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\textsuperscript{231} residue, located within the KIM. Upon phosphorylation of Ser\textsuperscript{231}, PTP-SL binding and tyrosine dephosphorylation of the MAP kinases extracellular signal–regulated kinase (ERK)\textsubscript{1/2} and p38\textalpha were impaired. Furthermore, treatment of COS-7 cells with PKA activators, or overexpression of the C\textalpha catalytic subunit of PKA, inhibited the cytoplasmic retention of ERK\textsubscript{2} and p38\textalpha 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)\textsuperscript{1} 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; Kryakis 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 activation in the cytoplasm of the effector kinases extracellular signal–regulated kinase (ERK)\textsubscript{1/2}, c-Jun NH\textsubscript{2}-terminal kinase (JNK), or p38, and their translocation to the nucleus, where phosphorylation of transcription factors takes place (Karin, 1995; Treisman, 1996). In addition, the crosstalk between distinct MAP kinases cascades as well as with protein kinases from other pathways, such as protein kinase A or C (PKA or PKC, respectively), cooperates in the integration of the signals delivered through the MAP kinases (Burgering and Bos, 1995; Rabinson and Cobb, 1997). The participation of the cAMP-dependent protein kinase, PKA, in the differential modulation of MAP kinase pathways has been documented. For instance, in T lymphocytes, PKA mediates the inhibition of the JNK but not of the ERK\textsubscript{1/2} pathway (Hsueh and Lai, 1995), whereas in Rat1 fibroblasts, adipocytes, and muscle cells, PKA inhibits ERK\textsubscript{1/2} by interfering with the activation of Raf-1 by Ras (Cook and Mccormick, 1993; Gavrilovic et al., 1993; Sevetson et al., 1993; Wu et al., 1993). Conversely, PKA cooperates in the sustained activation of ERK\textsubscript{1/2} in pheochromocytoma PC12 cells, in a process that involves the activation of the Rap1/B-Raf pathway (Frödin et al., 1994; Vossler et al., 1997).

The protein tyrosine phosphatases PTP-SL, STEP, and HePTP have emerged as major regulators of MAP kinase functions on the basis of their association with ERK\textsubscript{1/2} and p38 through a 16–amino acid kinase interaction motif (KIM), located in their cytosolic noncatalytic regions (Pulido et al., 1998; Oh-hora et al., 1999; Saxena et al., 1999a; Zúñiga et al., 1999). PTP-SL is encoded by a single gene, although different transcripts have been described that generate distinct transmembrane and nontransmembrane isoforms, which are excluded from the nucleus and whose expression is developmentally regulated in the brain (Hendriks et al., 1995; Ogata et al., 1995; Sharma and Lombroso, 1995; Shiozuka et al., 1995). Binding of ERK\textsubscript{1/2} to the KIM of PTP-SL blocks the nuclear translo-
cation of these MAP kinases, and favors their dephosphorylation and inactivation by the phosphatase in the cytoplasm (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 association of PTP-SL with the MAP kinases by KIM phosphorylation. In this report, we have investigated the involvement of PKA in the regulation of the association of PTP-SL with ERK1/2 and p38. We have found that phosphorylation 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 ERK2 cDNA constructs have been previously described (Pulido et al., 1999; Pulido et al., 1999). pCEV (PKA-α, mouse sequence; Uhler and McKnight, 1987) was provided by G. S. McKnight (University of Washington, Seattle, WA). pCE-HA-p38MAPK (p38, mouse sequence; Brunet and Pouyssegur, 1996) was provided by J. Pouyssegur (Centre de Biochimie-CNRS, Nice, France). pRK5-GST-PTP-SL cDNA mammalian expression vectors were made by PCR with a primer containing a Kozak sequence followed by a start codon and the S. japonicum 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 Biotechnology Inc. Dibutyryl-cAMP and okadaic acid (Boehringer Mannheim) were used at final concentrations of 2 mM and 1 μM, respectively. Forskolin (ICN Pharmaceuticals Inc.) was used at a final concentration of 40 μM. In the continuous presence of 1 mM IBMX (Sigma Chemical Co.), the PKA inhibitor H89 (Biomol) was used at a final concentration of 25 μM. When used, IBMX and H89 were added to the cells 30 min before stimulation. The bovine PKA catalytic subunit (cPKA) was purchased from Promega Corp.

In Vitro Kinase and Phosphatase Assays

For PKA in vitro kinase assays, GST fusion proteins (1 μg) were incubated at room temperature during the last 24 h of culture in the presence of 100 μM ATP, 15 mM MgCl₂, and 8 mM MgCl₂ (20 μl final volume). The reactions were stopped by adding SDS sample buffer and boiling, followed by SDS-PAGE and autoradiography. For in vitro association assays (see Fig. 2A), GST fusion proteins were phosphorylated with cPKA as above, in the presence of 200 μM cold ATP, and then mixed with cell lysates and precipitated with glutathione-Sepharose, followed by immunoblotting. In vitro phosphatase assays were performed in 25 mM Hepes, pH 7.5, 5 mM EDTA, and 10 mM DTT (40 μl final volume), at 37°C, during the indicated times, as described in Zúñiga et al. (1999).

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

Rat fibroblast 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 culture. In cells transfected with pCEV, the expression of cPKAα was induced by incubation during the last 24 h of culture in the presence of 100 μM ZnSO₄, for 14 h with phosphate-free DME, 2% FCS, and 50 μM cAMP. After 48 h, the cells were treated with dibutyryl-cAMP or forskolin plus IBMX during 1 h, or with okadaic acid during 30 min. HA-ERK2 or HA-p38 from transfected cells were collected by cell treatment with EGF (5 min, 50 ng/ml) or sorbitol (30 min, 0.5 M), respectively. Cell lysis, precipitation with GST-fusion proteins, immunoprecipitation, and immunoblotting were done as described (Pulido et al., 1999).
substrates of PKA. Substitution by alanine of the Ser231 residue (S231A mutant), abolished the phosphorylation of PTP-SL by cPKA (Fig. 1 A, 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. Phosphor 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 adenylate cyclase activator forskolin increased the phosphorylation of wild-type PTP-SL isoforms (Fig. 1 B, lanes 3, 4, and 8), but not of the S231A mutants (Fig. 1 B, lanes 6 and 10). Interestingly, the basal levels of phosphorylation were greatly diminished in the S231A mutants (Fig. 1 B, lanes 5 and 9) compared with the wild-type PTP-SL (Fig. 1 B, 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. 1 B, lanes 11–14). These results demonstrate that the Ser231 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 Raf-1 cell lysates and precipitated with glutathione-Sepharose. Samples were resolved by SDS-PAGE and the presence of the MAP kinases ERK1/2 or p38α was detected by immunoblot using specific antibodies. Remarkably, the phosphorylation of GST-PTP-SL wild type by cPKA abrogated its association with both ERK1/2 and p38α (Fig. 2 A, lane 2); however, no changes were observed with the GST-PTP-SL S231A mutant upon incubation with cPKA (Fig. 2 A, 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. 2 B, 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. 2 B, lane 4). Finally, the coprecipitation of ERK1/2 and p38α with the GST-PTP-SL S231E mutant, which mimics a phosphorylated Ser231 residue, was also tested. As shown, these MAP kinases did not associate in 293 cells with overexpressed GST-PTP-SL S231E (Fig. 2 C, lane 5), whereas association was efficiently detected with the GST-PTP-SL wild type, the S231A mutant or the plasmid 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 adenylate cyclase activator forskolin increased the phosphorylation of wild-type PTP-SL isoforms (Fig. 1 B, lanes 3, 4, and 8), but not of the S231A mutants (Fig. 1 B, lanes 6 and 10). Interestingly, the basal levels of phosphorylation were greatly diminished in the S231A mutants (Fig. 1 B, lanes 5 and 9) compared with the wild-type PTP-SL (Fig. 1 B, 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. 1 B, lanes 11–14). These results demonstrate that the Ser231 residue of PTP-SL is a substrate of PKA, and suggest a role for PP2A in the in vivo dephosphorylation of such a residue.

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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 Ser231 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. 4A). On the other hand, upon coexpression with the PTP-SL S231E mutant, the cytoplastematic retention of HA-ERK2 or HA-p38α was significantly reduced, as compared with wild-type PTP-SL (Fig. 4B 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. 4B 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 Cα catalytic subunit of PKA (cPKAα), also favored the nuclear localization of these MAP kinases in the presence of PTP-SL (Fig. 4B 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. 4B and C), demonstrating that phosphorylation of the Ser231 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 AKPK, 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)
Discussion

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 RapGDS (Vossler et al., 1997; Miller et al., 1998). In addition, PKA activity favors the nuclear translocation of ERK 1/2 in PC12 and hippocampal neurons, as well as in presynaptic sensory neurons from Aplysia (Impey et al., 1998; Martin et al., 1998; Yao et al., 1998). Our results, showing a crosstalk between the PKA and ERK 1/2 and p38α 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 ERK 1/2 (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 physiological 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

Figure 4. (A) PTP-SL retains HA-ERK2 in the cytoplasm independently of its PTP domain. COS-7 cells were cotransfected with pCDNA 3-HA-ERK2 plus pRK5 (mock), or plus pRK5 PTP-SL 147-549, 1-549, or 1-288 wild type or mutants, as indicated. Cells were costained and analyzed by immunofluorescence as in Fig. 3. PTP-SL 147-549 is located in the cytoplasm. Subcellular localization of PTP-SL 1-549 and 1-288 is identical (see Fig. 3). (B, C) Effect of PKA on the nuclear localization of HA-ERK2 and HA-p38α in the presence of PTP-SL. COS-7 cells were cotransfected with pCDNA 3-HA-ERK2 (B) or pECE-HA-p38MAPK (C) plus pRK5 PTP-SL 1-549 wild type or mutants, as indicated. Similar results were obtained with the double mutant C480S/ S231A and the single mutant S231A (not shown). In some points, cells were additionally transfected with pCαEV (cPKA α) (+), and were induced as described in Materials and Methods. Cells were left untreated (−) or were treated (+) with dibutyryl-cAMP plus H89, as indicated, and then were costained and analyzed by immunofluorescence. HA-ERK2 or HA-p38α nuclear localization is presented as the percentage of cells coexpressing PTP-SL and HA-ERK2, or PTP-SL and HA-p38α that showed the MAP kinase located into the nucleus. For each point, at least 100 double positive cells were scored. Bars represent the mean ± SD of at least two separate experiments.

Figure 5. Model of MAP kinase regulation by PKA and PTP-SL. Pools of MAP kinases are maintained in the cytoplasm and into the nucleus by the balance between activation stimuli and the PTP-SL (or other KIM-containing PTPs) inhibitory effects. Upon PKA activation, the association of PTP-SL with the MAP kinase is impaired, and MAP kinase tyrosine phosphorylation and nuclear translocation is favored. The putative regulatory role for serine/threonine phosphatases (PP) in the dephosphorylation of PTP-SL, is indicated (see details in the text).
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-mediated 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 on docking through the KIM (Pulido et al., 1998). Furthermore, the Thr253 residue is also a putative PKC phosphorylation site, and PTP-SP is phosphorylated in vitro by this kinase (our unpublished observations). In this context, the binding of MAP kinases to the KIM of PTP-SP could mask the PKA phosphorylation motif by steric hindrance, hampering the phosphorylation of PTP-SP 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 PKA-mediated phosphorylation of the Ser231 residue; accordingly, the basal phosphorylation of PTP-SP in COS-7 cells is found predominantly in such residue (Fig. 1 B). 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-SP from MAP kinases, as it has been suggested for HEP-PTP (Saxena et al., 1999a). However, the cytoplasmic retention of ERK2 by PTP-SP 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-SP 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). Also, 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., 1990; Chajry et al., 1998; Takekawa et al., 1998). 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 (Whitmarsh 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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