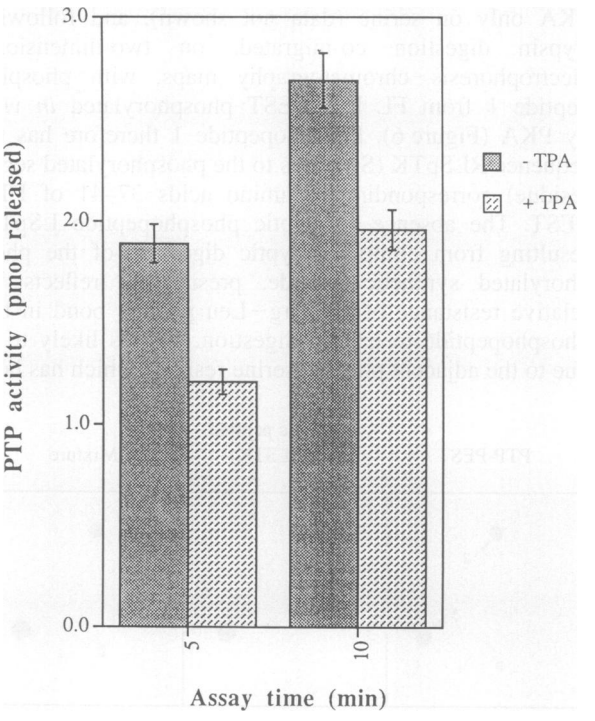

Fig. 9. Effect of TPA treatment on PTP-PEST activity *in vivo*. HeLa cell lysates were prepared from control or TPA stimulated cells as described in Materials and methods. PTP-PEST was then immunoprecipitated from aliquots containing 150 μg total protein and assayed for the indicated times using 0.1 μM tyrosine phosphorylated RCML as substrate. The results shown are expressed as pmol phosphate released in the assay, and represent the mean ± SD from assays performed in triplicate.

and PKC, which are known to have overlapping substrate specificities (Pearson and Kemp, 1991). The sequence surrounding Ser39 (RRLSTK) is that of a typical PKA phosphorylation site in that it contains two adjacent basic residues, N-terminal to, and separated by a single amino acid from, the phosphorylated serine. Ser435 has only a single basic amino acid to the N-terminal side (RNLSFEIKK). This is somewhat atypical for a PKA phosphorylation site, but a similar arrangement is found in the sequence around site 2 in glycogen synthase (RTLSVSS), a well established site of PKA phosphorylation (Embi *et al.*, 1981). The substrate specificity of PKC is rather less well defined than that of PKA, but PKC phosphorylation sites frequently have basic residues at both the N-terminal and C-terminal sides of the phosphorylated residue (Pearson and Kemp, 1991), a feature shared by both Ser39 and Ser435. The position of these phosphorylation sites in the structure of PTP-PEST is shown in diagrammatic form in Figure 10.

The inhibition of PTP-PEST observed following phosphorylation by PKA *in vitro* resulted from an increase in the apparent K_m of the enzyme for the substrate, while the V_{max} for the reaction was largely unaffected. This phosphorylation therefore affects enzyme activity predominantly against substrates present at concentrations below the K_m value. Since tyrosine phosphorylation is a relatively rare cellular event, it is highly likely that the typical intracellular concentrations of many phosphotyrosine-containing proteins are well below the K_m of PTP-PEST. An increase in K_m following the phosphorylation of PTP-PEST *in vivo* may therefore be expected to have a significant effect on the overall pattern of phosphotyrosine-containing proteins within the cell.

The effect of PTP-PEST phosphorylation on enzyme activity was found to be due to phosphorylation of Ser39, since a similar effect on activity was observed using a truncated form of PTP-PEST which was exclusively phosphorylated at Ser39. This result suggests that Ser435 phosphorylation has no direct effect on enzyme activity although it is also possible that phosphorylation of this site might affect activity against a different substrate. Alternatively, Ser435 phosphorylation could conceivably affect PTP-PEST activity indirectly, perhaps by influencing the proposed PEST sequence-directed degradation of the protein. Since the substrates for PTP-PEST *in vivo* are as yet unknown, the physiological effect of phosphorylation at these sites remains uncertain.

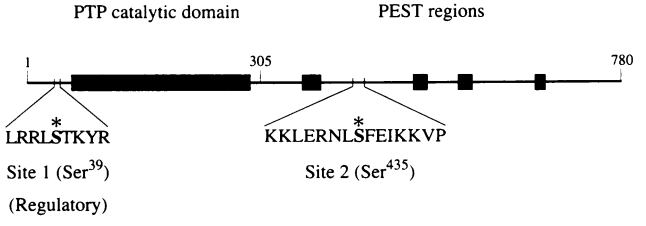

Fig. 10. Arrangement of PKA/PKC phosphorylation sites on PTP-PEST. The sequences surrounding the major PKA and PKC phosphorylation sites on PTP-PEST, and the positions they occur in the amino acid sequence of PTP-PEST, are shown. The phosphorylated residues are marked with an asterisk. Also shown are the positions of the PTP catalytic domain (filled bar), and the four PEST sequences (stippled bars).

The structural basis for the inhibition of PTP-PEST by Ser39 phosphorylation is unclear. Since phosphorylation had a negligible effect on the V_{max} of the reaction while reducing the affinity of the enzyme for its substrate, one possibility is that the phosphorylation site occurs at, or near to, a region of the enzyme involved in substrate binding, sterically hindering the enzyme–substrate interaction. The sequence of the N-terminal 300 amino acid residues of PTP-PEST is similar to that of PTP1B (33% amino acid identity), whose three-dimensional structure was recently determined by X-ray crystallography (Barford *et al.*, 1994). The structure revealed a clustering of basic residues on the surface of PTP1B surrounding the active site cleft. This is thought to account for the preference of PTP1B for substrates in which the phosphorylated tyrosine is adjacent to acidic residues (Hippen *et al.*, 1993), as found in many physiological sites of tyrosine phosphorylation (Pearson and Kemp, 1991). Interestingly, the region of PTP1B N-terminal to the catalytic domain is situated in close proximity to the presumed substrate binding surface. Assuming that PTP-PEST has a similar structure in this area to PTP1B, introduction of a negatively charged phosphate group at Ser39 in this region might be expected to interfere with the binding of substrates, resulting in the observed higher K_m of phosphorylated PTP-PEST compared with the non-phosphorylated form.

In HeLa cells grown in the presence of serum, PTP-PEST was predominantly phosphorylated on Ser435, while there was no phosphorylation of Ser39 under these conditions. Since both PKA and PKC phosphorylated Ser39 to a greater extent than Ser435 *in vitro* it is possible that a different protein kinase is responsible for Ser435 phosphorylation in asynchronously growing cells. Stimulation of HeLa cells with TPA, forskolin or IBMX resulted in phosphorylation of Ser39, accompanied by a slight net increase in Ser435 phosphorylation. This result demonstrates the ability of both PKC and PKA to phosphorylate *in vivo* the two major *in vitro* phosphorylation sites on PTP-PEST. In addition, immunoprecipitated PTP-PEST from TPA stimulated cells had lower activity than enzyme obtained from untreated cells, indicating that signal transduction pathways acting through PKC or PKA can potentially regulate the activity of PTP-PEST *in vivo*. Several recent observations suggest that the interplay between reversible serine/threonine and tyrosine phosphorylation may be a common feature of many signal transduction pathways. Our results indicate that PTP-PEST represents a novel physiological target for PKA or PKC through which these protein serine/threonine kinases may modulate cellular phosphotyrosine levels.

Materials and methods

Generation of baculoviruses, PTP-PEST expression and purification

cDNA encoding the entire sequence of PTP-PEST was subcloned into the baculovirus transfer vector pVL1393 using *Bam*HI and *Nsi*I restriction sites present outside of the coding sequence. A truncated cDNA encoding amino acid residues 1–305 inclusive, followed immediately by an in-frame stop codon, was generated by PCR amplification of this cDNA, and subsequently subcloned into the transfer vector pVL 1392; the absence of base incorporation errors resulting from the PCR amplification was verified by DNA sequencing. Recombinant baculoviruses encoding both full-length (FL PTP-PEST) and truncated (CD PTP-PEST) forms of the protein were then generated using the BaculoGold transfection

kit (Pharmingen, San Diego, CA). The recombinant baculoviruses were used to infect Sf9 cells (ATCC no. CRL 1711), which were grown in Grace's supplemented insect medium (GIBCO, Gaithersburg, MD) in the presence of 10% serum and 10 μg/ml gentamicin. Cells were harvested 4 days after infection, PTP-PEST was then purified from the cell lysate by successive FPLC on S-Sepharose Fast Flow and MonoQ columns (Pharmacia, Uppsala, Sweden).

Phosphorylation of PTP-PEST in vitro and assays of PTP activity

PKC and PKA were generously provided by Dr A.Nairn (Rockefeller University, New York) and Dr J.Scott (Vollum Institute, Portland, OR) respectively. Phosphorylation reactions were carried out at 30°C in 20 mM Tris−HCl (pH 7.4) in the presence of 5 mM $MgCl_2$, 1 mM DTT, 50 μM [γ-^{32}P]ATP (sp. act. 100–1000 c.p.m./pmol) and ~0.1 U of protein kinase (1 U incorporates 1 nmol ^{32}P into histone H1 per minute at 30°C). PKC phosphorylations also contained 50 μg/ml phosphatidyl serine, 100 nM TPA and 2 mM $CaCl_2$. Reactions were terminated by the addition of SDS−PAGE sample buffer; the extent of phosphorylation was then assessed following SDS−PAGE and autoradiography. Phosphorylation of the synthetic peptide LRRLSTK was performed under similar conditions, the reaction in this case being terminated by spotting onto P81 paper (Whatman) followed by washing in 1% (v/v) phosphoric acid. Non-radioactive phosphorylations of PTP-PEST, for subsequent assays of enzyme activity, were performed in parallel with ^{32}P-phosphorylations under identical conditions. These reactions were terminated by 300-fold dilution of aliquots into 25 mM Tris−HCl, pH 7.2, containing 30% (w/v) glycerol, 1 mg/ml BSA and 1 mM DTT, followed immediately by freezing the sample in dry ice/ethanol. These samples were stored at −70°C until required for assay; under these conditions, enzyme activity was stable for at least 1 month. Prior to assaying enzyme activity, the samples were diluted a further 10- to 50-fold in PTP assay buffer (25 mM Tris−HCl, pH 7.2, 1 mg/ml BSA, 1 mM DTT). Assays were performed as described previously (Flint *et al.*, 1993) using tyrosine phosphorylated, ^{32}P-labelled RCM-lysozyme as the substrate. One unit of enzyme activity catalyses the release of 1 nmol phosphate in 1 min at 30°C.

Phosphopeptide analysis by two-dimensional mapping, HPLC and protein sequencing

Following SDS−PAGE, macerated gel slices containing ^{32}P-phosphorylated PTP-PEST were incubated at 37°C for 15 h with 50 μg/ml trypsin (Worthington, Freehold, NJ) in 400 μl 0.1 M ammonium acetate, and the soluble phosphopeptides recovered following centrifugation at 10 000 r.p.m. The peptide yield was increased by a 2 h extraction with 400 μl water, resulting in 80–90% of the total initial radioactivity being recovered as soluble phosphopeptides. ^{32}P-phosphorylated synthetic peptide was digested with trypsin following extraction of the phosphopeptide from P81 paper with 0.1 M ammonium acetate. Two-dimensional phosphopeptide mapping was carried out as described by Boyle *et al.* (1991); electrophoretic separation was at pH 1.9 for 40 min at 1000 V, ascending chromatography was in butanol:pyridine:acetic acid:water (15:10:3:12).

[^{32}P]phosphopeptides were purified for protein sequencer analysis by reversed phase HPLC using C_8 and C_{18} columns. Samples were applied in 0.1% (v/v) trifluoroacetic acid (TFA), and the column was then washed with the same buffer until the eluate contained no detectable radioactivity. Phosphopeptides were then eluted with a linear gradient of acetonitrile containing 0.1% (v/v) TFA. The position of ^{32}P-phosphorylated residues in purified phosphopeptides was determined using an Applied Biosystems 473 protein sequencer following C-terminal covalent coupling of the peptide to the sample filter as described by Russo *et al.* (1992). Direct protein sequencing of purified phosphopeptides was carried out using an Applied Biosystems 477A sequencer.

Culture and labelling of HeLa cells and preparation of cell lysates

HeLa cells were cultured to 70% confluence in 6 cm plates at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). The cells were washed with phosphate-free DMEM and incubated for 30 min in 1.5 ml phosphate-free DMEM with 5% FBS prior to the addition of 1–5 mCi of [^{32}P]inorganic phosphate (NEN, Boston, MA). Cells were incubated for 4–5 h at 37°C and then either harvested or treated with TPA (180 ng/ml), forskolin (100 μM) or isobutyl methyl xanthine (IBMX) (5 mM) for 15 min at 37°C prior to harvesting. Labelled cells were washed twice in ice-cold PBS, and then duplicate plates were lysed together in 0.9 ml of buffer comprising 50 mM Tris−HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM NaF, 0.5 μM okadaic acid, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) SDS. The lysate was precleared prior to immunoprecipitation by addition of 100 μl IgGsorb (Enzyme Centre, Malden, MA) reconstituted in PBS, followed by rocking at 4°C for 30 min and centrifugation at 15 000 *g* for 5 min at 4°C. Lysates were also prepared under identical conditions from unlabelled cells in order to allow PTP-PEST activity to be assessed following immunoprecipitation.

Immunoprecipitation of PTP-PEST

PTP-PEST protein, expressed in Sf9 cells and purified as described above, was used to generate a polyclonal mouse antiserum following standard procedures. The serum was precoupled to protein A−Sepharose beads (Pharmacia, Uppsala, Sweden) (20 μl serum/50 μl beads) prior to use for immunoprecipitation of PTP-PEST. Cell lysates were rocked with 20 μl of coupled antibody for 15 h. Immune complexes were then collected by centrifugation at 1000 *g* for 15 s. Immunoprecipitates were then washed four times with 1 ml of buffer comprising 50 mM Tris−HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10% (v/v) glycerol and 1% (v/v) Triton X-100. The washed immune complexes from labelled cells were solubilized in SDS sample buffer for analysis of [^{32}P]PTP-PEST by SDS−PAGE and autoradiography. Immunoprecipitates prepared from unlabelled cells were used for assays of PTP-PEST activity.

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