A Novel Regulatory Mechanism of MAP Kinases Activation and Nuclear Translocation Mediated by PKA and the PTP-SL Tyrosine Phosphatase

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Abstract. Protein tyrosine phosphatase PTP-SL retains mitogen-activated protein (MAP) kinases in the cytoplasm in an inactive form by association through a kinase interaction motif (KIM) and tyrosine dephosphorylation. The related tyrosine phosphatases PTP-SL and STEP were phosphorylated by the cAMP-dependent protein kinase A (PKA). The PKA phosphorylation site on PTP-SL was identified as the Ser\(^{231}\) residue, located within the KIM. Upon phosphorylation of Ser\(^{231}\), PTP-SL binding and tyrosine dephosphorylation of the MAP kinases extracellular signal–regulated kinase (ERK)\(_{1/2}\) and p38\(_{\alpha}\) were impaired. Furthermore, treatment of COS-7 cells with PKA activators, or overexpression of the C\(_{\alpha}\) catalytic subunit of PKA, inhibited the cytoplasmic retention of ERK\(_2\) and p38\(_{\alpha}\) by wild-type PTP-SL, but not by a PTP-SL S231A mutant. These findings support the existence of a novel mechanism by which PKA may regulate the activation and translocation to the nucleus of MAP kinases.

Key words: MAP kinases • PKA • PTP-SL • tyrosine phosphatases • signal transduction

The mammalian mitogen-activated protein (MAP)\(^{1}\) kinase pathways are signaling cascades differentially activated by growth factors, mitogens, hormones, as well as by stress and inflammation, which contribute to the control of cell growth, differentiation, and survival (Cobb and Goldsmith, 1995; Kyrilakis and Avruch, 1996). Each pathway behaves as a multimolecular complex of receptors and regulatory and adapter proteins, which are functionally assembled around a modular core of three kinases (Whitmarsh and Davis, 1998; Schaeffer and Weber, 1999). A major mechanism of internal regulation and signal amplification of these cascades is the sequential phosphorylation and activation of the kinases within each three-kinase module, leading to the cytoplasmic retention of MAP kinases in an inactive form by association through a kinase interaction motif (KIM) and tyrosine dephosphorylation. The related tyrosine phosphatases PTP-SL and STEP were phosphorylated by the cAMP-dependent protein kinase A (PKA). The PKA phosphorylation site on PTP-SL was identified as the Ser\(^{231}\) residue, located within the KIM. Upon phosphorylation of Ser\(^{231}\), PTP-SL binding and tyrosine dephosphorylation of the MAP kinases extracellular signal–regulated kinase (ERK)\(_{1/2}\) and p38\(_{\alpha}\) were impaired. Furthermore, treatment of COS-7 cells with PKA activators, or overexpression of the C\(_{\alpha}\) catalytic subunit of PKA, inhibited the cytoplasmic retention of ERK\(_2\) and p38\(_{\alpha}\) by wild-type PTP-SL, but not by a PTP-SL S231A mutant. These findings support the existence of a novel mechanism by which PKA may regulate the activation and translocation to the nucleus of MAP kinases.

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1. Abbreviations used in this paper: cPKA, PKA catalytic subunit; ERK, extracellular signal–regulated kinase; GST, glutathione-S-transferase; HA, hemagglutinin; JNK, c-Jun NH\(_2\)-terminal kinase; KIM, kinase interaction motif; MAP, mitogen-activated protein; PKA, protein kinase A; PKC, protein kinase C; PTP, protein tyrosine phosphatase.
cation of these MAP kinases, and favors their dephosphor-
ylation and inactivation by the phosphatase in the cyto-
plasm (Zúñiga et al., 1999). Essential residues within the 
KIM of PTP-SL for the recognition of ERK1/2 include 
those within a PKA consensus phosphorylation sequence, 
raising the possibility that PKA could regulate the associa-
tion of PTP-SL with the MAP kinases by KIM phos-
phorylation. In this report, we have investigated the in-
volved of PKA in the regulation of the association of PTP-
SL with ERK1/2 and p38. We have found that phosphoryla-
tion of the KIM of PTP-SL by PKA is a major regulatory 
mechanism of the activities of these MAP kinases and 
their translocation to the nucleus.

Materials and Methods

Plasmids, Antibodies, and Reagents

PTP-SL, STEP, and ERK 2 cDNA constructs have been previously de-
scribed (Pulido et al., 1998; Zúñiga et al., 1999). pCEV (cPKAα, mouse 
sequence; Uhler and McKnight, 1987) was provided by G.S. McKnight 
(University of Washington, Seattle, WA). pCE- HA -p38 MAPK (p38α, mouse 
sequence; Brunet and Pouyssegur, 1996) was provided by J. 
Pouyssegur (Centre de Biochimie-CNRS, Nice, France). pRK5-GST-
PTP-SL mammalian expression vectors were made by PCR with a primer 
containing a Kozak sequence followed by a start codon and the 
S. japa-
num glutathione-S-transferase (GST) sequence. Antibodies and 
reagents were used as described (Pulido et al., 1998; Zúñiga et al., 1999).
Rabbit polyclonal anti-p38 (C-20) was purchased from Santa Cruz Bio-
technology Inc. Dibutyryl-cAMP and okadaic acid (Boehringer Mann-
heim) were used at final concentrations of 2 mM and 1 
M, respectively.

Cell Culture, Transfections, Precipitation with GST 
Fusion Proteins, Immunoprecipitation, 
and Immunoblotting

Rat fibroblasts Rat-1, human embryonic kidney 293, and Simian COS-7 
cell lines, were grown in DME containing high glucose supplemented with 
5% (for COS-7 cells) or 10% heat-inactivated FCS. Cells were transfected 
using the DEAE-dextran method (COS-7 cells) or the calcium phosphate 
precipitation method (293 cells), and were harvested after 48-72 h of cul-
ture. In cells transfected with pCEV, the expression of cPKAα was in-
duced by incubation during the last 24 h of culture in the presence of 100 
M ZnSO4. For -P labeling, transfected COS-7 cells were cultured for 4 h 
with phosphate-free DME, 2% FCS in the presence of 20 
P32-inorganic phosph-
ate (100 μCi/ml), and then cells were treated with dibutyryl-cAMP MP or 
 forskolin plus IBMX during 1 h, or with okadaic acid during 30 min. HA- 
ERK2 or H-P38α from transfected 293 cells were activated by cell treat-
ment with EGF (5 min, 50 ng/ml) or sorbitol (30 min, 0.5 M), respectively.

Immunofluorescence

COS-7 cells were processed for immunofluorescence as described (Zúñiga 
et al., 1999). In brief, after transfection, cells were rinsed with IPBS buffer 
(1.5 mM KH2PO4, 4.3 mM Na2HPO4, 137 mM NaCl, 2.7 mM KCl, 0.7 mM 
CaCl2, and 0.5 mM MgCl2; pH 7.4), and then fixed with methanol. 
Samples were incubated in blocking solution (IPBS 3% BSA), followed by 
incubation at 37°C for 90 min with the mixture of the anti-HA and anti-PTP-
SL primary antibodies. After washing with IPBS, cells were incubated for 
1 h at room temperature with a mixture of the anti-rabbit fluorescein 
isothiocyanate- and the anti-mouse tetramethylrhodamine B isothiocya-
nate-conjugated secondary antibodies, followed by washing with IPBS 
and mounting.

Results

PKA Phosphorylates PTP-SL and STEP

PTP-SL and STEP tyrosine phosphatases contain a con-
served KIM in their cytosolic noncatalytic regions that me-
diates association with MAP kinases (Pulido et al., 1998).
Since a consensus phosphorylation sequence for PKA 
occurs within the KIM (Arg280Arg282Gly283Ser284); amino 
acid numbering is according to PTP-SL; Hendriks et al., 
1995), the phosphorylation of these two phosphatases by 
PKA was tested. GST-PTP-SL or -STEP fusion proteins, 
were incubated in vitro with cPKA in the presence of 
γ32P ATP, followed by SDS-PAGE and autoradiography. 
As shown, a strong phosphorylation of the PTP-SL and 
STEP fusion proteins was detected (Fig. 1 A, lanes 2-4), 
whereas no phosphorylation took place with GST alone or 
a GST fusion protein containing a nonrelated PTP (Fig. 1 
A, lanes 1 and 5), indicating that PTP-SL and STEP are
substrates of PKA. Substitution by alanine of the Ser^{231} residue (S231A mutant), abolished the phosphorylation of PTP-SL by cPKA (Fig. 1A, lanes 8 and 10), demonstrating that this residue is the target of the kinase. Next, the in vivo phosphorylation of PTP-SL upon PKA activation conditions was investigated. Phosphorus 32 labeling was carried out on COS-7 cells transfected with plasmids encoding transmembrane (PTP-SL 1-549) or nontransmembrane (PTP-SL 147-549) PTP-SL isoforms, followed by treatment with PKA activators and immunoprecipitation with anti-PTP-SL antibody. Incubation in the presence of the cAMP analogues dibutyryl-cAMP or the adenylyl cyclase activator forskolin increased the phosphorylation of wild-type PTP-SL isoforms (Fig. 1B, lanes 3, 4, and 8), but not of the S231A mutants (Fig. 1B, lanes 6 and 10). Interestingly, the basal levels of phosphorylation were greatly diminished in the S231A mutants (Fig. 1B, lanes 5 and 9) compared with the wild-type PTP-SL (Fig. 1B, lanes 2 and 7), indicating that PKA phosphorylates this residue under the normal cell growth conditions of COS-7 cells. Furthermore, cell treatment with the PP2A serine/threonine phosphatase inhibitor, okadaic acid (1 μM), induced the hyperphosphorylation of wild-type PTP-SL, but not of the S231A mutant (Fig. 1B, lanes 11–14). These results demonstrate that the Ser^{231} residue of PTP-SL is a substrate of PKA, and suggest a role for PP2A in the in vivo dephosphorylation of such a residue.

**Phosphorylation of the KIM of PTP-SL by PKA Inhibits the Association and the Tyrosine Dephosphorylation of ERK1/2 and p38α**

Next, the effect of PTP-SL phosphorylation by PKA on its association with MAP kinases was analyzed. GST-PTP-SL fusion proteins were phosphorylated in vitro by cPKA as above, in the presence of cold ATP, and the fusion proteins were incubated with Rat-1 cell lysates and precipitated with glutathione-Sepharose. Samples were resolved by SDS-PAGE and the presence of MAP kinases ERK1/2 or p38α was detected by immunoblot using specific antibodies. Remarkably, the phosphorylation of GST-PTP-SL wild type by cPKA aborted its association with both ERK1/2 and p38α (Fig. 2A, lane 2); however, no changes were observed with the GST-PTP-SL S231A mutant upon incubation with cPKA (Fig. 2A, lanes 4 and 5). To test the effect of PTP-SL phosphorylation by PKA on the association with the MAP kinases in vivo, GST-PTP-SL fusion proteins were overexpressed in 293 cells and precipitated in one-step with glutathione-Sepharose, followed by immunoblot analysis, as above. Treatment of cells with dibutyryl-cAMP or forskolin resulted in the lack of coprecipitation of ERK1/2 or HA-p38α with PTP-SL (Fig. 2B, lanes 2 and 3); however, in dibutyryl-cAMP-treated cells that were preincubated with the PKA inhibitor H89, normal levels of association with the kinases were detected (Fig. 2B, lane 4). Finally, the coprecipitation of ERK1/2 and p38α with the PTP-SL S231E mutant, which mimics a phosphorylated Ser^{231} residue, was also tested. As shown, these MAP kinases did not associate in 293 cells with overexpressed GST-PTP-SL S231E (Fig. 2C, lane 5), whereas association was efficiently detected with the GST-PTP-SL wild type, the S231A mutant or the C480S catalytically inactive mutant (Fig. 2C, lanes 3, 4, and 6). The functional consequences of the phosphorylation of the Ser^{231} residue of PTP-SL, on the dephosphorylation of ERK1/2 and p38α by the phosphatase, were analyzed using the S231E PTP-SL mutant. GST-PTP-SL wild type or S231E fusion proteins were mixed with pellets containing activated HA-ERK2 or HA-p38α, and phos-
phatase assays were carried out, followed by SDS-PAGE and immunoblot with the anti-phosphotyrosine 4G10 mAb. As shown, the tyrosine dephosphorylation of HA-ERK2 and HA-p38α by GST-PTP-SL S231E mutant was impaired compared with that shown by GST-PTP-SL wild type, whereas equal activities of both fusion proteins were measured towards the nonspecific p-NPP substrate (Fig. 2D and data not shown). These findings demonstrate that phosphorylation of the Ser$^{231}$ residue of PTP-SL by PKA inhibits its association with ERK1/2 and p38α, and the subsequent tyrosine dephosphorylation of these MAP kinases.

**Nuclear Translocation of ERK2 and p38α, in the Presence of PTP-SL, Is Favored upon Activation of PKA**

PTP-SL retains ERK2 in the cytoplasm in a KIM-dependent manner (Zúñiga et al., 1999). To study the effect of phosphorylation of PTP-SL by PKA on its ability to retain MAP kinases outside of the nucleus, immunofluorescence analysis was performed on COS-7 cells cotransfected with HA-ERK2 or HA-p38α, and PTP-SL. Overexpression of HA-ERK2 or HA-p38α alone resulted in their accumulation in the nucleus (see Fig. 4A; and data not shown); however, in the presence of PTP-SL, the nuclear accumulation of these kinases was abolished, colocalizing with the phosphatase outside of the nucleus (Figs. 3 and 4A). Interestingly, neither the PTP activity nor the PTP domain of PTP-SL itself was required to retain HA-ERK2 outside of the nucleus, as observed by coexpression with PTP-SL catalytically inactive mutants (C480S or R486M) or with truncated PTP-SL forms lacking the PTP domain (PTP-SL 1-288) (Fig. 2D). On the other hand, upon coexpression with the PTP-SL S231E mutant, the cytoplasmic retention of HA-ERK2 or HA-p38α was significantly reduced, as compared with wild-type PTP-SL (Fig. 4, B and C). Also, when cells coexpressing wild-type PTP-SL and HA-ERK2 or HA-p38α were treated with dibutyryl-cAMP, the nuclear localization of both MAP kinases was partially restored, and such an effect was prevented by cell preincubation with H89 (Fig. 4, B and C). However, no effect was observed upon cell treatment with agents that activate other kinase pathways, such as EGF or PMA (data not shown). Furthermore, cotransfection with an inducible expression vector coding the Ca catalytic subunit of PKA (cPKA α), also favored the nuclear localization of these MAP kinases in the presence of PTP-SL (Fig. 4, B and C). Remarkably, the effect of PKA activation on the colocalization of HA-ERK2 and HA-p38α with wild-type PTP-SL was not observed with the PTP-SL S231A mutant (Fig. 4, B and C), demonstrating that phosphorylation of the Ser$^{231}$ residue of PTP-SL by PKA inhibits the in vivo association of PTP-SL with HA-ERK2 and HA-p38α, and favors the nuclear translocation of these kinases.

![Figure 3. Transmembrane PTP-SL retains ERK2 and p38α outside of the nucleus. COS-7 cells were cotransfected with pcDNA3-HA-ERK2 or pECE-HA-p38M A PK, plus pRK5-PTP-SL 1-549 wild type or ΔKIM (Δ224-239) mutant, as indicated. 48 h after transfection, cells were costained and analyzed by immunofluorescence. HA-ERK2 and HA-p38α were stained with the mouse anti-HA mAb 12CA5 plus rhodamine-conjugated goat anti-mouse antibody (red, A, D, G, and J). PTP-SL was stained with rabbit polyclonal anti-PTP-SL antibody plus fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (green, B, E, H, and K). Subcellular localization of PTP-SL 1-549 corresponds to perinuclear areas in the cytoplasm). In C, F, I, and L, double color staining is shown; yellow areas correspond to colocalization of HA-ERK2 or HA-p38α, and PTP-SL.](image-url)
PKA modulates the activity of MAP kinases in a cell type- and stimulus-specific manner by interfering with upstream events from signaling cascades activated through distinct Ras-like GTPases, including Ras, Rap1, and RalGDS (Vossler et al., 1997; Miller et al., 1998). In addition, PKA activity favors the nuclear translocation of ERK1/2 in PC12 and hippocampal neurons, as well as in presynaptic sensory neurons from Aplysia (Inpey et al., 1998; Martin et al., 1998; Yao et al., 1998). Our results, showing a crosstalk between the PKA and ERK 1/2 and p38 kinases through the tyrosine phosphatase PTP-SL, support the existence of a novel mechanism by which PKA can regulate the activity of the MAP kinases and their translocation to the nucleus (Fig. 5). Such a mechanism would involve the existence, in certain cell types, of a pool of inactive MAP kinases outside of the nucleus, which would be complexed with PTP-SL or other KIM-containing PTPs, including STEP and HePTP (see below). The dissociation equilibrium of the complex would depend upon the cell type– and the stimulus-specific conditions of PKA activity, and the lack of association would be favored by the PKA-mediated phosphorylation of the KIM regulatory residue on the PTP. Thus, upon conditions of PKA activation, both the tyrosine phosphorylation and the entry into the nucleus of the MAP kinases would be prevalent. It should be noted that the expression of PTP-SL and related isoforms is restricted to specialized areas of the brain, including the Purkinje cells in the postnatal cerebellum (Watanabe et al., 1998; van den Maagdenberg et al., 1999), suggesting the possibility of a differential regulation of MAP kinase functions by PTP-SL and PKA during brain development.

The mutational analysis of the KIM of PTP-SL has revealed that the residues involved in the PKA phosphorylation consensus sequence are also crucial for the docking of this phosphatase with ERK1/2 (Zúñiga et al., 1999). Such residues are conserved between the related tyrosine phosphatases PTP-SL, STEP, and HePTP, which have been found to associate with MAP kinases and regulate their activation (Pulido et al., 1998; Oh-hora et al., 1999; Saxena et al., 1999a). In this regard, while writing this manuscript, Saxena et al. (1999b) have reported the negative role of PKA phosphorylation of the KIM of HePTP in the physical and functional association of HePTP with MAP kinases. Also, a tyrosine phosphatase from Drosophila, PTP-ER, has been found that inactivates MAP kinase, and that contains three KIMs with consensus phosphorylation sites for PKA (Karim and Rubin, 1999). Finally, we have
found that the retention of ERK2 outside of the nucleus is efficiently achieved by PTP- SL catalytically inactive mutants, as well as by truncated PTP- SL molecules lacking the PTP domain, demonstrating that this domain is dispensable in a such process. Thus, PKA-medi ated KIM phosphorylations could have diverse regulatory effects on MAP kinase functions, depending on the functional properties of the affected KIM-containing molecule.

The involvement of distinct kinases in the in vivo phosphorylation of PTP- SL is likely to exist, which ultimately could control the biological functions of this PTP. Thus, the Thr253 residue of PTP- SL is phosphorylated in vivo by ERK1/2 upon EGF cell treatment in a manner dependent of docking through the KIM (Pulido et al., 1998). Furthermore, the Thr253 residue is also a putative PKC phosphorylation site, and PTP- SL is phosphorylated in vitro by this kinase (our unpublished observations). In this context, the binding of MAP kinases to the KIM of PTP- SL could mask the PKA phosphorylation motif by steric hindrance, hampering the phosphorylation of PTP- SL by PKA; conversely, phosphorylation of the KIM by PKA difficult the association of MAP kinases and the subsequent phosphorylation of the Thr253 residue. The results presented here indicate a major regulatory role on the PTP- SL functions for the PKA-mediated phosphorylation of the Ser231 residue; accordingly, the basal phosphorylation of PTP- SL in COS-7 cells is found predominantly in such residue (Fig. 1B). On the other hand, the functional significance of the phosphorylation of the Thr253 residue by ERK1/2 remains elusive. The possibility exists that phosphorylation of Thr253 regulates the dissociation of PTP- SL from MAP kinases, as it has been suggested for HePTP (Saxena et al., 1999a).

The cytoplasmic retention of ERK2 by PTP- SL was efficiently achieved upon conditions of EGF-induced phosphorylation of Thr253 (our unpublished observations). In addition, phosphorylation of this residue could account for the regulated binding of PTP- SL to other unidentified molecules. The participation of specific serine/threonine phosphatases in the in vivo dephosphorylation of the Ser231 and Thr253 residues of PTP- SL is also expected. In this regard, we have found that cell treatment with okadaic acid induces hyperphosphorylation of the Ser231 residue, suggesting an active role for PP2A in the in vivo dephosphorylation of this key residue (Fig. 5). A loss, PP2A and PP2C have been shown to interfere with the activation of the MAP kinase pathways by affecting the phosphorylation of MAP kinases or upstream phosphorylation events (Andersen et al., 1999; Chajry et al., 1996; Takekawa et al., 1999). Thus, a complex network of kinases and phosphatases could be envisioned within the MAP kinase pathways, which integrate the different signals to generate specific cell responses. The importance of the assembly of the molecular components that regulate the activation of the MAP kinases has been recently outlined (Whitmash and Davis, 1998; Schaeffer and Weber, 1999). The results reported here point to PKA as a major regulator of the physical and functional association between the ERK1/2 and p38α kinases and their inactivating tyrosine phosphatases.

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