Haematopoietic protein tyrosine phosphatase (HePTP) phosphorylation by cAMP-dependent protein kinase in T-cells: dynamics and subcellular location

Konstantina NIKA*, Huong HYUNH*, Scott WILLIAMS*, Surojit PAUL†, Nunzio BOTTINI*, Kjetil TASKÉN‡, Paul J. LOMBROSO† and Tomas MUSTELIN*[1]

*Program of Signal Transduction, Cancer Research Center, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, U.S.A., †Child Study Center, Yale University School of Medicine, 230 South Frontage Road, New Haven, CT 06520, U.S.A., and ‡The Biotechnology Centre, University of Oslo, N-0317 Oslo, Norway

The HePTP (haematopoietic protein tyrosine phosphatase) is a negative regulator of the ERK2 (extracellular signal-regulated protein kinase 2) and p38 MAP kinases (mitogen-activated protein kinases) in T-cells. This inhibitory function requires a physical association of HePTP through an N-terminal KIM (kinase-interaction motif) with ERK and p38. We previously reported that PKA (cAMP-dependent protein kinase) phosphorylates Ser-23 within the KIM of HePTP, resulting in dissociation of HePTP from ERK2. Here we follow the phosphorylation of this site in intact T-cells. We find that HePTP is phosphorylated at Ser-23 in resting T-cells and that this phosphorylation increases upon treatment of the cells with agents that elevate intracellular cAMP, such as prostaglandin E2. HePTP phosphorylation occurred at discrete regions at the cell surface. Phosphorylation was reduced by inhibitors of PKA and increased by inhibitors of protein phosphatases PP1 and PP2A, but not by inhibitors of calcineurin. *In vitro*, PP1 efficiently dephosphorylated HePTP at Ser-23, while PP2A was much less efficient. Activation of PP1 by treatment of the cells with ceramide suppressed Ser-23 phosphorylation, as did transfection of the catalytic subunit of PP1. Phosphorylation at Ser-23 is also increased in a transient manner upon T-cell antigen receptor ligation. In contrast, treatment of cells with phorbol ester had no effect on HePTP phosphorylation at Ser-23. We conclude from these results that HePTP is under continuous control by PKA and a serine-specific phosphatase, probably PP1, in T-cells and that this basal phosphorylation at Ser-23 can rapidly change in response to external stimuli. This, in turn, will affect the ability of HePTP to inhibit the ERK and p38 MAP kinases.

Key words: ceramide, phosphospecific antibody, prostaglandin E2, protein kinase A, protein phosphatase 1, T-cell antigen receptor.

INTRODUCTION

Ligation of the TCR (T-cell antigen receptor) together with appropriate co-stimulatory molecules causes a rapid activation of all three principal MAP kinases (mitogen-activated protein kinases), ERK (extracellular signal-regulated protein kinase), JNK (c-Jun N-terminal kinase) and p38, through distinct kinase cascades that result in the dual phosphorylation of a Thr-Xaa-Tyr motif in their activation loops [1]. MAP kinases are important integration points in signalling from a large number of cell-surface receptors that regulate growth, adaptation, differentiation and survival responses [1,2]. These kinases regulate directly or indirectly a number of transcription factors that control many important genes, including the c-*fos*, c-*jun*, FasL, interferon-γ and interleukin-2 genes in T-cells [2,3], as well as cell-cycle regulators and downstream kinases like MAPKAP (MAP kinase-activated protein) kinases 2 and 3.

Both the unphosphorylated and doubly phosphorylated forms of ERK2 have been crystallized and their three-dimensional structures solved [4,5]. In the unphosphorylated and catalytically inactive state, the side chain of Tyr-185 in the Thr-Xaa-Tyr motif is buried under the activation loop and blocks the catalytic cleft. Upon double phosphorylation, the loop moves away and the phosphates on both Tyr-185 and Thr-183 are brought into contact with Arg-rich clusters on the surface of the kinase [5–7]. This major conformational change of the activation loop results in a rotation of the upper lobe of the kinase domain with respect to the lower lobe, optimal positioning of the catalytic amino acid residues and opening of the substrate-binding groove. Once activated, some MAP kinases translocate to the nucleus [8], where many of their substrates reside.

Inactivation of MAP kinases can be achieved by dephosphorylation of either or both phosphoamino acid residues in the activation loop by a PTPase (protein tyrosine phosphatase), a DSP (dual-specificity PTPase) or a Ser/Thr-specific protein phosphatase [9–12]. The HePTP (haematopoietic protein tyrosine phosphatase) is a 38 kDa MAP kinase-specific enzyme [13,14] expressed throughout thymic development and in all T-cell lineages, as well as in other leucocytes [13–17]. HePTP consists of a catalytic domain with a $\approx$ 80-amino-acid residue non-catalytic N-terminal extension. As assessed by immunofluorescence staining and confocal microscopy, HePTP is cytosolic with a preference for a juxtamembranous location [17]. Two related PTPases, STEP (striatum-enriched phosphatase) [18] and PCPTP1 (PC12 cell-derived protein tyrosine phosphatase 1) [19–22], have been cloned, but they are not expressed in haematopoietic cells. Interestingly, both of these brain-specific enzymes exist as forms with many different molecular masses due to alternative mRNA splicing [23,24] or translational initiation [19]. This could also be true for HePTP.

Abbreviations used: ERK2, extracellular signal-regulated protein kinase 2; PTPase, protein tyrosine phosphatase; HePTP, haematopoietic PTPase; MAP kinase, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase; TCR, T-cell antigen receptor; PP1, protein phosphatase 1; JNK, c-Jun N-terminal kinase; KIM, kinase-interaction motif; HA, haemagglutinin; mAb, monoclonal antibody; GST, glutathione S-transferase; STEP, striatum-enriched phosphatase; PCPTP1, PC12 cell-derived protein tyrosine phosphatase 1; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP.

[1] To whom correspondence should be addressed (e-mail tmustelin@burnham-inst.org).

Expression of HePTP in T-cells results in a reduction in ERK2 and p38 kinase activation [25–27], but there are no effects on the upstream kinase MEK (MAP kinase/ERK kinase), on JNK or on overall tyrosine phosphorylation. Conversely, receptor-mediated activation of ERK2 and p38 are elevated 2- to 3-fold in HePTP$^{-/-}$ mice [28]. A key mechanism for the high degree of specificity of HePTP towards ERK and p38 resides within amino acids 15–30 in the non-catalytic N-terminus of HePTP, which form a KIM (kinase-interaction motif) that binds to ERK and p38 with high affinity [27]. A nearly identical motif is found in the related PTPases STEP and PCPTP [29,30]. Inactivation of ERK2 by HePTP is greatly facilitated by the formation of a 1:1 complex, in which HePTP efficiently dephosphorylates Tyr-185 of the activation loop of ERK2. Within the complex, HePTP can also be phosphorylated by ERK and p38 at two nearby residues, Thr-45 and Ser-72 [27].

Another important regulatory mechanism for HePTP is mediated by phosphorylation at Ser-23 within the KIM [31] by PKA (cAMP-dependent protein kinase; protein kinase A). This phosphorylation results in dissociation of the HePTP/ERK2 complex and reduced ability of HePTP to dephosphorylate ERK [31]. Using this mechanism, any stimuli that elevate the intracellular concentration of cAMP in T-cells, e.g. prostaglandin E2, lift the HePTP-mediated restriction on ERK (and p38) activation. Although it is clear that PKA phosphorylates HePTP at Ser-23 both *in vitro* and *in vivo* [31], the extent and dynamics of Ser-23 phosphorylation in intact T-cells have not been studied before.

Here we utilize a phospho-specific antibody to document a basal level of HePTP phosphorylation at Ser-23, its increase after prostaglandin E2 treatment, and responses to inhibition or stimulation of protein phosphatases. We also report that TCR triggering causes an increase in HePTP phosphorylation at Ser-23, which is sensitive to inhibitors of adenylate cyclase and PKA. Thus, the TCR not only initiates signalling pathways that activate ERK and p38, but also decreases the inhibition of these kinases by causing increased phosphorylation of HePTP at Ser-23.

MATERIALS AND METHODS

Antibodies and reagents

The 12CA5 anti-HA (haemagglutinin) mAb (monoclonal antibody) was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The C305 anti-TCR mAb was from ATCC and was used as culture supernatant. The polyclonal anti-ERK2 was from Santa Cruz Biotechnology and the polyclonal anti-phospho-ERK was from New England Biolabs. Anti-phospho-Ser-23 was as reported [32]. A series of mAbs were raised against recombinant full-length HePTP; mAb B3A/C9 was used in this study.

Plasmids and recombinant proteins

The expression plasmids for HePTP and HePTP-S23A in the pEF/HA vector, which adds an HA tag to the N-terminus of the insert, were as described in [25,27,31]. Recombinant HePTP proteins, with either GST (glutathione S-transferase) or 6 × His at the N-terminus, were produced as described in [27,31,33]. The catalytic subunit of PP1 (protein phosphatase 1) in the pcDNA-3-HA vector was kindly provided by Dr Marc Montminy (Salk Institute, La Jolla, CA, U.S.A.).

Cells and transfections

Jurkat leukaemia T-cells were kept in the logarithmic growth phase in RPMI 1640 medium with 5 % fetal calf serum, L-glutamine and antibiotics. These cells were transiently transfected with a total of 5 μg of DNA by electroporation at 950 μF and 240 V. Empty vector was added to control samples to make a constant amount of DNA in each sample. Cells were used for experiments 24–48 h after transfection.

Immunoprecipitation and immunoblotting

Cells were lysed in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl and 5 mM EDTA containing 1 % Nonidet P-40, 1 mM Na_3VO_4, 10 μg/ml aprotinin and leupeptin, 100 μg/ml soybean trypsin inhibitor and 1 mM PMSF and clarified by centrifugation at 20 800 ***g*** for 20 min. The clarified lysates were preabsorbed on Protein G–Sepharose and then incubated with antibody for 2 h, followed by Protein G–Sepharose beads. Immune complexes were washed three times in lysis buffer, once in lysis buffer with 0.5 M NaCl, again in lysis buffer and suspended in SDS sample buffer.

In vitro dephosphorylation of HePTP by protein phosphatases

HePTP–GST (0.5 μg) or 0.5 μg of GST bound to 40 μl of glutathione–Sepharose beads were incubated with 250 ng of recombinant PKA catalytic subunit in 20 μl of 10 mM Hepes, pH 7.4, containing 20 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 % Triton X-100 and 1 mM ATP for 30 min at 30 °C. The beads were washed three times with ice-cold phosphatase buffer (20 mM Mops, pH 7.5, with 150 mM NaCl and 10 % glycerol) and incubated with purified PP1 or PP2A (Upstate Biotechnology, Lake Placid, NY, U.S.A.) or buffer alone in 20 μl of phosphatase buffer at 30 °C for 30 min. The beads were washed three times in phosphatase buffer. The bound proteins were eluted in SDS sample buffer and analysed by immunoblotting.

RESULTS

Characterization of a phospho-Ser-23-specific antibody

An affinity-purified phospho-specific antibody was prepared against the synthetic phosphopeptide CQERRGpSNVSL, corresponding to amino acids 216–225 of STEP with phosphate at Ser-221 [32]. This peptide is identical to amino acid residues 18–27 of HePTP, except that residue 26 is an alanine in HePTP (serine in STEP), with Ser-23 being phosphorylated. Ser-23 is the only residue in HePTP phosphorylated by PKA *in vitro* and *in vivo* [31]. To test if this antibody reacts with Ser-23-phosphorylated HePTP, we incubated 1 μg of recombinant HePTP with recombinant PKA Cα catalytic subunit and ATP and immunoblotted the resulting material with the phospho-specific antibody. As shown in Figure 1(A), the antibody reacted very well with HePTP phosphorylated by PKA *in vitro*, but very faintly with unphosphorylated HePTP and not at all with PKA. Thus the antibody recognizes Ser-23-phosphorylated HePTP despite the one amino acid residue difference between HePTP and STEP.

To determine if the considerably smaller quantities of phospho-HePTP recovered from HePTP-transfected Jurkat T-cells can be detected with the phospho-Ser-23-specific antibody, we expressed HA-tagged HePTP and its S23A point mutant [31] in Jurkat T-cells, treated them with the membrane-permeable cAMP analogue 8-CPT-cAMP [8-(4-chlorophenylthio)-cAMP], and analysed cell lysates by immunoblotting with the phospho-specific antibody. These experiments (Figure 1B) showed that HePTP was phosphorylated at Ser-23 in untreated cells, but was clearly more phosphorylated upon cAMP elevation. The HePTP-S23A mutant did not react at all with the antibody (Figure 1B, lanes 5 and 6), demonstrating that the antibody reacts with the correct site and no other epitope in HePTP.

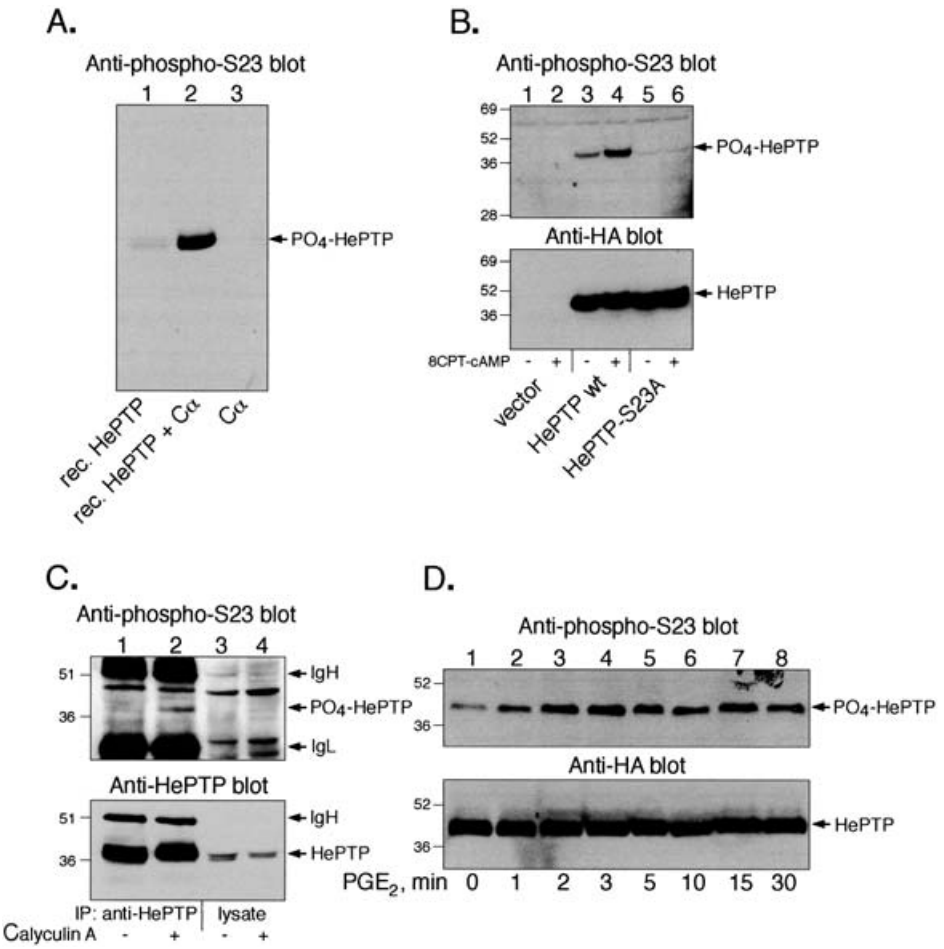

Figure 1 Characterization of the phospho-Ser-23-specific HePTP antibody

(**A**) Anti-phospho-Ser-23 immunoblot of recombinant (rec.) HePTP phosphorylated *in vitro* by purified PKA Cα catalytic subunit. (**B**) Total lysates of Jurkat cells transfected with empty vector, wild-type (wt) and the S23A mutant of HePTP. Prior to lysis the cells were stimulated with 1 mM 8-CPT-cAMP for 3 min. The filter was probed with the anti-phospho-Ser-23 antibody and the expression of the transfected proteins was visualized by an anti-HA blot (bottom panel). (**C**) Anti-phospho-Ser-23 immunoblot (upper panel) of anti-HePTP immunoprecipitates (lanes 1 and 2) or total cell lysates (lanes 3 and 4) from Jurkat cells treated with medium (lanes 1 and 3) or with 50 nM calyculin A for 30 min. The amount of HePTP was visualized by anti-HePTP immunoblotting of the same filter (lower panel). (**D**) Time course of stimulation with 10 μM prostaglandin E2 (PGE_2). Total cell lysates were probed with the anti-phospho-Ser-23 antibody. Bottom panel, anti-HA blot of the same filter.

Endogenous HePTP also reacted with the phospho-Ser-23 antibody, but only after the protein was enriched by immunoprecipitation (Figure 1C). Thus the detection limit of the affinity-purified antibody lies at higher concentrations of HePTP than the level of endogenous HePTP in a cell lysate. However, immunofluorescence staining showed that endogenous HePTP has the same subcellular location as transfected HePTP (results not shown), which was not more than 2–5-fold overexpressed in our experiments. Therefore, most of the subsequent experiments were performed with transfected HePTP.

Phosphorylation of HePTP at Ser-23 in T-cells in response to cAMP

Having characterized the anti-phospho-Ser-23 antibody, we began to explore the phosphorylation status of HePTP in cells subjected to various treatments. Addition of prostaglandin E2, a physiological agonist of G-protein-coupled receptors that elevates cAMP in T-cells, also resulted in a time-dependent phosphorylation of transfected HePTP at Ser-23 (Figure 1D). Peak phosphorylation was achieved in about 3 min and slowly declined thereafter. In many experiments, there was some oscillation in Ser-23 phosphorylation levels during the decline phase of the response. As shown in Figure 1(B), the membrane-permeable cAMP analogue 8-CPT-cAMP also elevated HePTP phosphorylation at Ser-23. Together, these findings are in agreement with cAMP-dependent kinase being the protein kinase that phosphorylates HePTP at Ser-23 in intact T-cells.

Phorbol ester does not induce Ser-23 phosphorylation, but TCR ligation does

Since protein kinase C isoforms often phosphorylate serine residues surrounded by basic amino acid residues similar to those phosphorylated by PKA, we decided to see if phorbol ester would induce Ser-23 phosphorylation of HePTP in T-cells. Jurkat cells were transfected with empty vector, HePTP, or HePTP-S23A, and treated for 1–30 min with 200 nM PMA, lysed and immunoblotted with anti-phospho-Ser-23. This treatment did not increase the reactivity of HePTP with the phospho-specific antibody (results not shown). Rather, the reactivity showed a slight decline over time. Lysates of cells transfected with empty vector or with HePTP-S23A mutant showed no reactivity at all with the phospho-specific antibody before or after addition of phorbol ester. Nevertheless, the mutant HePTP protein was expressed at

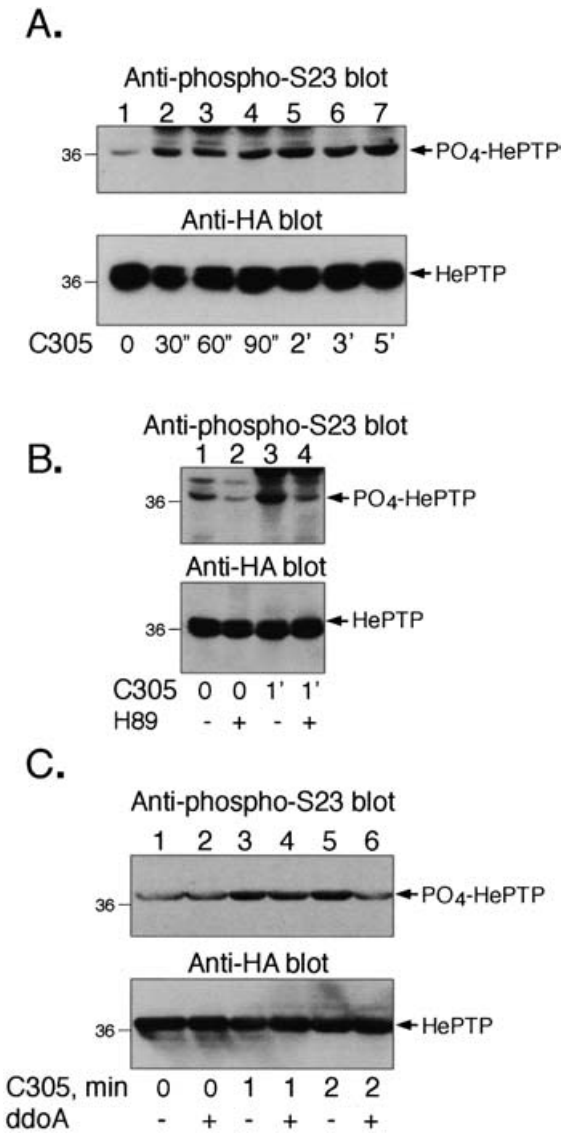

Figure 2 TCR engagement causes phosphorylation of HePTP at Ser-23 by PKA

(**A**) Anti-phospho-Ser-23 immunoblot (upper panel) of total cell lysates from Jurkat cells transfected with HePTP and stimulated with C305 anti-TCR mAb for the indicated times. The same filter was reprobed with anti-HA antibody (lower panel). (**B**) Anti-phospho-Ser-23 immunoblot (upper panel) of cells pretreated with 30 μM of the PKA inhibitor H89 for 30 min at 37 °C, as indicated, and then stimulated with the anti-TCR mAb C305 for 1 min. The same filter was reprobed with anti-HA antibody (lower panel). (**C**) Anti-phospho-Ser-23 immunoblot (upper panel) of cells pretreated with or without the adenylate cyclase inhibitor 2′-5′dideoxyadenosine (ddoA; 10 μM) for 30 min at 37 °C and then stimulated with the anti-TCR mAb C305, as indicated. The expression of HePTP was visualized by anti-HA immunoblotting of the same filter (bottom panel).

the same levels as the unmutated HePTP protein. Thus PKC activation does not result in Ser-23 phosphorylation. In agreement with this notion, we have found that although purified PKCγ can phosphorylate recombinant HePTP *in vitro*, tryptic peptide mapping showed that this phosphorylation occurred on a site different from Ser-23 (S. Williams and T. Mustelin, unpublished work). Clearly, this other site (which may not be phosphorylated *in vivo*) does not react with anti-phospho-Ser-23.

In contrast to phorbol ester, ligation of the T-cell antigen receptor resulted in a rapid, but transient, phosphorylation of HePTP at Ser-23 (Figure 2A). This response was seen as early as 30 s after receptor crosslinking, and peaked at around 2 min. As in the case of prostaglandin-induced HePTP phosphorylation, the response to TCR ligation often showed some oscillation after the peak and the time course varied a bit from experiment to experiment; in some experiments the highest phosphorylation was seen already at 1 min. In the experiment shown in Figure 2(A), the peak is at 2 min, followed by a decline at 3 min and again an increase at 5 min. This phenomenon is often observed for responses that contain a negative-feedback loop and may occur in a much more pronounced manner in individual cells, as in the case of calcium mobilization.

The phosphorylation of HePTP at Ser-23 in response to TCR ligation was sensitive to inhibitors of cAMP-dependent kinase, like H89 (Figure 2B) and 8-Rp-cAMP (results not shown), as well as to the adenylate cyclase inhibitor 2′-5′dideoxyadenosine (Figure 2C). Although our data do not allow us to conclude that TCR ligation causes HePTP phosphorylation at Ser-23 by activating PKA, we can say that adenylate cyclase and PKA are required for the response. In fact, it has been found that TCR ligation causes a transient activation of a lipid-raft-localized pool of adenylate cyclase, local cAMP elevation and activation of PKA. In the context of the present study, we cannot exclude the possibility that TCR ligation stimulates HePTP phosphorylation by inhibition of the relevant serine-specific protein phosphatase.

Inhibitors of PP1 and PP2A, but not PP2B, increase Ser-23 phosphorylation

Next, we used the phospho-Ser-23-specific antibody to gain some insight into the protein phosphatase that dephosphorylates HePTP. Treatment of cells with 2 μM okadaic acid or 50 nM calyculin A, the former inhibiting mostly PP2A (but also PP1 to some extent at this concentration) and the latter inhibiting PP1 better, caused a very strong increase in Ser-23 phosphorylation, while cyclosporin A (10 μg/ml), which inhibits PP2B (also known as calcineurin), had no effect (Figure 3A), Treatment of the cells with ionomycin (0.3 μM) to activate calcineurin also did not affect HePTP phosphorylation at Ser-23. Treatment of HePTP-expressing cells with both calyculin A and 8-CPT-cAMP (Figure 3B) showed that the effect of the phosphatase inhibitor was much more pronounced and that even maximal phosphorylation induced by cAMP was much lower than that seen with calyculin A. These results implicate PP1 and/or PP2A (more likely the former) in the dephosphorylation of HePTP at Ser-23 and suggest that the phosphatase plays a dominant role over cAMP-dependent kinase in maintaining the steady-state level of HePTP phosphorylation at Ser-23.

PP1 efficiently dephosphorylates HePTP at Ser-23 *in vitro*

To help us distinguish between PP1 and PP2A, we performed an *in vitro* experiment, in which recombinant HePTP was first phosphorylated by purified PKA Cα subunit in the presence of 1 mM ATP, washed and then subjected to dephosphorylation by purified PP1 and PP2A of very similar specific activity. These experiments showed that even doses of PP1 as low as 0.1 units removed all Ser-23 phosphate from 0.5 μg of phospho-HePTP in 30 min (Figure 4), while even five times higher activity of PP2A was unable to do so (Figure 4A, lane 9). The effect of PP1 was completely blocked by adding calyculin A to the assay (Figure 4A, lane 8). Although these experiments do not prove that PP1 is the phosphatase that dephosphorylates HePTP at Ser-23, we conclude that PP1 is more likely than PP2A.

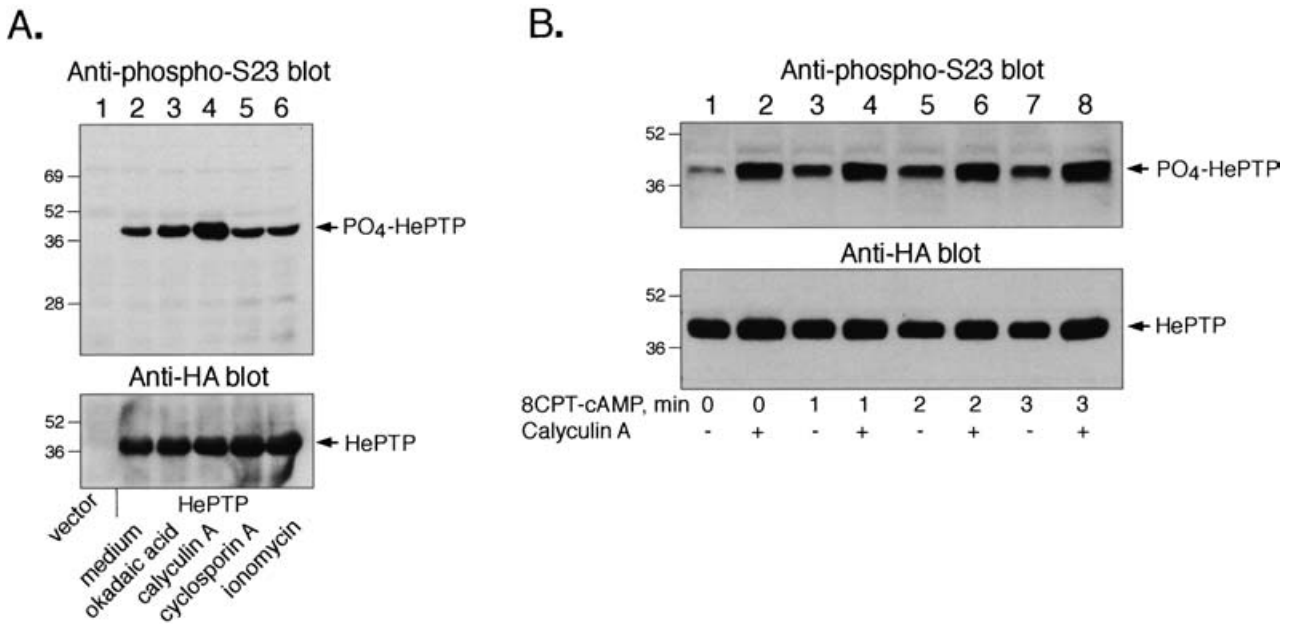

Figure 3 Inhibition of Ser/Thr protein phosphatases increases Ser-23 phosphorylation

(**A**) Anti-phospho-Ser-23 immunoblot (upper panel) of cells transfected with empty vector (lane 1) or HePTP (lanes 2–6) and treated for 30 min with medium alone (lane 2), 2 μM okadaic acid (lane 3), 50 nM calyculin A (lane 4), 10 μg/ml cyclosporine A (lane 5) or 0.3 μM ionomycin (lane 6). The same filter was reprobed with anti-HA (lower panel). (**B**) Anti-phospho-Ser-23 immunoblot (upper panel) of cells transfected with HePTP and pretreated with medium alone (lanes 1, 3, 5 and 7) or with 50 nM calyculin A (lanes 2, 4, 6 and 8) and then stimulated with 1 mM 8-CPT-cAMP for the indicated times. The same filter was reprobed with anti-HA (lower panel).

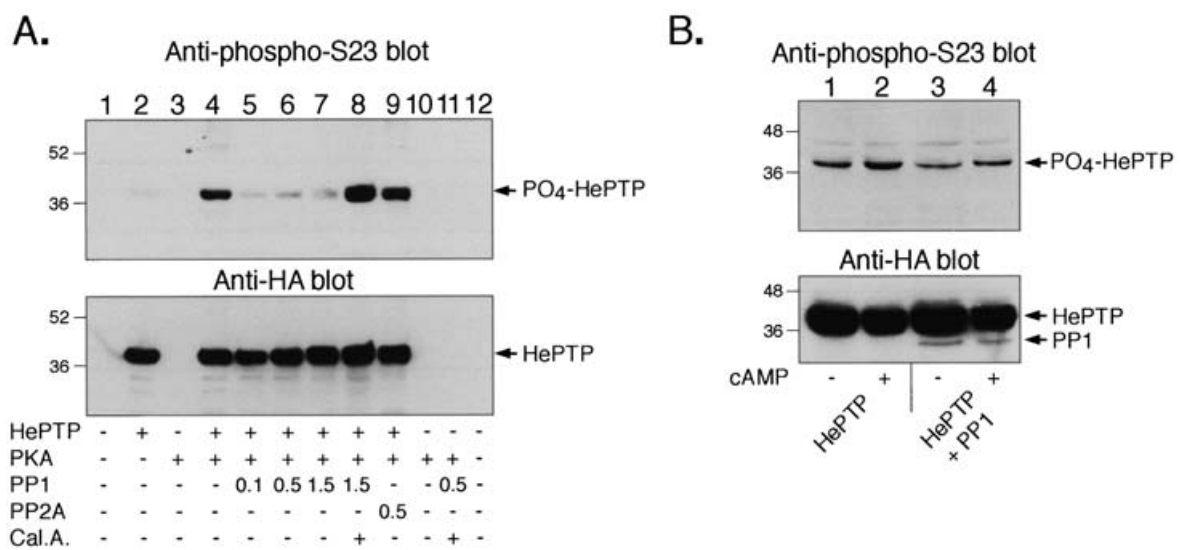

Figure 4 Dephosphorylation of HePTP by PP1 *in vitro* and in cells

(**A**) Anti-phospho-Ser-23 immunoblot (upper panel) of GST–HePTP (in lanes with +) or GST (lanes 10–12) pretreated with PKA Cα and ATP (PKA; where indicated) and then treated with the indicated amounts (in units) of purified PP1 or PP2A for 30 min at 30 °C. In lanes 8 and 11, 50 nM calyculin A was added. The same filter was reprobed with anti-HA (lower panel). (**B**) Anti-phospho-Ser-23 immunoblot (upper panel) of Jurkat cells transfected with HePTP (lanes 1 and 2) or HePTP and PP1 catalytic subunit (lanes 3 and 4) and kept in medium alone (lanes 1 and 3) or treated for 3 min with 1 mM 8-CPT-cAMP (lanes 2 and 4). The same filter was reprobed with anti-HA to show HePTP and PP1 (lower panel). Note that this exposure is longer than in other figures.

Expression of the catalytic subunit of PP1 causes reduced HePTP phosphorylation

Further support for a role of PP1 in dephosphorylation of HePTP at Ser-23 was obtained by transfecting Jurkat T-cells with the catalytic subunit of PP1. As shown in the lower panel of Figure 4(B), which represents a longer exposure of the anti-HA-epitope-tag immunoblot than in other figures, PP1 was expressed at much lower levels than HePTP. Nevertheless, the phosphorylation of HePTP both before and after addition of 8-CPT-cAMP was clearly reduced (Figure 4B, upper panel). This result supports a role for PP1 in dephosphorylation of HePTP at Ser-23.

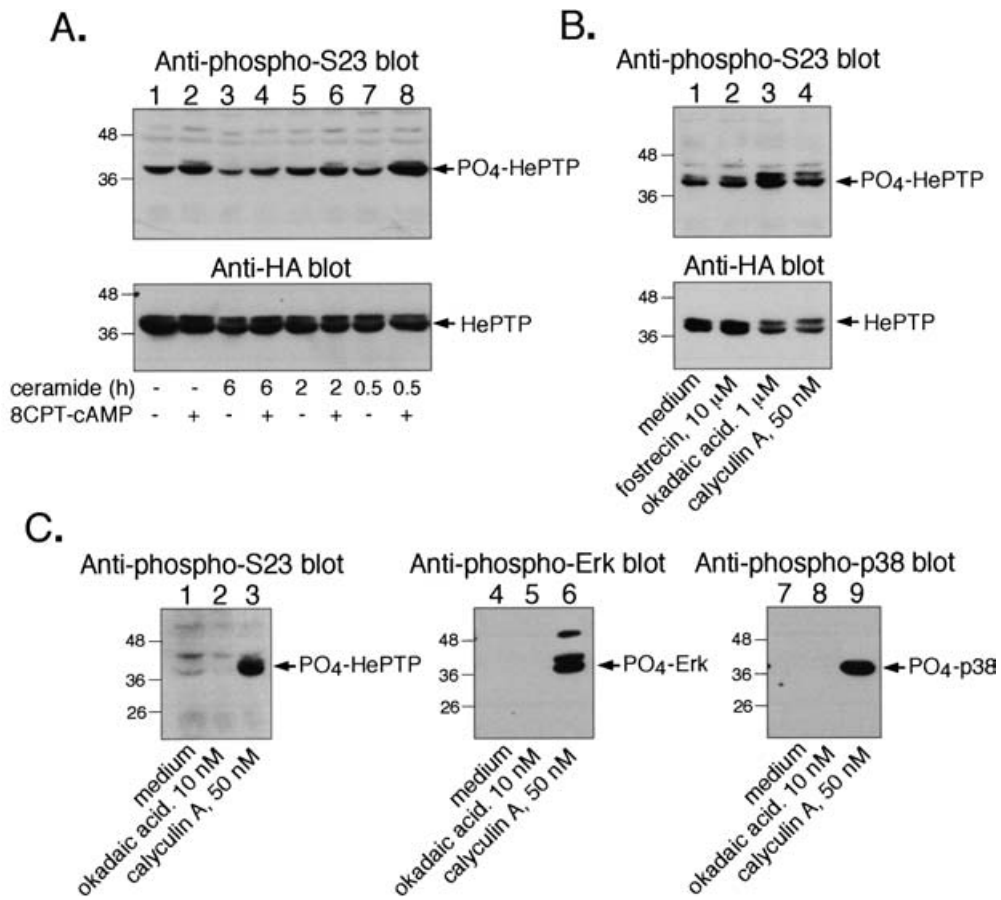

Figure 5 Effects on HePTP phosphorylation of PP1 activation and selective PP2A inhibition

(**A**) Anti-phospho-Ser-23 immunoblot (upper panel) of Jurkat cells transfected with HePTP and pretreated with 20 μM ceramide for the indicated times (h) and then stimulated with 8-CPT-cAMP for 3 min, as indicated. Anti-HA blot of the same filter is shown in the lower panel. (**B**) Anti-phospho-Ser-23 immunoblot (upper panel) of Jurkat cells transfected with HePTP and pretreated with medium alone (lane 1), 10 μM fostreicin (lane 2), 1 μM okadaic acid (lane 3) or 50 nM calyculin A (lane 4) for 30 min. Anti-HA blot of the same filter is shown in the lower panel. (**C**) Anti-phospho-Ser-23 immunoblot (lanes 1–3), anti-phospho-ERK blot (lanes 4–6) and anti-phospho-p38 blot (lanes 7–9) of Jurkat cells transfected with HePTP and treated with medium alone (lanes 1, 4 and 7), 10 nM okadaic acid (lanes 2, 5 and 8), or 50 nM calyculin A (lanes 3, 6 and 9).

Activation of PP1 by ceramide in T-cells reduces HePTP phosphorylation at Ser-23

The catalytic activity of PP1 can be increased by incubation of cells with ceramide in Jurkat T-cells [34]. When cells were preincubated with 20 μM ceramide for various times prior to addition of the membrane-permeable cAMP analogue 8-CPT-cAMP, it was clear that the reactivity of HePTP with the phospho-Ser-23 antibody declined and became much less responsive to 8-CPT-cAMP (Figure 5A). This effect was observed after 30 min of incubation, but became much more pronounced after 2 and 6 h. The expression of HePTP was not affected by these treatments (Figure 5A, lower panels) and the cells remained viable and appeared unchanged under the microscope.

Selective inhibition of PP1, but not PP2A, causes both HePTP phosphorylation at Ser-23 and MAP kinase activation

While the above experiments all suggest a role for PP1, they do not yet exclude a role for PP2A. To test if PP2A is involved, we treated T-cells with fostriecin, which inhibits PP2A at low nanomolar concentrations ($IC_{50} = 3.2$ nM), but PP1 at high micromolar concentrations ($IC_{50} = 131$ μM) [35,36], and found that this inhibitor had no effect at all on HePTP phosphorylation at Ser-23 (Figure 5B). In contrast, 1 μM okadaic acid and 50 nM calyculin A, which inhibit both PP1 and PP2A, increased HePTP phosphorylation in the same experiment (Figure 5B). Similarly, a low concentration (10 nM) of okadaic acid that selectively inhibits PP2A had no effects on HePTP phosphorylation (Figure 5C, lane 2), compared with 50 nM calyculin A, which inhibits PP1 efficiently and caused a strong increase in HePTP phosphorylation at Ser-23 (Figure 5C, lane 3). Interestingly, when the same samples were blotted for phospho-ERK and phospho-p38 MAP kinases, it was clear that these kinases were strongly phosphorylated in the calyculin A treated cells. Since PP2A rather than PP1 has been shown to dephosphorylate ERK [9,10], this response is most likely due to increased phosphorylation of HePTP at Ser-23 and thereby loss of ERK and p38 binding and suppression by HePTP [31]. However, we cannot exclude the possibility that PP1 inhibition activated ERK and p38 by some additional mechanisms.

Cellular hot spots of HePTP phosphorylation in response to prostaglandin E2

HePTP is a cytosolic protein with a clear enrichment under the plasma membrane [17]. To determine where in the cell phosphorylation at Ser-23 by PKA occurs, we treated cells with 10 μM prostaglandin E2 for 3 min and then fixed and stained

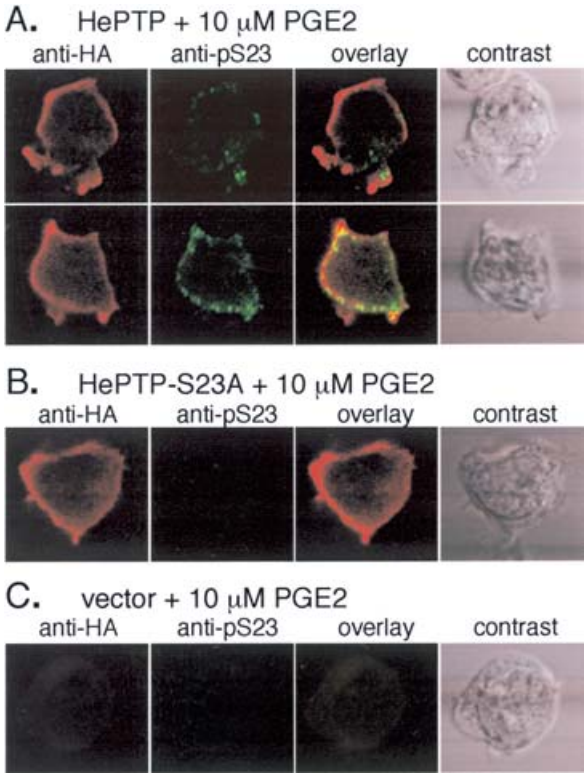

Figure 6 Hot spots of HePTP phosphorylation at Ser-23 in response to prostaglandin E2

(**A**) Confocal microscopy of cells expressing HePTP and treated with 10 μM prostaglandin E2 (PGE2) for 3 min. The cells were stained for the HA tag with a TRITC (tetramethylrhodamine β-isothiocyanate)-conjugated anti-HA mAb (red) and for phospho-Ser-23 with the rabbit antibody plus a FITC-conjugated sheep anti-rabbit antibody (green). The last panel in each row is a Nomarski phase-contrast image of the same cell. (**B**) Same experiments on cells expressing the HePTP-S23A mutant. (**C**) Same experiments on cells transfected with empty vector.

them with anti-HA to visualize HePTP and with anti-phospho-Ser-23 to see the pool of HePTP phosphorylated at Ser-23. Confocal microscopy of these cells (Figure 6A) showed that HePTP phosphorylation occurred in discrete regions at the plasma membrane, which contained only a fraction of the total HePTP. These hot spots of Ser-23 phosphorylation varied somewhat in number from cell to cell and probably represent the vicinity of clustered prostaglandin E2 receptors. As controls, we stained cells transfected with HePTP-S23A (Figure 6B) or empty vector (Figure 6C). While HePTP-S23A was well expressed and had the same subcellular location as wild-type HePTP, it did not stain at all with the phospho-Ser-23 antibody. Vector-transfected cells did not stain with either antibody. These controls demonstrate that the phospho-specific antibody does not recognize any other cellular protein in immunofluorescence staining.

DISCUSSION

HePTP is a cytosolic enzyme expressed in resting T-cells, where it forms a physical complex with ERK1, ERK2 and p38. In contrast, HePTP does not bind JNK [27], ERK3 or ERK7, but perhaps binds weakly to ERK5 (K. Nika and T. Mustelin, unpublished work). We speculate that upon activation of ERK or p38, a subset of the activated MAP kinase molecules dissociate from HePTP through a phosphorylation-dependent mechanism, and translocate to the nucleus. HePTP seems to remain exclusively cytosolic, and apparently does not follow the activated MAP kinases to the nucleus. Thus the MAP kinase molecules that escape HePTP and translocate to the nucleus are out of reach for HePTP and remain free to phosphorylate their targets until nuclear MAP kinase-specific phosphatases, such as Pac-1 (phosphatase of activated cells-1) or MKP-1 (MAP kinase phosphatase-1), are induced and synthesized $\approx$30–60 min after TCR triggering [9]. An additional layer of regulation is provided by the 20 kDa dual-specificity MAP kinase phosphatase VHR (vaccinia virus open-reading frame H-related), which is constitutively expressed in T-cells [37], but requires activation by ZAP-70 (ζ chain-associated protein of 70 kDa)-mediated tyrosine phosphorylation [38]. Together, these phosphatases provide a flexible mechanism for fine-tuning and timing of the MAP kinase response. We refer to this as the 'sequential phosphatase model' of MAP kinase regulation.

The molecular basis for release of ERK and p38 from HePTP upon TCR triggering of T-cells has remained unclear. Probable mechanisms include phosphorylation of HePTP at Ser-23 in the KIM by PKA or at Thr-45 and Ser-72 adjacent to the KIM by the MAP kinases themselves. It is clear that phosphorylation of HePTP at Ser-23 leads to dissociation of the complex [31], but it was previously not known if TCR triggering affects this event. We now report that TCR ligation indeed increased Ser-23 phosphorylation in a transient manner. This phosphorylation was not induced by phorbol esters or calcium ionophore making it unlikely that protein kinase C or other Ca^{2+}-dependent kinases were responsible. Even *in vitro*, purified protein kinase Cγ was unable to phosphorylate HePTP at Ser-23 (although it phosphorylated another serine). Expression of constitutively active c-Akt (often referred to as protein kinase B) in Jurkat T-cells also did not affect HePTP phosphorylation at Ser-23. In agreement with PKA being the kinase responsible for TCR-induced HePTP phosphorylation at Ser-23, we found that the two PKA inhibitors, 8-Rp-cAMP and H89, as well as an inhibitor of adenylate cyclase, blocked this TCR-induced response. However, since HePTP phosphorylation is a dynamic process, we cannot exclude the possibility that TCR triggering elevates HePTP phosphorylation by transiently decreasing the action of the protein phosphatase that dephosphorylates HePTP at Ser-23, which is probably PP1.

A recent paper [39] reported that HePTP exists in a cholesterol-dependent 440 kDa complex, which also contains PP2A, a Ser/Thr-specific phosphatase that can remove phosphate from Thr-183 of ERK2. Since HePTP only dephosphorylates Tyr-185, the two complementary activities can cause a complete dephosphorylation and inactivation of ERK [39]. In the context of the present study, it is interesting to note that HePTP was better dephosphorylated by PP1 than by PP2A. Perhaps the phosphatase that forms a complex with HePTP binds in a manner that makes Ser-23 inaccessible. In the end, however, both PP1 and PP2A have the same net effect on ERK: while PP2A directly dephosphorylates Thr-183 of ERK2, PP1 will dephosphorylate HePTP at Ser-23 and thereby promote the association of HePTP with ERK2, which in turn will facilitate dephosphorylation of ERK2 at Tyr-185. Thus our findings provide an example of a 'phosphatase network', in which a Tyr-specific phosphatase is dephosphorylated by a Ser/Thr-specific phosphatase and co-operates with another Ser/Thr-specific phosphatase to dephosphorylate a dually tyrosine- and threonine-phosphorylated substrate.

We are grateful to Dr M. Montminy for the kind gift of reagents and to Dr M. Croft and Dr M. Saxena for assistance with the preparation of monoclonal antibodies. This work was supported by a fellowship from American-Italian Cancer Foundation, and by grants AG00252 (H. H.), MH01527 (P. J. L.), MH52711 (P. J. L.), AI48032 (T. M.), AI53585

(T. M.), AI55741 (T. M.), AI35603 (T. M.) and CA96949 (T. M.) from the National Institutes of Health.

REFERENCES

1 Cobb, M. H., Xu, S., Hepler, J. E., Hutchison, M., Frost, J. and Robbins, D. J. (1994) Regulation of the MAP kinase cascade. Cell. Mol. Biol. Res. **40**, 253–256
2 Whitehurst, C. E. and Geppert, T. D. (1996) Mek1 and the extracellular signal-regulated kinases are required for the stimulation of IL-2 gene transcription in T cells. J. Immunol. **156**, 1020–1029
3 Karin, M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. J. Biol. Chem. **270**, 16483–16486
4 Zhang, F., Strand, A., Robbins, D., Cobb, M. H. and Goldsmith, E. J. (1994) Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution. Nature (London) **367**, 704–711
5 Canagarajah, B. J., Khokhlatchev, A., Cobb, M. H. and Goldsmith, E. J. (1997) Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. Cell **90**, 859–869
6 Zhang, J., Zhang, F., Ebert, D., Cobb, M. H. and Goldsmith, E. J. (1995) Activity of the MAP kinase ERK2 is controlled by a flexible surface loop. Curr. Biol. **3**, 299–307
7 Goldsmith, E. J. and Cobb, M. H. (1995) Protein kinases. Curr. Opin. Struct. Biol. **4**, 833–840
8 Khokhlatchev, A. V., Canagarajah, B., Wilsbacher, J., Robinson, M., Atkinson, M., Goldsmith, E. and Cobb, M. (1998) Phosphorylation of the MAP kinase ERK2 promotes its homodimerization and nuclear translocation. Cell **93**, 605–615
9 Saxena, M. and Mustelin, T. (2000) Extracellular signals and scores of phosphatases: all roads lead to MAP kinase. Semin. Immunol. **12**, 387–396
10 Alessi, D. R., Gomez, N., Moorhead, G., Lewis, T., Keyse, S. M. and Cohen, P. (1995) Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. Curr. Biol. **5**, 283–295
11 Keyse, S. M. (1998) Protein phosphatases and the regulation of MAP kinase activity. Semin. Cell. Dev. Biol. **9**, 143–152
12 Alonso, A., Rojas, A., Godzik, A. and Mustelin, T. (2003) The dual-specific protein tyrosine phosphatase family. Topics Curr. Genet. **5**, 333–358
13 Zanke, B., Suzuki, H., Kishihara, K., Mizzen, L., Minden, M., Pawson, A. and Mak, T. W. (1992) Cloning and expression of an inducible lymphoid-specific, protein tyrosine phosphatase (HePTPase). Eur. J. Immunol. **22**, 235–239
14 Adachi, M., Sekiya, M., Isobe, M., Kumura, Y., Ogita, Z.-I., Hinoda, Y., Imai, K. and Yachi, A. (1992) Molecular cloning and chromosomal mapping of a human protein-tyrosine phosphatase LC-PTP. Biochem. Biophys. Res. Commun. **186**, 1607–1615
15 Adachi, M., Sekiya, M., Ishino, M., Sasaki, H., Hinoda, Y., Imai, K. and Yachi, A. (1994) Induction of protein-tyrosine phosphatase LC-PTP by IL-2 in human T cells. FEBS Lett. **338**, 47–52
16 Sweiter, M., Berenstein, E. H., Swaim, W. D. and Siraganian, R. P. (1995) Aggregation of IgE receptors in rat basophilic leukemia 2H3 cells induces tyrosine phosphorylation of the cytosolic protein-tyrosine phosphatase HePTP. J. Biol. Chem. **270**, 21902–21906
17 Gjörloff-Wingren, A., Saxena, M., Han, S., Wang, X., Alonso, A., Renedo, M., Oh, P., Williams, S., Schnitzer, J. and Mustelin, T. (2000) Subcellular localization of intracellular protein tyrosine phosphatases in T cells. Eur. J. Immunol. **30**, 2412–2421
18 Lombroso, P. J., Murdoch, G. and Lerner, M. (1991) Molecular characterization of a protein-tyrosine-phosphatase enriched in striatum. Proc. Natl. Acad. Sci. U.S.A. **88**, 7242–7246
19 Shiozuka, K., Watanabe, Y., Ikeda, T., Hashimoto, S. and Kawashima, H. (1995) Cloning and expression of PCPTP1 encoding protein tyrosine phosphatase. Gene **162**, 279–284
20 Ogata, M., Sawada, M., Fujino, Y. and Hamaoka, T. (1995) cDNA cloning and characterization of a novel receptor-type protein tyrosine phosphatase expressed predominantly in the brain. J. Biol. Chem. **270**, 2337–2343
21 Sharma, E. and Lombroso, P. J. (1995) A neuronal protein tyrosine phosphatase induced by nerve growth factor. J. Biol. Chem. **270**, 49–53
21a Erratum (1995) J. Biol. Chem. **270**, 23234
22 Sharma, E., Zhao, F., Bult, A. and Lombroso, P. J. (1995) Identification of two alternatively spliced transcripts of STEP: a subfamily of brain-enriched protein tyrosine phosphatases. Mol. Brain Res. **32**, 87–93
23 Bult, A., Zhao, F., Dirkx, Jr, R., Raghunathan, A., Solimena, M. and Lombroso, P. J. (1997) STEP: a family of brain-enriched PTPs. Alternative splicing produces transmembrane, cytosolic and truncated isoforms. Eur. J. Cell. Biol. **72**, 337–344
24 Boulanger, L. M., Lombroso, P. J., Raghunathan, A., During, M. J., Wahle, P. and Naegele, J. R. (1995) Cellular and molecular characterization of a brain-enriched protein tyrosine phosphatase. J. Neurosci. **15**, 1532–1544
25 Saxena, M., Williams, S., Gilman, J. and Mustelin, T. (1998) Negative regulation of T cell antigen receptor signaling by hematopoietic tyrosine phosphatase (HePTP). J. Biol. Chem. **273**, 15340–15344
26 Oh-hora, M., Ogata, M., Mori, Y., Adachi, M., Imai, K., Kosugi, A. and Hamaoka, T. (1999) Direct suppression of TCR-mediated activation of extracellular signal-regulated kinase by leukocyte protein tyrosine phosphatase, a tyrosine-specific phosphatase. J. Immunol. **163**, 1282–1288
27 Saxena, M., Williams, S., Brockdorff, J., Gilman, J. and Mustelin, T. (1999) Inhibition of T cell signaling by MAP kinase-targeted hematopoietic tyrosine phosphatase (HePTP). J. Biol. Chem. **274**, 11693–11700
28 Gronda, M., Arab, S., Iafrate, B., Suzuki, H. and Zanke, B. (2001) Hematopoietic protein tyrosine phosphatase suppresses extracellular stimulus-regulated kinase activation. Mol. Cell. Biol. **21**, 6851–6858
29 Pulido, R., Zuñiga, A. and Ullrich, A. (1998) PTP-SL and STEP protein tyrosine phosphatases regulate the activation of the extracellular signal-regulated kinases ERK1 and ERK2 by association through a kinase interaction motif. EMBO J. **17**, 7337–7350
30 Zuñiga, A., Torres, J., Ubeda, J. and Pulido, R. (1999) Interaction of mitogen-activated protein kinases with the kinase interaction motif of the tyrosine phosphatase PTP-SL provides substrate specificity and retains ERK2 in the cytoplasm. J. Biol. Chem. **274**, 21900–21907
31 Saxena, M., Williams, S., Taskén, K. and Mustelin, T. (1999) Crosstalk between cAMP-dependent kinase and MAP kinase through hematopoietic protein tyrosine phosphatase (HePTP). Nat. Cell Biol. **1**, 305–311
32 Paul, S., Nairn, A. C., Wang, P. and Lombroso, P. J. (2003) NMDA-mediated activation of the tyrosine phosphatase STEP regulates the duration of ERK signaling. Nat. Neurosci. **6**, 34–42
33 Kholod, N. and Mustelin, T. (2001) Novel tools for studying protein complexes: vectors for co-expression of two proteins in *Escherichia coli*. Biotechniques **31**, 322–328
34 Chalfant, C. E., Ogretment, B., Galadri, S., Kroesen, B.-J., Pettus, B. J. and Hannun, Y. A. (2001) FAS activation induces dephosphorylation of SR proteins. Dependence on the de novo generation of ceramide and activation of protein phosphatase 1. J. Biol. Chem. **276**, 44848–44855
35 Chiang, C.-W., Harris, G., Ellig, C., Masters, S. C., Subramanian, R., Shenolikar, S., Wadzinski, B. E. and Yang, E. (2001) Protein phosphatase 2A activates the proapoptotic function of BAD in interleukin-3-dependent lymphoid cells by a mechanism requiring 14-3-3 dissociation. Blood **97**, 1289–1297
36 Sundaresan, P. and Farndale, R. W. (2002) p38 mitogen-activated protein kinase dephosphorylation is regulated by protein phosphatase 2A in human platelets activated by collagen. FEBS Lett. **528**, 139–144
37 Alonso, A., Saxena, M., Williams, S. and Mustelin, T. (2001) Inhibitory role for dual-specificity phosphatase VHR in T cell antigen receptor and CD28-induced Erk and Jnk activation. J. Biol. Chem. **276**, 4766–4771
38 Alonso, A., Rahmouni, S., Williams, S., van Stipdonk, M., Jaroszewski, L., Godzik, A., Abraham, R. T., Schoenberger, S. P. and Mustelin, T. (2003) Tyrosine phosphorylation of VHR phosphatase by ZAP-70. Nat. Immunol. **4**, 44–48
39 Wang, P.-Y., Liu, P., Weng, J., Sontag, E. and Anderson, R. G. W. (2003) A cholesterol-regulated PP2A/HePTP complex with dual specificity ERK1/2 phosphatase activity. EMBO J. **22**, 2658–2667

Received 14 August 2003/6 November 2003; accepted 13 November 2003
Published as BJ Immediate Publication 13 November 2003, DOI 10.1042/BJ20031244