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Inactivation of Mitogen-Activated Protein Kinases by a Mammalian Tyrosine-Specific Phosphatase, PTPBR7

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Mitogen-activated protein kinase (MAPK) is inactivated through dephosphorylation of tyrosyl and threonyl regulatory sites. In yeast, both dual-specificity and tyrosine-specific phosphatases are involved in dephosphorylation. In mammals, however, no tyrosine-specific phosphatase has been identified molecularly to dephosphorylate MAPK *in vivo.* Recently, we and others have cloned a murine tyrosine-specific phosphatase, PTPBR7/PTP-SL, which is expressed predominantly in the brain. Here we report inactivation of the extracellular signal-regulated kinase (ERK) family MAPK by PTPBR7. PTPBR7 made complexes with ERK1/ERK2 *in vivo* and dephosphorylated ERK1 *in vitro.* When overexpressed in mammalian cells, wild-type PTPBR7 suppressed the phosphorylation and activation of ERK by epidermal growth factor (EGF), nerve growth factor (NGF), and constitutively active MEK1, a mutant MAPK kinase. In contrast, catalytically inactive and ERK-binding-deficient mutants revealed little inhibition on the ERK cascade. These results indicate that PTPBR7 suppresses MAPK directly *in vivo.*

In response to various extracellular stimuli, mitogen-activated protein kinase (MAPK) is activated in the kinase cascade (1–4). ERK1 and ERK2 belong to one class of MAPK that is implicated in the regulation of proliferation, neuronal differentiation, long-term memory, cytoskeletal organization, and chemotaxis. The magnitude and the duration of MAPK activation are critical for the biological effects (2). MAPK is activated through phosphorylation of both tyrosine and threonine residues by MAPK kinase, a dual-specificity kinase, and inactivated by dephosphorylation of these residues [reviewed in (5)]. Thus, the activity of ERKs is determined by the balance between kinases and phosphatases.

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In mammals, dual-specificity phosphatases, such as MKP-1/CL100, are implicated in the dephosphorylation and negative feedback control of MAPK activity (5–7). In fact, MKP-1/CL100 expression is induced by various stimuli leading to MAPK activation. However, the down-regulation mechanism of ERKs has not been completely understood. In PC12 cells, though the expression of MKP-1/CL100 is induced rapidly after NGF stimulation, it does not correlate with the inactivation of ERKs (8). The presence of molecularly unidentified tyrosine-specific phosphatase activity that dephosphorylates ERKs in PC12 cells has been reported (8). This is in a good accordance with the observation that not only dual-specificity phosphatases but also tyrosine-specific phosphatases are involved in the dephosphorylation and inactivation of MAPK in yeast (5).

Recently, we have cloned a murine receptor-like protein tyrosine phosphatase (PTP) (Fig. 1A), PTPBR7, which is predominantly expressed in neuronal cells (9). There are several isoforms of PTPBR7. PTP-SL (10) is almost identical to PTPBR7 except for the most N-terminal amino acid sequence. In addition, an cytosolic isoform corresponded to a product translated from the fourth methionine of PTPBR7 was reported (11). This isoform reveals a structural similarity to two cytoplasmic PTPs, LC-PTP/HePTP (12, 13) and STEP (14, 15). A rat homologue of PTPBR7, PCPTP1/PC12-PTP, is expressed in PC12 cells and is increased about 10-fold after NGF stimulation for 8 h (11, 16). The biological function of PTPBR7 was not known.

Here we report the direct suppression of ERK family MAPK by PTPBR7. In our knowledge, PTPBR7 is the first molecularly identified mammalian tyrosine-specific phosphatase which suppresses MAPK directly *in vivo.*

MATERIALS AND METHODS

Generation of anti-PTPBR7 monoclonal antibody (mAb), 22-1. Lewis rat was immunized with PTPBR7-Fc (9), a fusion protein between extracellular domain of PTPBR7 and human IgG_1. Spleen cells were fused with SP2/O mouse myeloma cells and clones secret-

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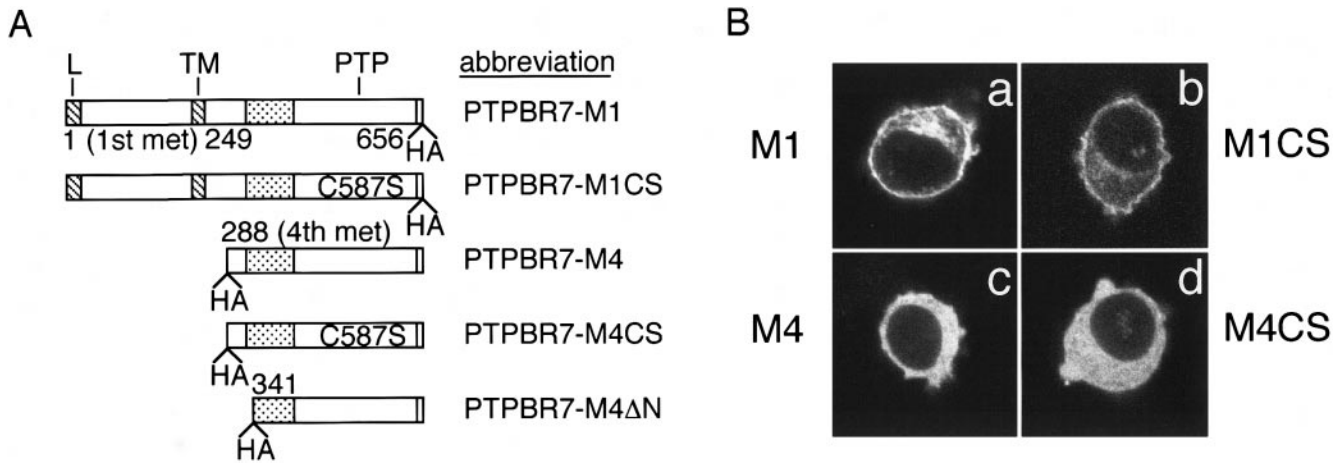

FIG. 1. Schematic drawings of PTPBR7 isoforms and mutants and their subcellular localization in PC12 cells. (A) PTPBR7-M1 and PTPBR7-M4 represent receptor-type and cytoplasmic isoforms of PTPBR7, respectively. The substitution of Cys587 by Ser inactivates the catalytic activity. In some cases, a tandem repeat of HA-tag (18 amino acids) is added at the C-terminal or N-terminal end. PTPBR7-M4ΔN is a deletion mutant of PTPBR7-M4. The shaded box indicates the region conserved among PTPBR7, LC-PTP/HePTP, and STEP. L, signal sequence; TM, transmembrane domain; PTP, protein tyrosine phosphatase domain. (B) Subcellular localization. PC12 cells were transiently transfected with plasmids encoding HA-tagged PTPBR7-M1 (a), PTPBR7-M4 (c), and their catalytically inactive mutants, PTPBR7-M1CS (b) and PTPBR7-M4CS (d). After 2 days, cells were fixed, permeabilized with 0.1% Triton X-100, and stained using an anti-HA monoclonal antibody (mAb), 12CA5, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG.

ing anti-PTPBR7 mAb were screened by PTPBR7-Fc coated ELISA plate.

Cell culture and transfection. 293T cells (1×10^6) in 6-cm dish were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and transfected with various PTPBR7 plasmids and ERK-Flag plasmids (total 10 μg) by calcium phosphate coprecipitation method. The cDNAs were subcloned in the pEF-BOS vector (17) and used for the transient expression experiment. PC12 cells were cultured on collagen coated dish in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum and 5% fetal calf serum (FCS). PC12 cells were transfected by Superfect transfection agent (Qiagen).

Two-hybrid screening. The cytoplasmic portion of PTPBR7 (amino acids 249 to 656) with a catalytically inactive mutation (Cys587 to Ser) was fused to GAL4 DNA binding domain and used as a bait. Human fetal brain (1.4×10^6 clones) and placenta (8×10^6 clones) cDNA libraries in the pACT2 plasmid were screened as described (18).

Immunoprecipitation and immunoblotting analysis. Two days after transfection, cells were lysed in 1% (v/v) Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM PMSF, 5 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, and 5 mM sodium pyrophosphate. Cell lysates were precleared and used for immunoprecipitation with protein G-Sepharose beads preloaded with various mAbs. Coprecipitated proteins were analyzed by immunoblotting using ECL system (Amersham). For immune complex kinase assay, cells were lysed in 1% (v/v) NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM PMSF, 5 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, and 5 mM sodium pyrophosphate.

Reporter assay. PC12 cells in 24-well tissue culture plates were transfected with 10 ng of pFA-Elk1 and 200 ng of pFR-Luc (*Firefly* luciferase reporter) with or without PTPBR7 plasmids (500 ng). Total amounts of DNA were kept constant (1 μg) by addition of empty pEF-BOS expression vector. To activate Elk-1/Gal4, 10 ng of pFC-MEK plasmid was used. Alternatively, cells were incubated with NGF (100 ng/ml) during the last 5 hr period of incubation. Two days after transfection, luciferase activity was measured. Transfection efficiencies were normalized by *Renilla* luciferase activity constitutively expressed from co-transfected 20 ng of pRL-TK.

RESULTS

Subcellular localization of PTPBR7 isoforms. PTPBR7 is a receptor-like PTP (Fig. 1A) (9), which is predominantly expressed in neuronal cells. An cytosolic isoform was also reported (11). In the following part, we abbreviate the receptor-like and cytoplasmic isoforms as PTPBR7-M1 and PTPBR7-M4, respectively.

HA-tagged PTPBR7-M1 and PTPBR7-M4 were transiently expressed in PC12 cells. As expected, PTPBR7-M1 was enriched in the plasma membrane (Fig. 1B, a), while PTPBR7-M4 localized diffusely in the cytosol (Fig. 1B, c). Catalytically inactive mutants, PTPBR7-M1CS and PTPBR7-M4CS, were made by altering conserved Cys587 to Ser. The distributions of these mutants were almost identical to that of wild-type molecules (Fig. 1B, b and d).

Complex formation of PTPBR7 with ERK family MAPKs. To study the function of PTPBR7, we investigated the interacting molecules by yeast two-hybrid screening. Four positive clones were finally obtained and all four clones encoded ERK1. The inserts started from nucleotide 2, 64, 70, and 110 according to the numbering of a human ERK1 cDNA (X60188).

The association of PTPBR7 with ERKs was confirmed in mammalian cells. 293T human fetal kidney cells were transiently transfected with expression plasmids encoding various forms of HA-tagged PTPBR7 and Flag-tagged ERK1 and ERK2. PTPBR7-M1 and

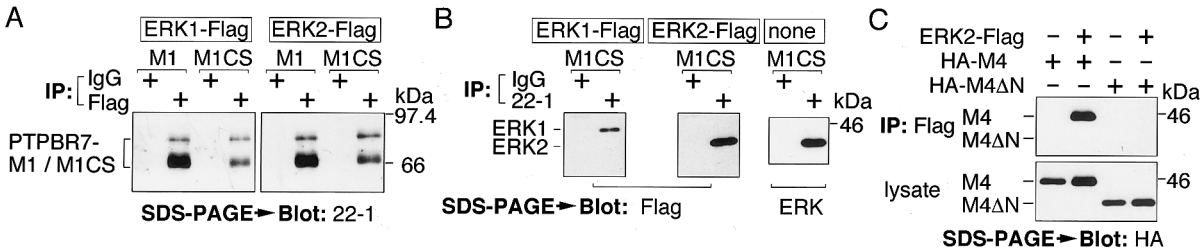

FIG. 2. Complex formation of PTPBR7 and ERK1/ERK2 *in vivo.* (A) Coprecipitation of PTPBR7-M1 and PTPBR7-M1CS with ERK1/ERK2. PTPBR7-M1 and PTPBR7-M1CS tagged with HA were transiently co-expressed with Flag-tagged ERK1 or ERK2 in 293T cells. After immunoprecipitation of ERK1 and ERK2 by anti-Flag mAb (M2), PTPBR7 coprecipitated was separated by SDS–PAGE and detected by immunoblotting using rat anti-PTPBR7 mAb (22-1). (B) Coprecipitation of ERK1/ERK2 with PTPBR7-M1CS. ERK1 and ERK2 were coprecipitated with PTPBR7-M1CS by 22-1 mAb, separated by SDS–PAGE, and detected by immunoblotting using biotinylated anti-Flag mAb (M2) (left and middle panels). Endogenous ERK2 coprecipitated with non-epitope-tagged PTPBR7-M1CS was detected by an anti-pan ERK mAb (E17120, Transduction Laboratories) (right panel). (C) Coprecipitation of cytoplasmic PTPBR7-M4 but not PTPBR7-M4ΔN with ERK2. HA-tagged PTPBR7-M4 and PTPBR7-M4ΔN were transiently expressed with or without ERK2-Flag in 293T cells. After immunoprecipitation of ERK2 by anti-Flag mAb, coprecipitated PTPBR7-M4 and PTPBR7-M4ΔN were separated by SDS–PAGE and visualized by immunoblotting using biotinylated anti-HA mAb (12CA5) (upper panel). The amounts of PTPBR7-M4 and PTPBR7-M4ΔN in the lysates were shown in the lower panel.

PTPBR7-M1CS with a molecular mass range between 70 and 85 kDa was coprecipitated with ERK1 and ERK2 (Fig. 2A). The molecular mass expected for the core protein of mature PTPBR7-M1 and PTPBR7-M1CS is 70 kDa. The difference in size could be explained by the difference in glycosylation. There are possible glycosylation sites in the extracellular domain of PTPBR7.

About 6% of PTPBR7-M1 was estimated to associate with ERK1. Association was further confirmed by the coprecipitation of ERK1 and ERK2 with PTPBR7-M1CS (Fig. 2B, left and middle panels). Also, endogenous ERK2 was coprecipitated with non-epitope-tagged PTPBR7-M1CS (Fig. 2B, right panel). PTPBR7-M4 and PTPBR7-M4CS (data not shown) coprecipitated with ERK1 (data not shown) and ERK2 too (Fig. 2C). On the contrary, PTPBR7-M4ΔN, a deletion mutant lacking small N-terminal portion (aa 288–340) of PTPBR7-M4 revealed no binding capacity (Fig. 2C).

Dephosphorylation and inhibition of ERK kinases by PTPBR7. To test whether PTPBR7 could dephosphorylate ERK1 *in vitro,* catalytic domain of PTPBR7 was expressed as a glutathione S-transferase (GST) fusion protein (GST-BR7) (9) and incubated with phos-

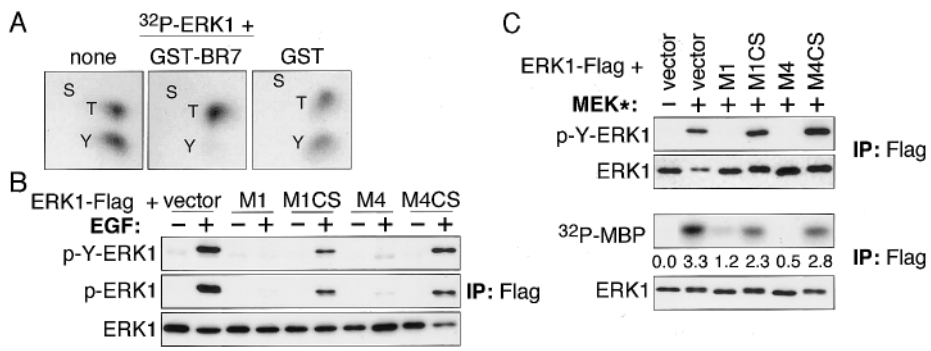

FIG. 3. Dephosphorylation and inhibition of ERK1 by PTPBR7. (A) Tyrosine-specific dephosphorylation of ERK1 *in vitro.* ERK1-Flag protein ^{32}P-labeled *in vivo* was incubated with 8 μM of GST-BR7 (containing aa. 363-656 of PTPBR7) or GST for 60 min at 37°C. After hydrolysis, phosphoamino acids were separated by two-dimensional electrophoresis on thin-layer chromatography plates. (B) Inhibition of EGF-mediated ERK1 phosphorylation *in vivo.* 293T cells transiently expressing various forms of PTPBR7 and ERK1-Flag were serum deprived for 14 h and then stimulated with 100 ng/ml of EGF for 5 min. ERK1-Flag was immunoprecipitated by anti-Flag mAb (M2) and the phosphorylation level was determined by anti-phosphotyrosine (PY20) or anti-phospho-ERK (#9105, New England BioLabs) mAbs. Phosphorylation of both tyrosine and threonine residues is essential for the binding of the #9105 antibody to ERK1 and ERK2. It does not cross-react with SAPK/JNK and p38 MAPK. The amounts of ERK1 precipitated were measured by M2 mAb. (C) Inhibition of mutant MEK1-mediated ERK1 activation in 293T cells. ERK1-Flag was activated by the co-transfection of pFC-MEK plasmid encoding constitutively active MEK1. Phosphorylation level and the amounts of ERK1 precipitated were determined as described above. ERK1 activity was measured by immune complex kinase assay using MBP as a substrate. Relative intensity of the band was quantitated by an imaging plate scanner BAS2000 and shown below.

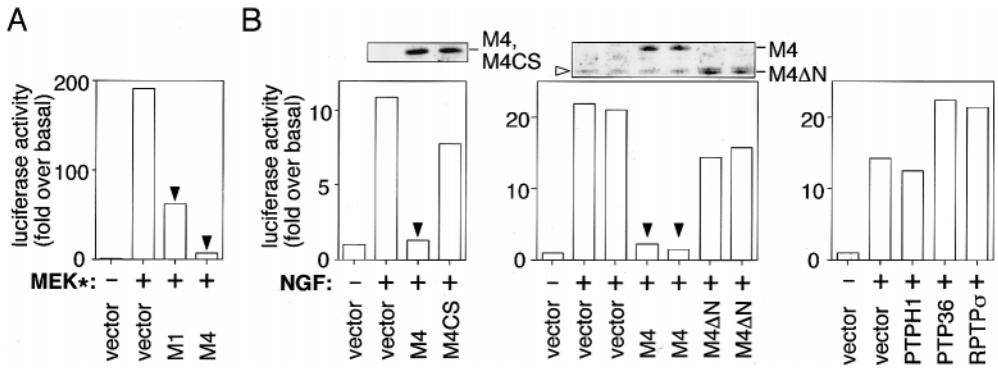

FIG. 4. The effects of PTPBR7 isoforms and mutants on ERK cascade in the PC12 cells. The activation levels of ERK were measured by the reporter assay using the Elk-1/Gal4 trans activator. Plasmids coding for HA-tagged PTPs were cotransfected with Elk-1/Gal4 (pFA-Elk1), Gal4-driven *Firefly* luciferase reporter (pFR-Luc), and control *Renilla* luciferase (pRL-TK). Reporter *Firefly* luciferase activity was normalized by *Renilla* luciferase activity and the basal activity (activity without NGF or pFC-MEK encoding active MEK1) was expressed as 1. Only the representative results from several independent experiments are shown. (A) Inhibition of mutant MEK1-induced Elk-1/Gal4 reporter activity by PTPBR7 isoforms. PC12 cells were transiently transfected with either control pEF-BOS vector or plasmid encoding PTPBR7-M1 or M4 (500 ng), and various reporter plasmids. After 2 days, reporter luciferase activity was measured. (B) The effects of PTPBR7 mutants and various PTPs on the NGF-induced Elk-1/Gal4 reporter activity. PC12 cells were transfected with control plasmid or plasmids encoding wild type PTPBR7-M4, catalytically inactive PTPBR7-M4CS, and PTPBR7-M4ΔN lacking ERK-binding activity (left and middle panels). After 2 days, Elk-1/Gal4 reporter was activated by incubating cells with NGF (100 ng/ml) for 5 h. The amounts of PTPBR7-M4 and its mutants expressed were analyzed by immunoblotting using anti-HA mAb (upper panels). The open arrowhead indicates the position of weak nonspecific band. In the right panel, PC12 cells were transiently transfected with plasmids (500 ng) encoding PTPH1, PTP36, and RPTPσ.

phorylated ERK1. ERK1 was dephosphorylated in the tyrosine-specific manner (Fig. 3A).

To study the effects of PTPBR7 on ERK *in vivo,* we transiently coexpressed ERK1-Flag and various forms of PTPBR7 in 293T cells. After stimulation with EGF for 5 min, ERK1-Flag was recovered by immunoprecipitation and its phosphorylation level was determined by the immunoblotting experiment (Fig. 3B). PTPBR7-M1 and PTPBR7-M4 strongly suppressed the phosphorylation of ERK1, whereas PTPBR7-M1CS and PTPBR7-M4CS, catalytically inactive mutants, had only marginal effects. PTPBR7 without HA-tag showed almost identical results (data not shown).

It is possible that PTPBR7 does not inactivate ERK1 directly but other signaling molecules between EGF receptor and ERK1. Thus, we activated ERK1 using a constitutively active mutant of MEK1 (19, 20). MEK1 is a MAPK kinase for ERK and the mutant MEK1 phosphorylates ERK1 regardless of upstream activation signals. Again, wild type PTPBR7 but not catalytically inactive mutants reduced the phosphorylation level and kinase activity of ERK1 (Fig. 3C) and ERK2 (data not shown).

Phosphatase and ERK-binding activities were crucial for the inhibition. PC12 cells express an endogenous rat homologue of PTPBR7, PCPTP1/PC12-PTP (11, 16). In PC12 cells, NGF is known to activate ERKs followed by the increased expression of PCPTP1/PC12-PTP. Thus, we tested the effects of PTPBR7 in PC12 cells using a reporter assay. Like in 293T cells, PTPBR7-M1 and PTPBR7-M4 inhibited the ERK cascade activated by constitutively active MEK1 (Fig. 4A).

PTPBR7-M4 also suppressed the activation of ERKs by NGF, a more physiological stimulator (Fig. 4B, left panel). In contrast, other phosphatases such as PTPH1 (21) and PTP36 (22), cytoplasmic phosphatases, and RPTPσ (23), a receptor-type phosphatase, revealed little inhibitory effects (Fig. 4B, right panel). Then, the effects of various mutants of PTPBR7 were tested. PTPBR7-M4CS, a catalytically inactive mutant, and PTPBR7-M4ΔN, a deletion mutant lacking ERK-binding capacity (Fig. 2C), revealed only marginal inhibition on the ERK cascade (Fig. 4B, left and middle panels). Thus, the phosphatase activity and ERK-binding capacity of PTPBR7 are crucial for the inhibition.

DISCUSSION

In this report, we demonstrated that PTPBR7, a mammalian tyrosine-specific phosphatase, suppresses ERK family MAPK. We concluded that ERK was the direct target of PTPBR7 because 1) PTPBR7 dephosphorylated ERK1 directly *in vitro,* 2) PTPBR7 suppressed the phosphorylation and activation of ERK by the mutant MEK1 that was active irrespective of upstream signals, and 3) ERK-binding activity was crucial for the *in vivo* suppression of ERKs.

Our results do not exclude the possibility that signaling molecules other than ERKs are also the target for PTPBR7. It should be noted however, no obvious change in the pattern of major tyrosine-phosphorylated proteins was observed when PTPBR7 was transiently overexpressed in more than 60% of 293T cells

(unpublished observation). Thus, we speculated that PTPBR7 dephosphorylated only a few signaling molecules including ERK1 and ERK2, if any.

For many PTPs, it was reported that catalytically inactive mutants but not wild-type PTPs made stable complexes with their phosphorylated substrates. However, several exceptions were reported. For example, wild type MKP-3/rVH6/Pyst1, a dual-specificity phosphatase, made stable complexes with its substrate, ERK1, which was not phosphorylated (24, 25). Like MKP-3/rVH6/Pyst1, wild type PTPBR7 made complexes with ERK1 and ERK2.

The inhibition by catalytically inactive PTPBR7-M4CS is weaker than wild type but reproducible (Fig. 4B, left panel). This is not surprising since similar observation has been reported for Ptp2p, a tyrosine-specific phosphatase in *S. cerevisiae* (26). It was explained that the binding of catalytically inactive PTP might somewhat interfere with the MAPK to interact with other signaling molecules.

The physiological role of PTPBR7 in neuronal cells remains to be elucidated. In PC12 cells, the PCPTP1/PC12-PTP, a rat homologue of PTPBR7, is expressed at a relatively low level and increased about 10-fold after NGF stimulation for 8 hr (16). Though MKP-1/CL100, a dual-specificity phosphatase, is also increased, the slow inactivation of ERK seems to correlate better with the slow increase of PCPTP1/PC12-PTP rather than rapid induction of MKP-1/CL100. In addition, MKP-1/CL100 localizes to the nucleus, while PTPBR7 localizes predominantly to cytosol or plasma membrane. These spatial and temporal differences may propose unique roles for each of these phosphatases.

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