

Tyrosine Phosphatases ϵ and α Perform Specific and Overlapping Functions in Regulation of Voltage-gated Potassium Channels in Schwann Cells

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Tyrosine phosphatases (PTPs) ϵ and α are closely related and share several molecular functions, such as regulation of Src family kinases and voltage-gated potassium (Kv) channels. Functional interrelationships between PTP ϵ and PTP α and the mechanisms by which they regulate K⁺ channels and Src were analyzed in vivo in mice lacking either or both PTPs. Lack of either PTP increases Kv channel activity and phosphorylation in Schwann cells, indicating these PTPs inhibit Kv current amplitude in vivo. Open probability and unitary conductance of Kv channels are unchanged, suggesting an effect on channel number or organization. PTP α inhibits Kv channels more strongly than PTP ϵ ; this correlates with constitutive association of PTP α with Kv2.1, driven by membranal localization of PTP α . PTP α , but not PTP ϵ , activates Src in sciatic nerve extracts, suggesting Src deregulation is not responsible exclusively for the observed phenotypes and highlighting an unexpected difference between both PTPs. Developmentally, sciatic nerve myelination is reduced transiently in mice lacking either PTP and more so in mice lacking both PTPs, suggesting both PTPs support myelination but are not fully redundant. We conclude that PTP ϵ and PTP α differ significantly in their regulation of Kv channels and Src in the system examined and that similarity between PTPs does not necessarily result in full functional redundancy in vivo.

INTRODUCTION

Reversible phosphorylation of tyrosine residues in proteins is a major mechanism for regulation of protein structure and function. Phosphorylation is regulated by the opposing activities of two superfamilies of enzymes—the protein tyrosine kinases (PTKs) and the protein tyrosine phosphatases (PTPs). More than 100 PTP genes are known in higher organisms, of which 38 belong to the classical, tyrosine-specific PTP family (Alonso *et al.*, 2004; Andersen *et al.*, 2004). The number of tyrosine phosphorylation sites in target proteins is significantly larger than the numbers of known PTPs or PTKs (Manning *et al.*, 2002; Alonso *et al.*, 2004). Each enzyme therefore interacts with several substrates, and a given substrate may be targeted by more than one kinase or phosphatase. Consequently, it is not always clear whether these enzymes fulfill molecular and physiological roles that are entirely independent or that overlap at least in part.

Molecular and physiological studies of specific PTPs in recent years indicate that the issue of functional independence versus redundancy is complex. In many cases, specific substrates are currently known to be targeted by an individual PTP; in other cases, however, substrate sharing clearly occurs. A prime example of this is Src, which can be activated by dephosphorylation of its C-terminal inhibitory tyrosine by several PTPs, including PTP1B, SHP1, PTP α , and PTP ϵ (Somani *et al.*, 1997; Ponniah *et al.*, 1999; Su *et al.*, 1999; Bjorge *et al.*, 2000; Gil-Henn and Elson, 2003; Pallen, 2003). Yet, targeting of a specific substrate by several PTPs may not necessarily imply that these PTPs are fully redundant in vivo, because their expression patterns, their physical access to the substrate, and regulation of their catalytic activities may vary.

Similar complexity is found at the physiological level when phenotypes of mice genetically lacking PTPs are analyzed. Several possible modes of functional interaction between pairs of PTPs may exist. Both PTPs may have physiological functions that are either partially or entirely overlapping, in which case single mutants may have relatively weak phenotypes due to functional compensation by the other PTP. Deletion of both PTPs might then exacerbate existing phenotypes or uncover novel phenotypes. We note that the developmental phenotypes of several single-gene PTP mutants, including some of the most informative ones, are in fact rather weak and do not lead to major histopathological abnormalities in mice (e.g., Schaapveld *et al.*, 1997; Elchebly *et al.*, 1999; Ponniah *et al.*, 1999; Su *et al.*, 1999; Klamann *et al.*, 2000; Peretz *et al.*, 2000). In other cases, both

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Abbreviations used: Kv channel, delayed rectifier, voltage-gated K⁺ channel; PTP, protein tyrosine phosphatase.

PTPs may antagonize each other's function, evident as phenotypes of reduced intensity in mice lacking both PTPs compared with mice lacking either PTP. Along these lines, simultaneous absence of CD45 and SHP-1 corrects several B-cell defects present in mice lacking either of these PTPs (Pani *et al.*, 1997). Antagonistic interactions have been demonstrated also in axon guidance between several *Drosophila* PTPs (Desai *et al.*, 1997; Schindelholz *et al.*, 2001; Sun *et al.*, 2001) and between PTPRO versus RPTP δ and RPTP γ in regulating nerve structure in chick lumbar spinal cord (Stepanek *et al.*, 2005). Last, PTPs may have separate and nonoverlapping physiological functions, in which case the phenotype of mice lacking both PTPs would seem to be a simple combination of the two single-knockout phenotypes. We note that functional redundancy or antagonism between PTPs does not necessarily imply similar interrelationships at the molecular level.

We have chosen to examine this issue by comparing the abilities of two closely related PTPs, PTP ϵ and PTP α , to regulate delayed rectifier voltage-gated potassium (Kv) channels in Schwann cells. The two major forms of PTP ϵ protein are the receptor-type form (RPTP ϵ) and the nonreceptor form (cyt-PTP ϵ), which are produced by distinct promoters of the *Ptpre* gene (Elson and Leder, 1995a,b; Nakamura *et al.*, 1996; Tanuma *et al.*, 1999). Other protein forms of PTP ϵ are p67 PTP ϵ and p65 PTP ϵ , whose production is regulated at the levels of transcription and posttranslational processing, respectively (Gil-Henn *et al.*, 2000, 2001). RPTP ϵ and cyt-PTP ϵ differ only at their amino termini, resulting in each having a distinct pattern of subcellular localization and rendering them physiologically nonequivalent. Demonstrated roles of RPTP ϵ include assisting Neu-induced mouse mammary tumor cells maintain their transformed phenotype by dephosphorylating and activating Src *in vivo* (Gil-Henn and Elson, 2003), and possibly down-regulating insulin receptor signaling (Moller *et al.*, 1995; Andersen *et al.*, 2001; Lacasa *et al.*, 2005; Nakagawa *et al.*, 2005). The cyt-PTP ϵ form is important for proper adhesion of osteoclasts to bone and for subsequent bone resorption (Chiusaroli *et al.*, 2004). cyt-PTP ϵ can also down-regulate signaling mediated by the mitogen-activated protein kinase (Wabakken *et al.*, 2002; Toledano-Katchalski *et al.*, 2003) and by the Janus tyrosine kinase-signal transducer and activator of transcription (Tanuma *et al.*, 2000, 2001, 2003) pathways and is required for proper functioning of mouse macrophages (Sully *et al.*, 2001).

cyt-PTP ϵ can also down-regulate the activity of Kv channels in Schwann cells (Peretz *et al.*, 2000). Kv channels comprise a large and ubiquitous family and are key regulators of cellular functions such as action potential waveforms, neuronal firing patterns, synaptic integration, neurotransmitter release, volume regulation, and cell proliferation. Kv channels are comprised of tetramers of membrane-spanning α subunits, which may associate with up to four cytosolic regulatory β subunits (Pongs, 1995; Martens *et al.*, 1999; Yi *et al.*, 2001). Kv channels are activated by membrane depolarization, but their activity can be modulated after tyrosine phosphorylation of their α subunits by Src family PTKs (Fadool *et al.*, 1997; Sobko *et al.*, 1998a; Levitan, 1999; Peretz *et al.*, 1999; MacFarlane and Sontheimer, 2000). We have shown that the α subunit Kv2.1 is a physiological substrate of cyt-PTP ϵ . Kv2.1 is hyperphosphorylated in Schwann cells and sciatic nerve tissue of PTP ϵ -deficient mice; phosphorylation of Kv2.1 by Src or Fyn up-regulates channel activity, whereas channel dephosphorylation by cyt-PTP ϵ counters this effect (Peretz *et al.*, 2000). The major site within Kv2.1 at which Src and cyt-PTP ϵ exert their opposing effects is Y124

in the cytoplasmic N terminus of the channel (Tiran *et al.*, 2003). A stable complex between Kv2.1 and the substrate-trapping mutant D245A cyt-PTP ϵ can be isolated readily; a significant part of the stability of this complex is linked to the presence of the Y124 phosphorylation site in Kv2.1 (Peretz *et al.*, 2000; Tiran *et al.*, 2003). Increased Kv channel activity in Schwann cells of mice lacking PTP ϵ is correlated with transiently reduced myelination of sciatic nerve axons of these mice (Peretz *et al.*, 2000).

The PTP most closely related to PTP ϵ is RPTP α , whose major protein form is a receptor-type molecule (Krueger *et al.*, 1990). RPTP α activates Src by dephosphorylating its C-terminal inhibitory tyrosine residue in a manner similar to PTP ϵ (for review, see Pallen, 2003). Activation of Src and related kinases by RPTP α mediates many of its physiological functions, such as regulation of integrin signaling and cell migration (Ponniah *et al.*, 1999; Su *et al.*, 1999; von Wichert *et al.*, 2003; Zeng *et al.*, 2003) and neurite elongation (Bodrikov *et al.*, 2005). RPTP α has been suggested to down-regulate insulin receptor signaling (Moller *et al.*, 1995; Lammers *et al.*, 1997; Andersen *et al.*, 2001; Lacasa *et al.*, 2005), although lack of RPTP α does not seem to affect glucose homeostasis *in vivo* (Le *et al.*, 2004). At the whole-animal level, gene-targeting studies have established that RPTP α functions in developmental positioning of hippocampal pyramidal neurons, learning, and neuroplasticity (Petroni *et al.*, 2003; Skelton *et al.*, 2003). Molecular targets of RPTP α responsible for these phenotypes remain to be identified. Interestingly, ectopically expressed RPTP α is capable of activating the Kv1.2 and Kv1.1 potassium channels upon signaling by the m1 muscarinic and serotonin receptors (Tsai *et al.*, 1999; Imbrici *et al.*, 2000). The difference between this effect and inhibition of Kv2.1 by cyt-PTP ϵ may stem from differences in the biophysical attributes of the two channel subtypes, the biochemical properties of either PTP, or from differences in the cellular context or specific signaling pathways involved in either system.

A significant part of the above-mentioned information has been obtained by use of mice genetically lacking either PTP α or PTP ϵ (Ponniah *et al.*, 1999; Su *et al.*, 1999; Peretz *et al.*, 2000; Sully *et al.*, 2001; Petroni *et al.*, 2003; Bodrikov *et al.*, 2005). Yet, lack of either PTP is tolerated well by mice and is not associated with major morbidity or mortality. Although PTPs α and ϵ are products of separate genes and have distinct expression patterns *in vivo*, these PTPs are highly related. PTPs α and ϵ are the only two members of the type IV family of receptor PTPs (Alonso *et al.*, 2004; Andersen *et al.*, 2004), and 83% of the amino acid residues in their cytosolic domains are either identical or similar, suggesting that functional redundancies may exist between them. To examine this issue *in vivo*, we derived and examined mice lacking both PTP ϵ and PTP α . In this study, we examine regulation of Kv channel activity and Src in Schwann cells in the absence of either or both PTPs and conclude that some of the functions of these closely related PTPs are clearly divergent within a single cell type.

MATERIALS AND METHODS

Plasmids

Full-length mouse cDNAs for cyt-PTP ϵ (Elson and Leder, 1995a), RPTP ϵ (Elson and Leder, 1995b), and RPTP α (kind gift of the late Dr. M. Thomas) were used in this study. Lck-cyt-PTP ϵ was prepared by polymerase chain reaction (PCR)-mediated addition of the N-terminal Lck myristoylation domain to the N terminus of cyt-PTP ϵ ; cyt-PTP α was prepared similarly by replacing the extracellular and transmembrane domains of RPTP α (amino acids 1–166) with the 12 N-terminal residues of cyt-PTP ϵ . The following mutants were prepared by site-directed mutagenesis: R340M RPTP ϵ , R283M

cyt-PTP ϵ , D245A cyt-PTP ϵ , D437A RPTP α , D245A Lck-cyt-PTP ϵ , and D437A cyt-PTP α . Numbering of the mutated residue in the latter two cDNAs is unchanged from cyt-PTP ϵ and RPTP α , respectively, for clarity. All products of PCR and of site-directed mutagenesis were sequenced before use, and all contained a C-terminal FLAG tag. Also used were the cDNAs for rat Kv2.1 (gift of Drs. J. Barhanin and M. Lazdunski), Y124F Kv2.1 (Tiran *et al.*, 2003), and chicken Y527F Src (gift of Dr. S. Courtneidge, Van Andel Research Institute, Grand Rapids, MI). All cDNAs were cloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA).

Generation and Analysis of Mice

Mice were housed (up to 5 per cage) in the Weizmann Institute barrier mouse facility. Animals were handled in accordance with National Research Council regulations, Israeli Law, and Weizmann Institute regulations; all studies were approved by the Institutional Animal Care and Use Committee. Mice lacking PTP ϵ (Peretz *et al.*, 2000) or PTP α (Su *et al.*, 1999) were crossed; progeny were interbred to generate the four genotypes used in this study—wild type (WT), PTP ϵ -deficient (EKO or *Ptpre*^{-/-}), PTP α -deficient (AKO or *Ptpra*^{-/-}), and deficient in both PTPs (DKO or *Ptpre*^{-/-}/*Ptpra*^{-/-})—in the same genetic background (C57Bl/6 \times 129Sv/Ev).

Genotyping of mice by PCR for the PTP ϵ -targeted allele was done using primers WT5' (5'-ACTCCAGACAGCTGCAAAGC-3') and WT3' (5'-CGC-TACAGTGAACCAATGG-3'), which amplify a 250-base pair fragment from the WT allele of PTP ϵ , and primers SL3015' (5'-GGATCCAATTGCAAT-GATCA-3') and NEOK03' (5'-ACTGAAGGCTCTTACTATTGC-3'), which amplify a 450-base pair fragment from the targeted PTP ϵ allele. Reactions included 1- μ l tail biopsy DNA, ~30 pmol of each oligonucleotide, 0.25 mM of each dNTP (Sigma-Aldrich, St. Louis, MO), 1 \times polymerase reaction buffer [1.5 mM MgCl₂, 20 mM (NH₄)₂SO₄, 75 mM Tris-HCl, pH 9.0, and 0.01% Tween 20], and 1.25 U of *Taq* polymerase (JMR Holdings, London, United Kingdom) in a final volume of 25 μ l. Samples were denatured at 93°C for 2 min followed by 30 cycles of 93°C for 30 s, 52°C for 1 min, and 72°C for 1 min. Genotyping by PCR for the RPTP α -targeted allele was performed as described previously (Su *et al.*, 1999). Mice were weaned at ~3 wk of age and were weighed once a week starting 40 d after birth through adulthood (5–7 mo). The following tissues were stained with hematoxylin and eosin and examined by light microscopy: liver, stomach, small and large intestines, kidney, adipose connective tissue, uterus, skeletal muscle (diaphragm), various peripheral nerves, pancreas, adrenal, brown fat, various blood vessels, heart, thymus, spleen, lung, lymph nodes (mesenteric, peripheral, and inguinal), skull (coronal sections), skin (head), marrow (sternum), thyroid, salivary glands, tongue, pancreas, oviducts, urinary bladder, and brain. No gross abnormalities beyond those described for AKO mice (Su *et al.*, 1999) were detected.

Electron Microscopy

Sciatic nerves from 5-d-old mice (6–11 mice from each genotype from at least 2 different litters born to different parents) were analyzed as described previously (Peretz *et al.*, 2000). Samples from different mice were always taken from the same location, 5 mm from the proximal end of the nerve. Briefly, samples were fixed with 3% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in 1% OsO₄ and 2% uranyl acetate, dehydrated in a graded series of ethanols, and embedded in Epon-812. Transverse cross-sections of 70–90 nm thickness were cut with a Leica Ultracut UCT ultramicrotome (Leica, Wetzlar, Germany), stained with uranyl acetate and lead citrate, and examined on a Tecnai 12 transmission electron microscope at 120Kv. Cross-sections were examined from each mouse at 4000 \times magnification. Pictures were digitized with a MegaView III charge-coupled device camera and analyzed with ANALYSIS software (SPSS, Chicago, IL). The thickness of the myelin sheath of all axon profiles in a given field was measured, and data for mice of the same genotype was pooled.

Cell Culture

Primary Schwann cells were prepared from sciatic nerves of 3- to 5-d-old pups as described previously (Sobko *et al.*, 1998a). Age-matched WT, EKO, AKO, or DKO pups were obtained from separate matings of homozygous mice of the same genotypes. Schwann cells were grown in DMEM/F-12 (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Invitrogen), 2 mM glutamine, and antibiotics (50 U/ml penicillin and 50 μ g/ml streptomycin) and analyzed 8–10 d after isolation. Human embryonic kidney (HEK)293 cells were grown in DMEM (Invitrogen) supplemented with 10% FCS and antibiotics as described above and transfected by the calcium phosphate technique.

Electrophysiology

Whole-cell currents were recorded from Schwann cells by using the patch-clamp technique as described previously (Sobko *et al.*, 1998a). Signals were amplified using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA), sampled at 2 kHz, and filtered below 0.8 kHz via a four-pole Bessel low-pass filter. Data acquisition was done using the pClamp 8.1 software (Molecular Devices). The patch pipettes were filled with 164 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 11 mM EGTA, 10 mM HEPES, and 11 mM glucose

at pH 7.4 and had a tip resistance of 4–8 M Ω . The external solution contained 140 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 2 mM MgCl₂, 11 mM glucose, and 10 mM HEPES at pH 7.4. Series resistances (3–13 M Ω) were compensated (75–90%) and periodically monitored.

Single-channel recordings were performed in Schwann cells using the cell-attached configuration of the patch-clamp technique. Cells were continuously perfused with a standard salt solution containing 150 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1.2 mM MgCl₂, 15 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). Pipettes were pulled from borosilicate glass, coated with Sylgard, and filled with the above-mentioned standard salt solution (8–10 M Ω). Signals were sampled at 10 kHz and low-pass filtered at 2 kHz. Data were analyzed using Clampfit 9.2 (Molecular Devices).

Biochemical Fractionation of Sciatic Nerve Tissue and Cells

For analysis of Kv2.1 phosphorylation in sciatic nerves, nerve tissue was dissected from ~3-d-old mice (30–40 mice per experimental point) and stored in liquid nitrogen until use. Tissue was homogenized in buffer containing 50 mM Tris, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM sodium fluoride, 0.5 mM sodium pervanadate, and protease inhibitors [1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 40 μ M bestatin, 15 μ M E-64, 20 μ M leupeptin, and 15 μ M pepstatin; Sigma-Aldrich], and centrifuged at 21,000 \times g for 30 min at 4°C. The pellet (crude membranous fraction) was resuspended and sonicated in solubilization buffer (10% glycerol, 50 mM HEPES, pH 7.4, 10 mM EDTA, 150 mM NaCl, 1.5 mM MgCl₂, 1% Triton X-100, 1 mM PMSF, 50 mM sodium fluoride, 0.5 mM sodium pervanadate, and protease inhibitors). The sonicate was incubated with shaking in solubilization buffer for 1 h at 4°C, spun at 21,000 \times g, and the supernatant was collected. Membranous extracts (1.5–3 mg) were incubated with shaking with anti-Kv2.1 monoclonal antibodies (Trimmer, 1991) at 4°C overnight. Immune complexes were collected by adding protein A/G-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) and incubating in solubilization buffer for 2 h at 4°C with shaking, followed by three washes in solubilization buffer. In other studies, cultured cells were fractionated into membranous and nonmembranous (i.e., combined cytosolic and nuclear) fractions by homogenization in hypotonic buffer as described previously (Elson and Leder, 1995a).

Immunoprecipitation and Protein Blotting

Cells were lysed in buffer A (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 1% NP-40), supplemented with protease inhibitors and either 0.5 mM sodium pervanadate (for phosphorylation studies) or 5 mM sodium iodoacetate (for substrate-trapping studies). In phosphorylation studies crude cellular proteins (1 mg) were reacted with anti-phosphotyrosine antibodies (clone PY20; BD Biosciences Transduction Laboratories, Lexington, KY) and protein A-Sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) for 2 h at 4°C, spun down, and washed three times in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). In substrate-trapping studies cellular proteins (1 mg) were reacted with anti-FLAG M2 affinity beads (Sigma-Aldrich) for 4–6 h, followed by two washes in buffer A and one wash in RIPA buffer. SDS gel electrophoresis, blotting, and antibody hybridization were done as described previously (Gil-Henn *et al.*, 2000). Primary antibodies used included rabbit polyclonal anti-PTP ϵ , which cross reacts with RPTP α (Elson and Leder, 1995b), monoclonal [clone D4/11, generous gift of Drs. E. Peles (Weizmann Institute of Science, Rehovot, Israel) and J. Trimmer (University of California, Davis, CA) (Trimmer, 1991)] or polyclonal (Alomone Labs, Jerusalem, Israel) anti-Kv2.1, monoclonal anti-v-Src (clone 327; Calbiochem, San Diego, CA), and monoclonal anti-phosphotyrosine (clone 4G10; Upstate Biotechnology, Lake Placid, NY). Band signals at non-saturating exposures were scanned and measured by image densitometry by using Image Gauge 4.0 (FujiFilm, Tokyo, Japan) and Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA).

Src Activity Assay

Sciatic nerve tissue was dissected from 3- to 5-d-old mice and stored frozen in liquid nitrogen until used. Tissue was homogenized in buffer A supplemented with protease inhibitors and sodium pervanadate as described above, sonicated, and spun to remove debris. Crude lysates (0.3–0.5 mg) were used to determine activity of immune-precipitated c-Src by using [γ -³²P]ATP and enolase as described previously (Gil-Henn and Elson, 2003).

In Vitro Dephosphorylation of pKv2.1

HEK293 cells expressing FLAG-tagged RPTP α or cyt-PTP ϵ were lysed in buffer A supplemented with protease inhibitors but without pervanadate. PTPs were immunoprecipitated with monoclonal anti-FLAG M2 antibodies (Sigma-Aldrich). Precipitated material was washed three times in buffer A, twice in buffer B (20 mM HEPES, pH 7.6, 100 mM KCl, 0.5 mM EDTA, 0.4% NP-40, and 20% glycerol), followed by two short (1.5-min) washes in buffer 54K (50 mM Tris, pH 7.9, 150 mM NaCl, and 0.5% Triton X-100). Proteins were eluted by two incubations in equal volumes of Tris-buffered saline buffer (20 mM Tris-Cl, pH 7.35, and 150 mM NaCl) containing 1 mg/ml FLAG peptide

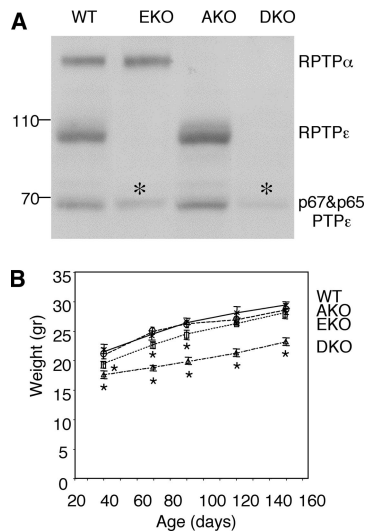


Figure 1. PTP expression and body weight of mice used in this study. (A) Expression of RPTP ϵ and RPTP α in brain lysates. Blots containing crude extracts of brain from adult WT, AKO, EKO, and DKO mice were reacted with anti-PTP ϵ antibodies, which also cross-react with RPTP α , to assess expression of PTP ϵ and PTP α in this tissue. Asterisks mark nonspecific bands; molecular mass standards are in kilodaltons. (B) Weights of mice of the indicated genotypes are shown between 40 and 150 d of age. The weight of DKO mice was significantly (15–25%) lower than that of WT, AKO, or EKO mice (Mann–Whitney *U*-test, $p < 0.0001$). EKO males weighed slightly less than WT males up to 90 d (Mann–Whitney *U*-test, $p < 0.01$). Each data point represents the average weight \pm SEM with $n = 20$ –51. Asterisks mark data points significantly different from WT. Similar results were obtained with female mice (our unpublished data).

(Sigma-Aldrich) and 0.1 mM EGTA, at 32°C for 3 min. Eluted material was pooled. FLAG-tagged phosphorylated Kv2.1 was purified in a similar manner from HEK293 cells expressing Kv2.1 and active (Y527F) Src, except that the lysis buffer A contained 0.5 mM sodium pervanadate. Protein purity and amounts were determined by gel electrophoresis and silver staining. pKv2.1 (15 ng) was incubated either alone or with 100 ng of purified PTP in PTP activity buffer [50 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 7.0, 0.5 mg/ml bovine serum albumin, and 0.5 mM dithiothreitol] at 32°C for 6 h, followed by adding SDS-PAGE sample buffer and boiling. Phosphorylation of Kv2.1 was analyzed by SDS-PAGE and protein blotting with anti-pTyr antibodies as described above.

RESULTS

Mice Lacking Both PTP ϵ and PTP α Are Viable

The effects of absence of PTP ϵ or PTP α on Kv channels were compared in mice lacking either PTP ϵ (EKO mice; Peretz *et al.*, 2000) or PTP α (AKO mice; Su *et al.*, 1999). To obtain additional information on the functional interactions between these PTPs, EKO and AKO mice were crossed to generate mice lacking both PTPs (DKO mice). DKO mice were viable and produced litters of normal size. Analysis of protein expression from various tissues confirmed that DKO mice lacked RPTP α and the various PTP ϵ proteins (Figures 1A and 3A). Interestingly, DKO mice of both genders weighed 15–20% less than WT, AKO, or EKO mice (Figure 1B). Reduced DKO body weight was detected before weaning and persisted throughout adulthood, indicating that DKO mice gain weight proportionally at the same rate as WT mice and consequently remain smaller throughout life. This result indicates that PTP α and PTP ϵ perform a redundant role in regulation of mouse body weight. Altered body weight is a complex phenotype that has been observed in

mice lacking various unrelated genes, among them PTPs (Klaman *et al.*, 2000; Cheng *et al.*, 2002; Zhang *et al.*, 2004). Further studies will therefore be needed to uncover its cellular and molecular bases in this particular case. Histological examination of several tissues of DKO mice did not reveal major gross structural abnormalities beyond those described for AKO mice (Su *et al.*, 1999; Petrone *et al.*, 2003); the tissues examined are listed in *Materials and Methods*. We conclude that abnormalities in these tissues, if they exist, are subtle; their detection may require use of more sensitive techniques, as shown below in the case of sciatic nerves.

Reduced Myelination of Sciatic Nerve Axons in AKO Mice

Mice lacking PTP ϵ exhibit transient but severe hypomyelination of sciatic nerve axons at an early postnatal age (Peretz *et al.*, 2000); the role of PTP α , the most closely related paralogue gene to PTP ϵ , in this system is unknown. To examine sciatic nerve myelination in the absence of RPTP α and/or PTP ϵ , we used transmission electron microscopy to quantify myelin sheath thickness in transverse cross-sections of sciatic nerves obtained from WT, AKO, and EKO, and DKO mice. Analysis of sciatic nerve axons of 5-d-old mice revealed significant reductions in the average myelin sheath thickness in AKO (down to 89% of WT control), EKO (to 82%), and DKO (to 76%) mice compared with WT mice (Figure 2). The effect was most evident in thinly myelinated axons (<150 nm), where increases of up to twofold in the number of axons in this category were seen in AKO, EKO, and DKO mice. Major changes were also observed in the most heavily myelinated sheaths (>350 nm), where a marked decrease in the number of axons in this category was observed in the various KO mice. The findings in EKO mice agree with previous studies (Peretz *et al.*, 2000). Importantly, lack of PTP α and PTP ϵ seem to have additive effects, because changes in myelin sheath thickness were more pronounced in the DKO mice than in AKO and EKO mice (Figure 2). The average diameter of sciatic nerve axons was increased from 1099 \pm 10 nm in WT mice to 1172 \pm 11 and 1164 \pm 12 nm in EKO and AKO mice, respectively, and it was decreased to 1046 \pm 11 nm in DKO mice (mean \pm SE, $n = 700$ –849, $p \leq 0.00056$ for all KO genotypes versus WT). This result suggests that sciatic nerve abnormalities are not limited to the myelin sheath alone. However, because thicker axons are usually associated with increased myelination, increased axon diameter in EKO and AKO mice most likely does not account for the reduced myelination observed in these mice. These results indicate that both PTP α and PTP ϵ support sciatic nerve myelination *in vivo* but that their roles in this process are at least in part nonredundant.

Adult mice did not display obvious difficulties indicative of neural dysfunction (e.g., in locomotion), indicating that myelination disruption was transient. This was confirmed by direct examination of sciatic nerves of mice 120 d old, which did not reveal myelination abnormalities (our unpublished data). Normal myelination in adult mice was also observed previously when EKO mice were examined (Peretz *et al.*, 2000). We conclude therefore that although loss of both PTP ϵ and RPTP α results in a more severe myelination phenotype than loss of either PTP alone, this phenotype is transient.

Increased Activation of Kv Channels in Schwann Cells of AKO Mice

Previous analyses revealed that Kv channel activity was increased in primary Schwann cells of EKO mice (Peretz *et al.*, 2000). To examine the effect of lack of PTP α on regulation of Kv channels, we examined the activity of Kv channels in

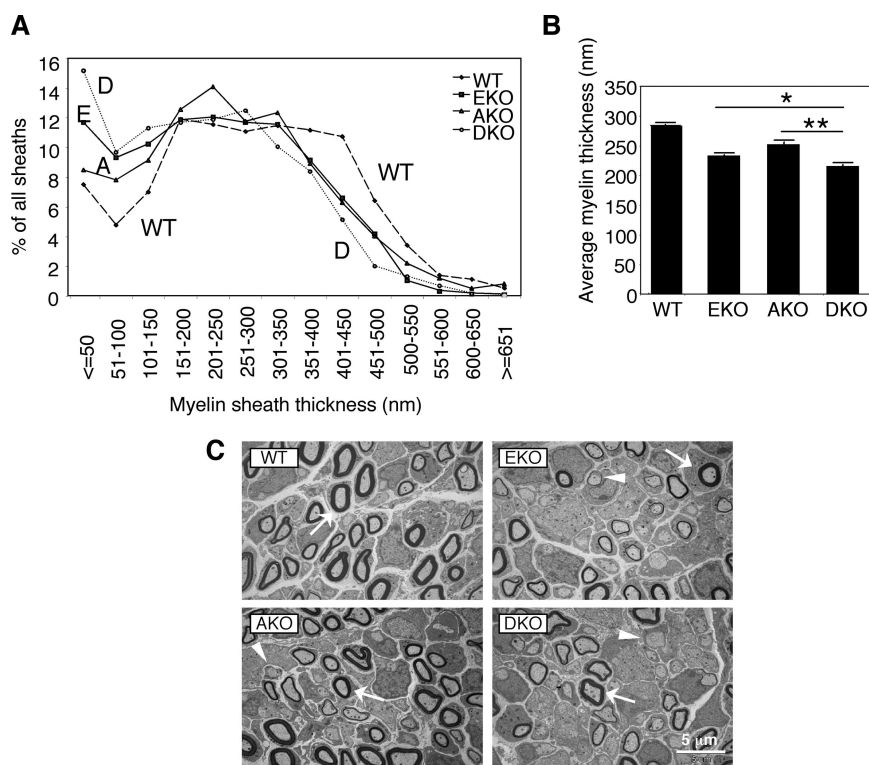


Figure 2. Reduced myelination of axons in sciatic nerves of 5-d-old AKO, EKO, and DKO mice. (A) Distribution of thicknesses of myelin sheaths in wild-type, AKO, EKO, and DKO sciatic nerves (WT, A, E, and D, respectively). $n = 1370$ – 2320 individual axons per genotype, as indicated below. (B) Bar diagram comparing the average sheath thickness (in nanometers, \pm SEM). WT, 284.2 ± 3.0 , $n = 2320$ sheaths; EKO, 232.6 ± 3.3 , $n = 1652$; AKO, 251.7 ± 3.8 , $n = 1370$; and DKO, 214.9 ± 5.4 , $n = 1601$. All knockout means are distinct from WT mean ($p < 0.0001$); EKO and AKO means are distinct from DKO mean (EKO, $*p = 0.0054$; AKO, $**p < 0.0001$). Statistical significance was determined by Welch's *t* test. (C) Representative electron microscope pictures of transverse cross-sections through sciatic nerves of 5 d-old WT, AKO, EKO, and DKO mice. Arrows mark several cross-sections of myelinated axons, evident as closed dark figures. Arrowheads mark several unmyelinated axons.

Schwann cells of AKO mice. Primary Schwann cells express both RPTP α and the nonreceptor form of PTP ϵ , cyt-PTP ϵ ; as expected, either PTP was absent from Schwann cells of the corresponding single knockout mice (Figure 3A). Primary Schwann cell cultures from 3- to 5-d-old mice of the various genotypes were prepared and used for recording voltage-gated potassium currents by using the whole-cell patch-clamp technique. Schwann cells of EKO and of AKO mice exhibited a marked increase in K⁺ current densities (Figure 3, B and C), with the effect in AKO mice much stronger than in EKO mice (47% increase in EKO Schwann cells compared with WT (at +60 mV), versus an increase of 150% in AKO Schwann cells). This result indicates that RPTP α and cyt-PTP ϵ each inhibit Kv channel activity in Schwann cells, but they do so most likely by distinct mechanisms. To determine the effects of simultaneous inactivation of both PTPs on current density we analyzed Schwann cells from DKO mice, which are devoid of both cyt-PTP ϵ and RPTP α proteins (Figure 3A). Kv channel activity in DKO Schwann cells was lower than in AKO cells and was slightly higher than in EKO cells (62% increase compared with WT (Figure 3, B and C). Thus, absence of both PTPs did not increase Kv currents in an additive manner, possibly suggesting that cyt-PTP ϵ may also promote Kv channel activity in the absence of RPTP α as discussed further below.

Lack of either or both PTPs affected only the amplitude of Kv currents; other biophysical parameters, such as the voltage dependence of activation and the kinetics of activation and inactivation, were not affected (Figure 3, B and C). Thus, absence of RPTP α and/or cyt-PTP ϵ could in principle increase the number of functional channels available in the membrane; alternatively, it might increase either the channel open probability P_o and/or the unitary conductance. To address this issue we performed single-channel recordings of K⁺ currents from Schwann cells using the cell-attached configuration of the patch-clamp technique. The resting

membrane potential (E_m) of Schwann cells was determined at a very short time after establishing the whole-cell configuration before dialysis of the cell. We used this value ($E_m = -50 \pm 12$ mV; $n = 21$) to determine the membrane potential of the intact patches. Single-channel currents could be detected easily at potentials 20 mV more positive than the resting membrane potential, i.e., above -30 mV, with current amplitudes increasing with further depolarization (Figure 4A). The unitary current-voltage relations indicated a mean single-channel conductance of 10 pS for the four mouse genotypes (Figure 4B; our unpublished data), a value very close to that obtained previously in Schwann cells from normal NMRI mice (Verkhatsky *et al.*, 1991). Analysis of the open probability, P_o , indicated that P_o was very high and not significantly different among the four mouse genotypes (Figure 4C). We conclude that the significant increase in Schwann cell K⁺ current density observed in PTP knockout mice relative to WT mice cannot be accounted for by differences in P_o or in unitary conductances; it most likely involves an increase in the number of functional channels available at the plasma membrane as discussed further below.

Hyperphosphorylation of Kv2.1 and Deregulation of c-Src in the Absence of Either or Both PTP ϵ and PTP α

To better understand the different effects of lack of PTP ϵ and PTP α on Kv currents, we examined the molecular mechanisms by which these PTPs may affect Kv channels. Kv2.1 is a substrate of cyt-PTP ϵ and is hyperphosphorylated in sciatic nerve tissue and in primary Schwann cells from PTP ϵ -deficient mice (Peretz *et al.*, 2000). Because cyt-PTP ϵ , RPTP ϵ , and RPTP α can dephosphorylate Kv2.1 in cultured cells (Gil-Henn *et al.*, 2000; Peretz *et al.*, 2000), we examined the phosphorylation state of Kv2.1 in sciatic nerves of AKO and DKO mice. For this purpose Kv2.1 was immunoprecipitated from membranous fractions obtained from sciatic nerve tissue

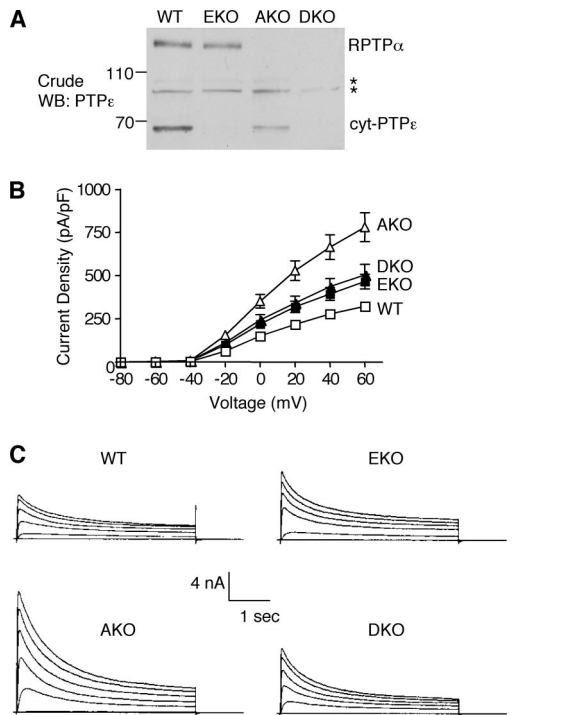


Figure 3. Increased Kv channel currents in primary Schwann cells of EKO, AKO, or DKO mice. (A) Blots containing crude extracts from Schwann cells from 3- to 5-d-old WT, AKO, EKO, and DKO mice were reacted with anti-PTP ϵ antibodies to assess expression of cyt-PTP ϵ and PTP α . Asterisks mark nonspecific bands; molecular mass markers are in kilodaltons. (B) The K⁺ current density (picoamperes/picofarads; mean \pm SEM) of WT (n = 12), AKO (n = 11), EKO (n = 14), and DKO (n = 9) Schwann cells plotted against voltage steps (millivolts). At +60 mV, EKO cells exhibit a 47% increase in current density compared with WT cells; analogous values for AKO and DKO cells are +150 and +62%, respectively. (C) Whole-cell K⁺ currents recorded from primary Schwann cells of the four genotypes. Cells were stepped from a holding potential of -80 to +60 mV in +10-mV increments for a 400-ms pulse duration.

of 3-d-old mice of all four genotypes. Phosphorylation of Kv2.1 was assessed by blotting with anti-phosphotyrosine antibodies, followed by stripping and reprobing with anti-Kv2.1 antibodies to normalize to Kv2.1 protein levels. As seen in Figure 5, A and B, Kv2.1 phosphorylation was elevated in AKO, EKO, and DKO compared with WT, supporting a general trend for increased phosphorylation and activation occurring together.

To provide further insight into the different ways by which both PTPs may regulate channel function, we examined their ability to regulate Src. It is well established that PTP ϵ and RPTP α activate Src and related kinases in several cell types by dephosphorylating their C-terminal inhibitory tyrosine (Ponniah *et al.*, 1999; Su *et al.*, 1999; Gil-Henn and Elson, 2003; Pallen, 2003; Granot-Attas and Elson, 2004). In turn, Src and Fyn can phosphorylate and up-regulate Kv channels in Schwann cells (Fadool *et al.*, 1997; Sobko *et al.*, 1998a; Levitan, 1999; Peretz *et al.*, 1999; MacFarlane and Sontheimer, 2000), raising the possibility that these PTPs might affect Kv channel activation in Schwann cells also indirectly by regulating kinase activity. To examine this possibility, we immunoprecipitated Src from cells of mice of the four genotypes and measured its kinase activity *in vitro* by using enolase as a substrate (Figure 5, C and D). Loss of cyt-PTP ϵ alone resulted in a small and statistically insignif-

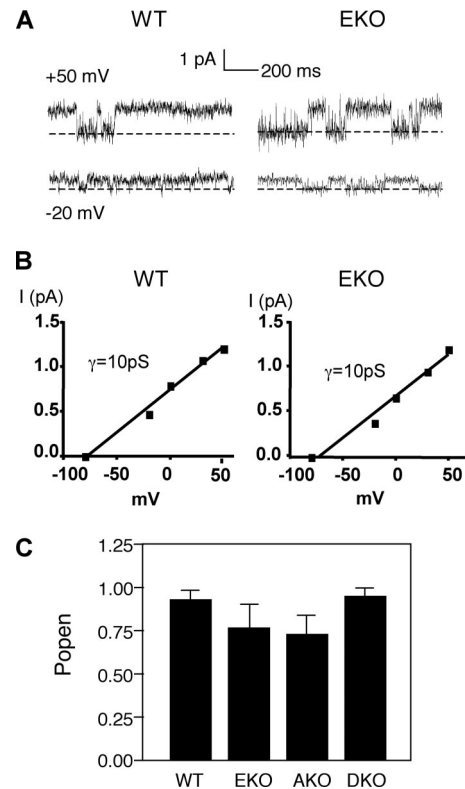


Figure 4. Single-channel properties of Schwann cell K⁺ currents measured in cell-attached patches. (A) Single channel current traces of WT and EKO Schwann cells are displayed at two different potentials evoked from the cell resting potential (-50 mV). The closed channel state is indicated by the dashed lines. The K⁺ concentration in the pipette and the bath was 2.5 mM. (B) Representative unitary current-voltage relations measured from WT and EKO Schwann cells (of 21 similar cells). The straight lines correspond to the fit of data points and indicate unitary conductance of 10 pS. (C) Open probability (mean \pm SEM) determined from the analysis of 17-21 cells of each the four genotypes. Differences between the four genotypes are not statistically significant.

icant reduction in c-Src activity, whereas loss of RPTP α reduced c-Src activity by \sim 50%. Interestingly, absence of both PTPs did not reduce c-Src kinase activity beyond the effect of ablation of RPTP α alone (Figure 5D), a finding compatible with a simple additive mode of regulation of Src by either PTP. These results establish RPTP α as a significant regulator of Src in this system and agree with previous results that indicate a much smaller role, if any, for cyt-PTP ϵ in this respect (Peretz *et al.*, 2000).

Kv2.1 Interacts Constitutively with RPTP α , but Not with cyt-PTP ϵ

The above-mentioned results suggest that RPTP α , but not cyt-PTP ϵ , may regulate Kv channels in Schwann cells also indirectly by affecting Src activity. However, this parameter is not sufficient to explain in full molecular detail the stronger inhibition of Kv channel activity by RPTP α . To further address this issue, we examined the abilities of either PTP to interact with and to dephosphorylate Kv channels.

Experiments using isolated cyt-PTP ϵ , RPTP α , and Src-phosphorylated Kv2.1 indicated that either PTP can dephosphorylate Kv2.1 *in vitro* (Figure 6A). However, their respective activities toward Kv2.1 may differ in a cellular context. RPTP α may have better access to Kv channel proteins be-

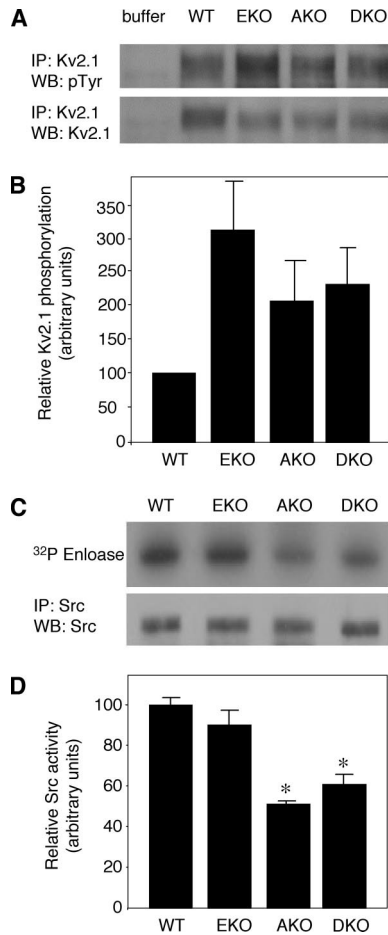


Figure 5. Phosphorylation of Kv2.1 and Src activity in sciatic nerves of WT, EKO, AKO, and DKO mice. (A) Crude membrane fractions of sciatic nerves from 3-d-old WT, EKO, AKO, and DKO mice were prepared and solubilized. After immunoprecipitation with anti-Kv2.1 antibodies, precipitates were blotted with anti-pTyr antibodies (top) followed by stripping and blotting with anti-Kv2.1 antibodies (bottom). Blots are from a representative experiment of four performed. (B) Bar diagram showing Kv2.1 phosphorylation intensity (\pm SEM) normalized to Kv2.1 protein content and presented relative to WT (= 100): EKO = 320.4 ± 105.8 , AKO = 216.0 ± 58.3 , and DKO = 231.8 ± 57.4 ; $n = 4$ repeats. (C) Src protein was immunoprecipitated from sciatic nerve extracts of 3- to 4-d-old mice and allowed to catalyze incorporation of ^{32}P into enolase. Reaction mixture was subject to SDS-PAGE and blotting. Blot was exposed to document enolase-associated radioactivity (top) and then probed with anti-Src antibody to document amount of Src protein present in the precipitate (bottom). (D) Bar diagram showing relative Src activity (\pm SEM) in the four genotypes studied: WT = 1.00 ± 0.04 ; EKO = 0.89 ± 0.09 ; AKO = 0.50 ± 0.02 ; and DKO = 0.61 ± 0.05 . $n = 3$ –5 experiments per genotype. AKO and DKO are statistically different (asterisks) by Student's *t* test from WT ($p \leq 0.0006$) and EKO ($p \leq 0.018$). WT versus EKO and AKO versus DKO are not statistically distinct.

cause it is an integral membrane protein; this is in contrast to cyt-PTP ϵ , which is predominantly cytosolic (Elson and Leder, 1995a). Alternatively, the membrane-spanning and extracellular domains of RPTP α , which have no counterparts in cyt-PTP ϵ , might interact with Kv2.1 and contribute to its regulation. Last, slight differences between the sequences of the active sites and regulatory regions of these highly related PTPs may make RPTP α a more effective down-regulator of Kv2.1.

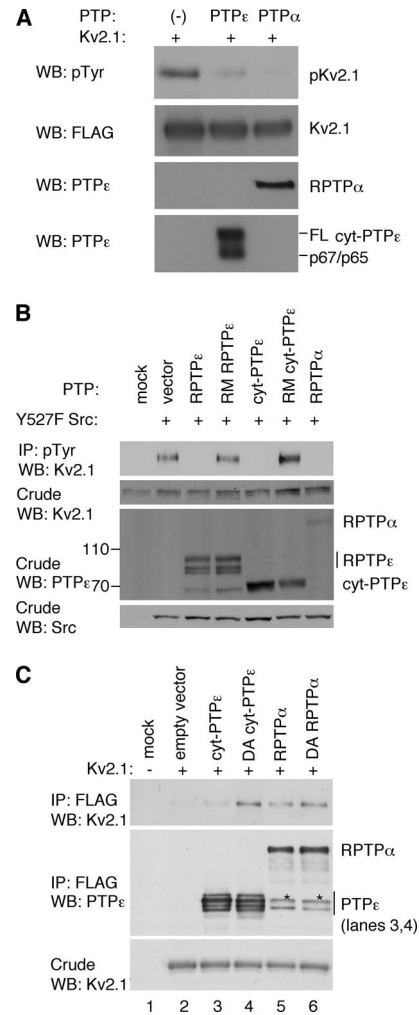
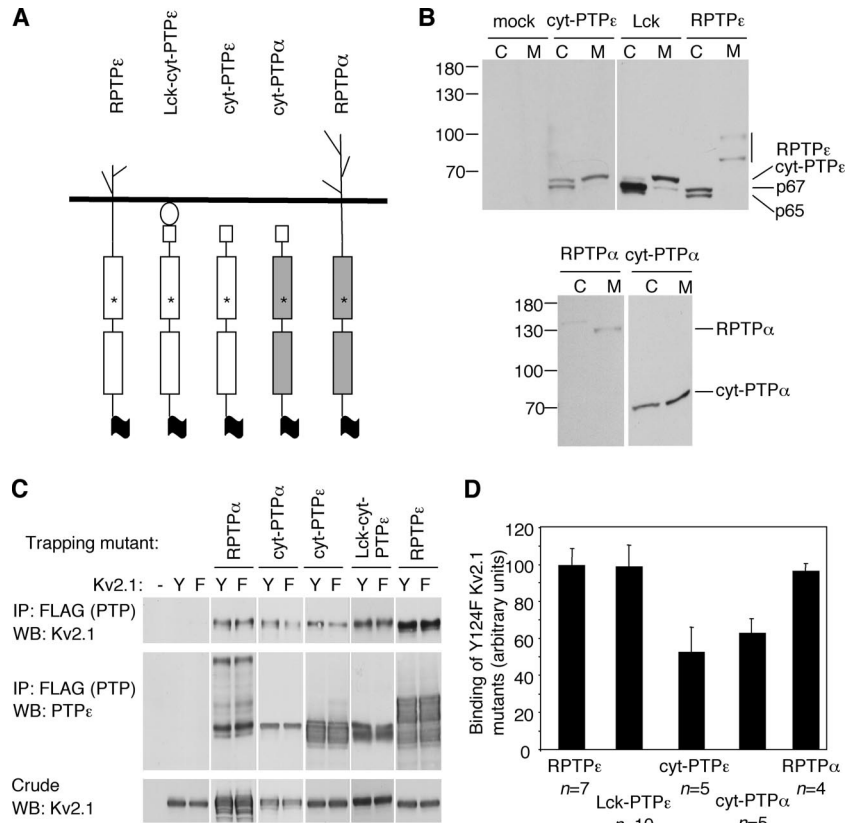


Figure 6. PTP ϵ and PTP α regulate tyrosine phosphorylation of Kv2.1. (A) RPTP α and cyt-PTP ϵ can dephosphorylate tyrosine-phosphorylated Kv2.1 *in vitro*. Top, phosphorylated Kv2.1 remaining after incubation without added PTP or after addition of purified cyt-PTP ϵ or RPTP α as indicated. Remaining panels document addition of purified pKv2.1, cyt-PTP ϵ , and RPTP α to the various reactions. FL, full-length cyt-PTP ϵ . Anti-PTP ϵ antibodies cross-react with RPTP α . (B) RPTP α and cyt-PTP ϵ reduce Src-mediated phosphorylation of Kv2.1 in cells. HEK293 cells stably expressing Kv2.1 were transiently transfected with activated (Y527F) Src, together with RPTP ϵ , cyt-PTP ϵ , RPTP α , or the inactive mutants R340M RPTP ϵ and R283M cyt-PTP ϵ . Cells were lysed and immunoprecipitated with anti-phosphotyrosine antibodies followed by blotting with anti-Kv2.1 antibodies. Blots containing crude extracts were reacted with anti-Kv2.1, anti-PTP ϵ , and anti-Src antibodies to assess expression of these proteins. Blots are from a representative experiment of three performed. (C) Constitutive association of Kv2.1 with RPTP α . HEK293 cells were transiently transfected with Kv2.1 together with FLAG-tagged cyt-PTP ϵ , RPTP α , or their substrate-trapping mutants (D245A cyt-PTP ϵ and D437A RPTP α), as indicated. Following cell lysis and anti-FLAG immunoprecipitation, coprecipitating Kv2.1 was detected by blotting with anti-Kv2.1 antibodies (top). Second and third panels depict amounts of precipitating PTPs and expression of Kv2.1 in the crude lysates, respectively. Asterisks denote p66 PTP α , a cytosolic cleaved form of RPTP α (Gil-Henn *et al.*, 2001).

To tell apart these possibilities, we examined PTP-Kv2.1 interactions in HEK293 cells. Preliminary experiments indicated that RPTP α , RPTP ϵ , and cyt-PTP ϵ can dephosphorylate Kv2.1 after its phosphorylation by activated (Y527F) Src;

Figure 7. Membranal localization of PTPs α and ϵ drives their constitutive interaction with Kv2.1. (A) Schematic representation of the various FLAG-tagged substrate-trapping D-to-A mutants of PTP ϵ and PTP α used in this study: D437A RPTP α , D437A cyt-PTP α , D245A cyt-PTP ϵ , D245A Lck-cyt-PTP ϵ , and D302A RPTP ϵ . The mutated aspartic residue (asterisk) is the same in all cases, although its exact numbering differs among the various molecules. Residue numbering in cyt-PTP α and Lck-cyt-PTP ϵ are as in RPTP α and cyt-PTP ϵ , respectively, for clarity. (B) The subcellular localization of molecules shown in A. After transfection into HEK293 cells, cells were fractionated and the cytoplasmic (C) and membranal (M) fractions were analyzed on 7% SDS-PAGE gels and blotted with anti-PTP ϵ/α antibodies. (C) Constitutive association with Kv2.1 is dependent on membrane localization. HEK293 cells were transiently transfected with WT Kv2.1 (Y) or Y124F Kv2.1 (F) together with the indicated FLAG-tagged substrate-trapping D-to-A mutants. After cell lysis and anti-FLAG immunoprecipitation, amount of coprecipitating Kv2.1 was assessed by blotting. Shown is a representative set of blots documenting coimmunoprecipitation of WT versus Y124F Kv2.1 with the various trapping mutants used. Also shown are amounts of precipitating PTP and Kv2.1 expression in the crude lysates (second and third panels, respectively). (D) Bar diagram showing Y124F Kv2.1 coimmunoprecipitated with substrate-trapping D-to-A mutants normalized to protein content. Values (mean \pm SEM) are relative to association of WT Kv2.1 with each phosphatase. D437A cyt-PTP α and D245A cyt-PTP ϵ exhibit reduced binding to Y124F Kv2.1 compared with WT Kv2.1 (63.3 ± 10.4 and $55.5 \pm 13.8\%$, respectively; $p = 0.03$ – 0.05 ; Welch's t test). In contrast, the membrane-bound mutants D437A RPTP α , D245A Lck-cyt-PTP ϵ , and D302A RPTP ϵ bind WT and Y124F Kv2.1 similarly.



as expected, the catalytically inactive mutants R283M cyt-PTP ϵ and R340M RPTP ϵ did not affect Kv2.1 phosphorylation (Figure 6B). Src phosphorylates Kv2.1 mainly at Y124 (Tiran *et al.*, 2003), indicating that all three PTP molecules examined efficiently dephosphorylate this site in Kv2.1. Activated Src was used here in order to prevent the PTPs from dephosphorylating and activating the kinase, thereby complicating interpretation of the results.

We next compared the ability of substrate-trapping mutants of cyt-PTP ϵ and RPTP α to bind WT Kv2.1. Mutants of this type (D245A cyt-PTP ϵ and D437A RPTP α) are almost entirely catalytically inactive, but their catalytic sites can still bind phosphorylated substrates (Flint *et al.*, 1997; Peretz *et al.*, 2000; Tiran *et al.*, 2003; Blanchetot *et al.*, 2005). Several-fold more Kv2.1 coprecipitated with the substrate-trapping D245A mutant of cyt-PTP ϵ than with WT cyt-PTP ϵ (Figure 6C), indicating that a significant part of the interactions between Kv2.1 and cyt-PTP ϵ is mediated by the active site of PTP ϵ . In contrast, similar amounts of Kv2.1 associated with the WT RPTP α and with its substrate-trapping mutant D437A RPTP α (Figure 6C). The interactions between Kv2.1 and RPTP α are therefore more stable and are likely to involve other regions in addition to the active site, in agreement with the stronger effect of RPTP α on Kv channel activity documented above.

RPTP α is an integral membrane protein with membrane-spanning and extracellular domains. cyt-PTP ϵ lacks such domains; although a fraction of cyt-PTP ϵ molecules are membrane associated, most are cytosolic. To examine the effects of membrane localization on the ability to interact with Kv2.1, we turned to a series of D-to-A substrate-trap-

ping mutants shown in Figure 7A. This series included cyt-PTP α , an artificial cytoplasmic derivative of RPTP α in which the transmembranal and extracellular regions of RPTP α were replaced with the first 12 amino acids of cyt-PTP ϵ . These 12 residues are unique to cyt-PTP ϵ and regulate membranal localization of this isoform (Gil-Henn *et al.*, 2000); access of cyt-PTP α to the cell membrane is similar to that of cyt-PTP ϵ (Figure 7B). In a second construct (Lck-cyt-PTP ϵ), cyt-PTP ϵ was targeted to the plasma membrane by an N-terminal Lck dual myristoylation motif (Zlatkine *et al.*, 1997). This construct is predominantly membrane-associated, but it lacks membrane-spanning and extracellular domains. The series also included RPTP ϵ , the receptor-type isoform of PTP ϵ , as well as cyt-PTP ϵ and RPTP α . All molecules were expressed in HEK293 cells, and their expected subcellular localization was verified by biochemical fractionation (Figure 7B). Full-length cyt-PTP ϵ was mostly localized in the cytoplasmic fraction with a portion present in the membranal fraction, as shown previously (Elson and Leder, 1995a). Adding the Lck motif in Lck-cyt-PTP ϵ resulted in a significant shift of full-length cyt-PTP ϵ to the membrane fraction; the receptor-type RPTP ϵ was found exclusively in the membrane fraction. In all three cases, p67 and p65, which are coexpressed with the full-length PTP ϵ molecules but which lack membrane-targeting sequences, were found in the cytosol. RPTP α was found exclusively at the cell membrane, and cyt-PTP α was expressed at similar levels in the cytoplasmic and membranal fractions. The cellular expression patterns of these two PTP α proteins are in good agreement with their PTP ϵ counterparts—RPTP ϵ and cyt-PTP ϵ , respectively.

We next examined the interactions between the molecules shown in Figure 7A and Kv2.1. We had shown previously that Y124 of Kv2.1 is a major site targeted by cyt-PTP ϵ and RPTP α ; accordingly, tyrosine phosphorylation of Y124F Kv2.1 is decreased by 70%, and binding of D245A cyt-PTP ϵ to this mutant is decreased by ~50% compared with WT Kv2.1 (Tiran *et al.*, 2003) (Tiran and Elson, unpublished data). We reasoned therefore that if binding of a particular D-to-A PTP mutant to Kv2.1 relies mainly on interactions mediated by the PTP catalytic domain, significantly less PTP would bind Y124F Kv2.1. In contrast, PTP-Kv2.1 binding in which interactions outside of the PTP catalytic domain are prominent would not be affected as strongly by the Y124F mutation in Kv2.1. The set of molecules of Figure 7A was therefore constructed as D-to-A substrate traps with a FLAG tag at their C termini. Each PTP was coexpressed with WT or Y124F Kv2.1 in HEK 293 cells. After PTP immunoprecipitation, levels of coprecipitated Kv2.1 were assessed by blotting the precipitated material with anti-Kv2.1 antibodies (Figure 7, C and D); the amount of precipitated Kv2.1 was normalized for expression levels of Kv2.1 protein. Care was taken to use data only from experiments in which for a given PTP expression levels of the PTP trapping mutant and of WT and Y124F Kv2.1 in the cells compared were similar.

Examination of binding of the various PTP substrate-trapping mutants to WT versus Y124F Kv2.1 revealed a clear distinction between receptor-type and nonreceptor-type forms of the PTPs (Figure 7D). Approximately 45% less Y124F Kv2.1 coprecipitated with D245A cyt-PTP ϵ compared with WT Kv2.1, despite similar expression of both forms of Kv2.1 and similar precipitation of D245A cyt-PTP ϵ in these cells (Figure 7C). Less Y124F Kv2.1 also coprecipitated with D437A cyt-PTP α compared with WT Kv2.1; in both cases the reductions in association were statistically significant. In contrast, associations of D302A RPTP ϵ with WT and Y124F Kv2.1 were comparable; similar results were obtained using D437A RPTP α (Figure 7D). Together, these results indicate strongly that the receptor-type forms of both PTPs can bind Kv2.1 constitutively, whereas their cytosolic forms do so much less efficiently; strong binding segregates with cellular localization and not with PTP identity. The differences observed between RPTP α and cyt-PTP ϵ in binding Kv2.1 are therefore not the result of sequence differences between these two distinct PTPs or in the phosphotyrosine residues they target in Kv2.1, but rather they are the result of one of them being a receptor-type molecule, whereas the other is not.

A question left unresolved was whether RPTP α and RPTP ϵ bind Kv2.1 constitutively because their membrane localization makes Kv2.1 more readily accessible or because their membrane-spanning and extracellular domains mediate additional interactions with Kv2.1. To resolve this issue, binding of D245A Lck-cyt-PTP ϵ to WT and to Y124F Kv2.1 was compared. This membrane-associated protein behaved similarly to the two receptor-type forms and bound similar amounts of WT and of Y124F Kv2.1, despite lacking membrane spanning and extracellular domains (Figure 7, C and D). We therefore conclude that the membrane-spanning and extracellular domains of RPTP ϵ and RPTP α do not play a significant role in binding Kv2.1. Localization at the cell membrane is sufficient to drive constitutive association between PTPs ϵ and α and Kv2.1. The inherent differences between the effects of either PTP on this channel protein are most likely the result of their differing patterns of subcellular localization in Schwann cells.

DISCUSSION

Comparison of the phenotypes of EKO and AKO mice indicates that the functional overlaps and interactions between PTPs ϵ and α in Schwann cells are complex and context dependent. Analyses of sciatic nerve axons of 5-d-old mice revealed significant reductions in myelin sheath thickness in AKO, EKO, and DKO mice compared with WT mice. The effects of lack of PTP α and PTP ϵ were additive, with DKO mice most severely affected. These results indicate that both PTP α and PTP ϵ support sciatic nerve myelination in a manner that is at least partially nonredundant and that the time period when this role is most significant is in early postnatal life. The transient nature of the myelination phenotype implies that as mice age, the need for PTP ϵ and PTP α in regulating myelination decreases or that other PTPs compensate for lack of these two PTPs. Possible candidates for such compensation include CD45 (Nakahara *et al.*, 2005), SHP-1 (Massa *et al.*, 2004), PTP ζ (Harroch *et al.*, 2002), and PTP σ (Wallace *et al.*, 1999), because mice separately lacking these PTPs exhibit defects in myelination and/or in recovery of myelination after damage to neurons. Nonetheless, existence of myelination defects in postnatal EKO, AKO, and DKO mice indicates that PTP ϵ and PTP α perform a unique function in Schwann cells and that other gene products cannot replace these enzymes at this developmental stage.

Previous reports have established a correlation between decreased Kv channel activation on the one hand, and exit of Schwann cells from the cell cycle and onset of myelination on the other hand (Sobko *et al.*, 1998a; MacFarlane and Sontheimer, 2000). Increased Kv channel activity observed in Schwann cells of AKO and EKO mice can suggest that loss of cyt-PTP ϵ or RPTP α reduces myelination by preventing the normal decrease in Kv channel activation that occurs as Schwann cells mature in early postnatal mice. However, this possibility does not hold up well in the more complex setting of DKO mice, where the largest decreases in myelination occur together with less-than-maximal increases in Kv channel activation. Together, our observations suggest that RPTP α and cyt-PTP ϵ can affect sciatic nerve myelination by additional mechanisms beyond regulation of Kv channels. For example, a possible additional mechanism for linking PTP activation and Schwann cell-mediated myelination is cell adhesion, which is necessary for proper axonal myelination. The involvement of RPTP α in cell adhesion is well established, evident in its ability to control integrin-mediated responses through activation of Src (Su *et al.*, 1999), to form a complex with contactin (Zeng *et al.*, 1999), to regulate integrin-stimulated focal adhesion kinase autophosphorylation and cytoskeletal rearrangement in cell spreading and migration (Zeng *et al.*, 2003), and to participate in neural cell adhesion molecule-mediated signaling (Bodrikov *et al.*, 2005). cyt-PTP ϵ is also involved in cell adhesion, because osteoclasts from EKO mice do not adhere well to matrix and display significantly disorganized adhesion structures (Chiusaroli *et al.*, 2004). Further studies will indicate whether these effects participate in regulating myelination in this system. Finally, we note that axons from AKO and EKO mice are slightly thicker than their WT counterparts. Thicker axons are often more heavily myelinated; hence, reduced myelination in AKO and EKO axons is probably not caused by this change in axon diameter. Conversely, absence of both PTPs results in a slight reduction in axon diameter in DKO mice, which may possibly contribute to the reduced myelination of these axons. In all, these results indicate that absence of either or both PTPs can affect the axons themselves as well. Further studies are required to better understand this issue.

The experimental system studied here allows better understanding of the molecular mechanisms by which PTPs ϵ and α regulate Kv channels. Loss of either *cyt-PTP ϵ* or *RPTP α* in primary Schwann cells resulted in marked increases in maximal K⁺ current density and in increased tyrosine phosphorylation of Kv2.1. Importantly, these results indicate that the ultimate physiological effect of either PTP in Schwann cells *in vivo* is to inhibit phosphorylation and activation of Kv channel proteins. In principle, increased K⁺ current density may be caused by an increase in the number of functional channels available at the plasma membrane, a rise in *P_o*, and/or elevated channel unitary conductance. Our single-channel analyses indicate that the increase in Schwann cell K⁺ current density observed in the PTP knockout mice cannot be accounted for by differences in *P_o* or in unitary conductances and most likely results from increased numbers of functional channels available at the plasma membrane. The gross expression levels of Kv2.1 and Kv1.5 in primary Schwann cells from all four genotypes are similar (Figure 5A; our unpublished data). However, channel availability may be regulated by more subtle means such as interactions with other proteins or subcellular localization of channel molecules. The latter phenomenon has been described recently in studies of hippocampal neurons, which showed that neuronal activity and ischemia cause rapid dephosphorylation of Kv2.1 channels and translocation from clusters to a more uniform localization (Misonou *et al.*, 2004, 2005). In this regard, we previously showed that Kv2.1 channels are among other delayed rectifier channel subunits, which prominently encode IK currents in Schwann cells (Sobko *et al.*, 1998a,b). Hence, our findings prompt the need to consider a possible role for phosphorylation and dephosphorylation (by PTPs α and ϵ) in dynamic modulation of IK/Kv2.1 localization.

By which molecular mechanisms do *RPTP α* and *cyt-PTP ϵ* regulate Kv channels? We have shown that both PTPs can dephosphorylate Kv2.1 *in vitro* and *in vivo*. However, the observation that absence of *RPTP α* increased Kv channel activity *in vivo* significantly more than absence of *cyt-PTP ϵ* indicates that there are additional molecular parameters that differ between both PTPs. One such likely parameter that was examined is Src activation. The data indicate that in the system examined here, there is clear distinction between both PTPs in this respect. The role of *cyt-PTP ϵ* seems to be direct: *cyt-PTP ϵ* dephosphorylates and down-regulates Kv channels without affecting Src. Loss of this PTP increases channel protein phosphorylation and current amplitude but does not change Src activity. In contrast, the role of *RPTP α* seems more complex and contains both direct and indirect components. Absence of *RPTP α* inhibits Src activity strongly but also activates Kv channels strongly. Src activates Kv channels in Schwann cells; hence, reduced Src activity in the absence of *RPTP α* would be expected to reduce Kv channel activity. Activation of Src by *RPTP α* is therefore not the only mechanism by which this PTP affects Kv channels in Schwann cells. We conclude that although *cyt-PTP ϵ* and *RPTP α* differ significantly in their abilities to regulate Src in this system, this factor is not sufficient to explain the full extent of their different abilities to regulate Kv channel activity.

The strong constitutive association that exists between *RPTP α* and Kv2.1 may provide an additional mechanism to account for the stronger effect of *RPTP α* on Kv channel activity. Interestingly, strong constitutive interactions do not exist between *cyt-PTP ϵ* and Kv2.1, with the main cause of this difference being the distinct cellular localizations of *RPTP α* and *cyt-PTP ϵ* . Constitutive binding between *RPTP α*

and Kv2.1 may affect Kv2.1 in several ways. Although Y124 of Kv2.1 is a major target of both *RPTP α* and *cyt-PTP ϵ* (Figure 6) (Tiran *et al.*, 2003), it is formally possible that its greater access allows *RPTP α* to target additional tyrosines besides Y124 in Kv2.1. Alternatively, constitutive interactions may influence channel activity by regulating access of regulatory molecules to channel proteins or by affecting channel protein conformation. Support for these latter possibilities comes from our demonstration in this study that WT *RPTP α* binds Kv2.1 constitutively at locations in addition to Y124. This notion is also consistent with interactions documented to exist between *RPTP α* and the N and C termini of the related channel protein Kv1.2, regions known to be important in regulating the activities of *Shaker*-type potassium channels (Tsai *et al.*, 1999).

Of particular interest is the observation that Kv channel activity in DKO Schwann cells is *reduced* relative to that measured in AKO cells (Figure 3B). Because results from the single-knockout cells indicate that removal of either PTP on its own activates the channels, this result is counterintuitive and suggests that *cyt-PTP ϵ* may also play a role in promoting Kv channel activity specifically in the absence of *RPTP α* . This role is most likely not mediated by c-Src, because *cyt-PTP ϵ* does not affect c-Src activity in these cells (Figure 5, C and D). One of several alternative possibilities is that *cyt-PTP ϵ* interacts with a negative regulator of Kv channels and prevents this regulator from affecting the channels. According to this model, removal of *cyt-PTP ϵ* in the absence of *RPTP α* prevents *cyt-PTP ϵ* -mediated dephosphorylation but also releases the negative regulator, which inhibits Kv channel molecules and decreases overall Kv channel activity in DKO mice relative to AKO mice. Presumably, removal of *cyt-PTP ϵ* in the presence of *RPTP α* does not cause this effect due to the already existing significant inhibition of Kv channels by *RPTP α* . This model may be challenged by identification of molecules that interact with *cyt-PTP ϵ* in Schwann cells.

A molecular scheme that summarizes the known differences between the functions of *cyt-PTP ϵ* and *RPTP α* versus Kv2.1 in this system is presented in Figure 8. Both PTPs down-regulate Kv2.1 activity by dephosphorylating the channel molecule (Figure 8A). This interaction between the active site of the PTP and Y124 of Kv2.1 is the major interaction between Kv2.1 and *cyt-PTP ϵ* , in agreement with its nonconstitutive nature and strong dependence on Y124 phosphorylation. *RPTP α* performs the same function, but in addition it interacts with Kv2.1 also at other sites. These latter interactions are constitutive and are not dependent upon phosphorylation of Kv2.1 at Y124. Further studies are required to identify specific domains of *RPTP α* that are involved, but the C-terminal PTP domain, which has been suggested to fulfill regulatory roles in several PTPs, is a possibility. Several such interactions can be hypothesized, and one is illustrated in Figure 8A. Introduction of Src into this model reveals that both PTPs can antagonize Src-mediated activation of Kv2.1 (Figure 8B). In agreement with our previous studies of EKO mice (Peretz *et al.*, 2000), a PTP-PTK-common substrate triangle exists therefore in Schwann cells, in which the PTP and PTK antagonize each other's function toward their common substrate, the Kv channel protein. The data suggest that this is a complete account of the events that occur between *cyt-PTP ϵ* , Src, and Kv2.1 in Schwann cells (Figure 8B, left). *RPTP α* , in contrast, can also strongly activate Src in this system (Figure 8B, right). *RPTP α* therefore fulfills two roles that can antagonize each other: direct inhibition of Kv2.1 by its dephosphorylation and indirect activation of Kv2.1 by dephosphorylation and activa-

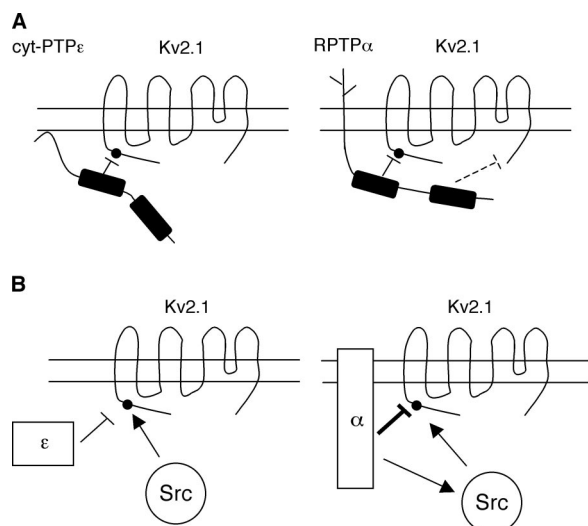


Figure 8. Schematic model illustrating the distinct mechanisms by which cyt-PTP ϵ and RPTP α regulate Src and Kv2.1 in Schwann cells. (A) Distinct modes of interaction with Kv2.1. Left, cyt-PTP ϵ down-regulates Kv2.1 by dephosphorylating the channel protein at Y124 (dark dot). Right, in addition to dephosphorylating Kv2.1 at Y124, RPTP α inhibits Kv2.1 activity by interacting with the channel protein at other locations. One such hypothetical interaction is marked by the dashed line. (B) Differences in the PTP-Kv-Src triangle. Left, cyt-PTP ϵ does not interact with Src in Schwann cells and acts only to counteract activation of Kv2.1 by Src-mediated phosphorylation at Y124. Right, RPTP α regulates Kv2.1 also, but not exclusively, by antagonizing Src activity at Y124 of Kv2.1. The overall inhibitory effect of RPTP α is stronger than that of cyt-PTP ϵ . RPTP α can also dephosphorylate and activate Src, thereby contributing indirectly to activation of Kv2.1. Y124 is noted by the dark dot near the N terminus of Kv2.1. Dark rectangles represent the PTP domains of cyt-PTP ϵ and RPTP α ; in both cases, activity is associated mainly with the N terminal PTP domain, whereas the C terminal PTP domain performs mainly regulatory roles.

tion of Src. Data presented in this study show that the overall effect of RPTP α is to inhibit Kv channel activity strongly under the conditions in which the Schwann cell system was studied here. These results raise the need to postulate a regulatory mechanism that can coordinate between these opposing effects of RPTP α . Recent studies have suggested that phosphorylation of RPTP α at its C-terminal Y789 may preferentially direct RPTP α to activate Src by exposing Y527 of the kinase to dephosphorylation by RPTP α (Zheng *et al.*, 2000). It is therefore tempting to speculate that the balance between the two opposing roles of RPTP α toward Kv2.1 may be regulated by phosphorylation of RPTP α by upstream kinases in response to physiological signals.

An additional conclusion from our studies is that the ability of PTP ϵ to activate c-Src is context dependent. Although cyt-PTP ϵ can activate c-Src in other systems (Gil-Henn and Elson, 2003), it does not do so in the system examined here, indicating somewhat unexpectedly that PTP ϵ and PTP α do not always regulate Src redundantly *in vivo*. Furthermore, the ability to bind Kv2.1 stably is linked with membranal localization, and the receptor form of PTP ϵ , RPTP ϵ , binds Kv2.1 stably as RPTP α . Because expression of cyt-PTP ϵ and RPTP ϵ differs among cell and tissue types, the manner in which PTP ϵ proteins regulate Kv channels and their redundancy with RPTP α may be tissue and isoform specific. We conclude that similarities between PTP activities at the molecular level may not always lead to full functional redundancy between them *in vivo*. Finally, we note that DKO mice are viable and

seem to tolerate well loss of both PTPs α and ϵ . It is therefore possible that some of the physiological functions of these PTPs are redundant with those of other PTPs, at least within a laboratory environment. Because genomic analyses have made it highly unlikely that additional members of the type IV subfamily of receptor-type PTPs exist (Alonso *et al.*, 2004; Andersen *et al.*, 2004), these functions may be shared with other, more distantly related PTPs.

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