Regulation of Protein-tyrosine Phosphatases α and ε by Calpain-mediated Proteolytic Cleavage*

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The precise subcellular localization of non-receptor tyrosine phosphatases is a major factor in regulating their physiological functions. We have previously shown that cellular processing of protein-tyrosine phosphatase ε (PTPε) generates a physiologically distinct, cytoplasmic form of this protein, p65 PTPε. Here we describe a novel protein form of the related receptor-type tyrosine phosphatase α (RPTPα), p66 PTPα, which is detected in nearly all cell types where RPTPα is expressed. Both p66 PTPα and p65 PTPε are produced by calpain-mediated proteolytic cleavage in vivo. Cleavage is inhibited in living cells by a variety of calpain inhibitors, can be induced in primary cortical neurons treated with calcium chloride, and is observed in lysates of brain or of induced in primary cortical neurons treated with calpain. Cleavage occurs within the intracellular juxtamembrane domain of RPTPα, releasing the phosphatase catalytic domains from their membranal anchors and translocating them to the cytoplasm. Translocation reduces the ability of PTPα to act on membrane-associated substrates, as it loses its ability to dephosphorylate Src at its C-terminal regulatory site, and its ability to dephosphorylate the Kv2.1 voltage-gated potassium channel is severely impaired. In all, the data indicate that control of phosphatase function via post-translational processing occurs also among receptor-type phosphatases, and demonstrate the molecular complexity of regulating these parameters within the PTPα/PTPε phosphatase subfamily.

Reversible phosphorylation of tyrosine residues in proteins plays a central role in regulation of cellular functions, and is a process controlled by the opposing actions of protein-tyrosine kinases (PTKs)† and protein-tyrosine phosphatases (PTPases) (1). Tyrosine phosphorylation can affect the structure of proteins, as well as their cellular localization, their abilities to associate with other proteins, and ultimately their functions.

Aberrant PTK activity has been repeatedly linked to cancer and to a wide variety of other human diseases, underscoring the pivotal role tyrosine phosphorylation plays in regulating function at the cell and whole-organism levels. Studies performed during the past decade have established that PTPases, which are molecularly, biochemically, and physiologically distinct from PTKs, are also central regulators of physiological processes (2). PTPases are a structurally diverse family of transmembranal and cytoplasmic enzymes, of which several dozen members have been identified in organisms ranging from viruses to man (3–8). In recent years it has become evident that the precise subcellular localization of PTPases is an important factor in regulating their physiological roles (2, 9). Along these lines, the non-receptor-type PTPases STEP, PTP1B, and PTP−PEST can undergo proteolytic cleavage, which alters their subcellular localization patterns and can result in their activation (10–14), while the Src homology 2 domains of SHIP1 and SHIP2 mediate recruitment of these PTPases to activated growth factor receptors (15).

The four forms of PTPε provide an additional example of correlation between subcellular localization and function (16). The transmembranal form of PTPε (tm-PTPε; 16, 29) is an integral membrane protein, which has been linked to transformation of mouse mammary epithelial cells (17, 18) and to down-regulation of insulin receptor signaling (19, 20). In contrast, the non-receptor form of PTPε (cyt-PTPε), which is expressed from the single PTPε gene by use of an alternative promoter (21–24), is predominantly a cytoplasmic protein, although it is found also at the cell membrane and in the nucleus (16). Accordingly, cyt-PTPε cannot inhibit insulin receptor signaling (19), but can dephosphorylate and down-regulate delayed rectifier, voltage-gated potassium (Kv) channels (25), which are integral membrane proteins. p67 PTPε, which is produced by internal initiation of translation from PTPε mRNA, and p65 PTPε, which is produced by proteolytic processing of the larger PTPε forms, are N-terminally truncated forms of PTPε and are localized only in the cytoplasm. As expected, their effects on Kv channels are much more limited than those of tm-PTPε and cyt-PTPε (16).

PTPε and the closely related RPTPα are the only known members of the type IV family of receptor-type PTPases. tm-PTPε and RPTPα both have short, highly glycosylated extra-cellular domains (18, 26, 27). Extensive sequence similarities exist between both molecules and extend well beyond their relatively conserved catalytic domains. RPTPα has been implicated in several signaling pathways, including C-terminal dephosphorylation and activation of Src and Fyn (28–32), cellular transformation (32), neuronal differentiation (29), cellular adhesion and spreading (31, 33), down-regulation of insulin receptor signaling (19, 20, 34, 35), and activation of the delayed rectifier, voltage-gated potassium channel Kv1.2 (36). Express-

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† The abbreviations used are: PTK, protein-tyrosine kinase; cyt-PTPε, cytoplasmic isoform of protein-tyrosine phosphatase ε; Kv channel, delayed rectifier, voltage-gated potassium channel; PTPase, protein-tyrosine phosphatase; RPTPα, receptor-type form of protein-tyrosine phosphatase α; tm-PTPε, receptor-type form of protein-tyrosine phosphatase ε; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.
sion of RPTPα has also been associated with low tumor grade in human breast cancer (37) and with late stage colon carcinomas (38). RPTPα is believed to undergo inhibitory dimerization, in the course of which the helix-turn-helix wedge domain of each RPTPα molecule interacts with and blocks access to the active site of its dimerization partner (39-42). No ligand for RPTPα is known, although the glycosylphosphatidylinositol-linked protein contactin has been shown to form a complex with RPTPα in neuronal cells (43), and recent studies have suggested that newborn calf serum may contain ligands for the phosphatase (44).

The existence of non-membranal forms of PTPε prompted us to search for similar forms of RPTPα, which might affect the nature of PTPα activity in cells. We report here that RPTPα can be processed in vivo to generate p66 RPTPα, an N-terminally truncated form analogous to p65 PTPε. Both p66 RPTPα and p65 PTPε are produced in vivo from larger RPTPα or PTPε molecules by calpain-mediated cleavage. As it lacks membrane-anchoring domains, p66 RPTPα is inherently a cytoplasmic molecule, although it can be detected in part at the cell membrane when expressed together with full-length RPTPα molecules. When absent from the cell membrane, p66 RPTPα is incapable of dephosphorylating Src at its C-terminal regulatory site and has significantly reduced activity toward the Kv2.1 potassium channel. These results demonstrate the importance of membrane association for the known functions of RPTPα, and underscore major functional differences between p66 RPTPα and its full-length RPTPα precursor.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The following cDNAs were cloned into the pcDNAs eukaryotic expression vector (Invitrogen) and used in transfection experiments: mouse tm-PTPε and cyt-PTPε (18, 22), ΔATG2.3 cyt-PTPε and p65 PTPε (16), mouse RPTPα (gift of Dr. Matt Thomas), rat κV2.1 (gift of Dr. M. Lazdunski), wild-type mouse Src (gift of Dr. J. den Hertog), chicken Y527F Src (gift of Dr. S. Courtneidge), and human calpastatin (Ref. 45; gift of Dr. M. Piechaczky). A cDNA construct for p66 PTPα was constructed by replacing all sequences upstream of Leu-198 in the mouse RPTPα cDNA with an ATG initiation codon. This was done by polymerase chain reaction, using the 5′ oligomer GCCGATATCGATGCT-

TCTGGACCGTCCTCCCA, in which the new initiator ATG and the Leu-198 codons are underlined, in conjunction with a vector-derivative 3′ oligomer. The polymerase chain reaction product was sequenced and cloned into the pcDNA3 plasmid. Primary antibodies used in this study included polyclonal anti-PTPε (EMID serum; Ref. 18), polyclonal anti-PTPα (serum 5479 (Ref. 46; gift of Dr. J. den Hertog)), polyclonal anti-Kv2.1 (Atlas Antibody, Lake Success, NY), anti-FLAG M2 monoclonal antibodies (Sigma), monoclonal anti-v-Src (Calbiochem), monoclonal anti-phosphotyrosine (Transduction Laboratories, clone PY20), and polyclonal anti-phospho-Y529Src (BIOSOURCE International, Camarillo, CA). E64, E64d, pepstatin, PMSF, chloroquine, and purified calpain II from skeletal muscle were purchased from Sigma; calpeptin, leupeptin, and MG-132 were purchased from Calbiochem. Knockout mice lacking PTPα or PTPε have been described previously (25, 31).

**Cell Culture**—Human embryonic kidney 293 cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 10% (v/v) fetal calf serum (Life Technologies, Inc.), 2 mm glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Transformed mouse fibroblasts deficient for the Src, Yes, and Fyn kinases (SYF cells; Ref. 47) were grown in similar medium containing 4 mm glutamine and 1 mm sodium pyruvate. 293 cells were transfected by the calcium phosphate method (48); SYF cells were transfected with LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer’s instructions. In some experiments cells were treated for 2 h with 50 μM MG132, 5-50 μM calpeptin, 100 μM leupeptin, or vehicle (up to 1.25% Me2SO). Fractionation of cell extracts and cytosolic fractions by mechanical disruption and centrifugation has been described (18). Primary cortical neurons were prepared by the trituration method as described (49). Following trituration and washing, neurons were resuspended in phosphate-buffered saline, 0.6% glucose, and aliquots of 1 × 10⁶ cells were incubated as such or exposed to 1 mm CaCl₂ for the times indicated in Fig. 4. For calpain inhibition, neurons were incubated for 5 min with 20 μM calpeptin prior to addition of calcium chloride. Neurons were then pelleted, frozen in liquid nitrogen, and extracted in RIPA buffer containing 10 μg/ml each of aprotinin, PMSF, and leupeptin, and 1 μg/ml pepstatin. Cell lysates were analyzed by protein blotting as described below.

**Immunoprecipitation and Protein Blot Analyses**—Unless noted otherwise, cells or mouse organs were lysed in buffer A (50 mm Tris-Cl, pH 7.5, 100 mm NaCl, 1% Nonidet P-40), supplemented with 0.5 mm sodium pervanadate and protease inhibitors (1 mm N-α-aminomethyl)benzenesulfonyl fluoride, 40 μM bestatin, 15 μM E64, 20 μM leupeptin, 15 μM pepstatin (Sigma). In several preliminary experiments, the lysis buffer also contained 5 μM EDTA; although this did not affect the detection of proteins detected and was therefore not used in later experiments. 5-20 μg of total protein were analyzed on 7% SDS-polyacrylamide gels, followed by transfer to nitrocellulose membranes (Protran, Schleicher & Schuell), and hybridization to antibodies. Complete protein transfer following blotting was verified routinely by noting transfer of pre-stained molecular size marker proteins of the proper size range; absence of lane-to-lane variations in blotting was verified by staining the blotted membranes with Ponceau S (Sigma). For immunoprecipitations, 0.5-1 mg of total cell protein were incubated with anti-FLAG M2 affinity beads (Sigma) or with anti-phosphotyrosine antibodies and protein A beads (Amersham Pharmacia Biotech) for 3-4 h, followed by three extensive washes with RIPA buffer. Experiments were repeated two to five times, and representative blots are shown.

Pulse-chase analysis was performed in 293 cells 24 h following transfection with a FLAG-tagged cyt-PTPε cDNA. Cells were washed in serum-free Dulbecco’s modified Eagle’s medium lacking methionine and cysteine (Sigma) and were then labeled in 4 ml of the same medium supplemented with 48 μCi (0.48 mCi) of [35S]methionine (1000 Ci/ mmol, 10 μCi/ml, Amersham Pharmacia Biotech) for 30 min. Following removal of the radioactive medium, cells were washed twice in phosphate-buffered saline and incubated for up to 8 h in serum-containing growth medium supplemented with 2% each of non-radioactive methionine and cysteine. At selected time points cells were lysed, immunoprecipitated with anti-FLAG beads, and blotted. Radioactivity present in each band of PTPε was quantified using a phosphorimager (Bas instrument; Ref. 31). The expression patterns, expression levels, and size of p66 PTPα were both detected in protein lysates from brains of wild-type or of PTPε-deficient mice, but were both absent from similar lysates prepared from brains of PTPα-deficient mice (Fig. 1B; Ref. 31). The expression patterns, expression levels, and size of p66 PTPα strongly resembled those of the p67 and p65 forms of the closely related PTPε (Ref. 16; Fig. 1). These forms of PTPε lack N-terminal sequences of full-length PTPε, suggesting that p66 PTPα might be an analogous N-terminal truncated form of RPTPα. In agreement with this, addition of a FLAG tag at the 3′ end of the coding region of the PTPα cDNA resulted in appearance of the tag in both p66 PTPα and RPTPα proteins (Fig. 1A), indicating that the C terminus of p66 PTPα was intact.

**p66 Is an N-terminal Truncated Form of RPTPα**—Anecdotal evidence of RPTPα expression in various cell lines and mouse tissues consistently revealed a protein (~66 kDa in addition to the previously described full-length unglycosylated (~100 kDa) and glycosylated (~130 kDa) forms of RPTPα (Fig. 1; Ref. 26). This protein, which we refer to as p66 PTPα, was clearly PTPα-derived, as it appeared together with full-length RPTPα in 293 cells transfected with a complete PTPα cDNA expression construct (Fig. 1A). Furthermore, p66 PTPα and RPTPα were both detected in protein lysates from brains of wild-type or of PTPε-deficient mice, but were both absent from similar lysates prepared from brains of PTPα-deficient mice (Fig. 1B; Ref. 31). The expression patterns, expression levels, and size of p66 PTPα strongly resembled those of the p67 and p65 forms of the closely related PTPε (Ref. 16; Fig. 1). These forms of PTPε lack N-terminal sequences of full-length PTPε, suggesting that p66 PTPα might be an analogous N-terminal truncated form of RPTPα. In agreement with this, addition of a FLAG tag at the 3′ end of the coding region of the PTPα cDNA resulted in appearance of the tag in both p66 PTPα and RPTPα proteins (Fig. 1A), indicating that the C terminus of p66 PTPα was intact.

**p66 PTPα Is Produced by Proteolytic Cleavage of Full-length RPTPα Molecules**—The p67 form of PTPε is produced by initiation of translation at an internal ATG codon corresponding to Met65 of tm-PTPα, whereas p65 PTPε is produced by proteolytic processing of tm-PTPε, cyt-PTPε, or p67 PTPε (16). Site-directed mutagenesis studies have shown that cleavage is independent of, but occurs in the immediate vicinity of, Met-99 of tm-PTPα (Fig. 2; Ref. 16). Examination of the juxtamembrane sequences of RPTPα revealed that neither Met-85 nor Met-99...
Proteolytic Processing of RPTPα and PTPε

Fig. 1. p66 PTPα is a PTPε-derived protein. A, 293 cells were transfected with expression vectors for cyt-PTPε (cyt-ε), tm-PTPε (tm-ε), or RPTPα (α) cDNAs, which had been FLAG-tagged at their 3′ ends. Lysates from cells were blotted with anti-PTPε (EMID serum, Ref. 18; left panel). Lysates were immunoprecipitated (IP) with anti-FLAG antibodies, blotted, and probed with anti-FLAG antibodies (right panel). WB, Western blot.

B, brain lysates of wild type (WT), PTPε-deficient (AKO), or PTPε-deficient (EKO) mice were blotted and probed with serum specific for PTPα (left panel) or with EMID serum (right panel). Note co-migration of p66 PTPε and p66 PTPε in these gels. Double and single asterisks denote the fully glycosylated and non-glycosylated forms, respectively, of RPTPα and PTPε.

of tm-PTPε were conserved in RPTPα and that this region contained no other close ATG codons, reducing the likelihood that p66 PTPα was the product of internal initiation of translation. However, sequences surrounding Met-99 in tm-PTPα were highly conserved in the RPTPα derived protein.

of p66 PTPα were detected in cells (Fig. 3A), similar to its ability to inhibit production of p65 PTPα from full-length PTPα (16). Additional studies, which we detail below, further strengthen the conclusion that p66 PTPα is produced by proteolytic cleavage of larger PTPα molecules and is therefore analogous to p65 PTPα. Of note, lysis of cells and tissues was routinely performed in the cold and in buffers containing inhibitors of proteases, including calpain inhibitors as explained below. p65 PTPα and p66 PTPα were observed also in protein blots prepared from cells that had been lysed directly in boiling SDS-PAGE loading buffer (data not shown), indicating that these shorter proteins were present in cells prior to lysis.

p66 PTPα and p65 PTPα Are Products of Calpain-mediated Processing in Vivo—MG132, which inhibits accumulation of p65 PTPα and p66 PTPα, is a known inhibitor of the proteasome and of several Ca2+-regulated and lysosomal proteases (50, 51). Previous studies have shown that lactacystin and epoxomicin, which are more specific inhibitors of proteasome function than MG132, did not affect accumulation of p65 PTPα (16). This finding indicated that MG132 affects p65 PTPα production not by inhibiting proteasome function, but by inhibiting another protease(s). As MG132 can also inhibit the major calcium-regulated cysteine protease calpain, we examined whether p65 PTPα and p66 PTPα are products of calpain-mediated cleavage. For this purpose we co-expressed PTPα or RPTPα in cells together with the highly specific calpain inhibitory protein calpastatin. Expression of calpastatin significantly reduced production of p65 PTPα from cyt-PTPε or tm-PTPε, and p66 PTPα from RPTPα (Fig. 3B and results not shown). Similar results were obtained when cells expressing cyt-PTPα were treated with the cell-permeable calpain inhibitor leupeptin, with significant inhibition of p65 PTPα accumulation evident in cells treated with as little as 5 μM calpain (Fig. 3C). Calpeptin also inhibited formation of p66 PTPα and p65 PTPε from RPTPα or tm-PTPε, respectively (Fig. 3A and results not shown). The cell-permeable cysteine protease inhibitors leupeptin and E64d also significantly reduced the amounts of p65 PTPα in cells, whereas peptatin, PMSF, chloroquine, and ammonium chloride, inhibitors known to act on other proteases and proteolytic systems, had no effect (Fig. 3D).

A separate set of experiments, leupeptin inhibited accumulation of endogenous p65 PTPα in NIH3T3 cells (data not shown).

Calpain-mediated cleavage of endogenous RPTPα and tm-PTPε could be induced in primary cortical neurons prepared from day 14.5 mouse embryos. These neurons express high levels of RPTPα and very low levels of tm-PTPε (Fig. 4).2 p66 PTPα was not detected in freshly isolated neurons prior to treatment, but was readily visible following addition of 1 mM CaCl2 to the cells. Cleavage was not detected in neurons, which had been exposed to calpeptin prior to CaCl2 treatment, attesting to the involvement of calpain in the cleavage event (Fig. 4).

In similar experiments the cell-impermeable calpain inhibitor E64d did not inhibit calcium-dependent cleavage, indicating that the cleavage observed occurred in intact cells. Interestingly, induction of cleavage by CaCl2 did not result in processing of all RPTPα molecules present in the primary neurons. This result did not change when the stimulus applied to the cells included 200 μM glutamate (not shown), suggesting that cleavage is regulated by additional cellular mechanisms as is discussed below. Taken together, these data indicate that p65 PTPα and p66 PTPα are produced in vivo by calpain-mediated cleavage of larger PTPα or RPTPα molecules, respectively.

Calpain Cleaves PTPα and PTPε in Vitro—In a manner consistent with the above results, addition of purified calpain to lysates of cells expressing cyt-PTPε resulted in cleavage of cyt-PTPε and increased amounts of a protein that co-migrated with p65 PTPα in SDS-PAGE gels (Fig. 5, A and B). Cleavage was clearly due to the added calpain, with the extent of cleavage dependent upon the amount of calpain added (Fig. 5A). Similar results were obtained when calpain was added to lysates of cells expressing tm-PTPε or RPTPα, or to extracts of mouse brain containing endogenous tm-PTPε and RPTPα (Fig. 5B). Cleavage of RPTPα and PTPα by calpain was specific and did not result in total degradation of these proteins. Addition of calpain to the protein extracts shown in Fig. 5B did not result in widespread, nonspecific cleavage of other cellular proteins, as judged by Coomassie Blue staining of SDS-PAGE gels prepared from these extracts. However, specific, known substrates of calpain, such as tubulin, were also cleaved following calpain treatment of these lysates (data not shown).

p65 PTPα Is a Stable Protein—Amounts of p65 PTPα and p66 PTPα are significantly decreased as early as 2 h after addition of MG132, calpain, or leupeptin, indicating that these processed proteins are either inherently short-lived or are destabilized following general inhibition of calpain. In order to compare the stability of the various forms of PTPα, we performed a series of pulse-chase experiments in which cyt-PTPε, p67, and p65 proteins were expressed in 293 cells from the cyt-PTPε cDNA. Following labeling with [35S]methionine, cells were washed and cultured for up to 8 h in the presence of excess cold methionine and cysteine. PTPε was then immunoprecipitated from cells at selected time points, subjected to SDS-PAGE, and...
FIG. 2. Sequence of the juxtamembrane regions of the RPTPα and tm-PTPα proteins from mouse and man. Vertical line indicates transition from the transmembranal to the intracellular, juxtamembrane domains; sequences downstream of this line are identical in both tm-PTPα and cyt-PTPα (16). Vertical arrows denote Met-85 and Met-99 of tm-PTPα; horizontal arrow depicts beginning of the wedge domain in RPTPα, which extends to residue 242 (39). + signs denote conservative amino acid changes. GenBank™ accession numbers of the sequences used are X54130, M33671, U35368, and X54134.

FIG. 3. In vivo inhibition of p66 PTPα and p65 PTPα production by calpain inhibitors. A, 293 cells expressing RPTPα cDNA were either untreated (−) or treated with 50 μM MG132 (MG) or 25 μM calpeptin (Cal) for 2 h. Note significant reductions in p66 PTPα in treated cells. RPTPα can be glycosylated (top band) or non-glycosylated (bottom band). B, 293 cells were transfected with cyt-PTPα (α, left panel) or RPTPα (α, right panel) cDNAs, either alone (−) or together with (+) calpain inhibitor(s). Cells were lysed 24 h after transfection and analyzed. C, 293 cells transfected with cyt-PTPα cDNA were either left untreated (−) or treated with 5, 25, or 50 μM calpeptin or with vehicle (DMSO; 0.5% Me2SO) for 2 h. D, as in C, except that cells were treated with 200 μM E64d, 100 μM leupeptin (Leu.), 1 μM pepstatin (Pep.), 2 mM PMSF, 2.5 mM chloroquine (Chl.), or 100 μM NH4Cl. In all cases cell lysates were blotted and probed with anti-PTPα or anti-PTPα (EMID) serum. Arrows denote p66 PTPα or p65 PTPα.

FIG. 4. Stimulation of primary cortical neurons from day 14.5 mouse embryos with CaCl2 induces calpain-mediated cleavage of RPTPα. Cells were incubated in assay buffer either with or without 20 μM calpeptin and were then stimulated with 1 mM CaCl2 for the indicated time periods. Cells were then lysed, blotted, and probed with anti-PTPα serum. Blot is representative of three similar experiments. N.S., nonspecific band.

As seen in Fig. 6, full-length cyt-PTPα, p67 PTPα, and p65 PTPα were stable to similar extents, with slight decreases detected in the normalized amount of radioactivity associated with them during the chase period shown. Interestingly, treatment of cells with leupeptin significantly reduced the amount of p65 PTPα protein present at the 2-h time point by 80%, together with a similar 67.3% reduction in the amount of radioactivity present in this band (Fig. 6B and results not shown). Consequently, leupeptin treatment did not change significantly the specific radioactivity associated with p65 PTPα (Fig. 6A), although amounts of p65 protein were significantly reduced (Fig. 6B). This last result indicates that a consequence of inhibition of calpain by leupeptin is destabilization of p65 PTPα, but that in the absence of calpain inhibition p65 PTPα is stable. These findings suggest that calpain also plays a role in stabilizing p65 PTPα once the protein has been produced. The demonstrated ability of exogenous and endogenous calpain to cleave PTPs and PTPα (Figs. 4 and 5) provides evidence, which is independent of calpain inhibitors, that calpain participates in cleaving both PTPases. Calpain then plays a dual role in stabilizing p65 PTPα, although amounts of p65 protein were significantly reduced (Fig. 6B). This last result indicates that a consequence of inhibition of calpain by leupeptin is destabilization of p65 PTPα.
amounts of radioactivity, normalized to protein content, for full-length Leu-198; the protein produced from this cDNA con-
which the initiating ATG codon was inserted immediately up-
that the cytosolic functions of both PTPases; hence, we chose to focus in this study on the consequences of cleavage on membrane-associated substrates. One of the best documented physiological functions of RPTPa is to dephosphorylate the membrane-associated kinase Src at its C-terminal tyrosine residue (Tyr-529 in mouse), thereby activating the kinase and initiating a broad series of cellular events (30, 31). In order to examine the ability of p66 PTPα to act on Src, mouse fibroblasts lacking Src, Yes, and Fyn (SYF cells; Ref. 47) were transfected with expression vectors for murine wild-type Src and for either RPTPa or the cytoplasmic truncated form of PTPα described above. Absence of endogenous Src and very low levels of endogenous PTPs in SYF cells ensured that Src molecules examined were only those present in cells co-transfected with PTPα, and that the effect of PTPα on Src would not be masked by endogenous Src from untransfected cells. Phosphorylation of Src at Tyr-529 was examined by probing protein blots prepared from the relevant cell extracts with an antibody specific for pY529Src. Co-expression of Src together with full-length RPTPa resulted in a 43% reduction in phosphorylation of Src at Tyr-529, in agreement with results obtained previously by several groups (Fig. 8; Ref. 52). In contrast, co-expression of truncated PTPα did not significantly affect Src phosphor-
ylation at Tyr-529, despite higher expression levels of this form as compared with RPTPa (Fig. 8, lane 3 versus lanes 4 and 5). As indicated under "Experimental Procedures", care was taken to ensure that protein blotting was complete and that increased levels of p66 PTPα versus RPTPa proteins faithfully reflect the situation within the cells.

We have documented previously that cyt-PTPe dephosphorylates and inactivates the delayed rectifier, voltage-gated po-
tassium channel Kv2.1, in a manner that correlates with re-
duced myelination of peripheral nerve axons in PTPe-deficient mice. In this system, PTPe and Src appear to counter each other’s activity toward their common substrate, Kv2.1 (25). In order to examine the possibility that RPTPa could also dephosphorylate Kv2.1, we transfected 293 cells with Kv2.1 and con-
sstitutively active (Y527F) chicken Src, either in the presence or absence of RPTPa (Fig. 9). Activated Src was used in this study to ensure phosphorylation of Kv2.1, as well as to prevent PTPα affecting Src activity via dephosphorylation of Tyr-527. Indeed, co-expression of Kv2.1 with Src resulted in massive tyrosine phosphorylation of the channel protein, whereas presence of RPTPa in these cells reduced Kv2.1 phosphorylation by 97% (Fig. 9).

Kv2.1 is an integral membrane protein. In order to deter-
mine whether the non-membranial localization of p66 PTPα impedes its ability to act upon Kv2.1, we examined the ability of p66 PTPα to reduce phosphorylation of Kv2.1 in transfected

**FIG. 6.** Pulse-chase experiment comparing stabilities of cyt-
PTPe, p67 PTPe, and p65 PTPe. 293 cells expressing full-length cyt-PTPe cDNA were labeled with [35S]methionine and then chased with cold methionine for up to 2 h, either in the presence or absence of 100 μM leupeptin. At the indicated time points, cells were lysed and blotted; radioactivity and protein content in each band of PTP protein was quantified as detailed under "Experimental Procedures." A, amounts of radioactivity, normalized to protein content, for full-length cyt-PTPe, p67 PTPe, and p65 PTPe at the indicated time points; data are presented relative to values obtained at the beginning of the cold chase (t = 0). B, protein blot documenting cyt-PTPe, p67 PTPe, and p65 PTPe amounts after 2 h of cold chase, either with (+) or without (−) 100 μM leupeptin. At this time point, leupeptin reduced the radioactivity and protein content of the p65 band by similar extents (67.3 and 80%, respectively). Specific radioactivities of all three PTPα forms in the absence of leupeptin remained over 50% throughout the remainder of the chase period of 8 h (data not shown). Experiment shown is representative of three performed.

**FIG. 7.** p66 PTPα is inherently a non-membrane protein. 293 cells expressing either PTPα cDNA beginning at Leu-198 (top panel) or full-length RPTPa (bottom panel) were fractionated into cytoplasmic (Cyt) or membranal (Mem) fractions. Proteins were blotted and probed with EMID serum. Double and single asterisks denote the fully glyco-
sylated and non-glycosylated forms, respectively, of RPTPa.
293 cells. Although RPTPα nearly eliminated Kv2.1 phosphorylation, expression of significantly higher levels of p66 PTPα reduced Kv2.1 phosphorylation by only 32% (Fig. 9, lane 5).

Strong dephosphorylation of Kv2.1 was observed only following RPTPα overexpression, expression of significantly higher levels of p66 PTPα, protein. All transfections contained the same total amount of DNA, with the difference made up with pCDNA3 plasmid. Lysates of cells were blotted and probed with antibodies against pY529Src, total Src, or PTP/PTPα, and were analyzed with a scanning densitometer. Blots are from a representative experiment; note strong effect of RPTPα on Y529Src phosphorylation, despite its much lower expression levels. Data represent average and standard deviation of three separate experiments. Differences between RPTPα and both p66 PTPα lanes are statistically significant (Student’s t test, p = 0.0085 (*) and p = 0.0046 (**)); differences between the two p66 PTPα lanes are insignificant. M, mock transfected cells; WB, Western blot.

DISCUSSION

Data presented in this study indicate that p66 PTPα and the analogous p65 PTPα are produced from larger RPTPα cDNAs, or with pCDNA3 vector alone (−). Lanes 2–5 were also transfected with Src cDNAs. Note that lanes 4 and 5 express different amounts of p66 PTPα protein. All transfections contained the same total amount of DNA, with the difference made up with pCDNA3 plasmid. Lysates of cells were blotted and probed with antibodies against pY529Src, total Src, or PTP/PTPα, and were analyzed with a scanning densitometer. Blots are from a representative experiment; note strong effect of RPTPα on Y529Src phosphorylation, despite its much lower expression levels. Data represent average and standard deviation of three separate experiments. Differences between RPTPα and both p66 PTPα lanes are statistically significant (Student’s t test, p = 0.0085 (*) and p = 0.0046 (**)); differences between the two p66 PTPα lanes are insignificant. M, mock transfected cells; WB, Western blot.

Fig. 9. Reduced ability of p66 PTPα to dephosphorylate the Kv2.1 voltage-regulated potassium channel. 293 cells were transfected with cDNAs for Kv2.1 and activated (Y527F) chicken Src, as well as with cDNAs for RPTPα or p66 PTPα. Note that lanes 3–5 contain different amounts of p66 protein. All transfections contained the same amount DNA, with the differences made up with pCDNA3 plasmid. Tyrosine-phosphorylated proteins were immunoprecipitated (IP) from cell lysates, blotted, and probed with antibodies against Kv2.1 or Src, and were then analyzed with a scanning densitometer. Bar diagram shows relative amounts of phospho-Kv2.1, normalized to Kv2.1 expression levels. Note the strong effect of RPTPα on Kv phosphorylation, despite its much weaker expression compared with p66 PTPα. Blots and diagram are from an experiment representative of three performed. WB, Western blot.

Fig. 8. p66 PTPα does not dephosphorylate Src at Tyr-529, SYF cells, which lack endogenous Src, Yes, and Fyn, were transfected with RPTPα or p66 PTPα cDNAs, or with pCDNA3 vector alone (−). Lanes 2–5 were also transfected with Src cDNA. Note that lanes 4 and 5 express different amounts of p66 PTPα protein. All transfections contained the same total amount of DNA, with the difference made up with pCDNA3 plasmid. Lysates of cells were blotted and probed with antibodies against pY529Src, total Src, or PTP/PTPα, and were analyzed with a scanning densitometer. Blots are from a representative experiment; note strong effect of RPTPα on Y529Src phosphorylation, despite its much lower expression levels. Bar diagram shows TyrP-P529 Src phosphorylation intensity normalized to Src protein content. Data represent average and standard deviation of three separate experiments. Differences between RPTPα and both p66 PTPα lanes are statistically significant (Student’s t test, p = 0.0085 (*) and p = 0.0046 (**)); differences between the two p66 PTPα lanes are insignificant. M, mock transfected cells; WB, Western blot.

Interestingly, calcium-induced cleavage of RPTPα in primary cortical neurons did not proceed to completion. A similar finding was noted previously in the case of calpain-mediated cleavage of the STEP tyrosine phosphatase in vivo. STEP is cleaved by calpain in primary neuronal cells following glutamate stimulation (13) and in perinatal rat brain following hypoxia/ischemia (12), but in both cases cleavage is rather limited and does not deplete the full-length STEP precursor molecules. This could indicate that cleavage is regulated by molecular mechanisms in addition to activation of calpain. In the case of RPTPα, it should be noted that cleavage occurs in close proximity to the wedge domain of the phosphatase (Fig. 2). This region participates in significant intermolecular interactions in the course of RPTPα dimerization (40), possibly impeding access of calpain to its site of action. Studies in transfected 293 cells have indicated that dimerization of RPTPα is quite prevalent, and that most RPTPα molecules present at the surface of these cells are found in dimers (40). Alternatively, the stimuli used in this study and in the studies of STEP might not have been sufficient to achieve full cleavage. Of note, addition of 200 mM glutamate together with CaCl2 to the cells examined here did not induce cleavage of RPTPα beyond that obtained with calcium alone.

Homodimerization of RPTPα (40) and of PTPε2 could also explain the finding that, although p66 PTPα and p65 PTPα are inherently cytoplasmic molecules, both can be found in part at the cell membrane when expressed together with their full-length RPTPα or PTPε precursors (Fig. 7; Ref. 16). Dimerization of RPTPα molecules is believed to be mediated by interactions throughout the entire RPTPα molecule, which involve the extracellular and transmembranal domains, the juxtamembranal wedge domain of the membrane-proximal catalytic domain, and the membrane-distal catalytic domain (40). Cleav-

3 H. Toledano-Katchalski and A. Elson, unpublished results.
Proteolytic Processing of RPTPa and PTPε

age of RPTPa within the juxtamembrane domain, which removes the transmembranal and extracellular domains of RPTPa but does not remove the wedge domain or the remainder of the catalytic domains, should therefore not necessarily abolish these interactions. We believe these interactions would withstand the process of cell fractionation, as PTPε homodimers can readily be detected following immunoprecipitation.3 It remains to be determined whether membrane-associated p66 RPTPa and p65 PTPε are molecules that have remained bound to their original uncleaved dimerization partner, or have been recruited to intact membrane-associated RPTPa or PTPε monomers.

Proteolytic cleavage and subcellular re-distribution of PTPε and PTPα is expected to affect the physiological functions of both PTPases in a significant and irreversible manner. Two possible outcomes of this process that are not mutually exclusive are loss of function toward membrane-associated substrates and gain of function toward substrates located in the cytosol. As very little is known concerning the cytosolic functions of both PTPases, we chose to focus here on the loss-of-function consequences of cleavage. Indeed, both p65 PTPε and p66 PTPα are severely limited in their ability to perform physiologically relevant roles, which depend on their being present at the cell membrane. Reduced ability of p66 PTPα to dephosphorylate Src at Tyr-529 is of particular significance, as regulation of Src phosphorylation and activity is perhaps the best characterized role of RPTPa to date. Dephosphorylation by RPTPa at their C-terminal negative regulatory tyrosine activates Src and the related Fyn kinase (28–32). This, in turn, causes several key physiological outcomes, including cellular transformation (32) and modulation of cell adhesion and spreading (31, 33). Altered phosphorylation and activation of Src by PTPα has been correlated with physiological consequences in RPTPa-deficient mice (30, 31), attesting to the relevance of our findings.

p66 PTPα is also significantly less able than RPTPa to reduce phosphorylation of the Kv2.1 voltage-gated potassium channel, an observation similar to that made previously in the case of p65 PTPε and p67 PTPε (16). Dephosphorylation of Kv2.1 by PTPε is of clear physiological importance in vivo, as it affects Kv2.1 channel activity in Schwann cells; it is also correlated with transient severe hypomyelination of sciatic nerves of PTPε-deficient mice (25). This study suggests that RPTPa might also affect Kv2.1 channel activity by altering its phosphorylation state, although this remains to be verified experimentally. Membrane association plays an important role in mediating RPTPa function in other systems as well. RPTPa can down-regulate insulin receptor signaling in baby hamster kidney cells (19, 20), possibly by dephosphorylating the β subunit of the receptor (34). RPTPa-induced inhibition of insulin receptor function has been shown to lead to decreased insulin-stimulated prolactin gene expression (53). Membrane association is crucial here as well, as removing the transmembranal and extracellular domains of tm-PTPε or RPTPa abolishes their ability influence insulin receptor signaling in this system (19).

Several points argue in favor of the possibility that cleavage and translocation allow PTPα and PTPε access to potentially new substrates in the cytosol. First, the cleaved products are ubiquitously expressed and stable, and studies have shown that p65 PTPε molecules, as well as PTPε molecules devoid of their extracellular and membrane-spanning domains, are catalytically active (16, 54).4 Second, a cytosolic gain of function could affect physiological processes even without full cleavage of tm-PTPε or RPTPa. However, direct testing of this possibility requires additional information concerning the cytosolic functions on both PTPases. A third potential consequence of cleavage is physical and irreversible separation between the catalytic domains of RPTPa and tm-PTPε and the extracellular domains of these molecules. Recent studies have suggested that interactions between the extracellular domain of RPTPa and extracellular molecules exist (43, 44). Their physiological implications, however, remain to be determined before this possibility can be adequately addressed.

A 75-kDa processed form of RPTPa, which is induced in NIH3T3 cells upon treatment with pervanadate, has been described recently (55). In contrast to this form, p66 PTPα is detected in a constitutive manner in most tissues and cell lines where RPTPa is detected, and neither it nor p65 PTPε is induced by pervanadate treatment (results not shown). Furthermore, our data indicate that p66 PTPα and p65 PTPε are the products of cleavage that occurs in the cytoplasmic juxtamembrane region of RPTPa, rather than on the outside of cells as has been suggested for the 75-kDa protein. The possible relationship between this protein and p66 PTPα therefore remains to be established.

This study highlights several new points of similarity and difference between PTPα and PTPε. Similarities extend to the existence of proteolytically processed forms of both phosphatases, to the mechanisms by which they are produced, and to the effects cleavage has on reducing the ability of both phosphatases to act on membrane-associated substrates. However, similarities between PTPα and PTPε are not absolute, as no forms of PTPα that are analogous to full-length cyt-PTPε or to the internal initiation product p67 PTPε have been found to date despite intensive searches. In all, expression of PTPε and PTPα, which together now include six distinct protein forms, is subject to complex regulation at the levels of transcription, translation, and post-translational processing. The concept that altering subcellular localization is a major factor in regulating the functions of non-membranal PTPases is well established (2). The existence of processed forms of PTPα and PTPε and their altered physiological properties as compared with their full-length precursors indicates that this principle operates among membrane-bound PTPases as well.

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Regulation of Protein-tyrosine Phosphatases α and ε by Calpain-mediated Proteolytic Cleavage

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