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Phosphotyrosine-specific Phosphatase PTP-SL Regulates the ERK5 Signaling Pathway*

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The duration and the magnitude of mitogen-activated protein kinase (MAPK) activation specifies signal identity and thus allows the regulation of diverse cellular functions by the same kinase cascade. A tight and finely tuned regulation of MAPK activity is therefore critical for the definition of a specific cellular response. We investigated the role of tyrosine-specific phosphatases (PTPs) in the regulation of ERK5. Although unique in its structure, ERK5 is activated in analogy to other MAPKs by dual phosphorylation of threonine and tyrosine residues in its activation motif. In this study we concentrated on whether and how PTP-SL, a kinase-interacting motif-containing PTP, might be involved in the down-regulation of the ERK5 signal. We found that both proteins interact directly with each other *in vitro* and in intact cells, resulting in mutual modulation of their enzymatic activities. PTP-SL is a substrate of ERK5 and independent of phosphorylation binding to the kinase enhances its catalytic phosphatase activity. On the other hand, interaction with PTP-SL not only down-regulates endogenous ERK5 activity but also effectively impedes the translocation of ERK5 to the nucleus. These findings indicate a direct regulatory influence of PTP-SL on the ERK5 pathway and corresponding downstream responses of the cell.

Mitogen-activated protein kinases (MAPKs)[1] are found in all eukaryotes and are expressed in virtually all mammalian cells. A broad variety of stimuli elicit MAPK activation, and MAPKs regulate a large number of distinct cellular responses (1–4). In the very same cell, MAPKs can even be involved in the control of different functions. In these cases the duration and the magnitude of MAPK activation are critical parameters that specify signal identity (5). In rat pheochromocytoma cells, for example, epidermal growth factor (EGF) as well as nerve growth factor activate MAPKs, however, cells proliferate in response to the first stimulus and differentiate in the presence of the latter (6). These distinct responses are due to the ability of nerve growth factor but not EGF to cause a sustained activation of MAPK. A tight and finely tuned regulation of MAPK activity is therefore critical for definition of a specific cellular response.

MAPKs can be grouped into three main subfamilies: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases, and the p38 stress-activated protein kinases. ERK5, which is also termed big MAPK 1, differs considerably from the other family members in that it contains an unique loop-12 domain within the kinase region that is followed by an unusually long C-terminal tail of hitherto unknown function (7, 8). ERK5 is activated by diverse stimuli such as cellular stress and growth factors (9, 10). The MAPK kinase MEK5 has been shown to specifically phosphorylate and thereby activate ERK5 (7, 11); the other components involved in the signaling cascade that ultimately leads to the activation of ERK5 are mostly unknown. Many of the ERK5 activating stimuli also affect other MAPK family members. The synergistic actions of both the ERK1/2 and the ERK5 pathways have for example been reported in the induction of cell transformation (12). On the other hand, downstream effects exerted by activated ERK5 and ERK1/2 can in many cases be clearly distinguished from each other (13, 14). Kato *et al.* (15) have for example demonstrated that ERK5 but not ERK2 is essential for proliferation and cell cycle progression in HeLa cells. More recently Karihaloo *et al.* (16) have shown that ERK5 mediates EGF-induced morphogenesis in renal epithelial cells, whereas ERK2 activity is critically involved in cell motility upon stimulation with hepatocyte growth factor.

MAPKs including ERK5 are generally activated by phosphorylation of threonine and tyrosine residues in their activation motif; however, dephosphorylation of either residue is sufficient for kinase inactivation (17). The present study concentrates on the question of whether and how PTP-SL might be involved in the down-regulation of the big MAPK 1/ERK5 signal. PTP-SL (18–21), like STEP (22) and HePTP (23), belongs to the kinase interacting motif (KIM)-containing phosphatases that have previously been shown by us and others to bind, dephosphorylate, and thereby inactivate signaling by ERK1/2 (24–26). KIM-containing PTPs are characterized by a very restricted expression pattern. They are not found in the nucleus and are generally considered to play a role in the short term inactivation of MAPKs (27).

Here we demonstrate that ERK5 and PTP-SL bind directly to each other. This interaction not only potently reduces kinase activity but also interferes with translocation of ERK5 to the nucleus. Our data show that ERK5 phosphorylates PTP-SL and that independent of phosphorylation binding the kinase stimulates phosphatase activity.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Rabbit polyclonal anti-PTP-SL was obtained by immunization of rabbits with the peptide CHSMVQPEQAPKVLN coupled to keyhole limpet hemocyanin (Calbiochem). For the generation of ERK5 antibodies, rabbits were immunized with a fusion protein of GST and ERK5 aa 410–558. The anti-HA monoclonal anti-

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; aa, amino acids; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GST, glutathione *S*-transferase; HA, hemagglutinin; KIM, kinase-interacting motif; PTP, protein-tyrosine phosphatase; WT, wild type; GFP, green fluorescent protein; CS, catalytic cysteine to serine mutated; PBS, phosphate-buffered saline; HEK, human embryonic kidney.

body 12CA5 (Roche Molecular Biochemicals) was used for immunoprecipitation and the HA.11 (BAbCo) for Western blot analysis. Phosphorylation state-specific antibodies for ERK5 and ERK2 were from BioSource Europe and New England BioLabs, respectively. Anti-ERK2 K23 and anti-histone H1 were from Santa Cruz Biotechnology. Anti-RanGAP antibody was kindly provided by Frauke Melchior (Martinsried, Germany). Horseradish peroxidase-conjugated goat anti-rabbit antibody was from Bio-Rad, goat anti-mouse antibody was from Sigma, and donkey anti-goat antibody was from Jackson ImmunoResearch Laboratories. Chemiluminescence reagents and [γ-^{32}P]ATP (6000 Ci/mmol) were from PerkinElmer Life Sciences. Protein A, protein G, and GSH-Sepharose beads were purchased from Amersham Biosciences. EGF was supplied by Invitrogen. The nitrocellulose membrane was from Schleicher & Schüll. All of the other reagents were obtained from Merck.

Plasmid Construction and Protein Purification—ERK5 and MEK5 were amplified from human placenta cDNA by PCR using primers flanking the coding regions. For the expression in eukaryotic cells, both fragments were cloned into pcDNA3 (Invitrogen). The protein sequence of the cloned human MEK5 differs from the published sequence (8) by an insertion of 10 amino acids between glutamate 348 and isoleucine 349, which is also present in rat and mouse MEK5 (28). N-terminally HA-tagged ERK5 was generated by PCR. ERK5 and the truncated form ERK5kin (aa 1–409) consisting only of the kinase domain, were subcloned into pcDNA3-Fc, a modified vector containing 3′ of the multiple cloning site the coding sequence of the human Fcγ chain. The Fc-tagged proteins were purified from crude cell lysates of transfected HEK 293 cells using protein A-Sepharose. MEK5(D), the dominant active mutant of MEK5 (11) was obtained by site-directed mutagenesis replacing serine 311 and threonine 315 by aspartate. Kinase inactive ERK5 KM was generated by exchanging lysine 83 for methionine. The expression constructs for PTP-SL WT and the catalytically inactive CS mutant in pRK5, a vector containing the cytomegalovirus early promotor, were described before (24). pcDNA3-GFP-PTP-SL WT and CS were generated by fusing cDNA coding for the GFP 5′ to the coding sequence of PTP-SL. GST-IA2-β (aa 641–1015) (29), GST-NC-PTP (aa 245–670) (GenBank^{TM} accession number Z79693), and GST-HePTP (aa 1–339) (30) were obtained by PCR using human placenta and glioblastoma cDNA. PCR fragments were cloned into pGEX5X vectors (Amersham Biosciences) in frame with the GST gene. The GST-STEP and the GST-PTP-SL fusion proteins were described elsewhere (24). The GST-ERK5 fusion protein was constructed by subcloning a cDNA fragment encoding ERK5 aa 410–558 into the pGEX5X vector. All of the GST fusion proteins were expressed in the BL21 DE3 codon + (Stratagene) and purified with glutathione-Sepharose beads. The sequences of the primers used for the construction of all plasmids and for mutagenesis are available upon request.

Cell Culture and Transfection—COS-7, HEK 293, and A431 cells were obtained from ATCC and cultivated following the supplier's instructions. PC12 cells (kindly provided by Philip Cohen) were grown in Dulbecco's modified Eagle's medium, 4500 mg/liter glucose, supplemented with 5% fetal calf serum and 10% horse serum. PC12 cells were generally grown on plastic dishes coated with collagen (Sigma). The cell culture reagents were purchased from Invitrogen. HEK 293 cells were transfected with 2 μg DNA/ml by the calcium phosphate precipitation method (31). For ectopic protein expression in COS-7 and PC12 cells, the cells were plated on 6-well dishes and were transfected with 1 μg of DNA/well and LipofectAMINE or LipofectAMINE Plus (Invitrogen), respectively, following the manufacturer's protocol. After 24 h, the cells were transferred to serum starvation medium and cultured for another 24 h before stimulation and lysis. Transfected PC12 cells were selected with 1 mg/ml neomycin.

Immunoprecipitation, in Vitro Binding Assays, and Western Blot—The cell cultures were washed with PBS and lysed with lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerine, and 1% Triton X-100) supplemented with phosphatase and protease inhibitors (10 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 1 mM NaF, and 0.5% aprotinin). The cellular debris was removed by centrifugation. The supernatants were precleared with 20 μl of Sepharose slurry. The immunoprecipitations were carried out as described before with slight modifications (32). In brief, anti-HA, anti-PTP-SL, or anti-ERK5 antibodies were added together with 20 μl of mixed protein A- and G-Sepharose and one volume of HNTG (20 mM HEPES, pH 7.5, 150 mM NaCl, 0,1% Triton X-100, 10% glycerine, and 10 mM $Na_4P_2O_7$). Fc-tagged proteins were directly precipitated with the mix of protein A- and G-Sepharose. For *in vitro* binding studies, 1 μg of each Fc and GST fusion protein were incubated in 250 μl of PBS (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7,3) for 20 min at room temperature under constant shaking. After the addition of 0.3 volumes of HNTG, the samples were precleared and finally precipitated with 20 μl of GSH-Sepharose beads.

In general, the precipitation samples were incubated for 3 h on a rotation wheel at 4 °C. The precipitates were washed three times with 0.5 ml of HNTG buffer, suspended in 2× SDS sample buffer, boiled for 3 min, and subjected to gel electrophoresis. For Western blot analysis, the proteins were transferred to nitrocellulose membranes and immunoblotted. If quantification was necessary, the filters were exposed to the LAS1000 chemiluminescence camera (Fujifilm) and analyzed with the program Image Gauge 3.3 (Fujifilm).

Kinase Assays—For *in vitro* kinase assays, the precipitates were washed twice with HNTG and twice with kinase assay buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.5 mM orthovanadate). The samples were suspended in 30 μl of kinase assay buffer containing 50 μM ATP and 2 μCi of [γ-^{32}P]ATP and incubated for 20 min at 30 °C under constant shaking. For the measurement of substrate phosphorylation, kinase reactions were also supplemented with 1 μg of GST fusion proteins or 10 μg of myelin basic protein. The reaction was extended to 30 min if 1 μg of purified ERK5 protein was used. The assay was stopped by the addition of 2× SDS sample buffer and boiling. The samples were resolved on SDS-PAGE and transferred to nitrocellulose. Phosphorylation was detected by phosphorus imaging using the BAS2500 Reader (Fujifilm) and quantified with Image Gauge 3.3 (Fujifilm). The amount of precipitated kinase was visualized by immunoblot analysis.

Phosphatase Assays—For the measurement of PTP activity, 1 μg of GST-PTP-SL was diluted into 20 μl of PBS and incubated with 1 μg of different proteins for 10 min at room temperature under constant shaking. The aliquots of 10 μl were then added to 100 μl of *p*-nitrophenyl phosphate buffer (25 mM HEPES, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA) containing 3.7 mg/ml *p*-nitrophenyl phosphate as unspecific PTP substrate. After 2 h at 37 °C, the absorption at 405 nm was determined. For performance of *in vitro* phosphatase assays (see Fig. 5, *A* and *B*), ERK5 precipitates were washed twice with HNTG and twice with PTP assay buffer (25 mM HEPES, pH 7.3, 10 mM dithiothreitol, 5 mM EDTA). The samples were resuspended in 20 μl of assay buffer containing the indicated amount of GST-PTP-SL fusion protein and were incubated for 20 min at 30 °C while shaking. Because these reaction were followed by *in vitro* kinase assays, they were stopped by washing with kinase assay buffer containing the phosphatase inhibitor orthovanadate. The kinase assay was carried out as described above.

Immunofluorescence—COS-7 cells were seeded at 2×10^4 cells/cm^2 on glass cover slips. The transfections were performed as described above, and the cells were processed for immunofluorescence after 24 h of further culture. The cells were washed twice with PBS and fixed with methanol at −20 °C for 5 min, rinsed once with −20 °C cold acetone, and washed twice with PBS. All further steps were carried out at room temperature. The samples were incubated in PBG (PBS containing 0.5% bovine serum albumin and 0.045% teleostean fish gelatin) supplemented with 5% normal goat serum for 1 h, washed twice with PBG, and incubated with a 1:1000 dilution of anti-ERK5 antibody for 1 h. After three more washes with PBG, the cells were incubated with the secondary Cy3-labeled goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories) in a 1:1000 dilution for 1 h. After one more PBG wash and three more PBS washes, the samples were rinsed in destilled water and mounted. DNA was stained for 10 min with 1 μg/ml bisbenzimid, which was included in the penultimate washing step.

Preparation of Nuclei—48 h after transfection 1×10^6 COS-7 cells were trypsinated and collected by centrifugation at 500 × *g*. After washing twice with PBS, the cells were lysed in hypotonic lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0,5% Nonidet P-40) on ice for 5 min. The nuclei were precipitated by centrifugation at 500 × *g*, washed once with hypotonic lysis buffer, and finally dissolved in Laemmli buffer.

RESULTS

Co-immunoprecipitation of ERK5 and PTP-SL—To address the relevance of PTP-SL function in the regulation of diverse MAPK cascades, we investigated the potential interaction between ERK5 and the cytosolic form of the STEP-like phosphatase. We performed co-precipitation experiments with polyclonal anti-PTP-SL antibody and lysates from transfected HEK 293 cells. Fig. 1*A* (*top panel*) shows PTP-SL association with hemagglutinin-tagged ERK5 (HA-ERK5) in those cells co-overexpressing both proteins.

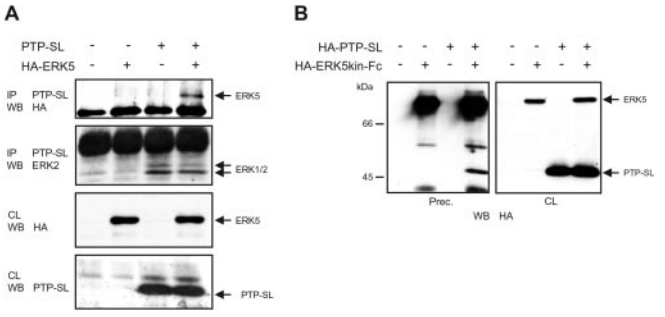

FIG. 1. **Co-immunoprecipitation of ERK5 and PTP-SL in transfected HEK 293 cells.** The cells were transfected with expression constructs of ERK5 and PTP-SL or the empty vectors and lysed after 48 h. *A*, pcDNA3 HA-ERK5 and pRK5 PTP-SL were transfected. PTP-SL immunoprecipitations (*IP*) and crude lysates (*CL*) were resolved by SDS-PAGE, followed by Western blot (WB) analysis using anti-HA, anti-ERK2, and anti-PTP-SL antibodies. The unspecific band migrating below ERK5 is due to the combination of antibodies used for precipitation and immunoblot (*top panel*). *B*, pRK5 HA-PTP-SL and pcDNA3 HA-ERK5kin-Fc were transfected. Protein A/G-Sepharose was used to precipitate Fc-tagged ERK5kin (aa 1–409). Precipitates and crude lysates were immunoblotted with anti-HA antibody.

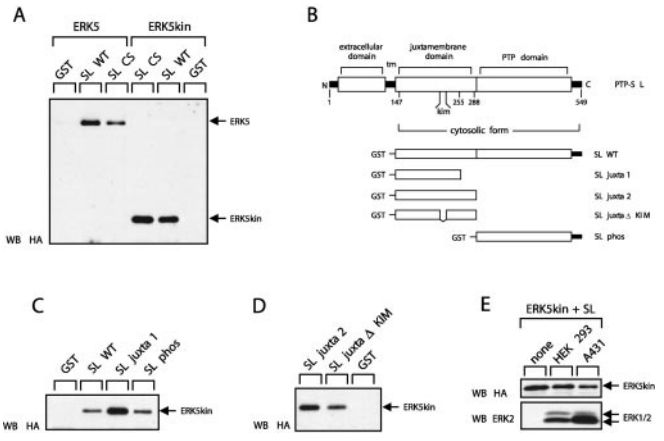

FIG. 2. **Direct binding of ERK5 and PTP-SL.** Purified Fc-tagged HA-ERK5 and HA-ERK5kin (Δaa 410–815) were incubated with GST fusion proteins of PTP-SL (*SL*). After precipitation with GSH-Sepharose, the samples were analyzed by anti-HA Western blot (*WB*). *A*, ERK5 and ERK5kin were incubated with wild type SL (*WT*) or a phosphatase-deficient mutant (*CS*). *B*, schematic representation of PTP-SL and the GST fusion proteins used in this study. Numbering of amino acids refers to the transmembrane form of PTP-SL and is according to Hendriks *et al.* (18). *C* and *D*, ERK5kin was precipitated with GST fusion proteins of different portions of PTP-SL, *e.g.* the juxtamembrane domain (*SL juxta 1*, aa 147–255, and *SL juxta 2*, aa 147–288), the phosphatase domain (*SL phos*, aa 256–549), the full cytosolic form SL WT (aa 147–549), or SL juxta 2 without the KIM (ΔKIM, Δaa 224–239). *E*, ERK5kin and PTP-SL WT were incubated in the presence of crude lysates of A431 and HEK 293 cells. The precipitates were additionally immunoblotted for ERK2 (*bottom panel*).

As can be seen in the *second panel* of Fig. 1*A*, in addition to HA-ERK5 endogenous ERK1/2 was also detected in the immunoprecipitates in the presence but not the absence of overexpressed PTP-SL. It is conceivable that ERK1/2 might compete with ERK5 for PTP binding. As expected from earlier studies describing the expression of PTP-SL predominantly in cell lineages of neuroendocrine origin (19, 20, 33), we did not detect endogenous PTP-SL in HEK 293 cell lysates (Fig. 1*A*, *bottom panel*).

The interaction of ERK5 and PTP-SL was further demonstrated by performing the converse experiment as shown in Fig. 1*B*. When a truncated version of HA-ERK5 (HA-ERK5kin) that contained only the kinase domain but lacked the unique C-terminal tail was co-expressed with HA-tagged PTP-SL (HA-PTP-SL), we were readily able to pull down PTP-SL by precipitating ERK5kin (Fig. 1*B*, *fourth lane*). Addition of the IgG-Fc portion to the C terminus of the ERK5kin construct was necessary to visualize the bound phosphatase, which would otherwise have been masked by the heavy chain of the precipitating antibody.

Direct Interaction of ERK5 and PTP-SL—The question of whether the proteins interact directly was addressed by performing *in vitro* binding experiments with bacterially expressed PTP-SL and purified Fc-tagged ERK5 from transfected HEK 293 cells. Correct folding of the proteins was verified by determining their phosphatase or kinase activities (data not shown). Fig. 2*A* shows that full-length ERK5 as well as the truncated ERK5kin protein directly interact with both wild type and catalytically inactive PTP-SL. Tarrega *et al.* (34) and Tanoue *et al.* (35, 36) identified docking motifs in ERK2 that mediate interaction with substrates and regulators including phosphatases. These docking motifs are also present and conserved in the ERK5 kinase domain and thus are likely to mediate the binding to PTP-SL.

We then asked which domain of PTP-SL would be responsible for binding to ERK5 by incubating ERK5kin with GST fusion proteins that contained different portions of PTP-SL (Fig. 2*B*). As shown in Fig. 2*C*, not only the full cytosolic form of PTP-SL but also the juxtamembrane and the phosphatase domain alone were both interacting with ERK5. Interestingly, even the PTP-SL juxtamembrane construct lacking the KIM, a motif that was shown to mediate the interaction with ERK2 (24–26), was still able to bind to ERK5 (Fig. 2*D*). When we further performed the binding experiment with ERK5kin and PTP-SL in the presence of crude lysates of HEK 293 and A431 cells as source for proteins that might possibly compete for PTP-binding sites, associated ERK5 decreased to a degree that correlated with the amount of endogenous ERK1/2 interacting with PTP-SL (Fig. 2*E*). Taken together, this set of data shows that the interaction between ERK5 and PTP-SL is direct and involves the kinase domain of ERK5 and, even though not exclusively, the KIM-containing juxtamembrane region of PTP-SL.

ERK5 Phosphorylates PTP-SL in Vitro—To examine whether PTP-SL itself could serve as ERK5 substrate, we performed *in vitro* kinase assays using activated ERK5 that was immunoprecipitated from EGF-stimulated COS-7 cells and as substrates several GST fusion proteins containing different portions of PTP-SL. Fig. 3*A* shows that wild type PTP-SL as well as those proteins containing the juxtamembrane domain were readily phosphorylated by ERK5. On the other hand, the phosphatase domain did not serve as a substrate. We then tested the ability of ERK5 to phosphorylate additional KIM-containing PTPs like NC-PTP, STEP (22), and HePTP (30). All of these phosphatases were phosphorylated; however, the cytosolic domain of the unrelated PTP IA2-β (29) was, as expected, not modified by ERK5 (Fig. 3*B*).

Binding of ERK5 Enhances the Activity of PTP-SL—We further asked whether complex formation between ERK5 and PTP-SL would alter enzymatic activity of the phosphatase. Therefore, we measured the activity of the bacterially expressed PTP-SL fusion protein upon binding to ERK5. As shown in Fig. 4*A*, ERK5 as well as ERK5kin enhanced PTP-SL activity ~3.5-fold, whereas GST and IgG alone had no effect.

Because PTP-SL was phosphorylated, although not quantitatively, by a preparation of ERK5 protein isolated from transfected HEK 293 cells (data not shown), we tested the influence of this phosphorylation on PTP-SL activity by performing *in vitro* kinase reactions in the presence and absence of ATP and subsequently quantified the resulting phosphatase activity. As shown in Fig. 4*B*, PTP-SL activity was again strongly enhanced

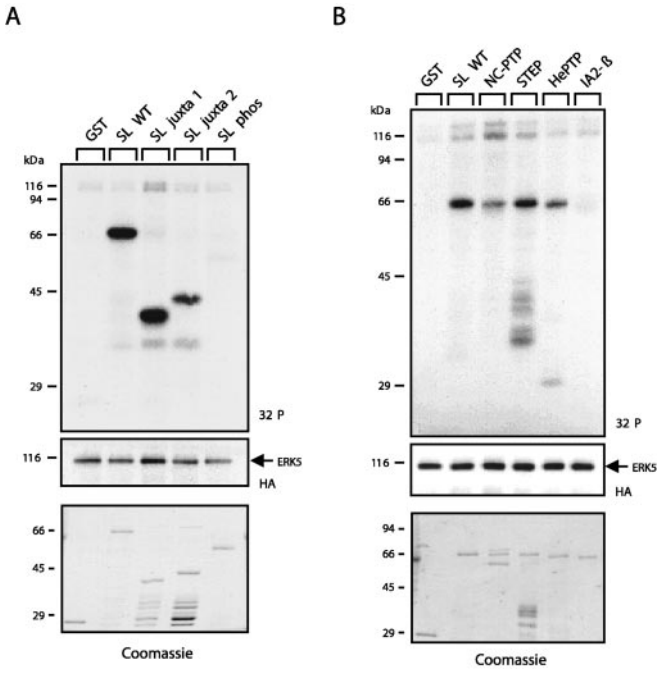

FIG. 3. **PTP-SL is an *in vitro* substrate for ERK5.** HA-ERK5 was immunoprecipitated from lysates of transfected COS-7 cells that were stimulated with 15 ng/ml EGF for 5 min. The samples were subjected to kinase assays using different GST fusion proteins as substrate. After separation, the samples were analyzed by autoradiography and anti-HA immunoblot. *A*, kinase substrates were GST-PTP-SL (*SL*) WT, SL juxta 1, SL juxta 2, and SL phos. *B*, GST fusion proteins of the full cytosolic forms of PTP-SL, NC-PTP, STEP, HePTP, and the unrelated IA2-β were used in the kinase assay as in *A*. The amount and the size of the fusion proteins used are shown in replica gels stained with Coomassie G250 (*A* and *B*, *bottom panels*).

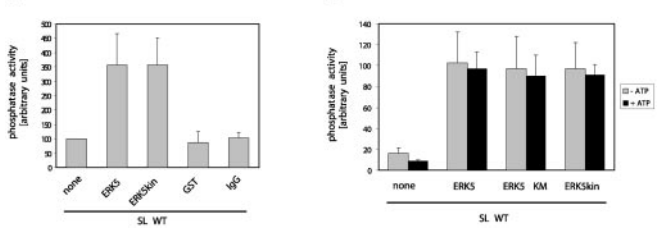

FIG. 4. **Activity of PTP-SL is enhanced by ERK5 binding.** *A*, 1 μg GST-PTP-SL (*SL*) WT was incubated with 1 μg of Fc-tagged ERK5, ERK5kin, or other proteins as indicated for 10 min at room temperature. Subsequently, PTP activity was measured with *p*-nitrophenyl phosphate as substrate. The data represent the means of three independent experiments ± S.D. The activity of SL WT alone was set as a reference. *B*, *in vitro* kinase reactions in the presence and absence of ATP were performed with 1 μg each of SL WT and purified Fc-tagged ERK5, kinase-deficient ERK5 KM, or ERK5kin. After 30 min at 30 °C, PTP activity was quantified in *p*-nitrophenyl phosphate assays. The values are the means of three separate experiments ± S.D. PTP activity was generally lower under these conditions compared with the data presented in *A*.

in the presence of either wild type ERK5, ERK5kin, or the kinase-inactive mutant ERK5 KM but was only very slightly decreased by the addition of ATP in all cases. These results indicate that binding to but not phosphorylation by ERK5 makes a major impact on PTP-SL activity.

PTP-SL Modulates ERK5 Activity in Vitro and in Transfected COS-7 Cells—To answer the question of whether PTP-SL might regulate ERK5 activity, we performed *in vitro* phosphatase reactions using various amounts of GST-PTP-SL fusion proteins together with a preparation of activated ERK5 and subsequently measured kinase activity. COS-7 cells co-

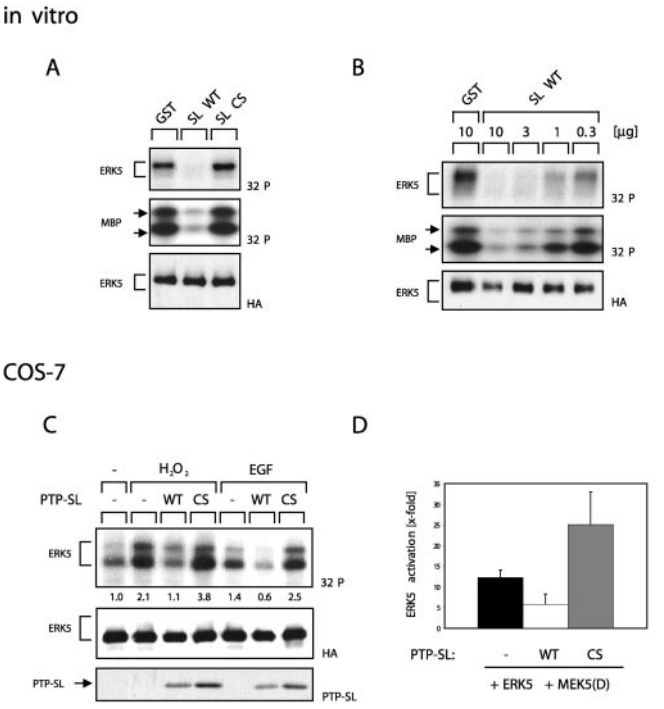

FIG. 5. **ERK5 inactivation by PTP-SL *in vitro* and in COS-7 cells.** *A* and *B*, pcDNA3 HA-ERK5-Fc and pcDNA3 MEK5(D) were co-transfected into COS-7 cells. After 48 h, Fc-tagged ERK5 was precipitated with protein A/G-Sepharose from crude cell lysates and subjected to *in vitro* phosphatase reactions in the presence of GST, GST-PTP-SL (*SL*) WT, or SL CS. Residual ERK5 activity was measured in a kinase assay with myelin basic protein as an additional substrate. The amount of SL protein was 3 μg if not indicated otherwise. The samples were analyzed by autoradiography (*top* and *middle panels*) and by anti-HA immunoblotting (*bottom panel*). *C*, COS-7 cells were transfected with pcDNA3 HA-ERK5, pRK5 PTP-SL WT, CS, or the empty vector. After serum starvation, the cells were stimulated for 15 min with 200 μM H_2O_2 or 5 min with 15 ng/ml EGF. Anti-HA immunoprecipitates were subjected to *in vitro* kinase assays and resolved on SDS-PAGE. Autophosphorylation and the amount of precipitated ERK5 were visualized by autoradiography and anti-HA immunoblot (*top* and *middle panels*). The *small numerals* below the lanes represent the relative autophosphorylation activities of ERK5. Expression of PTP-SL in crude lysates is shown by immunoblot using anti-PTP-SL (*bottom panel*). *D*, pcDNA3 HA-ERK5-Fc was transfected together with pcDNA3 MEK5(D) and pRK5 PTP-SL WT, CS, or the empty vectors into COS-7 cells. ERK5 was precipitated with protein A/G-Sepharose followed by kinase assays. The amount of precipitated and autophosphorylated ERK5 was quantified by anti-HA immunoblot and phosphorus imaging, respectively. The values represent the relative autophosphorylation activity normalized by the amount of precipitated ERK5 and are the means of two independent experiments ± S.D. ERK5 activity in the absence of exogenous PTP-SL and MEK5 was set as 1-fold.

expressing the dominant active form of MEK5 were used as source for activated ERK5. MEK5 was shown to be the MAPK kinase specifically activating ERK5 and to possess constitutive activity if serine 311 and threonine 315 were mutated to aspartate (8, 11). Wild type PTP-SL almost completely abolished ERK5 kinase activity, whereas the catalytically inactive CS mutant affected neither autophosphorylation of ERK5 nor phosphorylation of myelin basic protein (Fig. 5*A*). As shown in Fig. 5*B*, the degree of ERK5 inactivation correlated with the amount of PTP-SL protein utilized.

We then tested whether PTP-SL would be capable of inactivating ERK5 in transfected COS-7 cells by performing immunocomplex kinase assays after stimulation of cells with hydrogen peroxide or EGF. Whereas wild type PTP-SL reduced ERK5 kinase activity to basal levels, the PTP-SL mutant that lacked phosphatase activity seemed to further enhance ERK5 autophosphorylation (Fig. 5*C*, *upper panel*). ERK5 appeared as

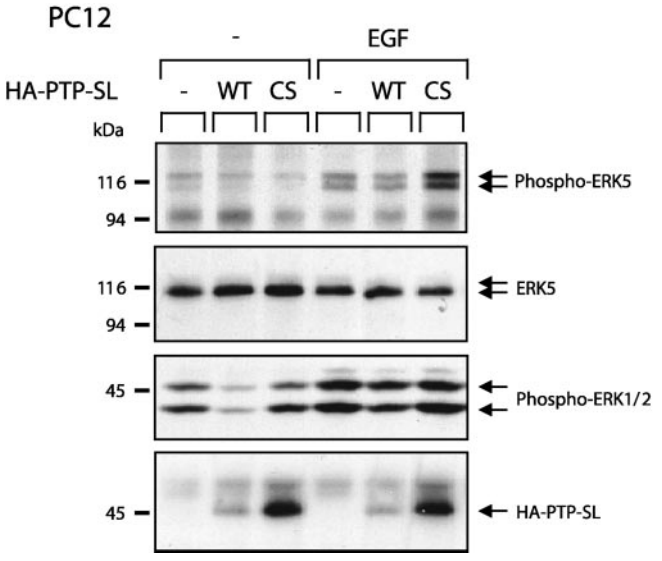

FIG. 6. **PTP-SL inactivates endogenous ERK5 in PC12 cells.** PC12 cells transfected with pcDNA3-HA-PTP-SL WT, CS, or mock DNA were serum-starved for 24 h followed by stimulation with 30 ng/ml EGF for 5 min. Endogenous ERK5 was immunoprecipitated. The split samples were analyzed by immunoblotting for ERK5 and for the active phosphorylated form phospho-ERK5 (*top two panels*). Activation of ERK2 was monitored by Western blot analysis of crude lysates using anti-phospho-ERK1/2. The expression of HA-PTP-SL was visualized by anti-HA immunoprecipitation and Western blot (*bottom panel*).

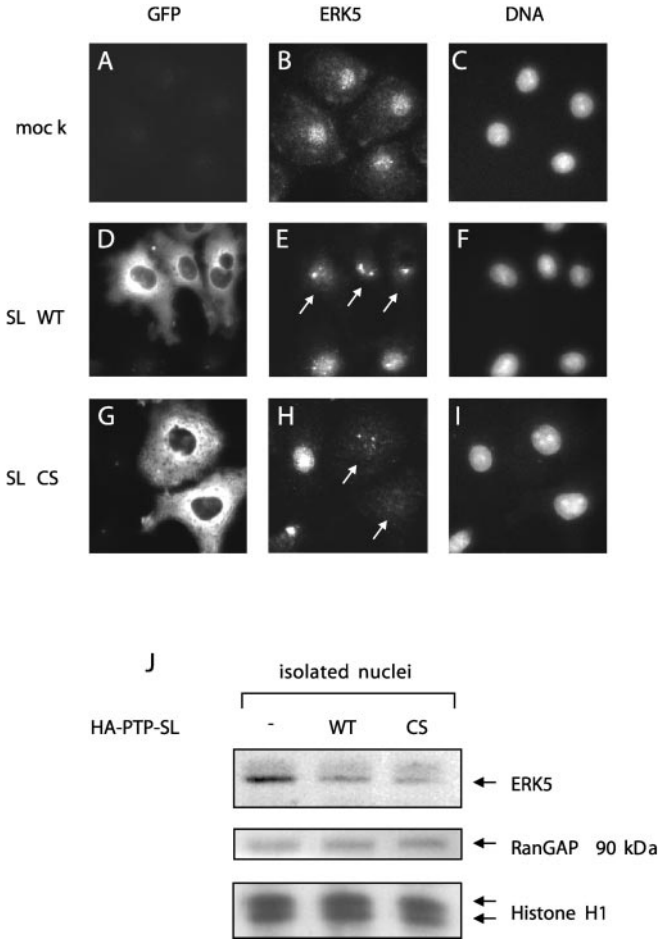

FIG. 7. **PTP-SL retains ERK5 in the cytoplasm.** COS-7 cells were transiently transfected with pcDNA3 GFP-PTP-SL (*SL*) WT, CS, or the empty vector and analyzed by fluorescence microscopy after 24 h. GFP autofluorescence is shown in the *left panels* (*A*, *D*, and *G*). ERK5 was visualized by immunofluorescence using anti-ERK5 antibodies (*B*, *E*, and *H*), and DNA was stained with bisbenzimid (*C*, *F*, and *I*). Each *row* shows identical sections of COS-7 cells. The *arrows* indicate nuclei with reduced ERK5 staining. *J*, isolated nuclei from the transfected COS-7 cells were separated on SDS-PAGE. The ERK5 content was analyzed by anti-ERK5 immunoblot. Equal loading was assured by reprobing the membrane with anti-RanGAP and anti-histone H1 antibodies.

a doublet resulting from a mobility shift of a small fraction of the enzyme that was hardly detectable in Western blot but clearly visible in the autoradiograph. This shift is probably due to phosphorylation of ERK5 because the amount of the upper band was found to correlate with the degree of kinase activation (14, 37); when dominant active MEK5 was co-expressed this shift was virtually quantitative (Fig. 5, *A* and *B*).

Because the relative activation of ERK5 after EGF and hydrogen peroxide treatment of cells was only moderate because of its high basal activity, we additionally expressed the constitutively active MEK5 construct to achieve a more pronounced ERK5 activation. Wild type PTP-SL partially counteracted the effect of MEK5 and reduced ERK5 activity to a level that may reflect the balance between the opposing actions of activating kinase and inactivating phosphatase in the cell. In turn, co-transfection of the catalytically impaired form of PTP-SL caused a profound increase in ERK5 activity that might be due to competition with endogenous phosphatases for ERK5-binding sites.

Endogenous ERK5 Is Inactivated by PTP-SL in PC12 Cells—To evaluate the potential of PTP-SL to inactivate endogenous ERK5, we stimulated PC12 cells stably expressing PTP-SL with EGF and analyzed the activation state of immunoprecipitated ERK5 by Western blot. After stimulation, a reduced amount of active ERK5 was detected in cells overexpressing wild type PTP-SL (Fig. 6, *top panel*), whereas in analogy to the effect seen in transfected COS-7 cells, the inactive CS mutant of PTP-SL further enhanced the activation of endogenous ERK5 (compare Fig. 5, *C* and *D*, and Fig. 6). Notably, two more or less equally phosphorylated forms of ERK5 were detected in PC12 cells, which indicates that additional modifications might be required for the mobility shift to the upper position. Fig. 6 (*top panel*) further shows that PTP-SL reduced the amount of both activated forms of ERK5 to a similar extent. As can be seen in the *third panel* of Fig. 6, ERK1/2 were, as expected, also inactivated by wild type PTP-SL. It has to be mentioned that the expression of wild type PTP-SL when compared with its inactive mutant decreased with time during the cultivation of cells, indicating growth selection for cells with low phosphatase expression (Fig. 6, *bottom panel*).

PTP-SL Retains ERK5 in the Cytoplasm—When we examined the subcellular localization of endogenous ERK5 in COS-7 cells by immunofluorescence, we found that a major portion of endogenous ERK5 resided in the nucleus (Fig. 7, *B* and *C*). To test whether PTP-SL would have an influence on this subcellular localization, we overexpressed the phosphatase fused to GFP. It can be clearly seen in Fig. 7 (*D–F*) that only those cells that express PTP-SL as indicated by the autofluorescence of GFP show reduced ERK5 staining in the nucleus when compared with neighboring untransfected cells. This reduction reflects the retention of ERK5 in the cytoplasm because expression of PTP-SL did not affect the overall levels of ERK5 protein in the cells (data not shown). Fig. 7 (*G–I*) further shows that not only the wild type PTP-SL but also its catalytically impaired CS mutant exerted this influence on ERK5. Accordingly, the amount of ERK5 protein was reduced in isolated nuclei from transiently transfected COS-7 cells expressing either form of PTP-SL (Fig. 7*J*). The influence of PTP-SL on the localization of ERK5 was independent of phosphatase activity, which demonstrates that PTP-SL is able to modulate ERK5 action not only by dephosphorylating the kinase but additionally by bind-

ing to it and thereby retaining it in the cytoplasmic department. Therefore, the cellular PTP-SL expression level is an additional and critical parameter for MAPK regulation.

DISCUSSION

The MAPK cascade is a major pathway by which cells transduce extracellular stimuli to the nucleus. ERK5 is one of the least examined members of the MAPK family. Similar to ERK1 and ERK2, dual phosphorylation of threonine and tyrosine residues in the kinase activating motif TEY is crucial for ERK5 activation. Because in analogy to ERK1/2 dephosphorylation of either residue is potentially sufficient for inactivation of ERK5 (17), we tested whether the protein-tyrosine phosphatase PTP-SL might be involved in the regulation of the ERK5 pathway.

PTP-SL not only bound to ERK5 but also modulated kinase activity *in vitro* and *in vivo*. Overexpression of wild type PTP-SL in PC12 and COS-7 cells reduced endogenous as well as exogenous ERK5 activity, whereas the catalytically inactive CS mutant of PTP-SL obviously enhanced kinase activity. The positive effect of the inactive phosphatase on kinase activity might be due to the competition between PTP-SL and endogenous phosphatases for ERK5-binding sites, which suggests that ERK5 might be under the permanent control of phosphatases that presumably belong to the KIM-containing PTP family.

Whereas inactivation of ERK5 by PTP-SL monitored *in vitro* is most probably simply due to the dephosphorylation of tyrosine 220 in the activating TEY motif (7, 8), the *in vivo* situation might be much more complex. PTP-SL is potentially able to affect the ERK5 pathway in several ways. Overexpression of wild type as well as catalytically inactive PTP-SL, for instance, reduced the amount of ERK5 in the nucleus. A similar influence of PTP-SL on the localization of ERK2 was described by Zuniga *et al.* (38). Because translocation of MAPKs to the nucleus is essential to phosphorylate transcription factors that subsequently regulate gene activity (5, 39), retention of ERK5 in the cytoplasm might reflect an additional mechanism by which PTP-SL can modulate the action of ERK5.

A further consequence of the interaction of ERK5 with PTP-SL is the phosphorylation of the phosphatase. The fact that phosphorylation could be localized to the juxtamembrane domain of PTP-SL alludes to threonine 253 as modified amino acid in view of the fact that it is also phosphorylated by ERK2. Not only PTP-SL but also other members of this PTP family were phosphorylated by both ERK2 (24, 25) and ERK5. Even though it has been shown that phosphorylation of a specific serine residue in the KIM of PTP-SL and HePTP by PKA triggers the release of ERK2 (40, 41), it is unlikely that the observed phosphorylation of PTP-SL by ERK5 directly influences the binding properties between these two enzymes because no change in complex formation of purified PTP-SL and ERK5 protein was observed after *in vitro* phosphorylation (data not shown). Interestingly, phosphorylation of PTP-SL by ERK5 had only a minor influence on enzymatic activity; binding of the kinase to the phosphatase, however, enhanced phosphatase activity substantially. A similar effect was shown for MAPK phosphatase 3, a dual specificity phosphatase that is readily activated by formation of a stable complex with ERK2 (42).

The effects on MAPK signaling exerted by MAPK phosphatases and by KIM-containing PTPs are generally considered to be temporarily and spatially distinct from each other (43). Saxena and Mustelin (27) have proposed a "sequential phosphatase model" in which KIM-containing PTPs control MAPK activity during their initial cytosolic phase, whereas, after translocation to the nucleus, termination of a sustained MAPK signal would be accomplished by MAPK phosphatases. We found that PTP-SL not only reduces ERK5 activity but also hampers its translocation to the nucleus. This result adds a new aspect to the regulation of MAPK by KIM-containing PTPs.

Unlike ERK5, which was detected in tissues and cell lines of various origin and thus seems to be ubiquitously expressed (9, 14, 44),[2] PTP-SL expression was shown to be restricted and predominantly found in brain and neuroendocrine cells (18–21, 33). Because other members of the same PTP family such as HePTP (30) show a similarly restricted expression pattern, it is conceivable that specificity of the ERK5 signal is further determined by different KIM-containing PTPs in a cell type-dependent manner.

Because MAPKs can generally play diverse physiological roles (1–4), ERK5 is supposedly also involved in a multitude of responses depending on the cellular context. For example in C2C12 cells, ERK5 was shown to be essential for differentiation and myotube formation, whereas in neurons the ERK5 pathway mediates cell survival (13, 44). In rat pheochromacytoma cells mitogenic stimuli as well as those leading to differentiation activate ERK5 in a similar manner as ERK2 (data not shown); however, no evidence for the involvement of ERK5 in neurite formation was found so far.

ERK5 but not ERK2 is required for epidermal growth factor-mediated cell cycle progression in HeLa and MCF10A breast cancer cells (15). Furthermore ERK5 was shown to possess constitutive activity in BT474 and SkBr3 breast carcinoma cells that also express activated forms of the oncogenic receptor human EGF receptor-2/neu (45). This provokes the intriguing speculation that ERK5 might be involved in cancer development, particularly in light of the fact that the gene encoding the transcription factor MEF2C, which is a well characterized substrate of ERK5 (11, 46, 47), is included in the mammary cancer susceptibility region Mcs 1 (48). Because disregulated EGF receptor activity is a hallmark of many tumors (49–51), the ERK5 pathway might be a potential target for cancer therapy.

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