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Protein phosphatase 1α associates with protein tyrosine phosphatase-PEST inducing dephosphorylation of phospho-serine 39

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Protein tyrosine phosphatase (PTP)-PEST is expressed in a wide variety of several cell types and is an efficient regulator of cell adhesion, spreading and migration. PTP-PEST-associating molecules are important in elucidating the function of PTP-PEST. Herein, we have identified protein phosphatase 1α (PP1α) as a novel PTP-PEST binding protein, and then we aimed to determine how PP1α contributes to the phosphorylation at Ser39 of PTP-PEST, whose phosphorylation suppresses PTP-PEST enzymatic activity. The HEK 293 cells overexpressing exogenous PTP-PEST were stimulated by 12-O-tetradecanoylphorbol 13-acetate (TPA) and the phosphorylation of PTP-PEST at Ser39 was evaluated using an anti-phospho-Ser39 PTP-PEST specific antibody (anti-pS39-PEST Ab). It was demonstrated that the phosphorylation at Ser39 detected by anti-pS39-PEST Ab was dependent on TPA treatment and a significant inverse correlation between the PTP activity of PTP-PEST and anti-pS39-PEST Ab-immunoreactive band intensity. The phosphorylation of Ser39 was suppressed by co-transfection of a plasmid encoding wild-type PP1α, but not by that of the dominant-negative PP1α mutant. Furthermore, TPA-induced phosphorylation could take place in PTP-PEST catalytic domain, but the phosphorylation of PTP-PEST catalytic domain could not be abrogated by co-transfection of a plasmid expressing wild-type PP1α. In conclusion, PP1α associates with the non-catalytic domain of PTP-PEST and regulates PTP activity via dephosphorylation of phospho-Ser39.

Keywords: Dephosphorylation/PP1α/PTP activity/PTP-PEST/yeast two-hybrid system.

Abbreviations: Ab, antibody; PBS, phosphate buffer saline; PP1α, protein phosphatase 1α; PTP, protein tyrosine phosphatase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

Protein tyrosine phosphorylation is an essential aspect of the regulation of a large number of diverse cellular events involved in growth, division, activation and differentiation. The opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) determine the net tyrosine phosphorylation state of cellular proteins. This suggests equally important roles for both PTKs and PTPs in the control of cellular events regulated by tyrosine phosphorylation. The PTPs, just as PTKs, are a structurally diverse family of enzymes that include receptor-type and soluble-type forms. They both have highly conserved catalytic domain but differ in their non-catalytic segments. The structural diversity presumably reflects differences in the regulation and function of each enzyme. It has been suggested that PTP activity must be stringently controlled within cell, in order to allow protein tyrosine phosphorylation that is necessary for cellular signaling by PTKs.

Protein tyrosine phosphatase-PEST (PTP-PEST), encoded by the *PTPN*12 gene, belongs to a subgroup of soluble-type PTPs, which also contains PEP/LYP (*PTPN*22) and PTP-HCSF (*PTPN*18). They all consist of an N-terminal PTP domain and an extended C-terminus with proline-rich motifs. In contrast to the restricted expression of PEP and PTP-HCSF, PTP-PEST is ubiquitously expressed, with higher levels found in haemopoietic tissues (*1*). A significant insight into the function of PTP-PEST has been provided by studies on fibroblast cells and in consequence PTP-PEST acts on integrin-mediated signal transduction such as cell adhesion, spreading and migration (*2*–*5*). The studies carried out in relation with PTP-PEST overexpression and PTP-PEST substrate-trapping mutants demonstrated that p130Cas is a major physiological substrate for PTP-PEST in non-lymphoid cells (*6*, *7*). PTP-PEST-deficient MEFs result in the hyperphoshorylation of p130Cas and exhibit a characteristic enhancement of migration phenotype (*2*), whereas overexpression of PTP-PEST abrogates motility (*5*). Other substrates have been surveyed by substrate-trapping or by direct association with PTP-PEST. Consequently, many proteins related to cytoskeletal and focal adhesion proteins such as Pyk2 (Cakβ, Fak2, RAFTK) (*8*), paxillin (*9*), Vav2 (*10*), p190RhoA GAP (*10*), c-Abl (*11*), PSTPIP (proline/serine/threonine-phosphatase-interacting protein) (*12*), WASP (Wiskott–Aldrich syndrome protein) (*12*) and others have been identified

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as PTP-PEST substrates. On the other hand, recent studies have also emphasized the importance of PTP-PEST in osteoclasts and lymphocytes. In osteoclasts, the inhibition of PTP-PEST was accompanied by an increase in tyrosine phosphorylation of WASP and other associated signalling molecules. These experiments indicate the involvement of PTP-PEST in sealing ring formation and bone resorption (*13*). In the B cell line A20, PTP-PEST is an efficient negative regulator of antigen receptor signalling as it reduces the phosphorylation of Shc, Pyk2, Fak and p130Cas (*1*). In T cells, PTP-PEST was found to dephosphorylate the WASP and the Lck kinase at its activation loop site Y394, resulting in the suppression of WASP-driven actin polymerization, synapse formation and TCR-signaling (*14*, *15*). These physiological roles of PTP-PEST are functionally coupled to PTP activity against a restricted set of tyrosine-phosphorylated substrates in several cell types. Despite the functional importance of PTP activity, little is known about the molecular mechanism and regulation of PTP-PEST enzymatic activity.

Here we have identified protein phosphatase 1α (PP1α) as a novel PTP-PEST-associated molecule by a yeast two-hybrid system to gain a better understanding of the function of PTP-PEST. PP1α is a member of protein serine/threonine-specific phosphatase family. Since the enzymatic activity of PTP-PEST is reported to be down-regulated by its serine residue at position 39 by protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) (*16*), we have examined the possible contribution of PP1α to the phosphorylation at Ser39 of PTP-PEST. Using an anti-phospho-Ser39 (pS39) PTP-PEST antibody (PTP-PEST Ab), we have found that PP1α selectively dephosphorylates pS39 of PTP-PEST and its dephosphorylation is required for PTP-PEST and PP1α binding. Our results strongly suggest that PP1α associates and regulates PTP-PEST enzymatic activity via dephosphorylation.

Experimental procedures

Generation of anti-pS39-phosphopeptide antibody and other antibodies

Phospho-specific antibody directed against the phosphorylated serine residue of PTP-PEST at position 39 was produced by immunizing rabbits with the following synthetic pS39-phosphopeptide, [^{36}RRL(pS^{39})TKYRTEK^{46}C] coupled to keyhole limpet haemocyanin. Enzyme-linked immunosorbent assay, using the pS39-phosphopeptide and corresponding non-phosphopeptide (^{36}RRLSTKYRTEK^{46}C), was employed to identify the rabbits responding best. Anti-pS39-phosphopeptide IgG was purified using the pS39-phosphopeptide affinity column, and the antibody reactive with the non-phosphopeptide was removed by adsorption to a non-phosphopeptide affinity column. The resulting antibody was characterized by an enzyme-linked immunosorbent assay against pS39-phosphopeptide and non-phosphopeptide to determine the extent of pS39-phosphopeptide-specificity, and subsequently by immunoblotting to examine the specificity against PTP-PEST from 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-treated or not treated cells ('Results'; Fig. 2A). The anti-PP1α Ab was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The anti-Flag M2 mAb was purchased from Sigma-Aldrich (St Louis, MO, USA). The anti-PTP-PEST Ab was kindly provided by Dr H. Yakura. The alkaline phosphatase (AP)-conjugated anti-rabbit IgG and AP-conjugated anti-mouse IgG were from Jackson Immunoresearch Laboratories (West Grove, PA, USA).

Screening of molecules that associate with PTP-PEST and plasmid construction

To isolate PTP-PEST-associated molecules, we used the DupLEX-A^{TM} yeast two-hybrid system (Origene Technology Inc.). For a bait, a fragment that encodes the non-catalytic domain of PTP-PEST (amino acids 304–775) was amplified by PCR using the PTP-PEST cDNA as a template. Primers were designed to create a 5′ *Bam* HI site and 3′ Not I site, and the fragment was inserted between Bam HI and Not I sites of pEG202 to express a fusion protein with LexA. Approximately 1×10^6 independent clones of mouse spleen cDNA library (Origene Technology Inc.) were screened using EGY48 yeast cells, and cDNA clones that interacted with the PTP-PEST non-catalytic domain were selected by growth on Leu-deficient plates. Approximately 200 positive clones on Leu-deficient plates were further screened for *LacZ* expression with pSH18-34 plasmid. Finally, we obtained 16 positive clones of which the nucleic acid sequences were determined.

For the expression of a His-tagged PTP-PEST protein in mammalian cells, a *Bam* HI site at the 5′ end and a *Xba* I site at the 3′ end of the mouse PTP-PEST cDNA full length or catalytic domain (amino acids 1–300) were introduced by a polymerase chain reaction (PCR), and the fragment was subcloned into the *Bam* HI and *Xba* I sites of the pcDNA3.1 Myc-His(−) vector in frame with the His-tag coding sequence. PP1α cDNA isolated by yeast two-hybrid screening was subcloned into the *Eco*RI and *Xho* I sites of the pCMV-Tag2B vector in frame with the Flag-tag coding sequence. To generate dominant negative PP1α (*17*), a point-mutated PP1α from aspartic acid at 95 to asparagine (PP1α D95N) was introduced by PCR-directed mutagenesis. The primers used were as follows:

PP1α forward primer: 5′-TGGGGGATTATGTAAATCGGGGCA AGCAG-3′;
PP1α reverse primer: 5′-CTGCTTGCCCCGATTTACATAATCC CCCA-3′.

The underlined base pairs represent the mutated amino acid. Collected clones were verified by DNA sequencing.

Cell culture, transfection and TPA-treatment

HEK 293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated FCS and antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) in a 5% CO_2 incubator. Transfections were performed using PolyFect transfection reagent (Qiagen Inc., Valencia, CA, USA) according to the manufacture's recommendations. For co-expression of PTP-PEST and PP1α, transfections were performed at a 1:2 ratio for PTP-PEST- and PP1α-expresion plasmids, respectively. For TPA stimulation, 40 h post-transfection, cells were cultured for 2 h in serum-free media and then treated with TPA for 15 min at 37°C.

Immunoblot analysis

HEK 293 cells were harvested with phosphate buffer saline (PBS), centrifuged at 1,000*g* for 5 min and lysed in the lysis buffer (10 mM Tris–HCl, pH 7.5, 1% Nonidet P-40, 0.15 M NaCl, 10 mM NaF, 2 mM Na_3VO_4, 10 mM $Na_4P_2O_7$ and 25 μg ml^{-1} benzylsulphonyl fluoride). Insoluble materials were removed by centrifugation for 20 min at 16,000*g*, and the resultant lysate was fractionated by SDS–PAGE and transferred to a PVDF membrane where the membrane-bound proteins were detected by immunoblot analysis with the appropriate antibodies. The fluorogenic substrate, DDAO (9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) phosphate (Molecular Probes, Eugene, OR, USA), was used for detection by the Typhoon 9210 (Laser excitation, 633 nm: Fluorescence filter, 670BP30) (GE Healthcare UK Ltd, Buckinghamshire, England). The densities of bands were analysed with ImageQuant software (Molecular Dynamics).

Phosphatase activity assay

For PTP activity of PTP-PEST *in vitro*, HEK 293 cells expressing His-tagged PTP-PEST were collected and sonicated in PBS for 30 s. Cell lysates were centrifuged for 20 min at 16,000*g*, and His-tagged PTP-PEST in supernatants were pulled down by Ni Sepharose 6 Fast Flow (GE Healthcare). Half of the PTP-PEST pull downs was processed for immunoblot analysis, and with the remaining half the PTP activity measured in 100 μl of 20 mM Hepes buffer (pH 7.0) containing 5 mM *p*-nitrophenylphosphate (*p*NPP),

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150 mM NaCl and 1 mM DTT at 37°C for 10 min. The production *p*-nitrophenol was measured colorimetrically at 405 nm.

For enzyme activity of PP1α against PTP-PEST *in vitro*, PP1α was immunoprecipitated with anti-Flag mAb from cell lysate containing Flag-tagged PP1α, and the phosphorylated PTP-PEST was pulled down from the lysate of TPA-treated cells. The reaction was started by mixing the immunoprecipitated PP1α with the pulled down phospho-PTP-PEST in 50 mM Tris–HCl buffer (pH 7.4) containing 0.1 mM EGTA and 1 mM DTT. After incubating for 30 min at 37°C, the reaction was stopped on the addition of SDS–PAGE loading buffer and the product was subjected to immunoblot analysis with anti-PTP-PEST and anti-pS39-PEST Abs.

Results

Isolation of cDNA encoding PTP-PEST-binding proteins

PTP-PEST is ubiquitously expressed and has a large non-catalytic C-terminus containing proline-rich sequences that mediate interactions with substrates/adaptors. In order to clarify the molecular basis for PTP-PEST cellular function, we attempted to isolate the PTP-PEST-binding proteins by the yeast two-hybrid screening, after which we utilized PTP-PEST non-catalytic segment (amino acid residues 304–775) fused bait protein LexA for screening cDNA encoding PTP-PEST-binding proteins on mouse spleen cDNA library. Sequences of seven clones out of 16 positives were identical to a part of a previously reported cDNA as PTP-PEST-associating molecules, Hic-5 and Leupaxin (*18*, *19*). A sequence of one clone was identified to be protein phosphatase 1α (PP1α), which has not yet been reported as the PTP-PEST-binding protein.

To validate the findings from yeast two-hybrid screening, we utilized an *in vivo* binding assay involving exogenous PTP-PEST and PP1α-expressed mammalian cells. HEK 293 cells were transiently transfected with plasmids encoding His-tagged PTP-PEST and Flag-tagged PP1α, as indicated in Fig. 1. PTP-PEST and PP1α expression in transfected cells was verified by immunoblotting with anti-PTP-PEST Ab and anti-Flag mAb (Fig. 1A). The binding capacity of PP1α to PTP-PEST was examined by pull-down experiments using a His-tagged PTP-PEST. In cells expressing both PTP-PEST and PP1α, we found that a significant amount of PP1α was recovered (Fig. 1B).

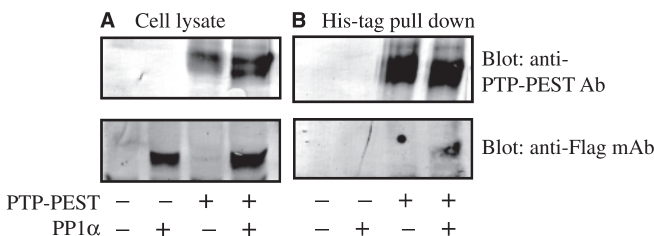

Fig. 1 PP1α physically interacts with PTP-PEST. (A) For *in vivo* binding assays, HEK 293 cells were transiently co-transfected with the indicated combinations of Flag-tagged PP1α and His-tagged PTP-PEST for 40 h. PTP-PEST and PP1α expression was verified by immunoblot analysis with anti-PTP-PEST Ab (upper panel) and anti-Flag mAb for PP1α (lower panel). (B) Pull downs of His-tagged PTP-PEST with Ni–sepharose were blotted with anti-PTP-PEST Ab (upper panel) and anti-Flag mAb for PP1α (lower panel). Similar results were reproduced in two independent experiments.

In comparison, there was no detection of the PP1α band in cells transfected with PP1α or PTP-PEST alone. These results are in accordance with the yeast two-hybrid data, demonstrating the association between PTP-PEST and PP1α *in vivo*.

Detection of PTP-PEST phosphorylation at Ser39 by anti-pS39-PEST Ab

It was also previously reported that PTP-PEST was phosphorylated at Ser39 by PKC and PKA *in vivo*, and the phosphorylated PTP-PEST at Ser39 decreased its activity by reducing its affinity for substrate (*16*). Thus, we generated an antibody against pS39 of PTP-PEST by immunizing rabbits with synthetic pS39-phosphopeptide, and affinity purified by the same phosphopeptide-coupled beads. Moreover, the specific antibody against pS39 of PTP-PEST (anti-pS39-PEST Ab) was isolated by removing nonphosphopeptide-reactive IgG with nonphosphopeptide-coupled beads. To verify the detection ability, the anti-pS39-PEST Ab was compared with the immunoblot of anti-PTP-PEST Ab. Herein, His-tagged PTP-PEST pulled down from each lysate of the TPA-treated HEK 293 cells was subjected to immunoblot analysis with anti-PTP-PEST and anti-pS39-PEST Abs (Fig. 2A). PTP-PEST bands were of almost the same levels, with only slight emerging pS39 bands observed in the TPA-untreated cells. On the other hand, in the TPA-treated cells, the band intensity of pS39 significantly increased dependent to the concentration. As expected, the anti-pS39-PEST Ab-immunoreactive band was not blocked by the non-phosphopeptide, however, hardly detected in the presence of its competitor, pS39-phosphopeptide, and in the Ser39-to-Ala mutant of PTP-PEST (data not shown). These results indicated that anti-pS39-PEST Ab specifically recognizes the pS39 of PTP-PEST. Additionally, we evaluated the relative correlation between PTP activity of PTP-PEST and the phosphorylation of Ser39, detected by anti-pS39-PEST Ab. HEK 293 cells expressing PTP-PEST were treated with TPA at several concentrations and PTP-PEST pulled down from each cell lysate was subjected to both immunoblotting analysis and the assay of PTP activity. The plots of the PTP activity against the relative phosphorylation of Ser39 calculated using immunoblotted bands are shown in Fig. 2B. In consequence, a decrease in the PTP activity that was the inverse proportion of the relative phosphorylation of PTP-PEST, indicating the anti-pS39-PEST Ab-immunoreactive band as a marker of the PTP-PEST enzyme activity.

PP1α dephosphorylated pS39 of PTP-PEST in vitro and in vivo

Here we report that PTP-PEST associated with PP1α, which is a member of serine/threonine specific protein phosphatase family. Therefore, to test the hypothesis that PP1α hydrolyzes the pS39 of PTP-PEST, we examined the phosphatase activity of PP1α against pS39 of PTP-PEST *in vitro* and *in vivo*. First of all, we evaluated *in vitro* the phosphatase activity of PP1α using pulled down phosphorylated PTP-PEST

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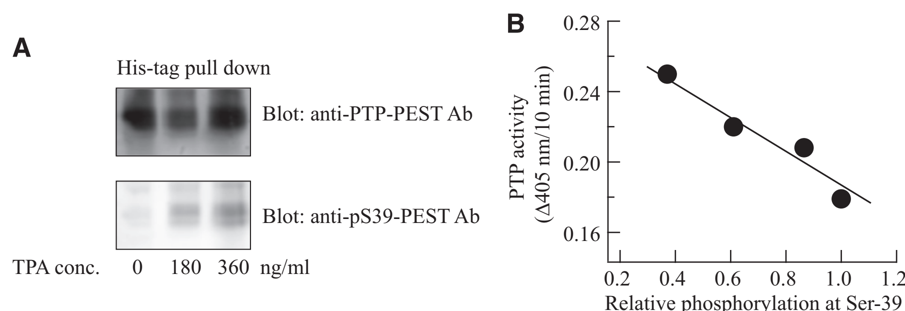

Fig. 2 Characterization of anti-pS39 PEST AB. (A) HEK 293 cells transfected with His-tagged PTP-PEST for 40 h were cultured in serum-free media for 2 h before TPA treatment at 0, 180 and 360 ng ml^{-1} for 15 min. Pull downs of His-tagged PTP-PEST with Ni–sepharose were applied to immunoblot analysis with anti-PTP-PEST Ab (upper panel) and anti-pS39-PEST Ab (lower panel). (B) To determine the relationship between the relative phosphorylation at Ser39 and the PTP activity, HEK 293 cells expressing His-tagged PTP-PEST were stimulated by TPA at several concentrations (50–400 ng ml^{-1}) for 15 min. Half of the pulled down PTP-PEST was subjected to immunoblot analysis with anti-PTP-PEST and anti-pS39-PEST Abs, and the relative phosphorylation of PTP-PEST was calculated by normalizing PTP-PEST band intensity against the band intensity of pS39. The PTP activity was measured with remaining half of the pulled down PTP-PEST using *p*NPP as a substrate, where the relative phosphorylation of PTP-PEST in cells treated with 400 ng ml^{-1} TPA was expressed as 1.

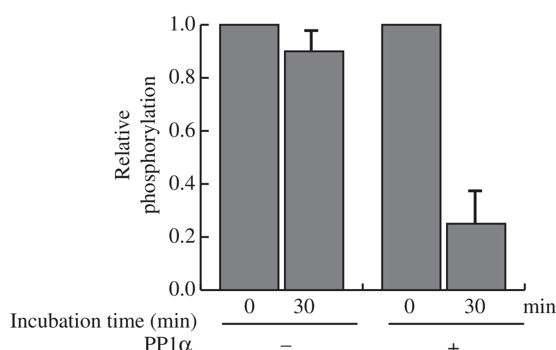

Fig. 3 PP1α dephosphorylates pS39 of PTP-PEST *in vitro*. To determine the phosphatase activity of PP1α against pS39 *in vitro*, the phosphorylated PTP-PEST prepared from cells treated with 360 ng ml^{-1} of TPA was mixed with anti-Flag immunoprecipitates. After 30 min incubation at 37°C, PTP-PEST was subjected to immunoblot analysis with anti-PTP-PEST and anti-pS39-PEST Abs. The relative phosphorylation of PTP-PEST at Ser39 was based on the band intensity as described in Fig. 2. Results represent the mean ± SE of values obtained from triplicate experiments.

and immunoprecipitate of PP1α (Fig. 3). When the phospho-PTP-PEST was incubated with or without PP1α, the pS39 of PTP-PEST was sufficiently dephosphorylated in the presence of PP1α and not affected in the absence of PP1α, showing that PP1α has an ability to dephosphorylate pS39 of PTP-PEST *in vitro*. Next, to elucidate phosphatase activity of PP1α against pS39 of PTP-PEST *in vivo*, HEK 293 cells co-transfected with the combinations of plasmids, as shown in Fig. 4A, were stimulated by TPA, and PTP-PEST was pulled down with Ni–sepharose and subjected to immunoblotting with anti-PTP-PEST and anti-pS39-PEST Abs, respectively (Fig. 4A). Immunoblot analysis with anti-PTP-PEST Ab showed that the PTP-PEST protein levels were equivalent in each of the samples; however, the pS39 levels were significantly reduced in PP1α-overexpressing cells (Fig. 4B). These results suggest that the hydrolysis activity of PP1α was involved in the diminution of the TPA-induced phosphorylation at Ser39 of PTP-PEST. To further examine the participation of PP1α enzymatic activity in the phosphorylation at Ser39, we tested whether TPA-induced pS39 was affected by replacing PP1α wild-type (wt) with dominant-negative PP1α (D95N) (Fig. 4C). In contrast to PP1α wt, PP1αD95N-overexpressing cells showed no reduction of pS39 (Fig. 4D). Taken together, these results suggest that PP1α dephosphorylates the pS39 of PTP-PEST *in vitro* and *in vivo*.

Dephosphorylation by PP1α required to PTP-PEST non-catalytic domain

Since PP1α is associated with PTP-PEST via its non-catalytic domain, we postulate that the binding of PP1α to the non-catalytic domain of PTP-PEST is prerequisite for hydrolysing the pS39. To test this hypothesis, we carried out experiments by replacing PTP-PEST full length (FL) with PTP-PEST catalytic domain (CD) (amino acid residues 1–300) since no interaction between PTP-PEST CD and PP1α was ascertained with *in vivo* binding experiments (data no shown). First, we determined whether PKC phosphorylates PTP-PEST CD at Ser39. In order to do this, PTP-PEST CD was purified by Ni–sepharose from TPA-treated cells, and subjected to immunoblot analysis (Fig. 5A). In consequence, the phosphorylation at Ser39 was dependent on TPA treatment in PTP-PEST CD (Fig. 5B), which was also observed in PTP-PEST FL (Fig. 2A), in agreement with the previous report (*16*). HEK 293 cells transfected with PTP-PEST plus/minus PP1α-expression plasmids were stimulated by TPA, and the pS39 levels in PTP-PEST CD was evaluated by immunoblot analysis (Fig. 5C). In consequence, the pS39 content of PTP-PEST CD was not significantly reduced by PP1α wt overexpression (Fig. 5D). These results show that PP1α contributes to the dephoshorylation of Ser39 via binding to PTP-PEST non-catalytic segment. Considering an inverse correlation between relative phosphorylation at Ser39 and PTP activity of PTP-PEST, PP1α can potentially contribute to the regulation of PTP-PEST enzymatic activity.

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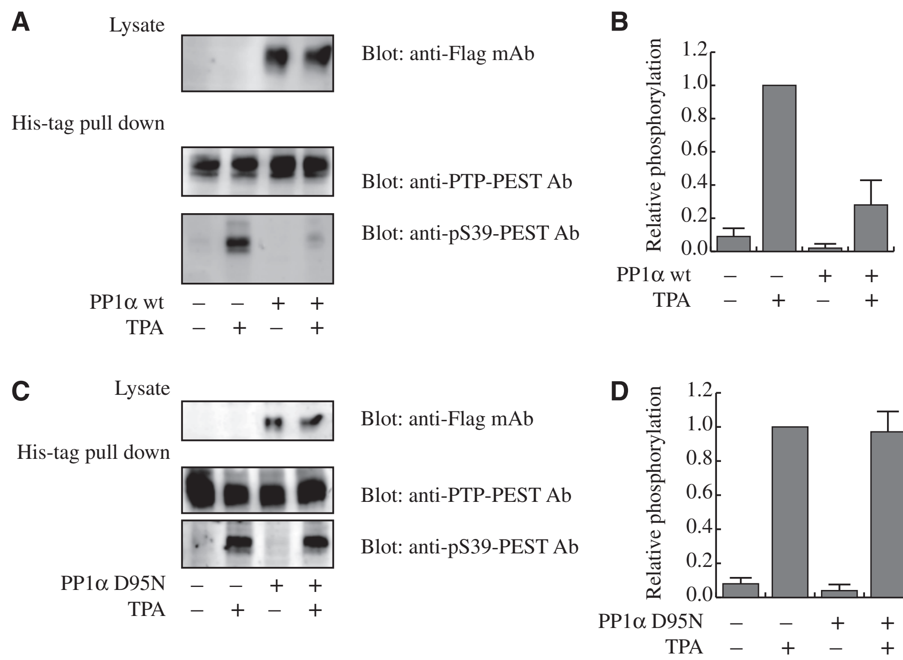

Fig. 4 PP1α suppresses phosphorylation of PTP-PEST at Ser39. (A) HEK 293 cells were transiently co-transfected with PTP-PEST alone or with PP1α wt for 40 h, after which cells were then cultured in serum-free media for 2 h. Co-transfected HEK 293 cells were treated (+) or not treated (–) with 180 ng ml^{-1} of TPA for 15 min. The expression of PP1α wt was verified by blotting with anti-Flag mAb (upper panel). Pull downs of His-tagged PTP-PEST with Ni–sepharose were blotted with anti-PTP-PEST Ab (middle panel) and anti-pS39-PEST Ab (lower panel). (B) The relative phosphorylation of PTP-PEST at Ser39, as shown in A, was determined by the band intensity described in Figure 2. The relative phosphorylation of cells not expressing PP1α wt, treated with 180 ng ml^{-1} of TPA for 15 min, was expressed as 1. The graph represents the mean ± SD of the relative phosphorylation from triplicate experiments. (C) The same experiments were carried out with the replacement of PP1α wt with dominant-negative PP1α (PP1α D95N). After confirmation of PP1α D95N expression (upper panel), pull downs of His-tagged PTP-PEST with Ni–sepharose were subsequently applied to immunoblot analysis with anti-PTP-PEST Ab (middle panel) and anti-pS39-PEST Ab (lower panel). (D) The relative phosphorylation of PTP-PEST at Ser39, as shown in C, was determined by the band intensity described in Fig. 2. The relative phosphorylation of cells not expressing PP1α D95N, treated with 180 ng ml^{-1} of TPA for 15 min, was expressed as 1. The graph presents the mean ± SD of the relative phosphorylation from triplicate experiments.

Discussion

Serine residues of PTP-PEST at positions 39 and 435 are the major phosphorylation sites *in vitro* by the PKC and PKA. Interestingly, pS39 of PTP-PEST is the main phosphorylation target site of PKC and PKA *in vivo* and phosphorylation of Ser39 down-regulates its activity by reducing its affinity for substrate, while Ser435 is constitutively phosphorylated in intact cells and has no apparent effect on PTP activity (*16*). To gain a better understanding of the function and regulation of PTP-PEST in several cell types, we reveal that PP1α is a novel PTP-PEST-associating molecule. The binding of PP1α to PTP-PEST emphasizes the contribution of PP1α to the reversible phosphorylation of PTP-PEST. In the present study, we aimed to determine the role of PP1α in relation with the pS39 of PTP-PEST, using an anti-pS39-PEST Ab. Results from *in vitro* and *in vivo* experiments, implemented that pS39 of PTP-PEST was hydrolyzed as a consequence of PP1α. Furthermore, PP1α-mediated dephosphorylation of pS39 PTP-PEST was dependent on non-catalytic C-terminal half of PTP-PEST.

Protein serine/threonine phosphatases comprise a family, which are mainly divided into type I (PP1) and type II (PP2), and PP2s are further classified into at least three groups, PP2A, PP2B (also known as calcineurin) and PP2C according to substrate specificity and metal ion dependence. PP1α, a novel PTP-PEST-binding molecule, belongs to the PP1 group, which consists of four isoforms PP1α, PP1β (also known as PP1δ) and the splice variants PP1γ1 and PP1γ2. With the exception of PP1γ2, which is predominantly expressed in testis, the other isoforms PP1α, PP1β/δ and PP1γ1 appear to be expressed in all cell types, and these PP1s regulate many cellular processes by dephosphorylating various key proteins (*20*). PP1 isoenzymes including PP1α appear to be associated with various proteins that regulate its activity and determine when and where PP1 acts (*21*). In this study, we identify PP1α as a novel PTP-PEST binding protein, and concurrently pS39 of PTP-PEST is a good substrate of PP1α. The PP1 isoenzymes are more than 90% identical at the protein level, raising the possibility that other PP1 family members participate in PTP-PEST dephosphorylation at Ser39. The potential contribution of other members to dephosphorylation of PTP-PEST is now under investigation. Previous reports have suggested that the motifs comprised of the amino acids (R/K)(I/V)xF and FxxR/KxR/K are involved in PP1/protein interaction (*21*, *22*). PTP-PEST amino acid sequence analysis has revealed close matches to FxxR/KxR/K motif: residues 757–763 (<u>F</u>GN<u>R</u>CG<u>K</u>). In this case, there is an

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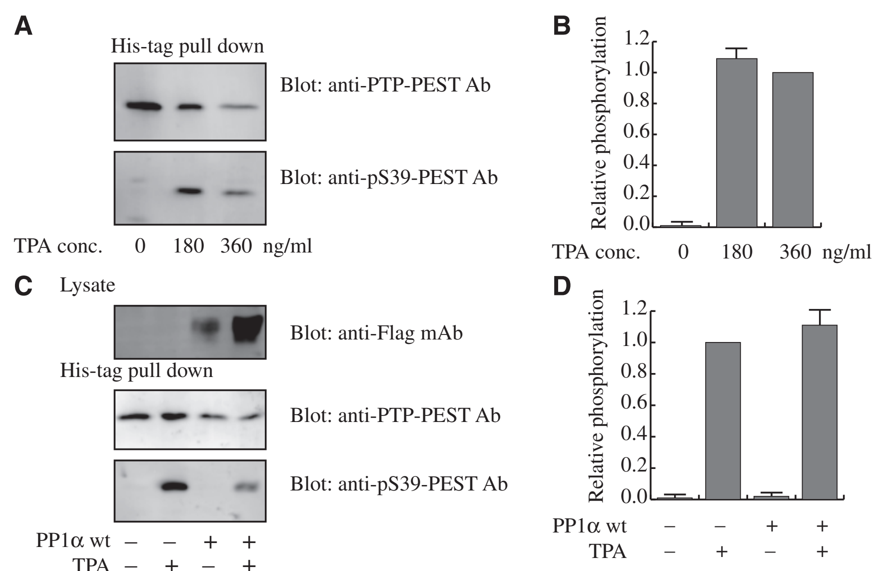

Fig. 5 Phosphorylation of PTP-PEST catalytic domain was not affect by PP1α. (A). HEK-293 cells transfected with His-tagged PTP-PEST CD for 40 h were then cultured in serum-free media for 2 h. Transfected cells were stimulated by TPA at 0, 180 and 360 ng ml^{-1} for 15 min. Pull downs of His-tagged PTP-PEST CD with Ni–sepharose were blotted with anti-PTP-PEST Ab (upper panel) and anti-pS39-PEST Ab (lower panel). (B) The relative phosphorylation of PTP-PEST CD at Ser39, as shown in (A), was determined by the band intensity as described in Figure 2. The relative phosphorylation of cells treated with 360 ng ml^{-1} of TPA was expressed as 1. The graph represents the mean ± SD of the relative phosphorylation from triplicate experiments. (C) HEK 293 cells co-transfected with His-tagged PTP-PEST CD alone or with PP1αwt for 40 h were then cultured in serum-free media for 2 h. Co-transfected HEK 293 cells were treated (+) or not treated (–) w. (B). ith 180 ng ml^{-1} of TPA for 15 min. PP1α wt expression was confirmed by blotting with anti-Flag mAb (upper panel). Pull downs of His-tagged PTP-PEST CD were blotted with anti-PTP-PEST Ab (middle panel) and anti-pS39-PEST Ab (lower panel). (D) The relative phosphorylation of PTP-PEST CD at Ser39, as shown in (C), was determined by the band intensity described in Fig. 2. The relative phosphorylation of cells not expressing PP1α wt, treated with 180 ng ml^{-1} of TPA, was expressed as 1. The graph represents the mean ± SD of the relative phosphorylation from triplicate experiments.

insertion of one residue within second and third conservative amino acids. Interestingly, the fragment of PTP-PEST (amino acid residues 420–775) containing this motif does not make a complex with PP1α using *in vitro* binding experiments (data not shown). The significance of this observation must await further mutagenesis studies.

PTP-PEST has a large non-catalytic C-terminus containing proline-rich motifs, which mediates interactions with substrates and/or adaptor/targeting proteins, belonging to the PEST motif PTP subgroup. PTP-PEST is expressed ubiquitously in numerous tissues and various cell types, including cells of the immune system, whereas the expression of the other member, PEP/LYP is restricted to leucocytes. In addition, *Ptp-pest*$^{-/-}$ mice die *in utero* (*23*), indicating that PTP-PEST has the fundamental biological role. To clarify the cellular function of PTP-PEST in several cell types, the substrates and/or binding molecules for PTP-PEST have been surveyed. In consequence, the focal adhesion protein, p130Cas, is identified as a major physiological substrate of PTP-PEST and the tyrosine phosphorylation of p130Cas is coupled to the regulation of cell motility. In addition, tyrosine-phosphorylated molecules, such as Shc, Fak, Pyk2, c-Abl, Vav2, p190RhoA, WASP, PSTPIP and others, are dephosphorylated by PTP-PEST. Among them, p130Cas, Shc, PSTPIP are directly associated with PTP-PEST, whereas Fak, Pyk2 and WASP might be recruited to PTP-PEST via adaptor/targeting molecules, such as the paxillin family (*8*, *24*, *25*) and PSTPIP (*12*); as a result, these molecules may become good substrates of PTP-PEST. In adherent cells, through these PTP-PEST substrates and associating molecules, PTP-PEST has a primary role in cytoskeletal rearrangement, that is, cell migration, cell spreading and cell division (*2*, *5*, *7*, *10*, *11*, *26*). In lymphocytes, since many of PTP-PEST substrates are involved in integrin-signalling, the formation of the immune synapse and T-cell receptor (TCR)-signalling (*1*, *15*, *27*), it seems that PTP-PEST may have a major impact on antigen recognition and antigen-induced lymphocyte activation.

It was previously reported that the enzyme activity of PTP-PEST in fibroblasts adhered to fibronectin is higher than that of not adhered cells (*4*), suggesting that its PTP activity is under control of integrin-mediated cell adhesion. Since the phosphorylation of PTP-PEST at Ser39 is an important regulatory modification of PTP activity, it is highly possible that PTP-PEST enzyme activity is regulated by phosphorylation at Ser39. Furthermore, considering that Ser39 of PTP-PEST is predominantly phosphorylated by PKC and/or PKA, the regulation of PTP-PEST enzyme activity via Ser39 may be dependent on integrin-mediated protein serine/threonine kinase, probably PKC or PKA, in fibroblasts. In addition, Lu *et al.* (*28*) reported that mammalian sterile 20-like kinase 3 (MST3) inhibits cell migration in a manner dependent on its autophosphorylation in human breast

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adenocarcinoma cell line MCF-7 cells. MST3 is a member of the serile-20 kinase family with a unique preference for manganese ion as a cofactor, which could phosphorylate PTP-PEST and inhibit its PTP activity, probably at Ser39. These reports suggest that PTP-PEST enzyme activity is regulated *in vivo* by protein serine/threonine kinases, presumably as a result of Ser39 phosphorylation. Considering that the phosphorylation of PTP-PEST at Ser39 may be controlled by protein serine/threonine kinases and protein serine/threonine phosphatases, elucidation of the reaction pathways involved in the dephosphorylation of pS39 is equally important as the phosphorylation for understanding the cellular function of PTP-PEST. Furthermore, although it has been reported that the phosphorylation of PTP-PEST at Ser39 is catalysed by PKC and PKA (*16*), and probably also by MST3 *in vivo* (*28*), elucidation of the dephosphorylation has not yet been reported. This is the first study to report that pS39 of PTP-PEST was dephosphorylated by a novel PTP-PEST-binding protein, PP1α, and these results contribute to the elucidation of the molecular function of PTP-PEST in various cells.

Conflict of interest
None declared.

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