Regulation of protein tyrosine phosphatases Alpha and Epsilon by calpain-mediated proteolytic cleavage

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Running title: proteolytic processing of RPTPα and PTPε
Summary

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 PTP epsilon generates a physiologically distinct, cytoplasmic form of this protein, p65 PTP\(\epsilon\). Here we describe a novel protein form of the related receptor-type tyrosine phosphatase alpha (RPTP\(\alpha\)), p66 PTP\(\alpha\), which is detected in nearly all cell types where RPTP\(\alpha\) is expressed. Both p66 PTP\(\alpha\) and p65 PTP\(\epsilon\) are produced by calpain-mediated proteolytic cleavage \textit{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 cultured cells following addition of purified calpain. Cleavage occurs within the intracellular juxtamembrane domain of RPTP\(\alpha\), releasing the phosphatase catalytic domains from their membranal anchors and translocating them to the cytoplasm. Translocation reduces the ability of PTP\(\alpha\) to act on membrane-associated substrates, as it looses 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 PTP\(\alpha\)/PTP\(\epsilon\) phosphatase subfamily.
Introduction

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 and phosphatases (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 SH2 domains of SHP1 and SHP2 mediate recruitment of these PTPases to activated growth factor receptors (15).

The four forms of PTP provide an additional example of correlation between subcellular localization and function (16). The transmembranal form of PTP (tm-PTP; 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 PTP (cyt-PTP), which is expressed from the single PTP 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 downregulate 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 extracellular 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), downregulation of insulin receptor signaling (19,20,34,35), and activation of the delayed-rectifier, voltage-gated potassium channel Kv1.2 (36). Expression 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 GPI-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 PTPα, an N-terminally truncated
form analogous to p65 PTPε. Both p66 PTPα and p65 PTPε are produced in vivo from larger RPTPα or PTPε molecules by calpain-mediated cleavage. As it lacks membrane-anchoring domains, p66 PTPα 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 PTPα is incapable of dephosphorylating Src at its C-terminal regulatory site, and has significantly reduced activity towards 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 PTPα and its full-length RPTPα precursor.
Experimental Procedures:

Reagents:
The following cDNAs were cloned into the pCDNA3 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 Kv2.1 (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 (45; gift of Dr. M. Piechaczyk). A cDNA construct for p66 PTPα was constructed by replacing all sequences upstream of Leu198 in the mouse RPTPα cDNA with an ATG initiation codon. This was done by PCR, using the 5' oligomer

GCGATATCGATGCTTCTGGCCAGGTCCCCA, in which the new initiator ATG and the Leu198 codons are underlined, in conjunction with a vector-derived 3' oligomer. The PCR product was sequenced and cloned into the pCDNA3 plasmid. Primary antibodies used in this study included polyclonal anti-PTPε (EMID serum; 18), polyclonal anti-RPTPα (serum #5478, (46; gift of Dr. J. den Hertog)), polyclonal anti Kv2.1 (Upstate Biotechnology, Lake Placid, New York), 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, California). E64, E64d, pepstatin, PMSF, chloroquine, and purified calpain II from rabbit skeletal muscle were purchased from Sigma; calpeptin, leupeptin, and MG-132 were purchased from Calbiochem. Knockout mice lacking PTPε or PTPα have been described previously (25,31).

Cell culture: Human embryonic kidney 293 cells were grown in DMEM (Gibco), supplemented with 10% (vol/vol) fetal calf serum (Gibco-BRL), 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, 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 (Gibco-
BRL) according to the manufacturer's instructions. In some experiments cells were treated
for two hours with 50 µM MG132, 5-50 µM calpeptin, 100µM Leupeptin, or vehicle
(up to 1.25 % DMSO). Fractionation of cells into membranal 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 1X10^6 cells were incubated as such or exposed to 1 mM CaCl2 for the times
indicated in Figure 4. For calpain inhibition, neurons were incubated for five minutes 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 aprotonin,
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 % NP-40),
supplemented with 0.5 mM sodium pervanadate and protease inhibitors (1 mM AEBSF,
40 µM Bestatin, 15 µM E-64, 20 µM Leupeptin, 15 µM Pepstatin; Sigma). In several
preliminary experiments the lysis buffer also contained 5 mM EGTA, although this did
not affect the pattern of proteins detected and was therefore not used in later
experiments. 5-20 µg total protein were analyzed on 7% SDS-polyacrylamide gels,
followed by transfer to nitrocellulose membranes (Protran, Schleicher and 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 (Pharmacia) for 3-4 hours, followed by 3 extensive washes with RIPA buffer. Experiments were repeated 2 to 5 times, and representative blots are shown.

Pulse-chase analysis was performed in 293 cells 24 hours following transfection with a FLAG-tagged cyt-PTPε cDNA. Cells were washed in serum-free DMEM medium lacking methionine and cysteine (Sigma), and were then labeled in 4ml of the same medium supplemented with 48 µl (=0.48 mCi) 35S-methionine (1000 Ci/mmol, 10 mCi/ml, Amersham Pharmacia Biotech) for 30 minutes. Following removal of the radioactive medium, cells were washed twice in phosphate-buffered saline and incubated for up to eight hours in serum-containing growth medium supplemented with 2 mM each of non-radioactive methionine and cysteine. At selected time points cells were lysed, immune-precipitated with anti-FLAG beads, and blotted. Radioactivity present in each band of PTPε was quantified using a Phosphorimager (BAS 2500, Fuji, Japan); the same blots were then probed with anti-PTPε serum and scanned with a scanning densitometer to correct for differences in PTPε protein amounts between lanes.
Results:

p66 is an N-terminal truncated form of RPTPα

Anecdotal examination of RPTPα expression in various cell lines and mouse tissues consistently revealed a protein of approximately 66kDa in addition to the previously described full-length unglycosylated (~100kDa) and glycosylated (~130kDa) forms of RPTPα (Figure 1; 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 (Figure 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 (Figure 1B; 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 (16; Figure 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 (Figure 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 Met85 of tm-PTPε, while 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, Met99 of tm-PTPε (Figure 2; 16). Examination of the juxtamembrane sequences of
RPTPα revealed that neither Met85 nor Met99 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 Met99 in tm-PTPε were highly conserved in the RPTPα protein (Figure 2), raising the possibility that the yet-unidentified cleavage site of PTPε is conserved in RPTPα. In a manner consistent with this, the proteasome and protease inhibitor MG132 reduced amounts of p66 PTPα detected in cells (Figure 3A), similar to its ability to inhibit production of p65 PTPε from full-length PTPε (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 PTPε. 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 PTPε and p66 PTPα were observed also in protein blots prepared from cells which had been lysed directly in boiling SDS-PAGE loading buffer (not shown), indicating that these shorter proteins were present in cells prior to lysis.

p66 PTPα and p65 PTPε are products of calpain-mediated processing in vivo

MG132, which inhibits accumulation of p65 PTPε and p66 PTPα, is a known inhibitor of the proteasome and of several Ca++-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 PTPε (16). This finding indicated that MG132 affects p65 PTPε 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 PTPε and p66 PTPα are products of calpain-mediated cleavage. For this purpose we co-expressed PTPε or RPTPα in cells together with the highly-specific calpain inhibitory
protein calpastatin. Expression of calpastatin significantly reduced production of p65 PTP\(\varepsilon\) from cyt-PTP\(\varepsilon\) or tm-PTP\(\varepsilon\), and p66 PTP\(\alpha\) from RPTP\(\alpha\) (Figure 3B and results not shown). Similar results were obtained when cells expressing cyt-PTP\(\varepsilon\) were treated with the cell-permeable calpain inhibitor calpeptin, with significant inhibition of p65 PTP\(\varepsilon\) accumulation evident in cells treated with as little as 5\(\mu\)M calpeptin (Figure 3C). Calpeptin also inhibited formation of p66 PTP\(\alpha\) and p65 PTP\(\varepsilon\) from RPTP\(\alpha\) or tm-PTP\(\varepsilon\), respectively (Figure 3A and results not shown). The cell-permeable cysteine protease inhibitors leupeptin and E64d also significantly reduced the amounts of p65 PTP\(\varepsilon\) in cells, while pepstatin, PMSF, chloroquine, and ammonium chloride, inhibitors known to act on other proteases and proteolytic systems, had no effect (Figure 3D). In a separate set of experiments, leupeptin inhibited accumulation of endogenous p65 PTP\(\varepsilon\) in NIH3T3 cells (not shown).

Calpain-mediated cleavage of endogenous RPTP\(\alpha\) and tm-PTP\(\varepsilon\) could be induced in primary cortical neurons prepared from day 14.5 mouse embryos. These neurons express high levels of RPTP\(\alpha\) and very low levels of tm-PTP\(\varepsilon\) (Figure 4, and unpublished results). p66 PTP\(\alpha\) was not detected in freshly-isolated neurons prior to treatment, but was readily visible following addition of 1 mM CaCl\(_2\) to the cells. Cleavage was not detected in neurons, which had been exposed to calpeptin prior to CaCl\(_2\) treatment, attesting to the involvement of calpain in the cleavage event (Figure 4). In similar experiments the cell-impermeable calpain inhibitor E64 did not inhibit calcium-dependent cleavage, indicating that the cleavage observed occurred in intact cells. Interestingly, induction of cleavage by CaCl\(_2\) did not result in processing of all RPTP\(\alpha\) molecules present in the primary neurons. This result did not change when the stimulus applied to the cells included 200 \(\mu\)M glutamate (not shown), suggesting that cleavage is regulated by additional cellular mechanisms as is discussed below. Taken together, these data indicate that p65 PTP\(\varepsilon\) and p66 PTP\(\alpha\) are produced \textit{in vivo} by calpain-mediated cleavage of larger PTP\(\varepsilon\) or RPTP\(\alpha\) 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 which co-migrated with p65 PTPε in SDS-PAGE gels (Figure 5A&B). Cleavage was clearly due to the added calpain, with the extent of cleavage dependent upon the amount of calpain added (Figure 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 (Figure 5B). Cleavage of RPTPα and PTPε by calpain was specific and did not result in total degradation of these proteins. Addition of calpain to the protein extracts shown in Figure 5B did not result in widespread, non-specific 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 (not shown).

p65 PTPε is a stable protein

Amounts of p65 PTPε and p66 PTPα are significantly decreased as early as two hours after addition of MG132, calpeptin, 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 PTPε 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 eight hours in the presence of excess cold methionine and cysteine. PTPε was then immune-precipitated from cells at selected time
points, subjected to SDS-PAGE, and blotted. Radioactivity present in each of the PTPε bands was quantified with the aid of a Phosphorimager, after which the same protein blots were probed with anti-PTPε antibodies to allow comparison of the measured radioactivity with the amounts of PTPε protein present in each band.

As seen in Figure 6, full-length cyt-PTPε, p67 PTPε, and p65 PTPε 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 PTPε protein present at the two hour time point by 80%, together with a similar 67.3% reduction in the amount of radioactivity present in this band (Figure 6B and results not shown). Consequently, leupeptin treatment did not change significantly the specific radioactivity associated with p65 PTPε (Figure 6A), although amounts of p65 protein were significantly reduced (Figure 6B). This last result indicates that a consequence of inhibition of calpain by leupeptin is destabilization of p65 PTPε, but that in the absence of calpain inhibition p65 PTPε is stable. These findings suggest that calpain also plays a role in stabilizing p65 PTPε once the protein has been produced. The demonstrated ability of exogenous and endogenous calpain to cleave PTPε and PTPα (Figures 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 production of p65 PTPε, both in the actual cleavage event and in stabilization of the resulting cleavage product. This effect of calpain inhibitors is limited to p65 PTPε (and presumably to p66 PTPα as well), as amounts of the full-length forms of PTPε and RPTPα and of p67 PTPε, which are known to be produced by mechanisms not involving proteolysis, were not reduced in the presence of leupeptin or of other calpain inhibitors (Figure 3; 16).
Proteolytic processing translocates PTPα to the cytoplasm

Full-length RPTPα is an integral membrane protein. Processing of RPTPα to yield p66 PTPα occurs downstream of the transmembranal domain of RPTPα, hence p66 PTPα lacks this domain and should be a cytoplasmic protein. In order to examine this possibility we performed a series of subcellular fractionation experiments in cells expressing PTPα (Figure 7). The precise amino terminus of p66 PTPα has yet to be determined, but comparison with p65 PTPε suggests that p66 PTPα should begin at or in the immediate vicinity of Leu198 (Figure 2). We therefore constructed a truncated PTPα cDNA molecule, in which the initiating ATG codon was inserted immediately upstream of Leu198; the protein produced from this cDNA construct co-migrated with p66 PTPα. Fractionation of cells expressing this truncated form of PTPα revealed that it was in fact entirely cytoplasmic (Figure 7). In contrast, fractionation of cells expressing full-length PTPα cDNA and in which both RPTPα and p66 PTPα proteins were present revealed that RPTPα was present exclusively in the membrane fraction, while p66 PTPα was detected in both the cytoplasmic and membranal fractions (Figure 7). Retention of p66 PTPα in the membrane fraction in the presence of RPTPα could be accounted for by the existence of PTPα dimers (40), with the cleaved product of one RPTPα molecule being associated with another uncleaved, membrane-bound RPTPα molecule.

Significantly reduced activity of p66 PTPα towards Src and the Kv2.1 potassium channel

Translocation of the catalytic domains of PTPε and PTPα to the cytosol could serve a regulatory function by either reducing the activity of these PTPαses towards membrane-associated substrates, or by increasing their activity towards substrates located in the cytosol. Very little is known about the cytosolic functions of both PTPαses, 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 RPTPα is to
dephosphorylate the membrane-associated kinase Src at its C-terminal tyrosine residue
(Y529 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, 47) were transfected with expression
vectors for murine wild-type Src and for either RPTPα or the cytoplasmic truncated form
of PTPα described above. Absence of endogenous Src and very low levels of endogenous
PTPα 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 Y529 was examined
by probing protein blots prepared from the relevant cell extracts with an antibody
specific for phospho-Y529Src. Co-expression of Src together with full-length RPTPα
resulted in a 43% reduction in phosphorylation of Src at Y529, in agreement with results
obtained previously by several groups (Figure 8; 52). In contrast, co-expression of
truncated PTPα did not significantly affect Src phosphorylation at Y529, despite higher
expression levels of this form as compared to RPTPα (Figure 8, lane 3 vs. lanes 4 and 5).
As indicated in Experimental Procedures, care was taken to ensure that protein blotting
was complete, and that increased levels of p66 PTPα vs. RPTPα proteins faithfully
reflect the situation within the cells.

We have previously documented that cyt-PTPε dephosphorylates and inactivates
the delayed-rectifier, voltage-gated potassium channel Kv2.1, in a manner which correlates
with reduced myelination of peripheral nerve axons in PTPε-deficient mice. In this
system, PTPε and Src appear to counter each other's activity towards their common
substrate, Kv2.1 (25). In order to examine the possibility that RPTPα could also
dephosphorylate Kv2.1, we transfected 293 cells with Kv2.1 and constitutively active
(Y527F) chicken Src, either in the presence or absence of RPTPα (Figure 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 Y527. Indeed, co-expression of Kv2.1 with Src resulted in massive tyrosine phosphorylation of the channel protein, while presence of RPTPα in these cells reduced Kv2.1 phosphorylation by 97% (Figure 9).

Kv2.1 is an integral membrane protein. In order to determine whether the non-membranal 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 293 cells. While RPTPα nearly eliminated Kv2.1 phosphorylation, expression of significantly higher levels of p66 PTPα reduced Kv2.1 phosphorylation by only 32% (Figure 9, lane 5). Strong dephosphorylation of Kv2.1 was observed only following massive overexpression of p66 PTPα, which resulted in several-fold more p66 PTPα protein being present in comparison with RPTPα (Figure 9, lane 3). These results indicate that membrane localization is a central determinant of the ability of RPTPα to act upon Kv2.1, although it appears not to be the only factor regulating this activity. Together with the inability of p66 PTPα to act upon Y529 of Src, these results indicate that translocation of PTPα to the cytoplasm significantly reduces its ability to act upon molecules located at the cell membrane and underscores an important functional difference between the p66 PTPα and RPTPα.
Discussion

Data presented in this study indicate that p66 PTPα and the analogous p65 PTPε are produced from larger RPTPα or PTPε molecules by calpain-mediated proteolytic processing. Accumulation of both molecules can be prevented in vivo by the same series of calpain inhibitors - calpastatin, calpeptin, MG132, and leupeptin. Furthermore, cleavage of RPTPα to yield p66 PTPα can be induced in primary cortical neurons by the presence of calcium cations, and this process can be prevented by prior inhibition of calpain in these cells. Exogenous calpain cleaves RPTPα and PTPε in a specific manner to yield proteins which co-migrate with p66 PTPα and p65 PTPε, respectively. Pulse-chase experiments indicate that p65 PTPε and most likely p66 PTPα are stable proteins, but are destabilized in the course of generalized calpain inhibition. The data then suggest that calpain activity is required for both production and subsequent stabilization of p65 PTPε and p66 PTPα.

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 (Figure 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 PTPε (Hila Toledano-Katchalski and A.E., unpublished) could also explain the finding that, while p66 PTPα and p65 PTPε 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 (Figure 7; 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). Cleavage of RPTPα within the juxtamembrane domain, which removes the transmembranal and extracellular domains of RPTPα but does not remove the wedge domain nor 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 immune-precipitation (Hila Toledano-Katchalski and A.E., unpublished). It remains to be determined whether membrane-associated p66 PTPα and p65 PTPε are molecules which have remained bound to their original uncleaved dimerization partner, or have been recruited to intact membrane-associated RPTPα 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 non mutually-exclusive possible outcomes of this process are loss of function towards membrane-associated substrates, and gain of function towards 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 Y529 is of particular significance, as regulation of Src phosphorylation and activity is perhaps the best-characterized role of RPTPα to date. Dephosphorylation by RPTPα 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 RPTPα-deficient mice (30,31), attesting to the relevance of our findings.

p66 PTPα is also significantly less able than RPTPα 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 RPTPα 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 RPTPα function in other systems as well. RPTPα can down-regulate insulin receptor signaling in BHK cells (19,20), possibly by dephosphorylating the beta subunit of the receptor (34). RPTPα-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 RPTPα abolishes their ability influence insulin receptor signaling in this system (19).

Several points argue in favor of the possibility that cleavage and translocation allow PTPε and PTPα 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 PTPα molecules devoid of their extracellular and membrane-spanning domains, are catalytically active (16,54; H. G-H., unpublished results). Second, a cytosolic gain of function could affect physiological processes even without full cleavage of tm-PTPε or RPTPα. 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 RPTPα and tm-PTPε and the extracellular domains of these molecules. Recent studies have suggested that interactions between the extracellular domain of RPTPα and extracellular molecules exist (43,44). Their physiological consequences, however, remain to be determined before this possibility can be adequately addressed.

A 75kDa processed form of RPTPα, which is induced in NIH3T3 cells upon treatment with pervanadate, has recently been described (55). In contrast to this form, p66 PTPα is detected in a constitutive manner in most tissues and cell lines where RPTPα is detected, and neither it nor p65 PTPε are induced by pervanadate treatment (results not shown). Furthermore, our data indicate that p66 PTPα and p65 PTPε are the products of cleavage which occurs in the cytoplasmic juxtamembrane region of RPTPα, rather than on the outside of cells as has been suggested for the 75kDa 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. Yet, similarities between PTPα and PTPε are not absolute as no forms of PTPα, which 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 to their full-length precursors indicates that this principle operates among membrane-bound PTPases as well.
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References:


Footnote:

Abbreviations used: BHK - Baby Hamster Kidney cells; cyt-PTPε - cytoplasmic isoform of PTPε; DMEM - Dulbecco's Modified Eagle's medium; DMSO – dimethyl sulfoxide; Kv channel - delayed-rectifier, voltage gated potassium channel; PTK - protein tyrosine kinase; PTPase: protein tyrosine phosphatase: RPTPα receptor-type form of PTPα: tm-PTPε: receptor-type form of PTPε.
Figure legends:

Figure 1: p66PTPα is a PTPα-derived protein. A. 293 cells were transfected with expression vectors for cyt-PTPε, tm-PTPε, or RPTPα cDNAs, which had been FLAG-tagged at their 3' ends. Lysates from cells were blotted with anti-PTPε polyclonal serum, which cross reacts also with PTPα (EMID serum, 18; left panel). Lysates were immunoprecipititated with anti-FLAG antibodies, blotted, and probed with anti-FLAG antibodies (right panel). B. Brain lysates of wild type (WT), PTPα-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 p67 PTPε and p66 PTPα in these gels. Double and single asterisks denote the fully-glycosylated and non-glycosylated forms, respectively, of RPTPα and PTPε.

Figure 2: Sequence of the juxtamembrane regions of the RPTPα and tm-PTPε 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-PTPε and cyt-PTPε (16). Vertical arrows denote Met85 and Met99 of tm-PTPε, and Leu198 of RPTPα; 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.

Figure 3: in vivo inhibition of p66 PTPα and p65 PTPε 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 two hours. 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-PTPε (left panel) or RPTPα (right panel) cDNAs, either alone (-) or together with (+) calpastatin cDNA. Cells were lysed 24 hours after transfection and analyzed. C. 293 cells transfected with cyt-PTPε cDNA were either left untreated (-) or treated with 5, 25, or 50 μM calpeptin or with vehicle alone (0.5% DMSO) for two hours. D. As in C, except that cells were treated with 200μM E64d, 100μM leupeptin, 1μM pepstatin, 2mM PMSF, 2.5mM chloroquine, or 100μM NH₄Cl. In all cases cell lysates were blotted and probed with anti-PTPε/PTPα (EMID) serum. Arrows denote p66 PTPα or p65 PTPε.

Figure 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 20μM calpeptin and were then stimulated with 1mM CaCl₂ for the indicated time periods. Cells were then lysed, blotted, and probed with anti-PTPα serum. Blot is representative of 4 similar experiments. NS- non-specific band.

Figure 5: Exogenous calpain cleaves PTPε and PTPα in vitro to generate p65 PTPε and p66 PTPα, respectively. A. Titration of the effect of calpain on processing of cyt-PTPε. 293 cells were transfected with the ΔATG2,3 mutant of cyt-PTPε, which cannot produce p67 PTPε (16). 50 μg of cell lysate protein were incubated with calpain solubilization buffer (20 mM Tris-Cl, 1 mM EDTA, 1 mM DTT, pH 7.5), buffer plus 5mM CaCl₂, or buffer/CaCl₂ and increasing amounts of purified calpain as indicated for 30 minutes at 37 degrees. B: 50 μg protein from lysates of 293 cells expressing cyt-PTPε, tm-PTPε, or RPTPα cDNAs were incubated at 37 degrees for 30 minutes after addition of buffer containing 5 mM CaCl₂ (B), buffer/CaCl₂ plus 0.001 units calpain (B&C), or no reagent at all (-). C. Same experiment as in B, using 50 μg protein from lysates of wild-type mouse brain. Asterisk indicates RPTPα and PTPε cleavage products. In all cases, lysates were blotted and probed with EMID (anti PTPε/PTPα) serum.
Figure 6: Pulse-chase experiment comparing stabilities of cyt-PTPε, p67 PTPε, and p65 PTPε. 293 cells expressing full-length cyt-PTPε cDNA were labeled with 35S-methionine and then chased with cold methionine for up to 2 hours, 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 PTPε protein was quantified as detailed in Experimental Procedures. **A.** Amounts of radioactivity, normalized to protein content, for full-length cyt-PTPε, p67 PTPε, and p65 PTPε at the indicated time points; data is presented relative to values obtained at the beginning of the cold chase (t=0). **B.** Protein blot documenting cyt-PTPε, p67 PTPε, and p65 PTPε amounts after 2 hours 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 PTPε forms in the absence of leupeptin remained over 50% throughout the remainder of the chase period of eight hours (not shown). Experiment shown is representative of three performed.

Figure 7: p66 PTPα is inherently a non-membrane protein. 293 cells expressing either PTPα cDNA beginning at Leu 198 (top panel) or full-length RPTPα (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-glycosylated and non-glycosylated forms, respectively, of RPTPα.

Figure 8: p66 PTPα does not dephosphorylate Src at Y529. SYF cells, which lack endogenous Src, Yes, and Fyn, were transfected with RPTPα or p66 PTPα cDNAs, or with pCDNA3 vector alone (-). 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 PTPε/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 pY529 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.

Figure 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 cDNA. Note that
lanes 3-5 contain different amounts of p66 protein. All transfections contained the same
amount DNA, with the difference made up with pCDNA3 plasmid. Tyrosine-
phosphorylated proteins were immune-precipitated 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.
Figure 1
Human RPTPα
Mouse RPTPα
Mouse PTPε
Human PTPε

Figure 2
Figure 3
Figure 4
### Figure 5

#### A

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#### B

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- RPTP$\alpha$
- p66
- tm-PTP$\varepsilon$
- *
Figure 6
Figure 7

Transfection: p66 PTPα

Transfection: Full-length PTPα
Figure 8
Figure 9
Regulation of protein tyrosine phosphatases α and β by calpain-mediated proteolytic processing
Hava Gil-Henn, Gloria Volohonsky and Ari Elson

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