The EMBO Journal Vol.17 No.24 pp.7337–7350, 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

Rafael Pulido[1,2,3], Ángel Zúñiga[2] and Axel Ullrich[1]

[1]Department of Molecular Biology, Max-Planck-Institut für Biochemie, Am Klopferspitz 18A, 82152 Martinsried, Germany and [2]Instituto de Investigaciones Citológicas, Amadeo de Saboya 4, 46010 Valencia, Spain

[3]Corresponding author
e-mail: rpulido@ochoa.fib.es

Protein kinases and phosphatases regulate the activity of extracellular signal-regulated kinases 1 and 2 (ERK1/2) by controlling the phosphorylation of specific residues. We report the physical and functional association of ERK1/2 with the PTP-SL and STEP protein tyrosine phosphatases (PTPs). Upon binding, the N-terminal domains of PTP-SL and STEP were phosphorylated by ERK1/2, whereas these PTPs dephosphorylated the regulatory phosphotyrosine residues of ERK1/2 and inactivated them. A sequence of 16 amino acids in PTP-SL was identified as being critical for ERK1/2 binding and termed kinase interaction motif (KIM) (residues 224–239); it was shown to be required for phosphorylation of PTP-SL by ERK1/2 at Thr^{253}. Co-expression of ERK2 with catalytically active PTP-SL in COS-7 cells impaired the EGF-induced activation of ERK2, whereas a PTP-SL mutant, lacking PTP activity, increased the ERK2 response to EGF. This effect was dependent on the presence of the KIM on PTP-SL. Furthermore, ERK1/2 activity was downregulated in 3T3 cells stably expressing PTP-SL. Our findings demonstrate the existence of a conserved ERK1/2 interaction motif within the cytosolic non-catalytic domains of PTP-SL and STEP, which is required for the regulation of ERK1/2 activity and for phosphorylation of the PTPs by these kinases. Our findings suggest that PTP-SL and STEP act as physiological regulators of the ERK1/2 signaling pathway.

Keywords: extracellular signal-regulated kinases/MAP kinases/protein tyrosine phosphatases/signal transduction

Introduction

A wide variety of extracellular stimuli, including growth factors, mitogens and cytokines, as well as environmental stress factors, result in the activation of mitogen-activated protein (MAP) kinase family members, which in turn phosphorylate different cytosolic, membrane-bound and nuclear substrates, thereby regulating the transcription of particular sets of genes (for reviews see Davis, 1993; Cobb and Goldsmith, 1995; Karin, 1995; Kyriakis and Avruch, 1996). MAP kinase activation requires phosphorylation by the dual specificity kinases, MAP kinase kinases, on both threonine and tyrosine residues located in the kinase domain VIII, near the substrate binding site of the enzyme (Kosako *et al*., 1992; Nakielny *et al*., 1992; Zhang *et al*., 1994). Protein serine/threonine phosphatases, as well as dual specificity phosphatases, are known to be involved in the inactivation of MAP kinases by dephosphorylating their phosphothreonine and phosphotyrosine regulatory residues (Anderson *et al*., 1990; Sun *et al*., 1993; Zheng and Guan, 1993; Ward *et al*., 1994). In particular, an expanding MAP kinase-specific, dual specificity phosphatase family, has been found, which is thought to account for the inactivation of distinct MAP kinases (Guan and Dixon, 1993; Ward and Kelly, 1994; Keyse, 1995). However, since dephosphorylation of the regulatory tyrosine residue is sufficient for MAP kinase inactivation, a role for protein tyrosine phosphatases (PTPs) in this process has been proposed (Anderson *et al*., 1990; Payne *et al*., 1991; Sarcevic *et al*., 1993). Similarly, the biological function of other signaling molecules involved in the control of cell growth and differentiation has been shown to be affected by the activity of various cytosolic and transmembrane PTPs (for recent reviews see Hunter, 1995; Tonks and Neel, 1996).

Protein–protein interaction motifs that are assembled in a modular fashion, sometimes in combination with enzymatic or DNA-binding functions, play a critical role in the transmission of cellular signals. Such a modular architecture, which combines conserved catalytic domains with targeting and/or regulatory domains, is also found in PTPs (for reviews see Fischer *et al*., 1991; Saito and Streuli, 1991; Trowbridge, 1991; Walton and Dixon, 1993; Mauro and Dixon, 1994). To elucidate further their regulatory function, we have searched for molecules that associate with PTPs and possibly represent substrates of their catalytic function. PTP-SL (Hendricks *et al*., 1995; Ogata *et al*., 1995; Sharma and Lombroso, 1995; Shiozuka *et al*., 1995) and STEP (Lombroso *et al*., 1991) are two related, non-nuclear PTPs, which exist in transmembrane and cytosolic forms and are mainly expressed in neuronal cells. We describe here that PTP-SL and STEP associate with, and are substrates and inactivators of members of the extracellular signal-regulated kinase (ERK) subfamily, ERK1 and ERK2. The binding of PTP-SL and STEP to ERK1/2 is dependent on a conserved kinase interaction motif (KIM) of 16 amino acids. Our results suggest a regulatory interaction between the PTPs-SL and STEP and ERK1/2 during signal transduction.

Results

PTP-SL tyrosine phosphatase associates with and is phosphorylated by an inducible kinase activity in vitro

To identify molecules that associate with PTP-SL, GST-fusion proteins containing the entire PTP-SL cytoplasmic

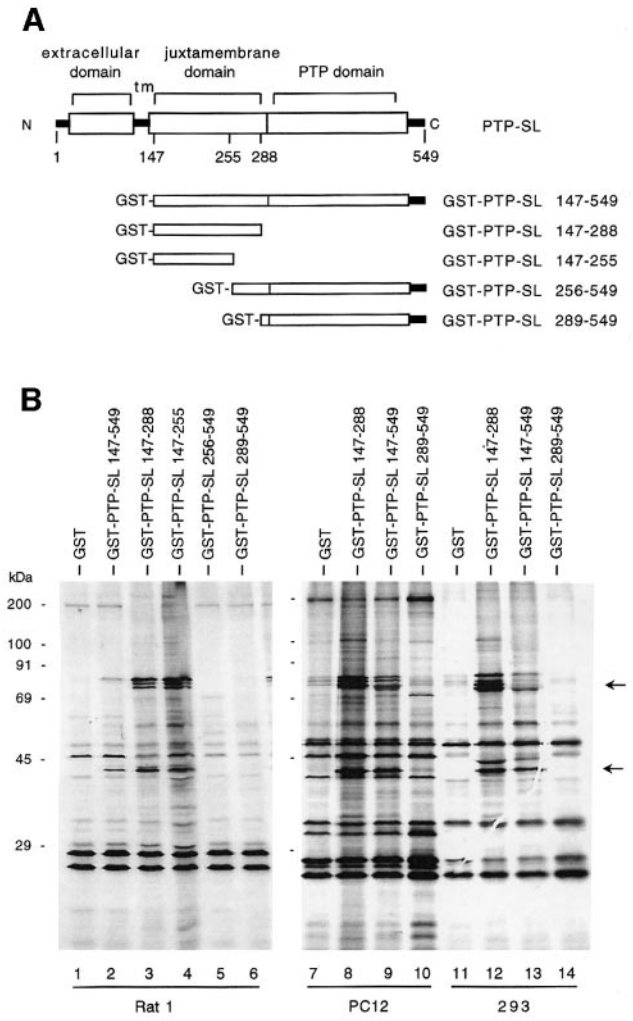


Fig. 1. Precipitation of [^{35}S]methionine-labeled cell lysates with GST–PTP-SL fusion proteins. (**A**) Schematic of the structure of PTP-SL and GST–PTP-SL fusion proteins used in this study. Amino acid numbering is shown according to Hendricks *et al*. (1995); tm, transmembrane. (**B**) Rat 1, PC12 and 293 cells were labeled and processed as described in Materials and methods, and lysates were precipitated with 1 μg of the different GST-fusion proteins, as indicated. The arrows indicate the proteins specifically precipitated by the GST–PTP-SL fusion proteins in lanes 2, 3, 4, 8, 9, 12 and 13. Samples were analyzed by 10% SDS–PAGE under reducing conditions, followed by autoradiography. Molecular mass standards are shown on the left in kDa.

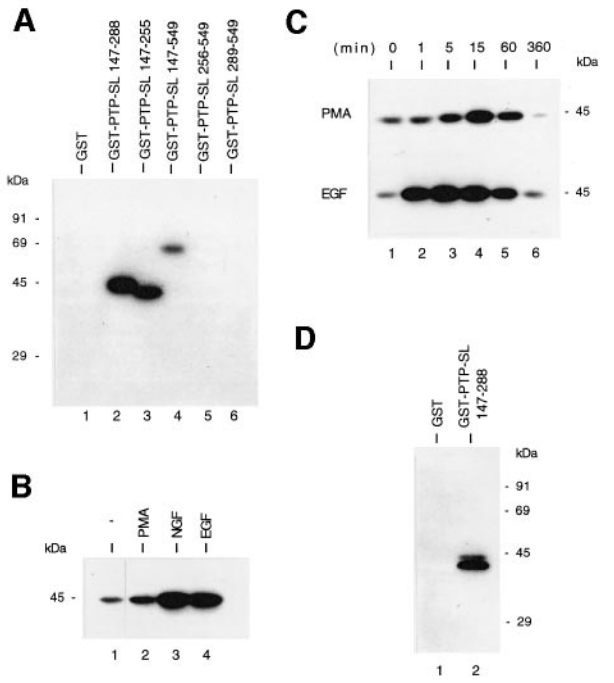


Fig. 2. Kinase activity of pellets containing GST–PTP-SL fusion proteins and associated proteins. (**A**) Rat 1 cell lysates were precipitated with 1 μg of the different GST fusion proteins, as indicated, and pellets were subjected to *in vitro* kinase assays in the presence of [γ-^{32}P]ATP. (**B**) PC12 cells were incubated during 15 min without stimulus (–), or in the presence of 10 ng/ml of PMA, 50 ng/ml of NGF or 50 ng/ml of EGF, as indicated. Cell lysates were precipitated with 1 μg of GST–PTP-SL 147–288, and pellets were subjected to *in vitro* kinase assays. (**C**) Rat 1 cells were incubated in the presence of 10 ng/ml of PMA or 50 ng/ml of EGF, for the times indicated. Cell lysates were precipitated with 1 μg of GST–PTP-SL 147–288, and pellets were subjected to *in vitro* kinase assays. (**D**) Rat 1 cell lysates were precipitated with 1 μg of GST or GST–PTP-SL 147–288, as indicated, and the complexes were loaded onto a SDS–PAGE gel containing GST–PTP-SL 147–255, followed by an *in gel* kinase assay in the presence of [γ-^{32}P]ATP. All samples were analyzed by 10% SDS–PAGE under reducing conditions, followed by autoradiography. Molecular mass standards are shown in kDa.

region, or truncation mutants thereof (see scheme in Figure 1A), were used as affinity reagents. Rat 1 fibroblasts were metabolically labeled in the presence of [^{35}S]methionine, and cell lysates were processed for precipitation with the different GST–PTP-SL fusion proteins, followed by SDS–PAGE analysis (Figure 1B, lanes 1–6). Two sets of proteins of 70–80 kDa and 42–44 kDa were specifically precipitated by fusion proteins containing the juxtamembrane region of PTP-SL [GST–PTP-SL 147–549, GST–PTP-SL 147–288 and GST–PTP-SL 147–255; amino acid numbering is according to Hendricks *et al*. (1995)] (Figure 1B, lanes 2–4, respectively), but not by GST protein alone (Figure 1B, lane 1) or fusion proteins containing the PTP-SL catalytic domain (GST–PTP-SL 256–549 and GST–PTP-SL 289–549) (Figure 1B, lanes 5 and 6, respectively). Similar results were obtained using other cell lines, including rat pheochromocytoma PC12, human embryonic kidney 293 and human neuroblastoma SK-N-SH cell lines (Figure 1B, lanes 7–14; data not shown), suggesting a broad distribution for the proteins precipitated by the GST–PTP-SL fusion proteins.

Since phosphorylation events play an important role in the regulation of the biological activity of protein kinases and phosphatases (Hunter, 1995), the possibility that a kinase activity was present in the pellets obtained after precipitation with the GST–PTP-SL fusion proteins was tested. Cell lysates from Rat 1 cells were subjected to precipitation, and after extensive washing, the pellets were resuspended in kinase assay buffer in the presence of [γ-^{32}P]ATP, and incubated for 15 min at room temperature, followed by SDS–PAGE analysis and autoradiography. As shown in Figure 2A, phosphorylation of some of the GST-fusion proteins used in the precipitation procedure was observed. This included those fusion proteins which contained the juxtamembrane region of PTP-SL, GST–PTP-SL 147–288, GST–PTP-SL 147–255 and GST–PTP-SL 147–549 (Figure 2A, lanes 2–4, respectively), but not proteins lacking these sequences, or containing GST alone (Figure 2A, lanes 1, 5 and 6). When the GST

control, or GST–PTP-SL 256–549 or GST–PTP-SL 289–549 fusion proteins were added (1 μg) to pellets obtained after precipitation with GST–PTP-SL 147–288, which co-precipitates the kinase activity (Figure 2A, lane 2), and then the kinase assay was performed, only phosphorylation of the latter protein (GST–PTP-SL 147–288) was observed (data not shown). This suggests that both the interaction site with the kinase and the phosphorylation site(s) on the PTP are located within the PTP-SL juxtamembrane region. Since this region (amino acids 144–288), does not contain any tyrosine residue, the enzyme responsible for its phosphorylation was predicted to be a serine/threonine kinase.

Experiments were performed to characterize and identify the kinase activity associated with PTP-SL. Cell lines were treated for 15 min with different mitogenic or differentiating agents, including phorbol 12-myristate 13-acetate (PMA), epidermal growth factor (EGF) and nerve growth factor (NGF). After lysis and precipitation with GST–PTP-SL 147–288, kinase assays were performed, and phosphorylation of the GST–PTP-SL 147–288 protein was visualized by SDS–PAGE. A representative experiment, obtained with PC12 cells, is shown in Figure 2B. Stimulation of PC12 cells with PMA, NGF or EGF (lanes 2–4, respectively) induced a strong increase in the kinase activity associated with PTP-SL. Results obtained with other cell lines were similar, although slight variations in the basal kinase activities, as well as in the degree of the increase of the kinase activity after the different treatments, were observed (data not shown; see also Figure 2C). A time course with EGF- or PMA-treated Rat 1 cells showed that the induction of the kinase activity associated with PTP-SL was rapid and transient, with a peak of activity after 5 and 15 min of stimulation, respectively (Figure 2C). The rapid increase of the kinase activity, as well as the absence of quantitative changes detected in the amount of protein associated with PTP-SL during the course of our experiments (data not shown; see also Figure 3B), suggested a post-translational regulation of the activity of the kinase associated with this PTP.

Finally, *in gel* kinase assays were performed to visualize the SDS–PAGE migration pattern of the protein kinase bound to PTP-SL (Figure 2D). Cell lysates from Rat 1 cells were precipitated with the GST protein alone or with GST–PTP-SL 147–288, and sample pellets were analyzed by SDS–PAGE with the GST–PTP-SL 147–255 protein as the kinase substrate, followed by an *in gel* kinase assay. Precipitation with GST–PTP-SL 147–288 (Figure 2D, lane 2), but not with GST alone (Figure 2D, lane 1), yielded a protein doublet with kinase activity of ~42–44 kDa.

ERK1/2 associate with and phosphorylate PTP-SL and STEP tyrosine phosphatases

The biochemical and functional properties of the kinase activity interacting with PTP-SL pointed to members of the MAP kinase family, which are known to mediate the cellular response to a wide array of extracellular stimuli (Davis, 1993; Cobb and Goldsmith, 1995; Karin, 1995; Kyriakis and Avruch, 1996). The ERKs, ERK1 and ERK2, are ubiquitous, mitogen-activated Ser/Thr kinases with electrophoretic mobilities of 44 and 42 kDa, respectively (Cobb *et al*., 1991). To test the possibility that ERK1/2 were the kinases interacting with and phosphorylating

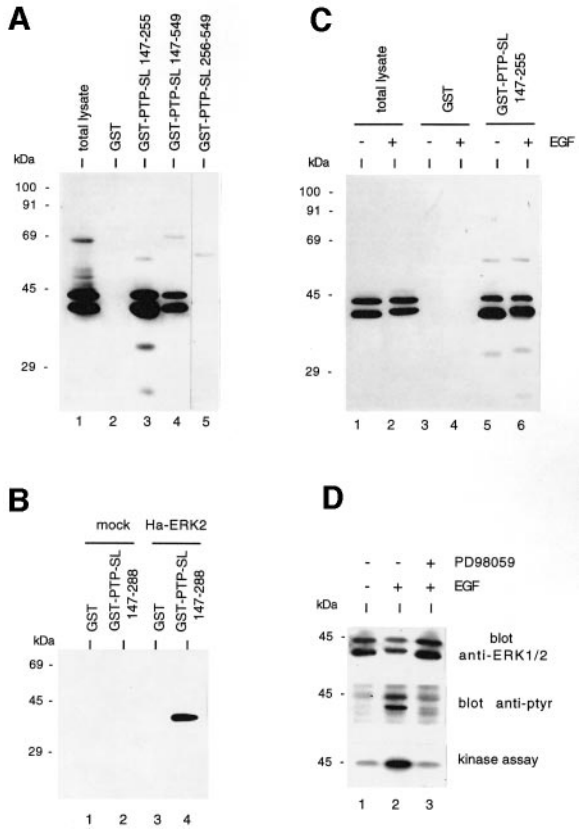


Fig. 3. GST–PTP-SL fusion proteins precipitate ERK1 and ERK2. (**A**) Rat 1 cell lysates were precipitated with 1 μg of the different GST-fusion proteins, as indicated. In lane 1, 10 μg of total lysate were loaded. Samples were resolved by 10% SDS–PAGE under reducing conditions, followed by immunoblot analysis with a mixture of anti-ERK1 and -ERK2 antibodies. (**B**) COS-7 cells were mock-transfected (pCDNA3 vector alone) or were transfected with pCDNA3 HA-ERK2. After 48 h, cells were lysed and precipitated with 1 μg of GST or GST–PTP-SL 147–288, as indicated. Samples were resolved by 10% SDS–PAGE under reducing conditions, followed by immunoblot analysis with the anti-HA mAb 12CA5. (**C**) Rat 1 cells were left untreated (–), or were treated for 10 min with 50 ng/ml of EGF (+), and cell lysates were precipitated with 1 μg of GST or GST–PTP-SL 147–255, as indicated. In lanes 1 and 2, 10 μg of total lysate were loaded. Samples were resolved by 10% SDS–PAGE under reducing conditions, followed by immunoblot analysis with a mixture of anti-ERK1 and -ERK2 antibodies. In all cases, reactive bands were visualized by chemiluminescence, as described in Materials and methods. Results obtained with GST–PTP-SL 147–288 or with GST–PTP-SL 147–255 were indistinguishable. (**D**) Rat 1 cells were left untreated (lanes 1 and 2) or were pre-incubated for 30 min with 50 μM of the MEK inhibitor PD98059 (lane 3), and then incubated for 10 min more (in the continuous presence of the inhibitor in the case of lane 3) with (lane 2 and 3) or without (lane 1) 50 ng/ml of EGF. Total lysate samples (upper and middle panels) were processed for ERK1/2 detection and phosphotyrosine content, respectively, as in (A) and (C). Samples in lower panel were precipitated with 1 μg of GST–PTP-SL 147–288, followed by *in vitro* kinase assays, 10% SDS–PAGE under reducing conditions and autoradiography. Molecular mass standards are shown in kDa.

PTP-SL, Rat 1 cell lysates were precipitated with the distinct fusion proteins, and the pellets were analyzed for the presence of ERK1/2 reactivity by immunoblot with a specific antibody (Figure 3A). Those samples precipitated with GST–PTP-SL containing the PTP-SL juxtamembrane region (GST–PTP-SL 147–255 and GST–PTP-SL 147–549; Figure 3A, lanes 3 and 4, respectively) showed the presence of anti-ERK1/2 immunoreactive proteins of

44 and 42 kDa, which co-migrated with the ERK1/2 proteins detected in the total lysates (Figure 3A, lane 1). The GST protein alone, or GST–PTP-SL lacking the juxtamembrane region of PTP-SL (GST–PTP-SL 256–549), did not precipitate any peptide recognized by the anti-ERK1/2 antibodies (Figure 3A, lanes 2 and 5, respectively). Additional confirmation of this result was obtained by precipitation of recombinant hemagglutinin (HA)-tagged ERK2 with GST–PTP-SL proteins, after transient expression in COS-7 cells, followed by immunoblot analysis with the anti-HA mAb 12CA5 (Figure 3B, lane 4 versus lanes 1–3). The association of the GST–PTP-SL fusion proteins with ERK1/2 was not affected after treatment of cells with EGF, which induced ERK1/2 activation, as determined by the shift in their apparent mobility and by *in vitro* kinase assays (Figure 3C, lanes 2 and 6 versus lanes 1 and 5; see also Figure 3D). To further demonstrate that ERK1/2 were the kinases phosphorylating PTP-SL in our *in vitro* kinase assays, Rat 1 cells were pre-incubated with an inhibitor of the ERK1/2 cascade (MEK1/2 inhibitor, PD98059; Dudley *et al*., 1995), and then stimulated with EGF for 10 min. Under these conditions, the activation of ERK1/2 due to EGF treatment was blocked, as indicated by the absence of a shift in their electropohoretic mobility and their reduced phosphotyrosine content [detected after immunoblot analysis of total lysate samples with anti-ERK1/2 or anti-phosphotyrosine 4G10 antibodies, respectively (Figure 3D, upper and middle panels, respectively)]. Cell lysate samples were precipitated in parallel with the GST–PTP-SL 147–288 fusion protein, and then subjected to *in vitro* kinase assays. As shown, pre-treatment with the MEK inhibitor abrogated the induction of PTP-SL phosphorylation by the associated kinase after EGF stimulation (Figure 3D, lower panel, lane 3 versus lane 2).

PTP-SL displays high sequence similarity with the STEP PTP (Lombroso *et al*., 1991; Hendricks *et al*., 1995; Ogata *et al*., 1995; Sharma and Lombroso, 1995; Shiozuka *et al*., 1995). Thus, the possibility that STEP also binds to and is phosphorylated by ERK1/2 was tested. [^{35}S]-methionine labeling of Rat 1 cells, followed by incubation with a GST–STEP fusion protein containing the region preceding the PTP domain [GST–STEP 1–107; numbering is according to Lombroso *et al*. (1991)] resulted in precipitation of identical sets of proteins of 70–80 kDa and 42–44 kDa (Figure 4A, lane 2), as in the GST–PTP-SL experiments (Figure 1). Furthermore, precipitation with GST–STEP 1–369 (GST–STEP full length) or GST–STEP 1–107 fusion proteins (Figure 4B, lanes 2 and 3, respectively), but not with GST alone or with a GST-fusion protein containing a non-related PTP (GST–HPTPδ-D1), yielded a mitogen-inducible kinase activity that phosphorylated the GST–STEP fusion proteins in *in vitro* kinase assays (Figure 4B, lanes 1 and 4, respectively; and data not shown). Finally, ERK1/2 were detected by immunoblot analysis using an anti-ERK1/2 antibody in samples precipitated with GST–STEP 1–107 and GST–STEP 1–369 fusion proteins (Figure 4C, lanes 2 and 3, respectively), but not in those precipitated using GST alone or GST–HPTPδ-D1 (Figure 4C, lanes 1 and 4, respectively). Taken together, these results demonstrate that ERK1/2 associate with PTP-SL and STEP PTPs, and phosphorylate their cytosolic N-terminal regions *in vitro*.

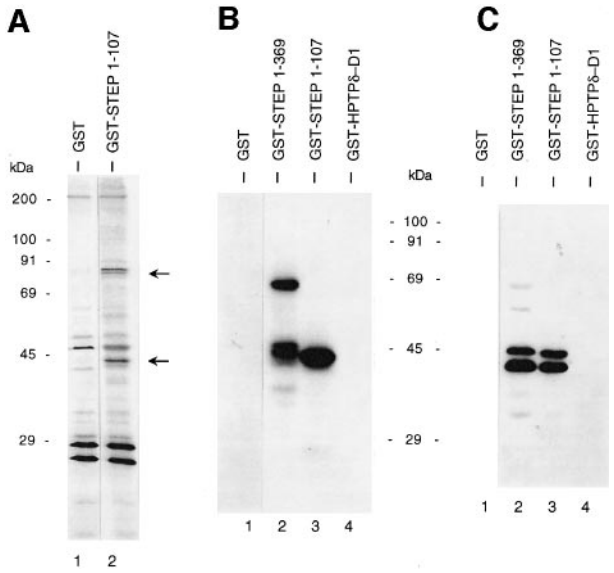


Fig. 4. GST–STEP fusion proteins precipitate and are substrates of ERK1/2. (**A**) [^{35}S]methionine-labeled Rat 1 cell lysates were precipitated with 1 μg of GST or GST–STEP 1–107 fusion protein, as indicated. The arrows indicate the proteins specifically precipitated by the GST–STEP fusion protein (lane 2). Samples were analyzed by 10% SDS–PAGE under reducing conditions, followed by autoradiography. Molecular mass standards are shown on the left in kDa. (**B**) Rat 1 cell lysates were precipitated with 1 μg of the different GST fusion proteins, as indicated, and the pellets were subjected to *in vitro* kinase assays, followed by 10% SDS–PAGE and autoradiography. GST–HPTPδ-D1 contains the juxtamembrane and PTP domain 1 of HPTPδ (Pulido *et al*., 1995). The smear in lane 2 probably correspond to degradation of the GST–STEP 1–369 fusion protein (upper band). (**C**) Rat 1 cell lysates were precipitated with 1 μg of the different GST fusion proteins, and samples were resolved by 10% SDS–PAGE under reducing conditions, followed by immunoblot analysis with a mixture of anti-ERK1 and -ERK2 antibodies. Reactive bands were visualized by chemiluminescence. Molecular mass standards are shown in kDa.

PTP-SL and STEP dephosphorylate and inactivate ERK1/2 in vitro

To test the capability of PTP-SL and STEP to dephosphorylate the regulatory phosphotyrosine and thereby inactivate ERK1/2, *in vitro* phosphatase and kinase assays were performed. Tyrosine phosphorylation of ERK1/2 was induced by treatment of Rat 1 cells with EGF for 10 min, cells were lysed and lysates were precipitated using the GST–PTP-SL 147–255 fusion protein, which lacks the PTP-SL PTP domain. Then, the pellets containing ERK1/2 were incubated in phosphatase assay buffer for 15 min with increasing concentrations of GST–PTP-SL 147–549 and GST–STEP 1–369 fusion proteins (containing the respective PTP domains), and the samples were resolved by SDS–PAGE, followed by phosphotyrosine content analysis by immunoblotting using the anti-phosphotyrosine mAb 4G10. As observed, both PTPs dephosphorylated ERK1/2 in a dose-dependent manner (Figure 5A, lanes 1–5 and lanes 7–11). GST protein alone (Figure 5A, lanes 6 and 12, and B, lane 2), or a PTP inactive mutant GST–PTP-SL fusion protein (GST–PTP-SL 147–549/C480S) (Figure 5B, lane 4), did not affect the ERK1/2 phosphotyrosine content. The controls (Figure 5A and B, lower panels) show the equal presence of ERK1/2 in all the samples subjected to the phosphatase assay, as revealed by reprobing the filters with the anti-ERK1/2 antibody.

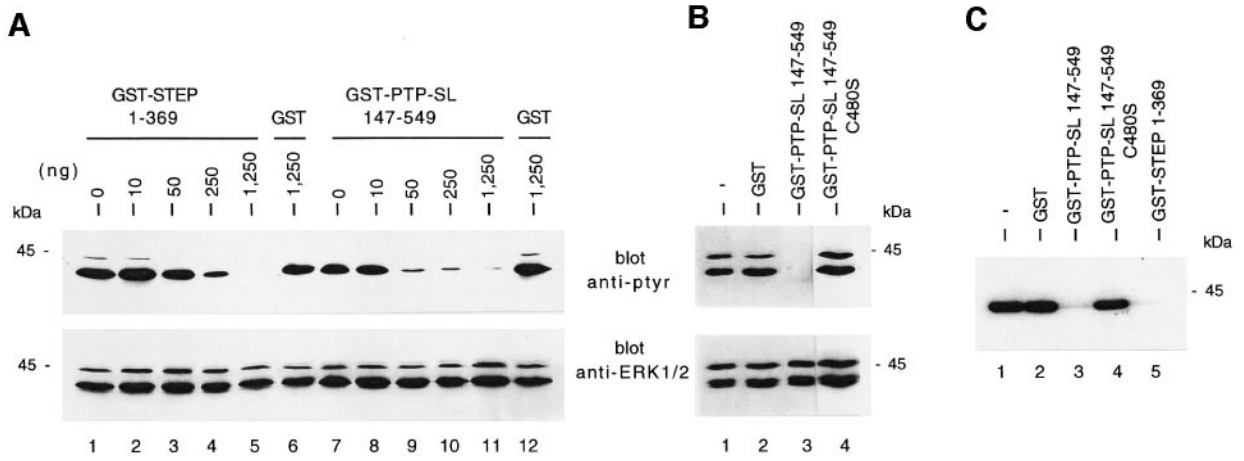


Fig. 5. Dephosphorylation and inactivation of ERK1/2 by GST–PTP-SL and GST–STEP fusion proteins *in vitro*. Rat 1 cells were treated with 50 ng/ml of EGF for 10 min, and cell lysates were precipitated with GST–PTP-SL 147–255 (1 μg/sample). (**A**) Increasing amounts of GST–STEP 1–369 or GST–PTP-SL 147–549 were added to the pellets, as indicated (amounts are in nanograms), and *in vitro* phosphatase assays were carried out. In control lanes (lanes 6 and 12), 1.25 μg of GST were added. (**B**) One microgram of the different GST-fusion proteins was added to the pellets to perform the phosphatase assay, as indicated. In lane 1, no protein was added to the pellets. After phosphatase assays, all samples were resolved by 10% SDS–PAGE under reducing conditions, followed by immunoblot analysis with the anti-phosphotyrosine mAb 4G10 (upper panels), or a mixture of anti-ERK1 and -ERK2 antibodies (lower panels), and reactive bands were visualized by chemiluminescence. (**C**) Pellets containing ERK1/2 were subjected to *in vitro* phosphatase assays in the absence (lane 1) or presence of 1 μg of the different GST fusion proteins, as indicated. After phosphatase assays were carried out, pellets were washed and subjected to *in vitro* kinase assays, using as substrate the GST–PTP-SL 147–255 used to precipitate ERK1/2. Samples were resolved by 10% SDS–PAGE under reducing conditions, followed by autoradiography.

Next, the effect of tyrosine dephosphorylation of ERK1/2 on the kinase activity towards PTP-SL was tested. Phosphatase assays were performed with the different GST-fusion proteins after ERK1/2 precipitation with GST–PTP-SL 147–255, as indicated in Figure 5A. Samples were washed with kinase assay buffer to remove the GST–PTP fusion proteins added to the phosphatase assay, and kinase assays were performed in the presence of [γ-^{32}P]ATP to measure the phosphorylation of GST–PTP-SL 147–255 (Figure 5C). Tyrosine dephosphorylation of ERK1/2 by GST–PTP-SL 147–549 or GST–STEP 1–369 fusion proteins, abrogated the kinase activity towards GST–PTP-SL 147–255 (Figure 5C, lanes 3 and 5, respectively), whereas no effect was observed after incubation of pellets containing ERK1/2 with GST alone or GST–PTP-SL 147–549/C480S (Figure 5C, lanes 2 and 4, respectively).

Identification of the ERK1/2-interaction and -phosphorylation sites on PTP-SL

To identify the region on PTP-SL responsible of the interaction with ERK1/2, as well as the PTP-SL residue(s) phosphorylated by these kinases, deletion and amino acid substitution mutants of GST–PTP-SL 147–288 were generated and used to perform precipitation and *in vitro* kinase assays with Rat 1 cell lysates. The alignment of the amino acid sequences of the cytosolic N-terminal regions of PTP-SL and STEP (see Figure 13B) shows a conserved 16 amino acid peptide (residues 224–239 in the mouse PTP-SL sequence; Hendricks *et al*., 1995), which could be involved in the interaction of both PTPs with ERK1/2. Therefore, deletion mutants were generated lacking this 16 amino acid peptide (GST–PTP-SL 147–288/Δ224–239), or lacking peptides flanking such conserved motif (GST–PTP-SL 147–288/Δ208–223 and GST–PTP-SL 147–288/Δ238–255). As hypothesized, the GST–PTP-SL 147–288/Δ224–239 mutant failed to precipitate ERK1/2 (Figure 6A, lane 4), whereas the Δ208–223 and Δ238–255 mutants did precipitate the kinases (Figure 6A, lanes 3 and 5, respectively). To check the effect of these deletions on phosphorylation by ERK1/2, the different deletion mutants were added to pellets containing endogenous ERK1/2 (obtained after precipitation with GST–PTP-SL 147–549/C480S, which lacks phosphatase activity; see Figure 5B), followed by *in vitro* kinase assays. Phosphorylation was only detected on the GST–PTP-SL 147–288 wild-type and the Δ208–223 mutant (Figure 6B, lanes 2 and 3, respectively), but not in the GST–PTP-SL 147–288/Δ224–239 or Δ238–255 mutants (Figure 6B, lanes 4 and 5, respectively), suggesting that the residue(s) phosphorylated by ERK1/2 could be localized in the 224–255 amino acid region of PTP-SL. Since a unique ERK1/2 Ser/Thr-Pro phosphorylation motif (Thr^{253}Pro^{254}) is found within the 224–255 amino acid region of PTP-SL, an amino acid substitution mutant was generated in PTP-SL 147–288 that changed the Thr^{253} residue to alanine (T253A). The mutant fusion protein GST–PTP-SL 147–288/T253A, although associating with ERK1/2 proteins (Figure 6A, lane 6), was not phosphorylated (Figure 6B, lane 6), indicating that Thr^{253} is a major ERK1/2 substrate site. Moreover, identical patterns of phosphorylation of the distinct GST–PTP-SL mutants were identified in pellets precipitated from HA-ERK2-transfected COS-7 cells with the anti-HA mAb 12CA5 (data not shown).

These results demonstrate the existence of separate ERK1/2-interaction and -phosphorylation sites in PTP-SL, and indicate that a docking site between residues 224 and 239 (KIM) is required for ERK1/2 in order to phosphorylate the downstream Thr^{253} residue.

PTP-SL associates with, and is phosphorylated in vivo by ERK1/2

The endogenous expression and phosphorylation of PTP-SL was analyzed in PC12 cells using anti-PTP-SL antibod-

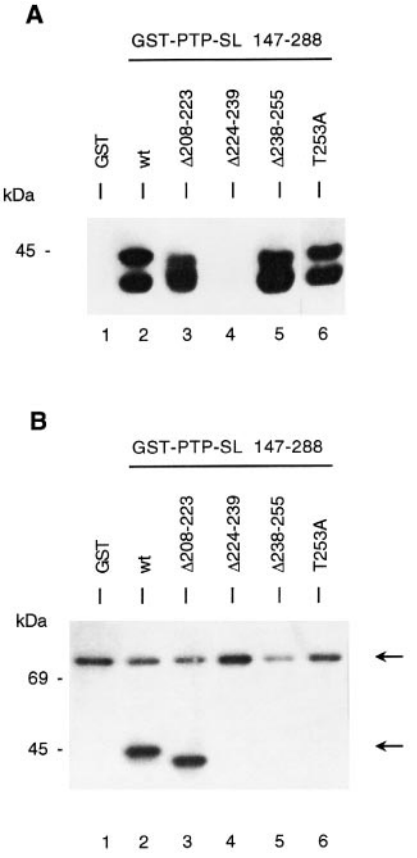


Fig. 6. Identification of the ERK1/2-interaction and -phosphorylation sites on PTP-SL. (**A**) Rat 1 cell lysates were precipitated with 1 μg of the distinct GST-fusion proteins, as indicated, and precipitates were processed for the presence of ERK1/2 as described above. (**B**) ERK1/2 were precipitated from Rat 1 cell lysates with 1 μg of GST–PTP-SL 147–549/C480S, and then 1 μg of the distinct GST fusion proteins was added to the pellets, as indicated, followed by *in vitro* kinase assays. Arrows in (B) indicate the migration of the GST–PTP-SL 147–549/C480S proteins used to precipitate ERK1/2 (upper bands), and the migration of the distinct GST fusion proteins added to the kinase assay (lower bands), as indicated at the top of the figure.

ies. PC12 cells were labeled with [^{35}S]methionine (Figure 7A) or ^{32}P (Figure 7B), and cell lysates were precipitated with pre-immune serum (Figure 7A and B, lane 1) or with anti-PTP-SL serum (Figure 7A and B, lane 2), followed by SDS–PAGE and autoradiography. PTP-SL proteins of ~80 and ~65 kDa were expressed on PC12 cells (Figure 7A, lane 2), probably corresponding to the two reported transmembrane PTP-SL isoforms (Hendricks *et al*., 1995; Ogata *et al*., 1995; Shiozuka *et al*., 1995). Remarkably, the PTP-SL proteins expressed on PC12 cells were found to be phosphorylated (Figure 7B, lane 2), demonstrating that endogenous PTP-SL is phosphorylated *in vivo*.

Next, the phosphorylation state of PTP-SL *in vivo* was analyzed in COS-7 cells after transfection with PTP-SL. Transfections were carried out using cDNAs encoding both transmembrane (residues 1–549; 62 kDa protein) and non-transmembrane PTP-SL isoforms (residues 147–549; 45 kDa protein) (Hendricks *et al*., 1995; Ogata *et al*., 1995; Sharma and Lombroso, 1995; Shiozuka *et al*., 1995). Immunofluorescence analysis confirmed that the non-transmembrane PTP-SL forms were present in the cytosol, whereas the transmembrane ones were found associated with membranes; in both cases, PTP-SL molecules were excluded from the nucleus (data not shown). Transfected cells were labeled with ^{32}P, and cell lysates were precipitated with anti-PTP-SL antibody (Figure 7C). Strong ^{32}P-labeling was observed in both PTP-SL 147–549 and PTP-SL 1–549 (Figure 7C, lanes 2 and 4, respectively), whereas only residual labeling was detected in the KIM-deletion mutants, PTP-SL 147–549/Δ224–239 and PTP-SL 1–549/Δ224–239 (Figure 7C, lanes 3 and 5, respectively). To assess the contribution of ERK1/2 to the *in vivo* phosphorylation of PTP-SL, cells transfected with transmembrane PTP-SL (PTP-SL 1–549) were labeled with ^{32}P and stimulated with EGF in the absence or presence of the MEK1/2 inhibitor PD98059, followed by precipitation of cell lysates with anti-PTP-SL antibody (Figure 7D). Phosphorylation of PTP-SL was increased upon EGF incubation (Figure 7D, lane 4 versus lane 2), and such increase was completely abolished by pre-incubation with PD98059 (Figure 7D, lane 6). Furthermore, the PTP-SL 1–549 T253A mutant was not hyperphosphorylated in response to EGF (Figure 7E, lane 3 versus lane 2), demonstrating that ERK1/2 phosphorylate the Thr^{253} residue of PTP-SL in intact cells.

Co-precipitation experiments were carried out to confirm that PTP-SL and ERK1/2 associate in intact cells. HA-tagged PTP-SL non-transmembrane proteins (HA-PTP-SL 147–549) were transiently expressed in COS-7 cells and immunoprecipitated by using the anti-HA mAb 12CA5, and the immune complexes were analyzed for the presence of endogenous ERK1/2 by immunoblot, using anti-ERK1/2 antibodies. As predicted from our previous findings (Figures 3 and 6), HA-PTP-SL 147–549 co-precipitated ERK molecules (Figure 8, upper panel, lanes 2 and 3), whereas HA-PTP-SL 147–549/Δ224–239 did not (Figure 8, upper panel, lanes 4 and 5). The expression of the different HA-PTP-SL proteins in the transfected cells is shown as a control (Figure 8, lower panel). All mock- and HA-PTP-SL-transfected COS-7 cells expressed identical levels of endogenous ERK1/2 (data not shown).

Taken together, these results indicate that the KIM sequence is essential for both *in vivo* phosphorylation of PTP-SL and association with ERK1/2, and demonstrate that ERK1/2 phosphorylates PTP-SL *in vivo* at the Thr^{253} residue.

PTP-SL regulates ERK2 activity in intact cells

To test for a functional association between PTP-SL and ERK1/2 in intact cells, co-transfection experiments were carried out in COS-7 cells using HA-tagged ERK2 and wild-type and mutant PTP-SL proteins. After co-transfection, cells were left untreated or were stimulated with EGF for 10 min to induce ERK2 phosphorylation and activation. Cell extracts were precipitated with the anti-HA mAb 12CA5, and tyrosine phosphorylation of HA-ERK2 was monitored by immunoblot using the anti-phosphotyrosine mAb 4G10 (Figure 9A, top panel). Both basal and EGF-induced tyrosine phosphorylation of HA-ERK2 were strongly diminished when cells co-expressed wild-type non-transmembrane PTP-SL (residues 147–549) (Figure 9A, top panel, lanes 3 and 4 versus lanes 1 and 2). However, when the inactive non-transmembrane PTP-SL C480S was co-expressed, HA-ERK2 tyrosine phosphorylation did not decrease, but rather increased somewhat

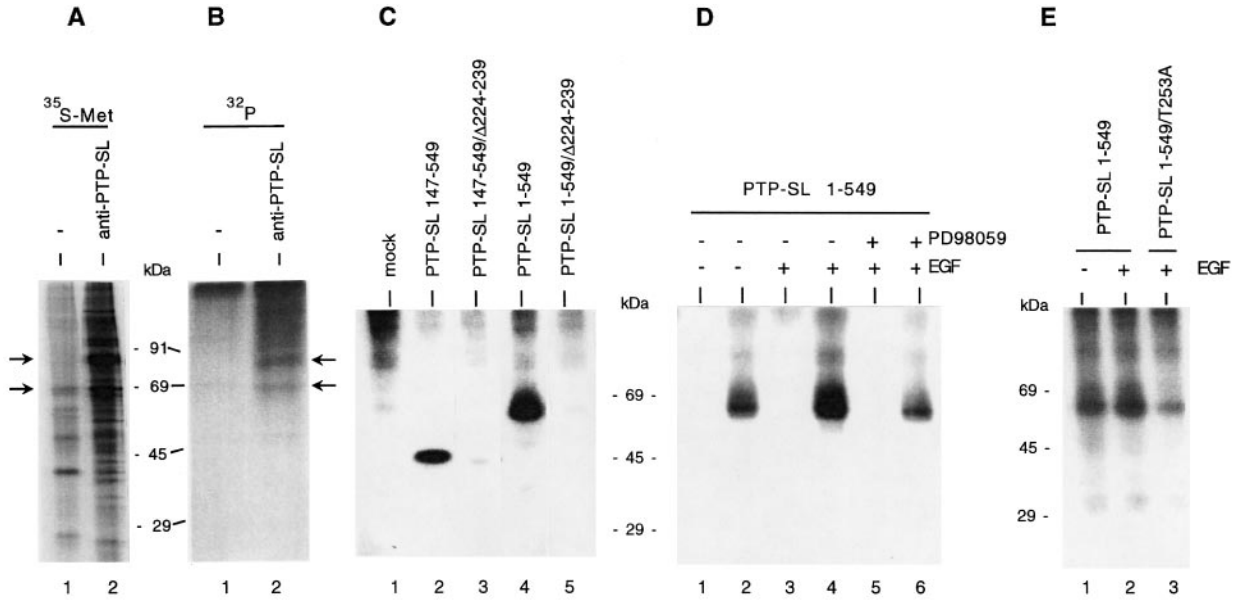


Fig. 7. *In vivo* phosphorylation of PTP-SL. (**A**) PC12 cells were labeled with [^{35}S]methionine, and cell lysates were precipitated with rabbit pre-immune serum (lane 1) or rabbit anti-PTP-SL serum (lane 2). (**B**) PC12 cells were labeled with ^{32}P, and cell lysates were precipitated with pre-immune serum (lane 1) or anti-PTP-SL antiserum (lane 2). The arrows in (A) and (B) indicate the 80 and 65 kDa PTP-SL proteins. (**C**) COS-7 cells were mock-transfected (pRK5 vector alone) (lane 1), or transfected with pRK5 PTP-SL 147–549 (lane 2), pRK5 PTP-SL 147–549/Δ224–239 (lane 3), pRK5 PTP-SL 1–549 (lane 4) or pRK5 PTP-SL 1–549/Δ224–239 (lane 5). After 48 h, cells were labeled with ^{32}P and cell lysates were precipitated with anti-PTP-SL antiserum. (**D**) COS-7 cells were transfected with pRK5 PTP-SL 1–549. After 48 h, cells were labeled with ^{32}P and left untreated (lanes 1 and 2) or were treated with 50 ng/ml of EGF for 10 min in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of 50 μM PD98059. Prior to stimulation with EGF in the presence of PD98059, cells were pre-incubated with the inhibitor for 30 min. Cell lysates were precipitated with preinmune serum (lanes 1, 3 and 5) or with anti-PTP-SL antiserum (lanes 2, 4, and 6). (**E**) COS-7 cells were transfected with pRK5 PTP-SL 1–549 (lanes 1 and 2) or pRK5 PTP-SL 1–549 T253A (lane 3). After 48 h, cells were labeled with ^{32}P and left untreated (lane 1), or were treated with 50 ng/ml of EGF for 10 min (lanes 2 and 3), and cell lysates were precipitated with anti-PTP-SL antiserum. All samples were resolved by 10% SDS–PAGE under reducing conditions, followed by autoradiography.

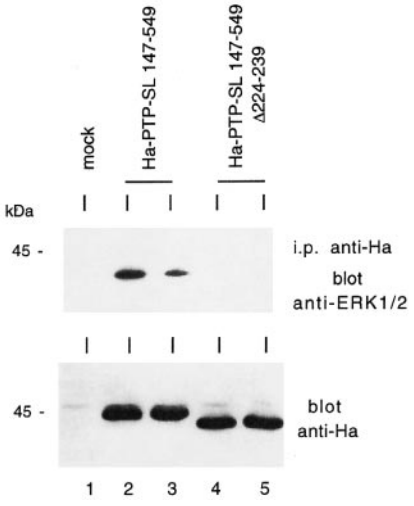


Fig. 8. *In vivo* association of PTP-SL with ERK1/2. COS-7 cells were mock-transfected (pRK5 vector alone) (lane 1), or transfected with pRK5 HA-PTP-SL 147–549 (lanes 2 and 3; 0.2 and 1 μg of DNA, respectively) or HA-PTP-SL 147–549/Δ224–239 (lanes 4 and 5; 0.2 and 1 μg of DNA, respectively). After 48 h, cells were lysed, and lysates were precipitated with the anti-HA mAb 12CA5 (upper panel). Samples were resolved by 10% SDS–PAGE under reducing conditions, followed by immunoblot analysis with anti-ERK1 and -ERK2 antibodies. In the lower panel, total lysate samples (10 μg) were loaded, and immunoblotting was performed with the anti-HA mAb 12CA5.

(Figure 9A, top panel, lanes 5 and 6 versus lanes 1 and 2). The levels of expression of HA-ERK2, and wild-type and mutant PTP-SL proteins, were identical in the different transfected cells (Figure 9A, middle and lower panels)

Finally, the kinase activity of HA-ERK2 in the different transfectants was analyzed by precipitation with the anti-HA mAb 12CA5, followed by *in vitro* kinase assays using the GST–PTP-SL 147–288 fusion protein as the substrate (Figure 9B). In cells co-expressing wild-type non-transmembrane PTP-SL, HA-ERK2 activation was remarkably decreased, whereas in cells co-expressing non-transmembrane PTP-SL C480S, this was not the case (Figure 9B, lanes 3 and 4, and lanes 5 and 6, respectively, versus lanes 1 and 2). Interestingly, the basal activity of HA-ERK2 was consistently increased in cells co-expressing non-transmembrane PTP-SL C480S (Figure 9B, lane 5 versus lane 1, and Figure 10).

The kinetics of the EGF-induced activation of HA-ERK2 in cells co-expressing HA-ERK2 and non-transmembrane PTP-SL was also investigated. As seen in Figure 10A, co-expression of HA-ERK2 with wild-type non-transmembrane PTP-SL resulted in a sustained inhibition of the EGF-induced HA-ERK2 activity. Remarkably, co-expression of HA-ERK2 with the catalytically inactive non-transmembrane PTP-SL C480S mutant, enhanced the EGF-induced HA-ERK2 activity, both quantitatively and temporally, in contrast to the very fast kinetics of inactivation observed in the controls (Figure 10A). When the PTP-SL double mutant lacking the KIM (C480S/Δ224–239) was co-expressed with HA-ERK2, the enhancing effect of PTP-SL C480S was completely lost (Figure 10C). Furthermore, the inhibitory effect of wild-type PTP-SL was also diminished compared with the effect of PTP-SL Δ224–239 (Figure 10B). *In vitro* phosphatase assays also

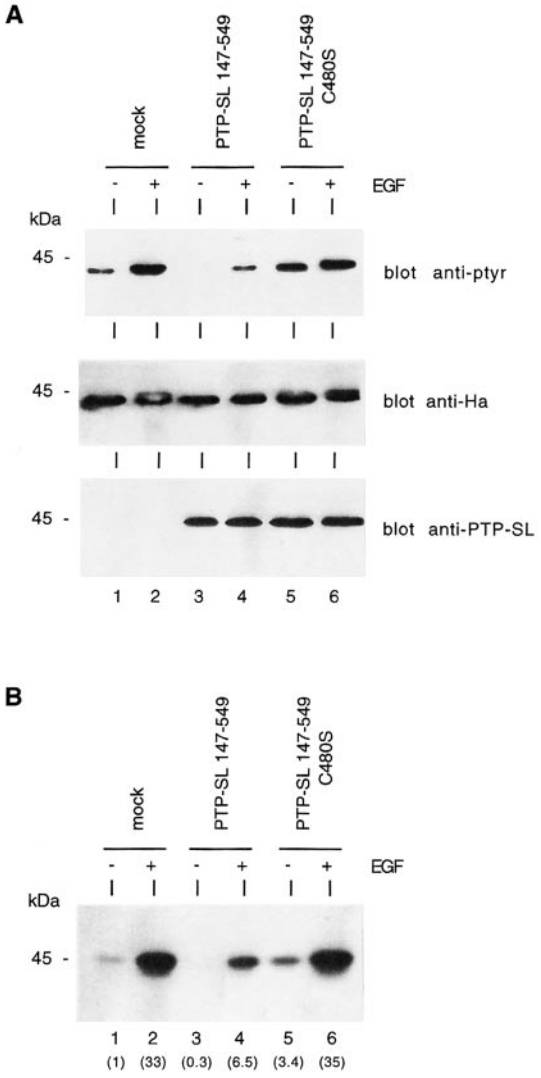


Fig. 9. Non-transmembrane PTP-SL dephosphorylates and inactivates ERK1/2 in intact cells. (**A**) COS-7 cells were co-transfected with pCDNA3 HA-ERK2 plus pRK5 vector alone (mock) (lanes 1 and 2), pCDNA3 HA-ERK2 plus pRK5 PTP-SL 147–549 (lanes 3 and 4) or pCDNA3 HA-ERK2 plus pRK5 PTP-SL 147–549/C480S (lanes 5 and 6). After 48 h, cells were left untreated (–) or were treated for 10 min with 50 ng/ml of EGF (+). HA-ERK2 was precipitated with the anti-HA mAb 12CA5, and samples were resolved by 10% SDS–PAGE under reducing conditions, followed by immunoblot analysis with the anti-phosphotyrosine mAb 4G10 (upper panel). The content of HA-ERK2 in all samples was analyzed after stripping of the filter shown in the upper panel, followed by immunoblotting with the anti-HA mAb 12CA5 (middle panel). In the lower panel, total lysate samples (10 μg) were loaded, and analyzed for the presence of PTP-SL recombinant molecules by immunoblotting with an anti-mPTP-SL serum. (**B**) COS-7 cells were transfected and treated with EGF as in (A). After precipitation with the anti-HA mAb 12CA5, 1 μg of GST–PTP-SL 147–288 was added to the pellets, and samples were processed for *in vitro* kinase assays, using GST–PTP-SL 147–288 as the substrate. Numbers in parentheses at the bottom correspond to the relative radioactivity incorporated into the substrate in the distinct samples (the value in sample 1 was 6500 c.p.m.).

showed that PTP-SL Δ224–239 dephosphorylates ERK2 to a lesser extent than wild-type PTP-SL (data not shown).

Co-expression of HA-ERK2 with transmembrane PTP-SL molecules (PTP-SL 1–549) similarly affected the activity of this kinase, although both the inhibitory and enhancing effects on such activity were consistently

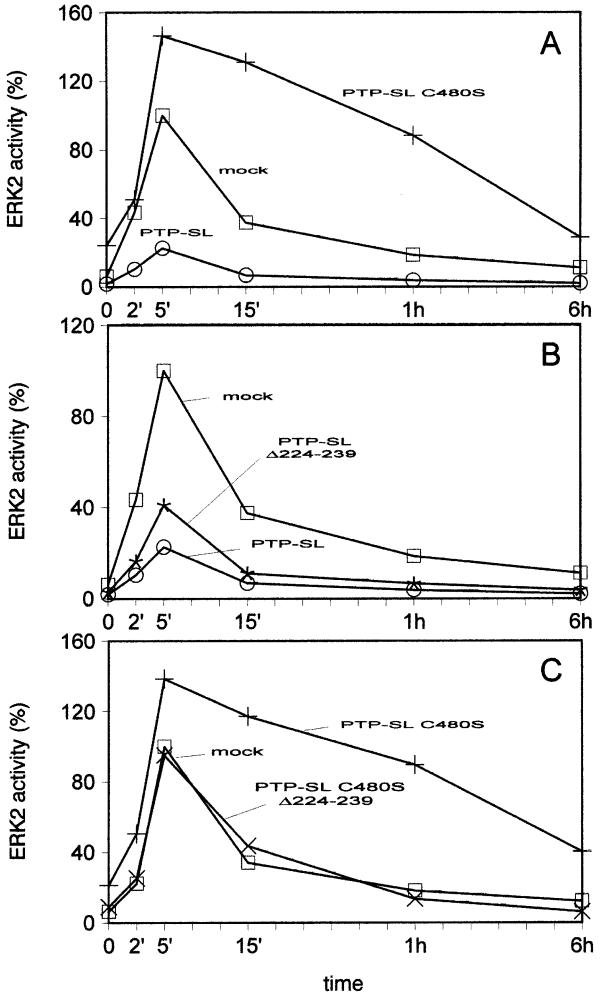


Fig. 10. Kinetics of EGF-induced ERK2 activation in COS-7 cells transfected with non-transmembrane PTP-SL. COS-7 cells were co-transfected with pCDNA3 HA-ERK2 plus pRK5 vector alone (mock) (□), pCDNA3 HA-ERK2 plus pRK5 PTP-SL 147–549 (○), pCDNA3 HA-ERK2 plus pRK5 PTP-SL 147–549/Δ224–239 (*), pCDNA3 HA-ERK2 plus pRK5 PTP-SL 147–549/C480S (+) or pCDNA3 HA-ERK2 plus pRK5 PTP-SL 147–549/C480S/Δ224–239 (×). After 48 h, cells were treated with 50 ng/ml of EGF during the times indicated, and HA-ERK2 was precipitated with the anti-HA mAb 12CA5. Then, 1 μg of GST–PTP-SL 147–288 was added to the pellets as the substrate and samples were processed for *in vitro* kinase assays. Data are presented as the percentage of ERK2 activity with respect to that shown for cells transfected with pCDNA3 HA-ERK2 plus pRK5 vector alone (mock) after 5 min of EGF stimulation (100% ERK2 activity). Values represent the mean of two separate experiments, and SDs were always <15% of the respective values. Results are shown in three sets of data [(**A**), (**B**) and (**C**)] to facilitate comparison.

weaker than that observed upon co-expression with the non-transmembrane PTP-SL forms (Figure 11).

Confirmation of the involvement of PTP-SL in the regulation of ERK1/2 activity was obtained in 3T3 cells stably expressing PTP-SL (Figure 12). Clones expressing non-transmembrane PTP-SL (PTP-SL 147–549) were shown to have diminished the activity of ERK1/2 (Figure 12, lower panel), in both the presence and absence of serum, as compared with mock-transfected cells. As a control, the expression levels of PTP-SL 147–549 in the distinct clones is shown (Figure 12, upper panel). Similar levels of ERK1/2 expression were observed in all clones (data not shown).

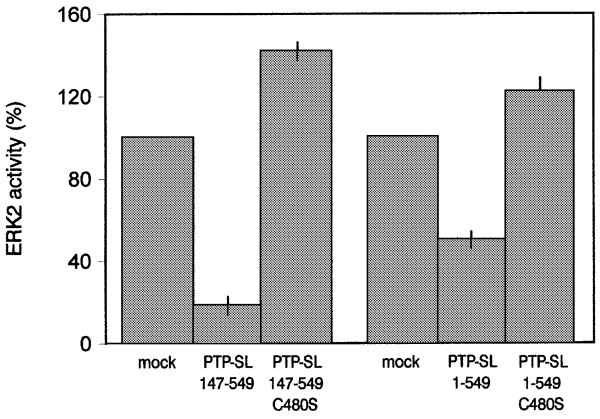


Fig. 11. Modulation of ERK2 activity by non-transmembrane and transmembrane PTP-SL. COS-7 cells were co-transfected with pCDNA3 HA-ERK2 plus pRK5 alone, or with pCDNA3 HA-ERK2 plus pRK5 containing the non-transmembrane (147–549) or transmembrane (1–549) PTP-SL wild-type or C480S forms, as indicated. After 48 h, cells were treated with 50 ng/ml of EGF for 5 min and processed to measure ERK2 activity as in Figure 9. Values represent the mean of two separate experiments ± SD.

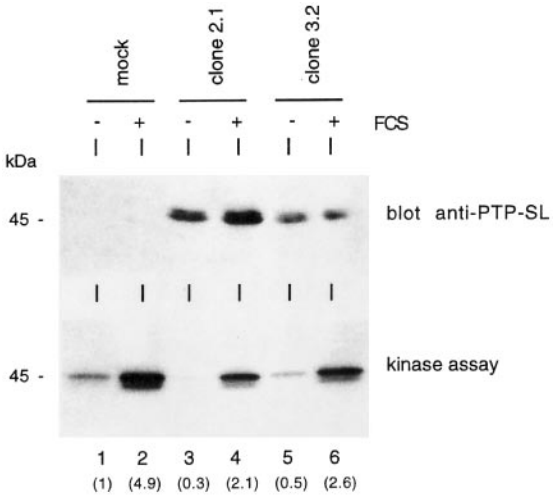


Fig. 12. Downregulation of ERK1/2 activity in 3T3 cells stably expressing PTP-SL. Independent clones of 3T3 cells, non-expressing (lanes 1 and 2) or expressing recombinant PTP-SL 147–549 (lanes 3–6), were grown in the presence of 10% fetal calf serum (FCS); then cells were left untreated (+), or were starved without FCS for 4 h (–). ERK1/2 were precipitated from cell lysates (400 µg) with 1 µg of GST–PTP-SL 147–288, and samples were processed for *in vitro* kinase assays, using GST–PTP-SL 147–288 as the substrate (lower panel). Numbers in parentheses at the bottom correspond to the relative radioactivity incorporated into the substrate in the distinct samples (the value in sample 1 was 44 500 c.p.m.). In the upper panel, 50 µg of total lysate samples were loaded and analyzed for the presence of PTP-SL by immunoblot with anti-PTP-SL antiserum.

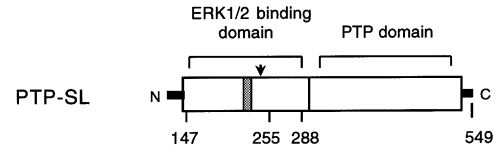
Taken together, our results indicate that PTP-SL associates with ERK1/2 *in vitro* and in intact cells through a specific docking site for these kinases (ERK1/2-KIM), and inactivate them by dephosphorylation of their tyrosine regulatory residues, and suggest that catalytically inactive splice forms of PTP-SL, by competing with active PTP forms for the docking site, could regulate the ERK1/2 activation signal.

Discussion

Following stimulus-specific phosphorylation and activation of ERKs, dephosphorylation and deactivation of these enzymes takes place with kinetics ranging from several minutes to several hours, depending on the cell type and the activating stimulus. The degree and duration of ERK activation during signal transduction have been postulated to be key parameters in the definition of the cell response to distinct extracellular stimuli (Traverse *et al*., 1992, 1994; Dikic *et al*., 1994; Marshall, 1995; Racke *et al*., 1997; Rouyez *et al*., 1997; Whalen *et al*., 1997). Protein serine/threonine phosphatase 2A has been found to be the major protein serine/threonine phosphatase that inactivates ERK2 (Alessi *et al*., 1995; Braconi-Quintaje *et al*., 1996; Chajry *et al*., 1996). In addition, the nuclear MAP kinase dual specificity phosphatases (MKPs) have been proposed to be involved in the inactivation of ERKs, after their translocation to the nucleus, in different mammalian and non-mammalian cell types (Anderson *et al*., 1990; Sun *et al*., 1993; Zheng and Guan, 1993; Doi *et al*., 1994; Ward *et al*., 1994; Lewis *et al*., 1995). However, the phosphotyrosine dephosphorylation and inactivation of ERKs in the cytoplasm is still unresolved (Wu *et al*., 1994), and recent reports suggest that vanadate-sensitive PTP activities may also play a role in the *in vivo* regulation of ERKs (Alessi *et al*., 1995; Zhao *et al*., 1996). In this report, we present evidence that the PTP-SL and STEP PTPs can associate with and inactivate cytosolic ERK1/2 in a rapid and specific manner by dephosphorylating the regulatory phosphotyrosine of the kinases. This physical and functional interaction is mediated by a 16 residue-ERK1/2 binding site (KIM), which is conserved in PTP-SL and STEP. In addition, we have found that association of ERK1/2 with the KIM sequence on PTP-SL leads to the phosphorylation of this PTP, predominantly on Thr^{253} (see Figure 13A for a summary), suggesting that the physical association of the two enzymes results in a transregulation of their biological activities. Serine/threonine phosphorylation of some cytosolic and transmembrane PTPs has been found to affect their intrinsic enzymatic activity, and it is conceivable that phosphorylation events regulate the subcellular localization and/or the association with substrates of these PTPs (Yamada *et al*., 1990; Garton and Tonks, 1994). For example, ERK2 has been shown to downregulate EGF receptor tyrosine phosphorylation through the activation of a PTP (Griswold-Prenner *et al*., 1993).

The results presented here suggest that PTP-SL and ERK1/2 are associated independently of the activation state of ERK1/2 (see Figure 3C). This is in agreement with recent crystallographic data indicating that the conformation of the extended substrate-binding region of ERK2 does not change upon phosphorylation of the kinase on the threonine and tyrosine regulatory residues (Canagarajah *et al*., 1997). However, one cannot exclude the possibility of such a regulation *in vivo*. Thus, phosphorylation of PTP-SL by ERK1/2 could account for a switch on/off mechanism of ERK1/2 tyrosine dephosphorylation, either by direct interference with the PTP activity or by affecting the stability of the complex between the two molecules. So far, we have been unable to detect modifications on the PTP activity of PTP-SL after *in vitro* phospho-

A



GST-PTP-SL fusion proteins	Association with ERK1/2	Phosphorylation by ERK1/2
GST alone	-	-
147-549	+	+
147-288	+	+
147-255	+	+
256-549	-	-
289-549	-	-
Δ208-223	+	+
Δ224-239	-	-
Δ238-255	+	-
T253A	+	-

B

PTP-SL	--	(224)	G	L	Q	E	R	R	G	S	N	V	S	L	T	L	D	M	(239)	--					
STEP	--	(42)	G	L	Q	E	R	R	G	S	N	V	S	L	T	L	D	M	(57)	--					
LC-PTP	--	(37)	R	L	Q	E	R	R	G	S	N	V	A	L	M	L	D	V	(52)	--					

Fig. 13. Schematic showing the ERK1/2-association and -phosphorylation sites of PTP-SL. (**A**) The top is a depiction of the structure of the cytosolic portion of PTP-SL. Amino acid numbering is shown according to Hendricks *et al.* (1995). The gray box in the ERK1/2 binding domain indicates the position of the ERK1/2 interaction motif (KIM) (peptide 224–239). The arrow indicates the position of the residue (Thr^{253}) phosphorylated by ERK1/2. The table below summarizes the association and phosphorylation by ERK1/2 of the distinct GST–PTP-SL fusion proteins, as described in Results. (**B**) Alignment of the ERK1/2-KIM peptide of PTP-SL (residues 224–239; Hendricks *et al*., 1995) with the equivalent STEP (residues 42–57; Lombroso *et al*., 1991) and LC-PTP/HePTP (residues 37–52; Adachi *et al*., 1992) sites. Amino acids are indicated using the one-letter code. Identical residues are boxed.

rylation by ERK1/2 (our unpublished observations); however, the interpretation of these experiments is hampered by the fact that the PTP activity itself, through dephosphorylation of the regulatory tyrosine, can interfere with the kinase activity of ERK1/2 during the course of the kinase assays. Further work will be necessary to elucidate the role of phosphorylation on the function of PTP-SL in the context of a complex with ERK1/2.

Interestingly, our findings regarding the docking site-dependent phosphorylation of PTP-SL by ERK1/2 are analogous to that for the phosphorylation of the Ste7 kinase by the yeast ERKs homologs Kss1/Fus3 (Bardwell *et al*., 1996), and that of the transcription factors c-Jun and Elk-1 by the c-Jun N-terminal kinases (JNKs) and ERK2, respectively (Hibi *et al*., 1993; Karin, 1995; Yang *et al*., 1998). Thus, one could hypothesize that the existence of a KIM, required for phosphorylation of a proximal Ser/Thr residue, could be a common feature of some physiological substrates of MAP kinases. These KIMs could confer different specificities for the recruitment of substrates and/or regulatory molecules, allowing them to operate through distinct signal transduction pathways and/or at distinct subcellular compartments. In this regard, other PTPs have recently been identified in yeast that selectively associate and dephosphorylate Hog1/Sty1, the yeast homologue of the mammalian p38 MAPK (Millar *et al*., 1995; Jacoby *et al*., 1997; Wurgler-Murphy *et al*., 1997).

Our observation that the EGF-induced tyrosine phosphorylation and activation of ERK2 are strongly diminished in COS-7 cells co-expressing wild-type PTP-SL suggests an active role for this PTP in the physiological regulation of the early steps of the ERK signaling cascade. Furthermore, we have found that the basal levels of ERK1/2 activities are downregulated in 3T3 cells stably expressing wild-type PTP-SL, both in the presence and in the absence of serum. Phosphorylation of cytosolic substrates, as well as feedback phosphorylation, is a known property of ERK1/2 (Lee *et al*., 1992; Mukhopadhyay *et al*., 1992), which may contribute to the regulation of the Ras signaling pathway and could be a target site for cross-talk with other signaling pathways. Interestingly, the cytosolic Pyst1/MKP-3 phosphatase has also been found to be tightly associated with ERK1/2 and to specifically dephosphorylate these kinases (Groom *et al*., 1996; Muda *et al*., 1998). Thus, different compartmental levels of ERK1/2 inactivation might exist in the cell, which would involve either different forms of PTP-SL and STEP, other cytosolic phosphatases or nuclear MKPs.

We have also found that the basal levels of ERK2 activity are increased by co-expression of the non-transmembrane catalytically inactive C480S PTP-SL mutant, and that the kinetics of EGF-induced ERK2 activation under these conditions is remarkably sustained, in a KIM sequence-dependent manner (Figures 9 and 10C). These findings suggest that binding to the non-catalytic PTP-SL would prevent the dephosphorylation of ERK2 by an endogenous, catalytically active, PTP. Together, our results support the hypothesis that: (i) endogenous PTP-SL could be responsible of the rapid *in vivo* inactivation of ERK1/2 in the cytosol after activation by the MEKs; and (ii) endogenous forms of PTP-SL molecules, lacking PTP activity but containing the KIM peptide, could be *in vivo* modulators of ERK1/2 activities, by inhibiting its association with an active PTP. In this regard, truncated forms of STEP have been described which contain the ERK1/2-KIM and lack the PTP domain (see below) (Sharma *et al*., 1995; Bult *et al*., 1997). In addition, the association of ERK1/2 with the different forms of PTP-SL could contribute to the regulation of their subcellular localization upon distinct cell activation conditions (González *et al*., 1993; Lenormand *et al*., 1993).

PTP-SL and STEP are enzymes that exist in transmembrane and cytosolic forms (Lombroso *et al*., 1991; Ogata *et al*., 1995; Hendricks *et al*., 1995; Sharma and Lombroso, 1995; Shiozuka *et al*., 1995). Different STEP isoforms have been described which seem to be generated by alternative splicing of a unique precursor STEP mRNA, and whose expression is developmentally regulated in the brain (Boulanger *et al*., 1995; Sharma *et al*., 1995; Bult *et al*., 1997). Similarly, distinct transmembrane and non-transmembrane PTP-SL (also named PTPBR7, PC12-PTP1 and PCPTP1) isoforms have also been documented (Hendricks *et al*.,

1995; Ogata *et al*., 1995; Sharma and Lombroso, 1995; Shiozuka *et al*., 1995). In addition, another related PTP, LC-PTP (also designated HePTP) (Adachi *et al*., 1992; Zanke *et al*., 1992), is encoded by a distinct gene and is mainly expressed in lymphoid tissues. All these PTPs have in common the existence of a large, cytosolic non-catalytic N-terminal region of unknown function. Remarkably, the KIM sequence, which is required for interaction of PTP-SL with ERK1/2, is located in this region and is highly conserved in these three PTPs (Figure 13B). Truncated forms of STEP, containing the KIM peptide, also associate to and are phosphorylated by ERK1/2 (Figure 4), and putative serine or threonine ERK1/2 phosphorylation sites are present in both STEP and LC-PTP/HePTP, which are located in positions analogous to the PTP-SL Thr^{253} ERK1/2 phosphorylation site. From these results, it can be predicted that STEP and LC-PTP/HePTP associate with and are phosphorylated by ERK1/2 in a similar manner as PTP-SL. In this regard, it has been shown that LC-PTP/HePTP reduces the T-cell antigen receptor-induced activation of ERK2 (Saxena *et al*., 1998). Interestingly, the ERK1/2-KIM sequence (see Figure 13B) contains a phosphorylation consensus sequence for cAMP-dependent protein kinase substrates (Kemp and Pearson, 1990), suggesting the possibility that phosphorylation of the KIM peptide could regulate the functional interaction between the PTPs containing the KIM and ERK1/2. This model is supported by our finding that phosphorylation of PTP-SL in intact cells is dependent on the presence of the KIM sequence on the PTP. It should also be mentioned that high expression levels of HA-PTP-SL were necessary to co-precipitate ERKs with the anti-HA mAb (Figure 8), and that association of truncated GST–PTP-SL proteins with ERK1/2 was consistently more pronounced than that shown by GST–PTP-SL protein containing the intact intracellular portion of the PTP (Figures 1 and 2). Thus, additional regulatory mechanisms might exist in intact cells that regulate the association of PTP-SL with ERK1/2.

PTP-SL and STEP are likely to associate with other proteins than ERK1/2, which may contribute to the regulation of their subcellular localization and function. The as yet unidentified proteins of 70–80 kDa that we also find associated with the juxtamembrane domain of PTP-SL and STEP (Figures 1 and 4) may be of significance in this context. Our *in gel* kinase assays suggest that, other than ERK1/2, these proteins do not phosphorylate PTP-SL. One could consider that these proteins represent linkers to other signaling proteins or other cellular components. Interestingly, the existence of molecular complexes that physically link multiple kinases in the Fus3/MAPK/ERK1/2 signaling cascades, have been reported (Scimeca *et al*., 1992; Choi *et al*., 1994; Hsiao *et al*., 1994; Fukuda *et al*., 1997; Fukunaga *et al*., 1997; Waskiewicz *et al*., 1997). The recruitment of PTP-SL and related proteins into such multimolecular signaling complexes through direct association with ERK1/2 could be pivotal for regulating the kinase activities of their elements, and therefore for the control and definition of a wide variety of biological signals.

Materials and methods

Plasmid construction, mutagenesis and purification of GST-fusion proteins

The cDNA encoding mouse PTP-SL (mPTP-SL) was obtained by RT–PCR amplification of a cDNA library from mouse brain, using primers flanking the mPTP-SL coding region (Hendricks *et al*., 1995). Rat STEP cDNA was kindly provided by P.Lombroso (Lombroso *et al*., 1991). For expression in COS-7 cells, cDNAs coding the wild-type or mutant sequences of the full-length mPTP-SL [amino acids 1–549; amino acid numbering of all PTP-SL proteins used in this study is according to Hendricks *et al*. (1995)], the cytosolic portion of mPTP-SL (amino acids 147–549) or the entire sequence of mERK2 (Her *et al*., 1991) were subcloned into the expression vectors pRK5 (based on the cytomegalovirus early promoter) and pCDNA3 (Invitrogen Corp., Carlsbad, CA). For construction of pRK5-mPTP-SL 147–549, PCR amplifications were made using a primer which contains a Kozak sequence for initiation of translation (Kozak *et al*., 1988), followed by a start codon and the mPTP-SL sequence. For N-terminal HA-tagging of mPTP-SL and mERK2, PCR amplifications were made using a primer which contain a Kozak sequence, followed by the immunodominant peptide sequence from influenza hemagglutinin HA1 (Wilson *et al*., 1984). mPTP-SL deletion mutants and amino-acid-substitution mutants were performed by oligonucleotide site-directed mutagenesis, and mutations were confirmed by DNA sequencing. For the construction of plasmids encoding GST-fusion proteins, the complete or truncated forms of the cytoplasmic portions of mPTP-SL or STEP (see scheme in Figure 1) were subcloned into the expression vector pGEX-5X (Pharmacia Biotech, Uppsala, Sweden). GST–HPTPδ-D1 has been described previously (Pulido *et al*., 1995). Purification of all GST-fusion proteins was done using glutathione–Sepharose beads (Pharmacia Biotech), as described previously (Smith and Johnson, 1988). The apparent molecular weight in SDS–PAGE gels of all purified GST-fusion proteins was in accordance with their predicted sizes, as follows: GST, 29 kDa; GST–PTP-SL 147–549, 74 kDa; GST–PTP-SL 147–288, 45 kDa; GST–PTP-SL 147–255, 41 kDa; GST–PTP-SL 289–549, 58 kDa; GST–PTP-SL 256–549, 62 kDa; GST–STEP 1–369, 71 kDa; GST–STEP 1–107, 41 kDa. Sequences of primers used for construction of all plasmids and for mutagenesis are available upon request.

Antibodies and reagents

Rabbit polyclonal anti-mPTP-SL was obtained by immunization of rabbits with GST–mPTP-SL 147–549. Rabbit polyclonal anti-ERK1 (C-16) and anti-ERK2 (C-14) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The 12CA5 anti-HA mAb was from Boehringer Mannheim (Mannheim, Germany). The 4G10 anti-phosphotyrosine mAb was from Upstate Biotechnology Inc. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were from Oncogen Research Products (Cambridge, MA). Chemoluminescence reagents were from Boehringer Mannheim, and were used following the manufacturer's instructions. PMA (Sigma Chemical Co., St Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at 1 mg/ml, and was used at a final concentration of 10 ng/ml. EGF was from Gibco-BRL (Gaithersburg, MD), and NGF was from Boehringer Mannheim; both were used at a final concentration of 50 ng/ml. The MEK1/2 inhibitor PD98059 (Dudley *et al*., 1995) (New England Biolabs Inc., Beverly, MA) was dissolved in DMSO at 50 mM, and was used at a final concentration of 50 μM. Glutathione–Sepharose and protein A–Sepharose were from Pharmacia Biotech. [^{35}S]methionine and [γ-^{32}P]ATP were from Amersham (Little Chalfont, UK).

Cell culture and transfections

Rat fibroblast Rat 1, rat pheochromocytoma PC12, human embryonic 293, simian COS-7 and mouse fibroblast 3T3 cell lines (all from ATCC) were grown in Dulbecco's minimal essential medium (DMEM) containing high glucose (4.5 g/l) supplemented with 10% heat-inactivated FCS. Human neuroblastoma SK-N-SH cell line (ATCC) was grown in MEM supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% FCS. All media were purchased from Gibco-BRL. For [^{35}S]methionine labeling, cells were cultured for 4 h with L-methionine-free DMEM and 2% dialyzed FCS in the presence of [^{35}S]methionine (0.1 mCi/5×10^5 cells). For ^{32}P-labeling, cells were cultured for 4 h with phosphate-free DMEM 2% FCS in the presence of [^{32}P]inorganic-phosphate (25 μCi/10^5 cells). COS-7 cells were transfected with the pRK5-mPTP-SL and pCDNA3-mERK2 expression plasmids using the DEAE–dextran method, and were harvested after 48–72 h of culture. 3T3 cells were transfected with pRK5-mPTP-SL plus pSV2neoSP (3:1) by the calcium phosphate precipitation method, and clones were selected by growing in the presence of 0.4 mg/ml geneticin sulfate (Gibco-BRL).

Precipitation with GST-fusion proteins, immunoprecipitation and immunoblot

Cell cultures were washed with ice-cold PBS, and cells were lysed in lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40)

supplemented with protease inhibitors (1 mM PMSF and 1 μg/ml aprotinin) and phosphatase inhibitors (100 mM NaF, 2 mM Na_3VO_4, and 20 mM $Na_4P_2O_7$). Lysates were centrifuged for 10 min at 14 000 r.p.m. in an Eppendorf microcentrifuge 5415C and post-nuclear supernatants were pre-cleared with glutathione–Sepharose or protein A–Sepharose (20 μl of slurry). For GST-fusion protein precipitations, 1 μg of GST-fusion protein was added per sample, followed by 20 μl of glutathione–Sepharose. For immunoprecipitation, mAb 12CA5 was added, followed by 20 μl of protein A–Sepharose. Precipitates were washed 4 times with HNTG buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100). For SDS–PAGE analysis, SDS sample buffer was added to the pellets before boiling. For immunoblot analysis, the separated proteins were transferred to nitrocellulose and incubated with the primary antibody. After washing, filters were incubated with the secondary peroxidase-conjugated antibody, and reactivity was visualized by chemiluminescence. Dilution of antibodies and washing of nitrocellulose filters for immunoblot was done in 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100 and 0.25% gelatin.

In vitro kinase and phosphatase assays

For *in vitro* kinase assays, pellets obtained after precipitation with the GST-fusion proteins and washing with HNTG buffer, were washed once with kinase reaction buffer (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 2 mM Na_3VO_4, 0.3 μM ATP), and then were resuspended in 20 μl of kinase reaction buffer containing 0.5–2 μCi [γ-^{32}P]ATP, and incubated at room temperature for 15 min, under constant shaking. When samples were precipitated with the anti-HA mAb 12CA5, 1 μg of the GST–PTP-SL 147–288 fusion protein was added to the pellets as the substrate, before starting the kinase assay. Reactions were stopped by adding SDS sample buffer and boiling. The reaction mix was loaded and resolved on SDS–PAGE gels, and radioactivity incorporated into the GST-fusion proteins was visualized by autoradiography. For quantification, the GST–PTP-SL 147–288 fusion proteins used as the substrate, were excised from the gel and counted in a scintillation counter. *In gel* kinase assays were essentially performed as described by Hibi *et al*. (1993). Briefly, precipitated samples were resolved by SDS–PAGE on a gel polymerized in the presence of GST–PTP-SL 147–255 (40 μg/ml). After electrophoresis, washing and renaturation, the gel was incubated at 30°C for 1 h in kinase reaction buffer containing 1 μCi/ml of [γ-^{32}P]ATP, and incorporated radioactivity was visualized by autoradiography. For *in vitro* phosphatase assays, pellets obtained after precipitation and washing with HNTG buffer, were washed once with phosphatase reaction buffer (25 mM HEPES pH 7.3, 5 mM EDTA, 10 mM DTT). Then, 20 μl of phosphatase reaction buffer containing the indicated amount of GST-fusion proteins was added, and incubated at room temperature for 15 min, under constant shaking. Reactions were stopped by adding SDS sample buffer and boiling. Samples were resolved on SDS–PAGE gels and phosphotyrosine content of ERK1/2 was determined by immunoblot with the anti-phosphotyrosine mAb 4G10. When kinase *in vitro* assays were performed after phosphatase *in vitro* assays (Figure 5C), samples were washed twice, after the phosphatase assay, with kinase reaction buffer to remove the added phosphatase, and then kinase assays were performed as indicated above.

Acknowledgements

We thank Dr P.Lombroso for providing STEP cDNA, and our colleagues at the Max-Planck-Institut für Biochemie: H.Daub, T.Müller, C.Bagowski, J.-F.Giot and P.Knyazev, for providing plasmids and reagents, and for their suggestions and stimulating discussions. We also thank members of Instituto de Investigaciones Citológicas for their support, and I.Roglá for her excellent technical assistance. Á.Z. is the recipient of a postdoctoral fellowship from Bancaja (Valencia, Spain). This work was partially supported by a grant from Ministerio de Educación y Cultura (Spain) (PB96-0278) and from Sugen Inc.

References

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.

Alessi,D.R., Gómez,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.

Anderson,N.G., Maller,J.L., Tonks,N.K. and Sturgill,T.W. (1990) Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature*, **343**, 651–653.

Bardwell,L., Cook,J.G., Chang,E.C., Cairns,B.R. and Thorner,J. (1996) Signaling in the yeast pheromone response pathway: specific and high-affinity interaction of the mitogen-activated protein (MAP) kinases Kss1 and Fus3 with the upstream MAP kinase kinase Ste7. *Mol. Cell. Biol*., **16**, 3637–3650.

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.

Braconi-Quintaje,S., Church,D.J., Rebsamen,M., Valloton,M.B., Hemmings,B.A. and Lang,U. (1996) Role of protein phosphatase 2A in the regulation of mitogen-activated protein kinase activity in ventricular cardiomyocites. *Biochem. Biophys. Res. Commun.*, **221**, 539–547.

Bult,A., Zhao,F., Dirkx,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.

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.

Chajry,N., Martin,P.-M., Cochet,C. and Berthois,Y. (1996) Regulation of p42 mitogen-activated-protein kinase activity by protein phosphatase 2A under conditions of growth inhibition by epidermal growth factor in A431 cells. *Eur. J. Biochem*., **235**, 97–102.

Choi,K.-Y., Satterberg,B., Lyons,D.M. and Elion,E.A. (1994) Ste5 tethers multiple protein kinases in the MAP kinase cascade required for mating in *S. cerevisae*. *Cell*, **78**, 499–512.

Cobb,M.H. and Goldsmith,E.J. (1995) How MAP kinases are regulated. *J. Biol. Chem.*, **270**, 14843–14846.

Cobb,M.H., Boulton,T.G. and Robbins,D.J. (1991) Extracellular signal-regulated kinases: ERKs in progress. *Cell Regul*., **2**, 965–978.

Davis,R.J. (1993) The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem*., **268**, 14553–14556.

Dikic,I., Schlessinger,J. and Lax,I. (1994) PC12 cells overexpressing the insulin receptor undergo insulin-dependent neuronal differentiation. *Curr. Biol.*, **4**, 702–708.

Doi,K., Gartner,A., Ammerer,G., Errede,B., Shinkawa,H., Sugimoto,K. and Matsumoto,K. (1994) MSG5, a novel protein phosphatase promotes adaptation to pheromone response in *S.cerevisae. EMBO J*., **13**, 61–70.

Dudley,D.T., Pang,L., Decker,S.J., Bridges,A.J. and Saltiel,A.R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl Acad. Sci. USA*, **92**, 7686–7689.

Fischer,E.H., Charbonneau,H. and Tonks,N.K. (1991) Protein tyrosine phosphatases: a diverse family of intracellular and transmembrane enzymes. *Science*, **253**, 401–406.

Fukuda,M., Gotoh,Y. and Nishida,E. (1997) Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase. *EMBO J*., **16**, 1901–1908.

Fukunaga,R. and Hunter,T. (1997) MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J*., **16**, 1921–1933.

Garton,A.J. and Tonks,N.K. (1994) PTP-PEST, a protein tyrosine phosphatase regulated by serine phosphorylation. *EMBO J*., **13**, 3763–3771.

González,F.A., Seth,A., Raden,D.L., Bowman,D.S., Fay,F.S. and Davis,R.J. (1993) Serum-induced translocation of mitogen-activated protein kinase to the cell surface ruffling membrane and the nucleus. *J. Cell Biol.*, **122**, 1089–1101.

Griswold-Prenner,I., Carlin,C.R. and Rosner,M.R. (1993) Mitogen-activated protein kinase regulates the epidermal growth factor receptor through activation of a tyrosine phosphatase. *J. Biol. Chem*., **268**, 13050–13054.

Groom,L.A., Sneddon,A.A., Alessi,D.R., Dowd,S. and Keyse,S.M. (1996) Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. *EMBO J*., **15**, 3621–3632.

Guan,K.-L. and Dixon,J.E. (1993) Bacterial and viral protein tyrosine phosphatases. *Sem. Cell Biol*., **4**, 389–396.

Hendricks,W., Schepens,J., Brugman,C., Zeeuwen,P. and Wieringa,B. (1995) A novel receptor-type protein tyrosine phosphatase with a

single catalytic domain is specifically expressed in mouse brain. *Biochem. J.*, **305**, 499–504.
Her,J.-H., Wu,J., Rall,T.B., Sturgill,T.W. and Weber,M.J. (1991) Sequence of pp42/MAP kinase, a serine/threonine kinase regulated by tyrosine phosphorylation. *Nucleic Acids Res.*, **19**, 3743.
Hibi,M., Lin,A., Smeal,T., Minden,A. and Karin,M. (1993) Identification of an oncoprotein- and UV-reponsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.*, **7**, 2135–2148.
Hsiao,K.-M., Chou,S.-Y., Shih,S.-J. and Ferrel,J.E. (1994) Evidence that inactive p42 mitogen-activated protein kinase and inactive Rsk exist as a heterodimer *in vivo*. *Proc. Natl Acad. Sci. USA*, **91**, 5480–5484.
Hunter,T. (1995) Protein kinases and phosphatases: the Ying and Yang of protein phosphorylation and signaling. *Cell*, **80**, 225–236.
Jacoby,T., Flanagan,H., Faykin,A., Seto,A.G., Mattison,C. and Ota,I. (1997) Two protein-tyrosine phosphatases inactivate the osmotic stress responsive pathway in yeast by targeting the mitogen-activated protein kinase, Hog 1. *J. Biol. Chem.*, **272**, 17749–17755.
Karin,M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.*, **270**, 16483–16486.
Kemp,B.E. and Pearson,R.B. (1990) Protein kinase recognition sequence motifs. *Trends Biochem Sci.*, **15**, 342–346.
Keyse,S.M. (1995) An emerging family of dual specificity MAP kinase phosphatases. *Biochim. Biophys. Acta*, **1265**, 145–160.
Kosako,H., Gotoh,Y., Matsuda,S., Ishikawa,M. and Nishida,E. (1992) *Xenopus* MAP kinase activator is a serine/threonine/tyrosine kinase activated by threonine phosphorylation. *EMBO J.*, **11**, 2903–2908.
Kozak,M. (1988) An analysis of 5′-noncoding sequences from 699 vertebrate messengers. *Nucleic Acids Res.*, **15**, 8125–8148.
Kyriakis,J.M. and Avruch,J. (1996) Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.*, **271**, 24313–24316.
Lee,R., Cobb,M.H. and Blackshear,P.J. (1992) Evidence that extracellular signal-regulated kinases are the insulin-activated Raf-1 kinase kinases. *J. Biol. Chem.*, **267**, 1088–1092.
Lenormand,P., Sardet,C., Pagès,G., L'Allemain,G., Brunet,A. and Pouysségur,J. (1993) Growth factors induce nuclear translocation of MAP kinases ($p42^{mapk}$ and $p44^{mapk}$) but not of their activator MAP kinase kinase ($p45^{mapkk}$) in fibroblasts. *J. Cell Biol.*, **122**, 1079–1088.
Lewis,T., Groom,L.A., Sneddon,A.A., Smythe,C. and Keyse,S.M. (1995) XCL100, an inducible nuclear MAP kinase phosphatase from *Xenopus laevis*: its role in MAP kinase inactivation in differentiated cells and its expression during early development. *J. Cell Sci.*, **108**, 2885–2896.
Lombroso,P.J., Murdoch,G. and Lerner,M. (1991) Molecular characterization of a protein-tyrosine-phosphatase enriched in striatum. *Proc. Natl Acad. Sci. USA*, **88**, 7242–7246.
Marshall,C.J. (1995) Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, **80**, 179–185.
Mauro,L.J. and Dixon,J.E. (1994) 'Zip codes' direct intracellular protein tyrosine phosphatases to the correct cellular 'address'. *Trends Biochem. Sci.*, **19**, 151–155.
Millar,J.B., Buck,V. and Wilkinson,M.G. (1995) Pyp1 and Pyp2 PTPases dephosphorylate and osmosensing MAP kinase controlling cell size at divison in fission yeast. *Genes Dev.*, **9**, 2117–2130.
Muda,M. *et al.* (1998) The mitogen-activated protein kinase phosphatase-3 N-terminal noncatalytic region is responsible for tight substrate binding and enzymatic specificity. *J. Biol. Chem.*, **273**, 9323–9329.
Mukhopadhyay,N.K., Price,D.J., Kyriakis,J.M., Pelech,S., Sanghera,J. and Avruch,J. (1992) An array of insulin-activated, proline-directed serine/threonine protein kinases phosphorylate the p70 S6 kinase. *J. Biol. Chem.*, **267**, 3325–3335.
Nakielny,S., Cohen,P., Wu,J. and Sturgill,T.W. (1992) MAP kinase activator from insulin-stimulated skeletal muscle is a protein threonine/tyrosine kinase. *EMBO J.*, **11**, 2123–2129.
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.
Payne,D.M., Rossamando,A.J., Martino,P., Erickson,A.K., Her,J.-H., Shabanowitz,J., Hunt,D.F., Weber,M.J. and Sturgill,T.W. (1991) Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *EMBO J.*, **10**, 885–892.
Pulido,R., Serra-Pagès,C., Tang,M. and Streuli,M. (1995) The LAR/PTPδ/PTPσ subfamily of transmembrane protein-tyrosine-phosphatases: multiple human LAR, PTPδ and PTPσ isoforms are expressed in a tissue-specific manner and associate with the LAR-interacting protein LIP.1. *Proc. Natl Acad. Sci. USA*, **92**, 11686–11690.
Racke,F.K., Lewandowska,K., Goueli,S. and Goldfarb,A.N. (1997) Sustained activation of the extracellular signal-regulated kinase/mitogen-activated protein kinase pathway is required for megakaryocytic differentiation of K562 cells. *J. Biol. Chem.*, **272**, 23366–23370.
Rouyez,M.-C., Boucheron,C., Gisselbrecht,S., Dusanter-Fourt,I. and Porteu,F. (1997) Control of thrombopoietin-induced megakaryocytic differentiation by the mitogen-activated protein kinase pathway. *Mol. Cell. Biol.*, **17**, 4991–5000.
Saito,H. and Streuli,M. (1991) Molecular characterization of protein tyrosine phosphatases. *Cell Growth Differ.*, **2**, 59–65.
Sarcevic,B., Erikson,E. and Maller,J.L. (1993) Purification and characterization of a mitogen-activated protein kinase tyrosine phosphatase from *Xenopus* eggs. *J. Biol. Chem.*, **268**, 25075–25083.
Saxena,M., Williams,S., Gilman,J. and Mustelin,T. (1998) Negative regulation of T cell antigen receptor signal transduction by hematopoietic tyrosine phosphatase (HePTP). *J. Biol. Chem.*, **273**, 15340–15344.
Scimeca,J.-C., Nguyen,T.T., Filloux,C. and Van Obberghen,E. (1992) Nerve growth factor-induced phosphorylation cascade in PC12 pheochromocytoma cells: association of S6 kinase II with the microtubule-associated protein kinase, ERK1. *J. Biol. Chem.*, **267**, 17369–17374.
Sharma,E. and Lombroso,P.J. (1995) A neuronal protein tyrosine phosphatase induced by nerve growth factor. *J. Biol. Chem.*, **270**, 49–53.
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. *Brain Res. Mol. Brain Res.*, **32**, 87–93.
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.
Smith,D.B. and Johnson,K.S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene*, **67**, 31–40.
Sun,H., Charles,C.H., Lau,L.F. and Tonks,N.K. (1993) MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase *in vivo*. *Cell*, **75**, 487–493.
Tonks,N.K. and Neel,B.G. (1996) From form to function: signaling by protein tyrosine phosphatases. *Cell*, **87**, 365–368.
Traverse,S., Gómez,N., Paterson,H., Marshall,C. and Cohen,P. (1992) Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. *Biochem. J.*, **288**, 351–355.
Traverse,S., Seedorf,K., Paterson,H., Marshall,C.J., Cohen,P. and Ullrich,A. (1994) EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. *Curr. Biol.*, **4**, 694–701.
Trowbridge,I.S. (1991) CD45, a prototype for transmembrane protein tyrosine phosphatases. *J. Biol. Chem.*, **266**, 23517–23520.
Walton,K.M. and Dixon,J.E. (1993) Protein tyrosine phosphatases. *Ann. Rev. Biochem.*, **62**, 101–120.
Ward,Y. and Kelly,K. (1994) The MAP kinase phosphatases. *Adv. Prot. Phosphatases*, **8**, 87–97.
Ward,Y., Gupta,S., Jensen,P., Wartmann,M., Davis,R.J. and Kelly,K. (1994) Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1. *Nature*, **367**, 651–654.
Waskiewicz,A.J., Flynn,A., Proud,C.G. and Cooper,J.A. (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J.*, **16**, 1909–1920.
Whalen,A.M., Galasinski,S.C., Shapiro,P.S., Stines Nahreini,T. and Ahn,N.G. (1997) Megakaryocytic differentiation induced by constitutive activation of mitogen-activated protein kinase kinase. *Mol. Cell. Biol.*, **17**, 1947–1958.
Wilson,I.A., Niman,H.L., Hougten,R.A., Cherenson,A.R., Connolly,M.L. and Lerner,R.A. (1984) The structure of an antigenic determinant in a protein. *Cell*, **37**, 767–778.
Wu,J., Lau,L.F. and Sturgill,T.W. (1994) Rapid deactivation of MAP kinase in PC12 cells occurs independently of induction of phosphatase MKP-1. *FEBS Lett.*, **353**, 9–12.
Wurgler-Murphy,S.M., Maeda,T., Witten,E.A. and Saito,H. (1997) Regulation of the *Saccharomyces cerevisae* HOG1 mitogen-activated protein kinase by the PTP2 and PTP3 protein tyrosine phosphatases. *Mol. Cell. Biol.*, **17**, 1289–1297.
Yamada,A., Streuli,M., Saito,H., Rothstein,D.M., Schlossman,S.F. and Morimoto,C. (1990) Effect of activation of protein kinase C on CD45

isoform expression and CD45 protein tyrosine phosphatase activity in T cells. *Eur. J. Immunol*., **20**, 1655–1660.

Yang,S.-H., Yates,P.R., Whitmarsh,A.J., Davis,R.J. and Sharrocks,A.D. (1998) The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif. *Mol. Cell. Biol*., **18**, 710–720.

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.

Zhang,F., Strand,A., Robbins,D., Cobb,M. and Goldsmith,E.J. (1994) Atomic structure of the MAP kinase ERK2 at 2.3 Å resolution. *Nature*, **367**, 704–711.

Zhao,Z., Tan,Z., Diltz,C.D., You,M. and Fischer,E.H. (1996) Activation of mitogen-activated protein (MAP) kinase pathway by pervanadate, a potent inhibitor of tyrosine phosphatases. *J. Biol. Chem*., **271**, 22251–22255.

Zheng,C.-F. and Guan,K.-L. (1993) Dephosphorylation and inactivation of the mitogen-activated protein kinase by a mitogen-induced Thr/Tyr protein phosphatase. *J. Biol. Chem*., **268**, 16116–16119.

Received August 4, 1998; revised October 22, 1998; accepted October 23, 1998