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Molecular Cloning of Neural Receptor-like Protein Tyrosine Phosphatase Zeta/Beta and Regulation of its mRNA Expression During Development and Following Sciatic Nerve Injury

by

Ji Li

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York

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To my parents,
Yu Li and Zhuo Zhou,
my wife Yiling,
my daughter Sophia,
with love

Abstract

Molecular cloning of neural receptor-like protein tyrosine phosphatase zeta/beta and regulation of its mRNA expression during development and following sciatic nerve injury

by

Ji Li

Advisor: Stephen R.J. Salton, M.D., Ph.D.

Protein tyrosine phosphorylation is recognized as one of the main eukaryotic cell signaling mechanisms. The overall level of cellular protein tyrosine phosphorylation is determined by the dynamic balance between the competing actions of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). Identification of a number of neurotrophin receptors as receptor tyrosine kinases has emphasized the integral role that tyrosine phosphorylation is likely to play within the nervous system. To identify PTP genes that regulate tyrosine phosphorylation during development and regeneration of the nervous system, this thesis describes the molecular cloning of the rat neural receptor-like protein tyrosine phosphatase (RPTP) RPTPζ/β, the molecular mechanisms responsible for generating various isoforms of RPTPζ/β, and the regulation of expression of various RPTPζ/β mRNAs during neural development and following peripheral nerve injury.

I have isolated overlapping rat RPTPζ/β cDNA clones. Analysis indicates that the RPTPζ/β coding sequence has been highly conserved during mammalian evolution.

I have determined that three major RPTPζ/β transcripts are generated through alternative mRNA splicing and transcribed from the same gene. These three transcripts are co-expressed extensively in the developing and mature CNS and PNS. I have also characterized additional RPTPζ/β isoforms that differ in the juxtamembrane region, suggests that regulation of RPTP dimerization may control enzymatic activity of the phosphatase.

Lastly, I have demonstrated that RPTPζ/β mRNAs are greatly induced in distal segments of sciatic nerve following crush lesions. The time course of this induction correlates well with the response of Schwann cells to injury in this paradigm. Furthermore, I found a significant difference in the relative abundance of RPTPζ/β isoforms expressed in the PNS and CNS. The short transcript, which has been shown to be able to induce neurite outgrowth and promote neuronal differentiation, is most abundant in the PNS, whereas the phosphacan transcript, which is often being associated with inhibition of neuronal adhesion and neurite outgrowth, is most abundant in the CNS. These differences could account in part for the strikingly different regeneration capabilities of the PNS and CNS.

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Table of Contents

List of Figures

Chapter 1: Introduction

The protein tyrosine phosphatase (PTP) gene family

Protein tyrosine phosphorylation is recognized as one of the main eukaryotic cell signaling mechanisms. The overall level of cellular protein tyrosine phosphorylation is determined by the dynamic balance between the competing actions of protein tyrosine kinases (PTKs), which catalyze the phosphorylation of specific tyrosine residues, and protein tyrosine phosphatases (PTPs), which remove phosphate groups from tyrosine residues. Both PTKs and PTPs have been shown to play important roles in regulation of intracellular signal transduction pathways.

Protein tyrosine phosphatase (PTP) gene structure

The protein tyrosine phosphatases (PTPs) represent a rapidly growing and diverse family of enzymes that share highly conserved cytoplasmic catalytic domain(s), and they exist in both receptor-like and non-transmembrane intracellular forms (Tonks and Neel 1996). At least 75 PTPs have been identified to date, and data from human genome sequencing predicts the existence of as many as 500 human PTPs. These enzymes are characterized by the presence of one or two conserved catalytic domains of 230-250 residues, within which is contained the consensus catalytic active site. This active site is defined by a unique signature motif, [I/V]HCxAGxxR[S/T]G (where X is any amino acid), that is shared by almost all the PTPs (Fischer *et al.* 1991). Within this motif the invariant cysteine residue has

proven to be essential for phosphatase activity by virtue of its action as a nucleophile during the catalytic reaction (Barford *et al.* 1994b; Zhang *et al.* 1994). Recent crystal structure data from several PTPs has provided further insights into the catalytic mechanisms and regulation of PTP enzymes (Barford *et al.* 1994a; Stuckey *et al.* 1994; Jia *et al.* 1995; Bilwes *et al.* 1996).

The unique feature of the intracellular PTPs is the diversity of domains flanking the single catalytic domain (Mauro and Dixon 1994). These flanking sequences appear to target PTPs to specific intracellular locations, and include the following: 1) Membrane targeting domains, such as the carboxyl-terminal 35 amino acid sequence of PTP1B, which localizes the phosphatase to the cytoplasmic face of the endoplasmic reticulum (ER) (Frangioni *et al.* 1992). 2) Nuclear targeting domains, such as the one that is found in the alternatively-splicing carboxyl-terminal isoform of *Drosophila* DPTP61F, which localizes this PTP to the nucleus (McLaughlin and Dixon 1993). 3) The amino-terminal domains in PTP Meg1 (Gu *et al.* 1991) and PTPH1 (Yang and Tonks 1991) have been shown to be homologous to the band 4.1 superfamily of cytoskeletal proteins, and might therefore function to regulate cytoskeleton-plasma membrane interactions. 4) Several intracellular PTPs containing Src homology 2 (SH2) domains (SHPs) have been identified in various species (Neel 1993; Feng and Pawson 1994). They each have two SH2 domains at their amino terminus, and a single phosphatase domain. The presence of SH2 domains in PTPs implies possible interaction between protein tyrosine kinases (PTKs) and PTPs in cellular signal transduction. At least two SHPs, SHP-1 and SHP-2, are expressed in

vertebrates (Tonks and Neel 1996). Both of these SHPs have been demonstrated to regulate different sets of signaling pathways, and several potential targets have been identified. In addition, depending on the specific signaling pathway, these SHPs may have either positive or negative effects. SHP-1 is expressed at high levels in hematopoietic cells, and the naturally occurring mouse mutant *motheaten (me/me)* has been shown to be caused by the absence of SHP-1 (Shultz *et al.* 1993; Tsui *et al.* 1993). This murine model of SHP-1 deficiency displays broad hematopoietic abnormalities, affecting almost every lineage (Tsui and Tsui 1994). It has been demonstrated that SHP-1 negatively regulates several hematopoietic signal transduction pathways, including those downstream of cytokine receptors, receptor tyrosine kinases (RTKs), and oligomeric receptors (Tonks and Neel 1996). SHP-2 and its *Drosophila* homologue Corkscrew (CSW) are expressed ubiquitously. They have been shown to be mostly positive regulators of RTK signal transduction (Streuli 1996). CSW is a required positive component of several *Drosophila* RTK signaling pathways, including the Torso pathway that is critical for embryonic head and tail development (Perkins *et al.* 1992). Recent data has also suggested that CSW can enhance signaling activation of Sevenless, probably by dephosphorylating the Daughter of Sevenless (DOS) protein (Herbst *et al.* 1996; Raabe *et al.* 1996). Therefore, targeting of intracellular PTPs is an important mechanism that modulates PTP activity in the cell, and may serve to define substrate specificity and phosphatase function.

The receptor-type protein tyrosine phosphatases (RPTPs) possess an extracellular domain, a single transmembrane domain and normally two tandem catalytic domains followed by a short carboxyl-terminal segment. At present, three RPTPs, HPTPβ (Krueger *et al.* 1990), DPTP10D (Krueger *et al.* 1990; Yang *et al.* 1991), and DPTP4E (Oon *et al.* 1993) which have only one catalytic domain have been identified. For most RPTPs, only the N-terminal catalytic domain is thought to be active, while the C-terminal domain is believed to be inactive, serving as a regulator of the catalytic activity and substrate specificity of the N-terminal domain. However, in RPTPs such as HPTPα (Wang and Pallen 1991) and CD45 (Tan *et al.* 1993), the C-terminal domains have been shown to possess catalytic activity, raising the possibility that each catalytic domain might be regulated differently or might display different substrate specificity in response to the binding of distinct ligands.

In contrast to the similarity within the intracellular domains, the extracellular domains of these RPTPs are highly divergent. Interestingly, many extracellular domains of RPTPs contain structural features, such as immunoglobulin domains and fibronectin type III repeats, suggesting possible roles in cell-cell or cell-matrix adhesion. At least five subtypes of RPTPs can be distinguished based on the structural diversity within their extracellular domains (Fischer *et al.* 1991; Barnea *et al.* 1993). The type I RPTP, CD45, has a large unique extracellular segment that is highly glycosylated. The type II RPTPs are characterized by the presence of multiple immunoglobulin (Ig) like domains near the N-terminus that are adjacent to multiple fibronectin type III-like repeats (FN III), with

an arrangement of motifs that resembles the neural cell adhesion molecule (CAM) gene family products such as N-CAM and Ng-CAM/L1/NILE. This raises the possibility that type II RPTPs may take part in cell adhesion processes, thereby controlling growth and differentiation in response to cell-cell or cell-matrix interactions. It has been proposed that binding of these CAM domains of RPTPs to cell matrix constituents or to other CAMs could potentially regulate the phosphatase activity, transducing this interaction into an alteration in the state of phosphorylation of key intracellular regulatory proteins. Two type II RPTPs, RPTPμ and RPTPκ, were shown to mediate cell-cell adhesion through a homophilic binding mechanism in transfected cells (Brady-Kalnay *et al.* 1993; Gebbink *et al.* 1993; Sap *et al.* 1994) However, no change in phosphatase activity was associated with homophilic binding. Rather than regulating activity directly through binding interactions, RPTP activity may therefore be indirectly controlled by restricting the mobility or local concentration of phosphatase on the cell surface, which may in turn restrict the substrates available for dephosphorylation. However, recent data has suggested an alternative functional mechanism for the adhesion of these RPTPs. Both RPTPμ and RPTPκ have been shown to associate with cadherin-catenin complexes in various tissues and cell lines (Brady-Kalnay *et al.* 1995; Fuchs *et al.* 1996; Kypta *et al.* 1996). Cadherins are Ca++ dependent cell adhesion molecules and are a major adhesive component in cell adherens junctions. Cadherins have been shown to play important roles during tissue development and morphogenesis (Takeichi 1991). The association between the intracellular segments

of cadherins and the actin cytoskeleton, mediated by catenin, is important for adhesion. Several protein tyrosine kinases (PTKs) can phosphorylate components of the cadherin-catenin complex with concomitant disruption of adhesion (Gumbiner 1996). RPTPμ and RPTPκ may regulate the tyrosine phosphorylation, and thus the adhesive properties of cadherin-catenin complexes. Therefore, these PTPs probably do not directly promote cell adhesion themselves, but rather transduce signals generated by cell adhesion, possibly by regulating cadherin-catenin function.

The type III RPTPs, such as HPTPβ (Krueger *et al.* 1990) and DPTP99A (Hariharan *et al.* 1991; Tian *et al.* 1991; Yang *et al.* 1991), are characterized by the presence of multiple FN-like repeats. The type IV RPTPs, such as HPTPα (Krueger *et al.* 1990) and HPTPε (Krueger *et al.* 1990) are distinguished by short extracellular segments. These short extracellular segments are highly glycosylated. The functional implication for HPTPα in the nervous system will be discussed in detail below. The type V RPTPs now include RPTPβ/HPTPζ (Krueger and Saito 1992; Levy *et al.* 1993) and RPTPγ/HPTPγ (Kaplan *et al.* 1990; Krueger *et al.* 1990; Barnea *et al.* 1993) which are characterized by the presence of an extracellular domain near the N-terminus which is very similar to the enzyme carbonic anhydrase. Because the RPTPβ/HPTPζ gene is the focus of my thesis, I will discuss this family of RPTPs in greater detail in the next section of this chapter.

Identification of ligands of RPTPs

There are two major challenges in the PTP research field. The first one is to identify potential ligands of RPTPs. The highly divergent extracellular domains suggest that the activity of these RPTPs might be modulated by potential ligands, which upon binding to these extracellular domains can regulate the activity of the intracellular catalytic domains. No such ligand has yet been identified. Because several RPTPs, including CD45 (Tonks *et al.* 1990) display high basal activity *in vitro*, in the absence of ligand binding, it is possible that these RPTPs are constitutively active *in vivo*, and that ligand binding might suppress rather than increase their catalytic activity. Transfection of a chimeric receptor which included the extracellular domain of the EGF receptor and the intracellular domain of CD45 followed by EGF treatment resulted in the partial inhibition of CD45 phosphatase activity (Desai *et al.* 1993). The first success in ligand hunting so far comes from the identification of the adhesion-like homophilic interaction of the two type II RPTPs, RPTPμ (Brady-Kalnay *et al.* 1993; Gebbink *et al.* 1993) and RPTPκ (Sap *et al.* 1994). The receptor tyrosine phosphatase ζ/β (RPTPζ/β) is the first RPTP for which heterophilic ligands have been identified. In fact, a group of proteins including many members of the neural cell adhesion molecule family, the extracellular matrix protein tenascin-C and the novel neurotrophic factor pleiotrophin has been shown to be able to bind to RPTPζ/β (as detailed in the next section of this chapter). However, alteration in phosphatase activity was not detected following homophilic and heterophilic binding. An

alternative proposal is that ligand binding could cause the receptors to aggregate or come into contact with other cell surface antigens or intracellular substrates, generating a positive or negative response depending upon the molecules with which they interact.

Searching for specific substrates of PTPs

The second major challenge is to define the substrate specificities of individual PTPs. The PTPs have been shown to have activities *in vitro* that are 10 to 1000 times greater than those of the PTKs (Fischer *et al.* 1991). Therefore, it is crucial to identify specific substrates for particular PTPs in defined cellular environments. The alternative, that PTPs act as non-specific constitutively active housekeeping enzymes which function solely to antagonize the action of PTKs in the cell seems unlikely. In T lymphocytes, CD45 directly dephosphorylates the C-terminal tyrosine phosphorylation sites of the Src-family tyrosine kinases, activating $p56^{lck}$ and $p59^{fyn}$, which are absolutely required to transmit signals through the T-cell and B-cell antigen receptors (Thomas 1994). The identification of these substrates and other data including the development of CD45-deficient cell lines (Weaver *et al.* 1991) and "knock out " mice (Kishihara *et al.* 1993) have clearly indicated that CD45 functions to positively regulate lymphocyte signal transduction.

However, for most members of the PTP family, specific substrates are unknown. A promising and powerful approach to identify physiological substrates of individual PTPs has recently been developed based on the detailed structural features generated from high resolution crystal analysis of several PTPs (Barford *et al.* 1994a;

Stuckey *et al.* 1994; Jia *et al.* 1995; Bilwes *et al.* 1996). This approach involves the design of mutants for 'trapping' PTP substrates. The ideal substrate-trapping mutants should retain similar affinity to that of the wild type PTP but their catalytic activity should be greatly reduced, so that the enzyme-substrate complex, once formed, is stable enough to withstand isolation and to allow identification of the specific substrate. Some early studies suggested that PTP mutants of the catalytically-essential cysteine reside (Cys—>Ser or Ala mutants) are enzymatically inactive but can retain the ability to bind to substrate in certain situations (Bliska *et al.* 1991; Sun *et al.* 1993). Detailed analysis of the PTP-1B catalytic domain crystal structure identified an invariant aspartate residue, which functions as a general acid to protonate the leaving tyrosyl group on the substrate during catalysis (Zhang *et al.* 1994). This finding has led to the development of PTP mutants of this aspartyl residue (Asp—>Ala mutants), which seem to be a much more efficient substrate traps than the Cys mutants of the same PTP. Use of this Asp—>Ala mutant in the intracellular PTP-PEST has already led to the identification of $p130^{cas}$ as a highly specific substrate (Garton *et al.* 1996). Since this aspartate residue is invariant among PTP family members, this substrate-trapping strategy should be generally applicable to all PTPs, and may be especially important in the identification of additional RPTP substrates.

PTPs in the nervous system

In the nervous system, several PTPs have been shown to play extremely important roles during various events of neural

development. In *Drosophila*, four of the five identified RPTPs were shown to be expressed selectively in the developing CNS axons in late stage embryos, suggesting that these RPTPs may have important functions during neuronal development (Zinn 1993). Recently, mutant *Drosophila* embryos lacking expression of all four axonal RPTPs have been generated (Desai *et al.* 1996; Krueger *et al.* 1996; Desai *et al.* 1997). Disruption of RPTP69D (Desai *et al.* 1996) results in pupal lethality. In the mutant embryos, motor neuron growth cones stop growing before reaching their muscle targets, or follow incorrect pathways that bypass their muscle targets. In the mutant embryos lacking DLAR protein (Krueger *et al.* 1996), similar severe defects in motor axon guidance were found on subsets of different, but overlapping motor neurons. On the other hand, mutants lacking the other two RPTPs DPTP99A or DPTP10D are viable, fertile, and show no detectable phenotype in the embryonic CNS (Hamilton *et al.* 1995; Desai *et al.* 1997). In addition, axonal guidance defects observed in mutants lacking DPTP69D or DLAR are incompletely penetrant (Desai *et al.* 1996; Krueger *et al.* 1996). However, these defects are dramatically enhanced in certain double- and triple-mutant combinations (Desai *et al.* 1996; Desai *et al.* 1997), strongly suggesting that RPTP99A and RPTP10D are also involved in motor axonal guidance, and that all four *Drosophila* RPTPs have overlapping functions during development. These functionally redundant pathways could compensate to some extent for their individual loss. Such redundancy and compensation would ensure proper nervous system connectivity.

In the mammalian nervous system, RPTPα (Krueger *et al.* 1990) has been linked to neuronal differentiation. RPTPα is a ubiquitously expressed type III RPTP with two active intracellular catalytic domains and a very short, highly glycosylated extracellular domain. It has been shown that RPTPα mRNA expression is enhanced during neuronal differentiation of three distinct cell lines, and that overexpression of RPTPα in pluripotent P19 embryonic carcinoma (EC) cells alters the differentiation fate of these cells in favor of a neuronal phenotype (den-Hertog *et al.* 1993). The cytoplasmic PTK c-Src is activated in these RPTPα-overexpressing P19 cells, possibly as a result of direct dephosphorylation of the inhibitory tyrosine site (Tyr-527) of c-Src by RPTPα (Zheng *et al.* 1992). A role for c-Src activation in neuronal differentiation has been demonstrated. So consequently, these data strongly link RPTPα to neuronal differentiation. Furthermore, RPTPα has been shown to be phosphorylated constitutively *in vivo* on tyrosine residue 789, which is 5 amino acids away from the C-terminus (den-Hertog *et al.* 1994; Su *et al.* 1994). Phosphorylation of Tyr-789 creates a binding site for the SH3-SH2-SH3 adaptor protein Grb2, a key component in the RTK-Ras signaling pathway (den-Hertog *et al.* 1994). Auto-dephosphorylation of RPTPα has been observed *in vitro* (den-Hertog *et al.* 1994), and may regulate RPTPα activity. In addition to tyrosine phosphorylation, RPTP-α has also been shown (Tracy *et al.* 1995) to be phosphorylated by protein kinase C (PKC) on two serine residues in the juxtamembrane domain. This phosphorylation may be responsible for the induction of a rapid, transient increase in RPTP-α catalytic activity by treatment of RPTP-α transfected cells

with phorbol ester (a direct activator of PKC) (den-Hertog *et al.* 1995). Additional studies have demonstrated that c-Jun and MAP kinase are activated, possibly by c-Src, in fibroblasts overexpressing RPTPα (Zheng and Pallen 1994). Therefore, a signal transduction pathway for RPTPα leading from cell membrane to the nucleus is beginning to emerge.

Summary

Less than a decade after the determination of the first amino acid sequence for a PTP (Tonks *et al.* 1988; Charbonneau *et al.* 1989), much progress has been made toward our understanding of the biological functions for this family of enzymes. In addition to the 'classical' PTPs discussed above, there are at least two other families of molecules, the dual-specificity phosphatases and the low molecular weight (acid) phosphatases that can also dephosphorylate phosphotyrosyl proteins. These three groups of enzymes together form the tyrosine phosphatase superfamily (Neel and Tonks 1997). There is little overall sequence similarity amongst the three groups other than the conserved catalytic signature motif, but they have similar tertiary structures and share the same general catalytic mechanism, characterized by the formation of a thiophosphate intermediate involving the essential catalytic cysteinyl residue (Denu *et al.* 1996). In addition, the most recent molecular cloning of CEL-1, an RNA 5'-triphosphatase (mRNA capping enzyme) from C. elegans, revealed that it has a domain highly homologous to the catalytic domain of the classical PTPs (Takagi *et al.* 1997). Although CEL-1 has

no detectable PTP activity, the catalytic mechanism of the RNA triphosphatase is similar to that of PTPs: the active site contains a conserved nucleophilic cysteine required for activity. This new result broaden the tyrosine phosphatase superfamily to include enzymes with RNA substrates. PTPs can not only negatively influence protein tyrosine kinase (PTK) signaling transduction, but they can also act as positive signal transducers in other pathways, and even the same PTP can have positive and negative effects in different signaling pathways. Over the past ten years many researchers had hoped to clone PTPs as tumor suppressor genes based on the simple assumption that PTPs directly counter the actions of PTKs which are often discovered to be encoded by proto-oncogenes. Very recently, finally the first such PTP gene, a cytoplasmic PTP named *PTEN* or *MMAC1*, was cloned as a human tumor suppressor gene (Li *et al.* 1997; Steck *et al.* 1997). Multiple mutations in its coding region, including within the PTP signature motif, were detected in human brain gliomas, as well as in prostate, breast and kidney cancers. It appears that this PTP might rank in importance with *p53*, RB, and *p16*, tumor suppressors that have been linked to many types of human tumors. At last, it seems that the critical roles that PTPs play in normal cell function can no longer be ignored.

Receptor protein tyrosine phosphatase ζ/β (RPTPζ/β)

RPTPζ/β gene structure

Receptor tyrosine phosphatase ζ/β was originally cloned independently by two laboratories, and named PTPζ (Krueger and Saito 1992) and RPTPβ(Levy *et al.* 1993), and now is commonly referred to as RPTPζ/β. In the extracellular region, RPTPζ/β is characterized by the presence of a carbonic anhydrase (CA)-like domain at its N-terminus. The CA-like domain is immediately followed by a fibronectin type III (FN III) repeat and a long cysteine-free segment (called spacer domain) extending to the single transmembrane segment. CA is a family of enzymes that catalyze reversible hydration of carbon dioxide to form bicarbonate ion. The physiological functions of CA include CO_2 transport by blood, pH regulation in kidney and CO_2 exchange in mitochondria and chloroplasts. In CAs, a catalytically essential zinc ion is bound to three conserved histidine residues. In RPTPζ/β, two of the three equivalent histidine residues have been changed, presumably preventing the binding of zinc (Krueger and Saito 1992; Levy *et al.* 1993). Therefore, it is unlikely that RPTPζ/β has carbonic anhydrase activity. However, the RPTPζ/β sequence contains most of the other amino acids that are well conserved among CA family members. In addition, molecular modeling studies have suggested that the amino acid sequence of the CA-like domain in RPTPζ/β can be superimposed onto the CA II structure without major distortion (Krueger and Saito 1992; Levy *et al.* 1993). Therefore, the CA-like domain of RPTPζ/β

probably forms a hydrophobic pocket similar to CAs that could be used as a ligand binding site for the phosphatase. Later studies have now validated this hypothesis (see detail below).

The fibronectin type III (FN III) repeat that follows the CA-like domain in RPTPζ/β is 100% conserved among several mammalian species, suggesting the potential functional importance of this domain. FN III repeats are widely found in numerous cell adhesion molecules (CAMs), and extracellular matrix (ECM) proteins and are also present in extracellular regions of many RPTPs. It could be potentially another ligand binding site for RPTPζ/β, just as in some of the other CAMs and ECMs. In fact, there has been report that a protein on neural glial cells is found to bind to the FN III repeat of RPTPζ/β, although the identity of this glial ligand has not been revealed (Peles *et al.* 1995).

The FN III repeat is then followed by the spacer domain, a long stretch of 1048 amino acids that is characterized by a lack of cysteine residues. The length of this spacer is regulated by alternative mRNA splicing, generating an isoform of RPTPζ/β that lacks a stretch of 860 amino acids within the cysteine-free domain. This RPTPζ/β isoform (called the short isoform) with the shortened spacer domain has been shown to have a dramatically different glycosylation pattern, compared to the full length RPTPζ/β isoform (see Discussions in Chapter 4). Furthermore, several possible ligands of RPTPζ/β have been identified that may bind to the spacer segment that is deleted in the short isoform. Therefore, the spacer domains appear to be another very important extracellular functional domain for RPTPζ/β.

RPTPζ/β has two PTPase catalytic domains in their cytoplasmic segment. When expressed in *E. coli*, the cytoplasmic segment of RPTPζ/β had PTPase activity *in vitro* (Krueger and Saito 1992). However, the carboxyl-terminal PTPase domain lacks the catalytically essential cysteine residue, being replaced by an aspartate residue. This critical change makes it very unlikely that this PTPase domain has tyrosine phosphatase activity. Consistent with this prediction, a cysteine —> serine mutation in the catalytically active N-terminal PTPase domain completely inactivates RPTPζ/β (Krueger and Saito 1992).

RPTPζ/β tissue distribution

RPTPζ/β is one of the few mammalian PTPs whose expression is primarily restricted to the nervous system, as demonstrated by results from early Northern blot and RNase protection analysis (Levy *et al.* 1993; Li *et al.* 1993; Maeda *et al.* 1994; Maurel *et al.* 1994). In addition, these analysis also revealed that there are three major RPTPζ/β transcripts (Levy *et al.* 1993; Li *et al.* 1993; Maurel *et al.* 1994). A couple of which encode the two transmembrane RPTPζ/β isoforms that differ in their length of the extracellular spacer domain. The third transcript predicts a soluble isoform that terminates at the end of the spacer domain, lacking the single transmembrane domain and the whole intracellular segment (including the two PTPase catalytic domains).

Detailed *in situ* hybridization analysis has shown that the levels of RPTPζ/β mRNA expression are regulated in a temporal and region-specific fashion during the development and maturation of the

nervous system (Canoll *et al.* 1993; Li *et al.* 1993; Li *et al.* 1994; Engel *et al.* 1996; Snider *et al.* 1996). In rodent, the expression of RPTPζ/β mRNAs can first be detected in the neuroepithelium throughout the length of the newly closed neural tube at embryonic day 11.5 (E11.5). Additional expression can also be seen in the optic vesicles and in the primordia of the dorsal root ganglia of the developing PNS. From E13.5 to E15.5, RPTPζ/β mRNAs can be detected throughout the developing brain and spinal cord, with particularly high levels of expression seen in areas of active cell proliferation, such as the ventricular and subventricular zones. By E16.5 to E19.5, the highest level of expression for RPTPζ/β mRNAs in the ventricular and subventricular zones is detected in the developing CNS. In addition, expression is also seen throughout the developing layers of the cerebral cortex during this period. From postnatal day 0 (P0) to P5, RPTPζ/β mRNA levels are generally higher and more uniformly distributed throughout the CNS. Intense expression can be seen in the subventricular zone of the lateral ventricle, the Purkinje cell layer of cerebellum, the hippocampal pyramidal and dentate granule layers, and the olfactory bulbs and olfactory tubercle. By P10, levels of RPTPζ/β mRNA have markedly decreased to a diffuse pattern throughout the brain. However, those areas with intense expression during the first postnatal week (see above) persist with relatively high levels of expression. In the adult, the overall expression pattern of RPTPζ/β mRNAs seen postnatally was retained. Therefore, the expression of RPTPζ/β mRNAs is dynamically regulated during the timecourse of neural development: high levels of expression correlate

with the peaks of neuronal and glial proliferation, and levels of expression decrease as the nervous system matures.

During development and in adult, three alternative RPTP transcripts are generally co-expressed, predominantly in astrocytes, although relative levels vary. There are, however, several differences in their expression patterns during development. For example, during late embryonic and early postnatal period (Canoll *et al.* 1996; Li *et al.* 1996; Snider *et al.* 1996), the two transmembrane RPTPζ/β mRNAs are found more abundantly in the deepest portion of the neuroepithelium than in the superficial portion, while the expression of soluble RPTPζ/β mRNA is high in both regions, suggesting that the more mature cells which have migrated out of the deep neuroepithelium may preferentially downregulate the two transmembrane RPTPζ/β isoforms while they continue to synthesize the soluble isoform. In postnatal brains, much higher levels of transmembrane RPTPζ/β mRNAs have been found in the ventricular ependyma, neuroepithelium, and subependymal zone relative to their levels elsewhere in the brain (Li *et al.* 1996; Snider *et al.* 1996). In contrast, the soluble RPTPζ/β mRNA is found at low levels in these periventricular regions relative to its expression in the remainder of the brain (Snider *et al.* 1996). These differences in expression are most likely a reflection of regional and possibly cell-type specific RNA splicing, the likely mechanism by which various RPTPζ/β transcripts are generated (see Chapter 4).

The distribution of RPTPζ/β proteins has been investigated by immunocytochemistry. Comparison of the distributions of RPTPζ/β polypeptides, determined by indirect immunofluorescence (Maeda *et*

al. 1995; Meyer-Puttlitz *et al.* 1996), with that of RPTPζ/β mRNA, localized by *in situ* hybridization, suggest that RPTPζ/β polypeptide is more widely distributed than the corresponding mRNA at comparable developmental ages. This is consistent with the possibility that after secretion of the soluble isoform, extensive redistribution of this isoform occurs which may account for the sometimes significantly different results obtained using these two methodologies.

Morphological studies demonstrate that RPTPζ/β is primarily synthesized and expressed in glial cells (Canoll *et al.* 1993; Engel *et al.* 1996; Meyer-Puttlitz *et al.* 1996; Snider *et al.* 1996), such as in the embryonic radial glial cells and in the postnatal Golgi epithelial cells. In addition, data from the study of primary CNS glial cell cultures has shown that the three RPTPζ/β transcripts are also differentially expressed within the glial cell lineage (Canoll *et al.* 1996). O2A oligodendrocyte progenitors express high levels of the full length and soluble RPTPζ/β transcripts, whereas type 1 astrocyte progenitors predominantly express the short transmembrane RPTPζ/β transcript. Moreover, when O2A progenitors differentiate into oligodendrocytes, there is a marked decrease in the expression of the transmembrane isoforms of RPTPζ/β.

Whether there is neuronal localization of RPTPζ/β in addition to the abundant glial RPTPζ/β expression is still controversial. Immunostaining with the 3F8 antibody in brain slices shows no expression at all in neuronal populations (Meyer-Puttlitz *et al.* 1996), whereas staining with another antibody 6B4 in both brain slices and primary neuronal cell cultures shows a predominantly neuronal

expression pattern, as well as some glial staining (Maeda *et al.* 1992; Maeda *et al.* 1995; Maeda and Noda 1996). *In situ* hybridization histochemistry is generally considered a better method for determining the cellular sites of protein synthesis, especially when soluble extracellular protein is involved. Recent results from *in situ* hybridization (Li *et al.* 1996; Snider *et al.* 1996) suggest that RPTPζ/β mRNAs is synthesized by discrete populations of neurons in addition to glia. In rodent, by E19.5, developing neurons and glia in the trigeminal ganglia and cortex express RPTPζ/β mRNAs (Li *et al.* 1996; Snider *et al.* 1996). In the P10 and adult hippocampus, the dentate granule cells and the pyramidal cells of Ammon's horn express the transmembrane RPTPζ/β mRNAs while the soluble RPTPζ/β mRNA is not detectable in these cells until adulthood (Li *et al.* 1996; Snider *et al.* 1996). In the adult CNS, RPTPζ/β mRNAs are found in several subsets of neurons, such as cerebellar Purkinje cells, neurons of the olfactory tubercle and neocortex, dentate granule cells, hippocampal pyramidal cells, photoreceptors, and α-motor neurons, often at levels comparable to those seen in astrocytes (Li *et al.* 1996; Snider *et al.* 1996). The soluble RPTPζ/β isoform, on the other hand, while detectable in many of the same sets of neurons (it was undetectable in α-motor neurons) is invariably expressed at lower levels in neurons than in astroglia (Li *et al.* 1996; Snider *et al.* 1996). The dramatically different immunostaining pattern generated by different antibodies is probably reflects specificity to different epitopes. Both of these monoclonal antibodies were generated against the soluble RPTPζ/β isoform (which has been identified and cloned as a chondroitin sulfate proteoglycan from brain, see below)

in a chondroitin sulfate proteoglycan preparation (Rauch *et al.* 1991; Maeda *et al.* 1992). Therefore, each of the two antibodies may recognize distinct epitopes present on only a subpopulation of RPTPζ/β molecules.

The level of expression of the three RPTPζ/β isoforms are differentially regulated during neural development. This has been investigated by Western blotting using several different antibodies that allow the three isoforms to be distinguished (Sakurai *et al.* 1996). In the CNS, the level of the soluble RPTPζ/β isoform increases dramatically during rat embryogenesis and it is the most prevalent form of RPTPζ/β at most stages of embryonic development and postnatally. Expression of the short transmembrane isoform increases moderately during brain development, whereas the full length RPTPζ/β isoform is least prevalent and is detected primarily during early stages of neural development. On the other hand, analysis of the three RPTPζ/β transcripts by RNase protection assay has revealed that there are major differences in their relative abundance in the CNS and PNS. Unlike in the CNS where the soluble RPTPζ/β isoforms predominantly are expressed, the short transmembrane RPTPζ/β isoform is the most abundant form (see Chapter 4). This differential expression of RPTPζ/β transcripts between CNS and PNS may contribute to the striking difference in regeneration capabilities between the two nervous systems.

Expression of RPTPζ/β as proteoglycan

Molecular cloning of a major chondroitin sulfate proteoglycan (CSPG) purified from rat brain revealed that it is the soluble isoform

of RPTPζ/β (Maeda *et al.* 1994; Maurel *et al.* 1994). This neural tissue specific CSPG was originally purified using the 3F8 monoclonal antibody, and was renamed phosphacan (Maurel *et al.* 1994). Phosphacan accounts for about 40% of the total soluble CSPG proteins in rat brain. It contains a 173 kDa core protein and three 28 kDa chondroitin sulfate chains that show a developmental increase in 4- versus 6-sulfation (Rauch *et al.* 1991). There are also chondroitin/keratan sulfate-containing glycoforms of phosphacan (phosphacan-KS) that seems to account for all of the PBS-soluble keratan sulfate proteoglycans of rat brain (Rauch *et al.* 1991).

Phosphacan concentrations rapidly increase during the late embryonic and early postnatal period, remaining high in mature brain (Meyer-Puttlitz *et al.* 1995). The keratan sulfate on phosphacan-KS is not detectable until early postnatal days, and its peak concentration is reached about one week later than that of the phosphacan core protein. In adult brain, phosphacan-KS account for more than half of the total phosphacan expressed.

The full length form of RPTPζ/β has been shown to be expressed as a CSPG, and its overall glycosylation pattern is similar to that of phosphacan (Sakurai *et al.* 1996). However, the short RPTPζ/β isoform is primarily not expressed as a proteoglycan (Sakurai *et al.* 1996), presumably due to the fact that all of the potential glycosaminoglycan (GAG) attachment sites that are actually utilized are located within the deleted spacer segment.

In addition, RPTPζ/β isoforms also undergo other types of glycosylation (Margolis *et al.* 1996). RPTPζ/β contains many potential serine and threonine O-glycosylation sites, most of which are located

within the deleted spacer segment in the short RPTPζ/β isoform. RPTPζ/β also bears the HNK-1 carbohydrate epitope, which is found in many neuronal adhesion molecules and has been implicated in cell recognition and axonal guidance.

Therefore, various isoforms of RPTPζ/β undergo complicated developmental changes in glycosylation, sulfation and relative abundance. Although the exact functional significance for many of these modulations remains to be determined, the magnitude and complexity of these changes suggest that they represent a very important mechanism for the regulation of RPTPζ/β interactions with its heterophilic ligands, including members of the cell adhesion molecules (CAMs) and extracellular matrix (ECM) proteins.

Interactions of RPTPζ/β with ligands and neural cell function

One of the major challenges of PTP research is to identify specific extracellular ligands for individual RPTPs. RPTPζ/β is the first and the only RPTP whose heterophilic ligands have been clearly identified. In fact, a group of proteins including many members of the immunoglobulin (Ig) superfamily neural cell adhesion molecules (CAMs), the extracellular matrix (ECM) protein tenascin-C and the novel growth factors pleiotrophin and midkine have been shown to be able to bind to RPTPζ/β. The interactions between various isoforms of RPTPζ/β and its ligands have been shown to have differential effects on neural cell function.

The initial evidence for possible interaction between RPTPζ/β and neural CAMs came before the molecular cloning of phosphacan. In a Covasphere aggregation assay, phosphacan was found to inhibit the

homophilic aggregation of microbeads coated with N-CAM and Ng-CAM/L1/NILE (Grumet *et al.* 1993). Subsequent radioligand binding assays demonstrated that phosphacan specifically bound to the two neural CAMs (Milev *et al.* 1994). Binding was saturable, reversible and of high affinity, with a K_d of about 0.1 nM. Phosphacan binding was reduced by only about 15% after chondroitinase treatment (which digests the chondroitin sulfate GAG chains) and free chondroitin sulfate was merely inhibitory, strongly suggesting that the phosphacan core glycoprotein accounts for most of the binding activity to the two neural CAMs. In addition, immunocytochemical localization of phosphacan in embryonic spinal cord and postnatal cerebellum has been shown to overlap with that of the two CAMs, suggesting the biological relevance of their interaction *in vivo* (Milev *et al.* 1994).

The interaction between phosphacan and the two neural CAMs has been shown to have a profound effects on neural cell functions such as neural adhesion and neurite outgrowth. Phosphacan can inhibit neuronal adhesion to Ng-CAM when mixtures of the two proteins are absorbed to culture dishes prior to plating of dissociated neurons (Grumet *et al.* 1994). Similar result have also been obtained using rat C6 glioma cells which express high levels of RPTPζ/β isoforms. Phosphacan specifically inhibited adhesion of C6 cells to Ng-CAM (Milev *et al.* 1994). Direct binding of neurons to phosphacan has also been shown, and the binding was completely inhibited by antibodies against Ng-CAM and N-CAM, suggesting that these CAMs are major receptors for phosphacan on these neurons (Milev *et al.* 1994). Under similar experimental condition as in the neuronal adhesion

assay, phosphacan also inhibited neurite outgrowth mediated by Ng-CAM in a dose-dependent manner, as evidenced by the dramatic decrease of mean neurite length in dishes coated with mixtures of phosphacan and Ng-CAM (Milev *et al.* 1994). Both the native phosphacan and its core glycoprotein were effective in inhibiting neurite outgrowth (Milev *et al.* 1994). Because it is known that phosphacan is expressed abundantly in areas of CNS in which neuronal migration and neurite outgrowth occur during development, it is not likely that phosphacan simply inhibits these processes. Instead, phosphacan may modulate CAM-mediated adhesion and neurite growth, and that relative concentrations of phosphacan and CAMs and the sequence of their expression under specific cellular context will influence neuronal migration and process outgrowth. In addition, phosphacan molecules (including phosphacan-KS), isolated using 6B4 antibody, have been shown to be a general repulsive substratum for neurons (Maeda and Noda 1996). However, these phosphacan can still stimulate neurite outgrowth of embryonic cortical neurons, when these neurons were cultured on polylysine or fibronectin substrates mixed with phosphacan (Maeda and Noda 1996). On the other hand, these phosphacan molecules have no obvious effect on thalamic neurons. Since phosphacan molecules identified with different antibodies show dramatically different expression patterns during CNS development, different subpopulations of phosphacan glycoforms are probably recognized by these various antisera. Therefore, various glycoforms of phosphacan may have distinct effects on neural cell functions, depending on the specific cell types and on the effects of other CAMs

and ECM proteins that may be present in their immediate environment.

Potential interaction sites on phosphacan/RPTPζ/β that are responsible for its binding to N-CAM and Ng-CAM have been identified. In one study, two glycopeptides generated by tryptic digest of phosphacan were shown to bind to N-CAM and Ng-CAM (Milev *et al.* 1995). These two peptides were located at the end of CA-like domain and in the FN III repeat. Further analysis of the two peptides suggested that the interactions of phosphacan/RPTPζ/β with N-CAM and Ng-CAM are mediated by complex-type asparagine-linked oligosaccharides present in their CA-like domain and FN III repeat (Milev *et al.* 1995). However, there may be additional binding sites responsible for the high affinity interactions between phosphacan/RPTPζ/β and the two neural CAMs, because the affinity for most oligosaccharide-lectin interactions are several orders of magnitude lower than those observed (Margolis *et al.* 1996). In another study (Sakurai *et al.* 1997), recombinant proteins containing the spacer (S) domain from the short RPTPζ/β isoform have been shown to bind to N-CAM and Ng-CAM, and S domain protein was able to potentiate neurite outgrowth when mixed with CA-like and FN III domains. While the S domain by itself had no effect on neurite outgrowth, it did seem to be repulsive for cell adhesion (Sakurai *et al.* 1997). Thus, multiple binding sites on phosphacan/RPTPζ/β may exist for the same ligand, and each of the binding site may exert different effects on neural cell function.

Another member of the Ig superfamily of neural cell adhesion molecules, TAG-1/axonin-1, was also found to be a high affinity

ligand of RPTPζ/β in radioligand binding assays *in vitro*, with a k_d of 0.04 nM (Milev *et al.* 1996). Unlike the interaction with N-CAM and Ng-CAM, phosphacan binding to TAG-1 was decreased by about 70% following chondroitinase treatment, suggesting important contributions of chondroitin sulfate GAG chains to the binding interaction. In addition, N-deglycosylation of phosphacan had no effect on its binding to TAG-1, indicating no involvement of N-linked oligosaccharides on phosphacan to the binding. Overlapping localization patterns in nervous tissue between phosphacan/RPTPζ/β and TAG-1 further support their possible interaction *in vivo*.

In order to search for additional RPTPζ/β ligands, fusion proteins containing individual extracellular RPTPζ/β domains have been used in an expression cloning strategy (Peles *et al.* 1995). The CA-like domain bound specifically to a 140 kDa protein on the surface of neuronal cells. Expression cloning revealed that this protein is contactin/F3/F11, a GPI membrane-anchored neural cell adhesion molecule that also belongs to the Ig superfamily. In addition, the CA-like domain has also been shown to induce cell adhesion and neurite outgrowth of primary neurons (Peles *et al.* 1995). These responses were blocked by antibodies against contactin, indicating that contactin is a neuronal ligand of RPTPζ/β.

As a GPI-linked cell adhesion molecule, contactin has been implicated in transmembrane signal transduction, but most likely through an indirect mechanism. It may associate with other transmembrane proteins within the same plasma membrane to relay the signal into the cytoplasm. Such lateral (*cis*) interactions have been shown within the context of the trans-interaction of contactin

with RPTPζ/β. Nr-CAM, a neural CAM closely related to Ng-CAM, was found to form a complex laterally with contactin (Sakurai *et al.* 1997). This complex was found to bind to RPTPζ/β through direct binding between contactin and the RPTPζ/β CA-like domain. Neurite outgrowth induced by extracellular domains of the short RPTPζ/β isoform was inhibited by antibodies against Nr-CAM and contactin, indicating that binding of RPTPζ/β to the contactin/Nr-CAM complex is critical for signal transduction of neurite growth. Another candidate that may serve as a signaling subunit of contactin has recently been identified as a novel transmembrane receptor Caspr (Contactin-associated protein) (Peles *et al.* 1997b). Caspr represents a mammalian homologue (Peles *et al.* 1997a) for *Drosophila* Neurexin IV, a member of the neurexin gene family that encode a large number of neuronal cell surface proteins that are involved in cell-cell interactions and target recognition (Ullrich *et al.* 1995). Caspr interacted with contactin by means of lateral association in the plasma membrane (Peles *et al.* 1997b). Contactin can bind simultaneously to both Caspr and the CA-like domain of RPTPζ/β, forming a ternary complex of these three proteins. The cytoplasmic domain of Caspr contained a proline-rich sequence capable of binding to a subclass of SH3 domains of signaling molecules(Peles *et al.* 1997b). Therefore, Caspr may function as a signaling component of contactin to recruit and activate intracellular signaling pathways in response to interactions of contactin to RPTPζ/β with the opposing cells.

In addition to the four neural CAMs identified as ligands for RPTPζ/β, the extracellular matrix (ECM) protein tenascin-C has also

been demonstrated to bind to phosphacan with high affinity (apparent k_d = 3 nM) (Grumet *et al.* 1993; Barnea *et al.* 1994; Grumet *et al.* 1994). Similar to the interaction of phosphacan with N-CAM and Ng-CAM, the binding to tenascin-C was saturable and reversible. The binding was also insensitive to chondroitinase treatment, and was mediated at least in part by N-linked oligosaccharides on phosphacan. Recently, the fibrinogen-like globe of tenascin-C has been demonstrated to mediate its interaction with phosphacan/RPTPζ/β (Milev *et al.* 1997). Phosphacan also inhibited the adhesion of C6 glioma cells to tenascin-C, an effect that is apparently mediated by a direct repulsive action of phosphacan on the cells rather than by its interaction with the phosphacan binding site on tenascin-C (Milev *et al.* 1997).

Besides its abilities to bind to high molecular weight proteins such as CAMs and ECM components, phosphacan/6B4 proteoglycan has also been shown to bind to low molecular weight proteins, pleiotrophin/heparin-binding growth-associated molecule (HB-GAM) and midkine, members of a novel mitogenic and neurite-promoting growth factor gene family (Rauvala and Peng 1997). Pleiotrophin was identified as a ligand by means of affinity chromatography using a matrix coupled with 6B4 proteoglycan/phosphacan (Maeda *et al.* 1996). It bound to phosphacan with low (K_d = 3 nM) and high (K_d = 0.25 nM) affinity, and chondroitinase treatment of phosphacan decreased the binding affinity to a single value (K_d = 13 nM), suggesting the presence of two subpopulations of proteoglycan with different chondroitin sulfate chain structures. Moreover, antibodies against 6B4 proteoglycan added to the culture medium suppressed

pleiotrophin-induced neurite outgrowth of cortical neurons (Maeda *et al.* 1996). Therefore, interaction between 6B4 proteoglycan and pleiotrophin is required for the action of pleiotrophin, and chondroitin sulfate GAG chains on 6B4 proteoglycan play regulatory roles in its binding.

The highly complicated interactions of RPTPζ/β to its heterophilic ligands revealed to date indicate that this receptor protein tyrosine phosphatase is a very important component of cell-cell and cell-ECM regulatory machinery in the developing and mature nervous system. In the future, it will be critical to evaluate the effects of these ligand interactions on the enzymatic activity of RPTPζ/β, and to identify specific intracellular substrates dephosphorylated by activated RPTPζ/β.

Chapter 2: Materials and Methods

Isolation of PTP cDNA clones

Fully degenerate oligonucleotide primers, corresponding to the amino acid sequences KCAQYWP and VHCSAGV that are conserved in a number of tyrosine phosphatases, were used to specifically amplify PTP sequences from postnatal rat cerebellar cDNA by the polymerase chain reaction (PCR). RNA was isolated from P1, P3, P6, P9 and P11 Sprague-Dawley rat cerebellum (Cathala *et al.* 1983). cDNA was synthesized from 30 µg of total RNA from each developmental age, using Superscript reverse transcriptase (BRL), in a 50 µl reaction. Vent polymerase (NEB) was used to separately amplify 3 pools of cDNA (P1 and P3; P6; P9 and P11), in the presence of 5 µg of each primer and a buffer containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100, for 30 cycles (94°Cx1min/55°Cx2min/72°Cx5min). PCR products of the expected size were excised from NuSeive GTG agarose (FMC) and were directionally cloned into Bluescript SK and transformed into *E. coli.* Transformants were replated in a gridded array and nitrocellulose replicas of the plates were hybridized with 100 ng of the gel-purified PTP PCR products, amplified from either P6 or adult cerebellar cDNA, and labeled with $[^{32}P]$-ATP using polynucleotide kinase. After washing to a stringency of 0.1x SSPE at 56°C and autoradiography, clones which were differentially labeled by the two probes were picked and sequenced. Distribution and developmental regulation of the corresponding mRNAs were investigated by RNase protection

analysis, and one of the clones (2-3-14) was found to hybridize to an RNA that was expressed exclusively in nervous tissues.

Isolation of RPTPζ/β cDNA clones

A P15 rat brain directional cDNA library (Stratagene) was screened using the 2-3-14 PTP insert, and 20 clones were isolated on repeated screening. Sixteen additional clones were isolated from a random and oligo-dT primed adult rat brain cDNA library (Stratagene). Select cDNA clones were sequenced on both strands by the method of Sanger (Sanger *et al.* 1977), using Sequenase (U.S. Biochemical), and through the use of an Applied Biosystems Automated Sequencer. After characterizing all the rat RPTPζ/β clones that we had obtained from repetitive screening of both libraries, we found our sequence terminated 24 bp 3' of the predicted initiator methionine of the human RPTPζ/β. We obtained additional sequence overlapping and extending 5' of that determined from analysis of our cDNA clones by 'rapid amplification of cDNA ends' (RACE) (Frohman *et al.* 1988; Loh *et al.* 1989; Ohara *et al.* 1989) (GibcoBRL) and direct sequencing of the PCR-amplified product (Promega).

Cell culture

Primary cultures of dissociated E16 rat midbrain were grown on 35 mm tissue culture dishes in defined medium as described (Casper *et al.* 1991), and where indicated, 50 ng/ml NGF (Boehringer Mannheim), 10 ng/ml EGF (Collaborative), 30 ng/ml bFGF (Collaborative). Primary Schwann cell cultures, grown in the presence or absence of glial growth factor (GGF) and 2μM of forskolin

as described (Einheber *et al.* 1993), were the generous gift of Dr. James Salzer, NYU School of Medicine. PC12 cells (generously provided by Dr. Lloyd A. Greene, Columbia University) were grown as previously detailed (Greene and Tischler 1976) in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal calf serum (Gemini) and where indicated 50 ng/ml 2.5S nerve growth factor (NGF; Boehringer-Mannheim). Rat C6 glioma cells were grown in DMEM supplemented with 10% fetal calf serum (Gemini).

Northern blot analysis

RNA was prepared from tissues by lysis in guanidinium thiocyanate and LiCl precipitation (Cathala *et al.* 1983), and from cells by NP40 lysis and organic extraction (Greenberg *et al.* 1985). Northern analysis was performed as previously described (Salton *et al.* 1991), employing poly-A (+) RNA that was isolated from total RNA using Dynabeads, according to the manufacturer's instructions. One or two microgram samples of poly-A (+) RNA per lane were loaded, co-electrophoresed on 1% agarose gels with RNA standards of 0.24, 1.4, 2.4, 4.4, 7.5 and 9.5 kb (BRL), and transferred to nylon membranes. Blots were hybridized in 5x SSPE (Sambrook *et al.* 1989), 50% formamide at 42°C and washed to high stringency (0.1x SSPE, 56°C).

RNase protection analysis

RNase protection analysis was carried out as previously detailed (Salton *et al.* 1988; Salton *et al.* 1991). [^{32}P]-labeled RPTPζ/β and cyclophilin (internal control) RNA antisense probes were synthesized

using T3 and T7 RNA polymerases, as specified by the manufacturer (Stratagene). Samples containing 5 or 10 μg total cytoplasmic RNA were hybridized with ~1 ng labeled probes in 80% formamide-40 mM PIPES-400 mM NaCl-1 mM EDTA for 18 h at 45°C. Samples were treated for 30 min at 37°C with RNase A (40 μg/ml) and RNase T1 (2 μg/ml) in 10 mM Tris (pH 7.5)-5 mM EDTA-300 mM NaCl, and the protected fragments were resolved on nondenaturing 5% polyacrylamide gels, using *Msp*I-digested pBR322 DNA as molecular weight marker. All assays were carried out in duplicate or triplicate, and dried gels were analyzed by autoradiography and direct quantitation using a Molecular Dynamics PhosphorImager. Standard curves, constructed by hybridizing ^{32}P-labeled antisense probes with known amounts of sense-stand RNA, were used to convert the arbitrary volume number PhosphorImager reading of protected fragments into picograms RPTPζ/β or cyclophilin mRNA per 5 or 10 μg of total RNA. Before converting to the picogram value, PhosphorImager readings of the partial protected fragments were corrected according to the number of labeled nucleotide contained within these partial fragments.

Southern blot analysis

Genomic DNA was isolated from rat liver and from phage (Sambrook *et al.* 1989). Restriction digest of 10 μg genomic DNA and 5 μg of phage clone DNA were electrophoresed on 0.8% agarose gels and transferred to nitrocellulose (Sambrook *et al.* 1989). The blots were probed with PCR-labeled cDNA fragments that encode different

regions of rat RPTPζ/β protein (see Fig. 2B and 5B). Blots were progressively washed to a stringency of 0.1x SSPE-0.1% SDS at 55°C.

Isolation of RPTPζ/β genomic clones

Ten genome equivalents (4 x 10^6 plagues) of a rat lambda genomic library DASH® II (Stratagene) were plated by using the host *E. coli* strain LE392, and duplicate nitrocellulose filters were screened with PCR-labeled cDNA fragments that encode different regions of rat RPTPζ/β protein. Hybridization was carried out in 5x SSPE-1x Denhardt's solution-0.1% SDS-100 μg of sonicated salmon sperm DNA per ml-50% formamide for 24 h at 42°C. Filters were progressively washed to a stringency of 0.1x SSPE-0.1% SDS at 55°C. Phage were plaque purified by two additional rounds of plating at limited dilution and rescreening.

RT-PCR analysis

First strand cDNA synthesis with 1 or 2 μg of total RNA and oligo (dT) primer were performed using reagents and protocols included in the SuperScript™ Preamplification System for First Strand cDNA Synthesis (GibcoBRL). PCR was performed for 30 cycles using the *Pfu* DNA polymerase (Stratagene) as follows: denaturing, 94°Cx1min; annealing, 55°Cx2min; extension, 72°Cx5min. Specific primers were used as described in the figure legend. Appropriate negative and positive controls were included with all reverse transcription (RT) and PCR reactions.

Sciatic nerve crush lesion

Experiments were performed on adult, female Sprague-Dawley rats weighing 200-250 g at the time of lesion. Animals were housed and cared for pre- and post-operation according to the guidelines established by Institutional Animals Care and Use Committee. For surgery, animals were anesthetized intraperitoneally using chloral hydrate (30 mg/kg). Bilateral sciatic nerve crush was performed by crushing with a pair of hemostatic forceps for 30 s at the midthigh level after exposing of the nerve. To prevent nerve laceration, the tips of the forceps were covered with tightly fitting polyethylene tubings. For sham operated animals, their sciatic nerves were exposed but not crushed. The skin incision was closed with wound clips. At 1, 4, 7, 14, 21, 28, and 70 days post-surgery, animals were sacrificed, and proximal and distal sciatic nerve segments of 8 mm immediately adjacent to the lesion sites were collected. In addition, lumbar L4-L6 dorsal root ganglia (DRGs) and L4-L6 segments of spinal cord from these animals were also collected. Tissues from at least three animals were collected for each time point studied. Total RNA was isolated separately from collected tissues of individual animal using the RNA Stat-60™ reagent (Tel-Test "B", Inc.) and used in RNase protection assay.

Chapter 3: Molecular Cloning of Rat RPTPζ/β cDNA

Introduction

Identification of a number of neurotrophic growth factor receptors as receptor tyrosine kinases has emphasized the integral role that tyrosine phosphorylation is likely to play in differentiation and cell survival within the nervous system. The state of protein tyrosine phosphorylation within the cell is regulated by both protein tyrosine kinases, enzymes which catalyze the phosphorylation of specific tyrosine residues, and by protein tyrosine phosphatases (PTPs), proteins which remove phosphate groups from tyrosine residues. Similar to the tyrosine kinases, PTPs can be divided into two classes, cytoplasmic and membrane associated (Fischer *et al.* 1991; Saito and Streuli 1991; Charbonneau and Tonks 1992; Pot and Dixon 1992). Binding of a growth factor to its receptor tyrosine kinase activates a cascade of second messenger pathways, triggering changes in the state of tyrosine, threonine, and serine phosphorylation of a number of target proteins, which ultimately lead to alterations in gene expression. Dephosphorylation of the intracellular Src family of tyrosine kinases by the CD45 PTP results in their catalytic activation, suggesting that both the addition and removal of key phosphate residues is important in the regulation of intracellular signaling pathways (reviewed in Mustelin *et al.* 1992).

Extracellular signals, resulting from the interaction of a cell with neighboring cells or matrix, can be transduced into intracellular signals through the binding of ligands to receptor tyrosine kinases

and also by the association of cell surface adhesion molecules with one another or with matrix constituents. Clustering of cell surface β_1 integrins results in increased protein tyrosine phosphorylation (Kornberg *et al.* 1991). Interestingly, pp60c-src-dependent phosphorylation of membrane associated tubulin at tyrosine residues is reduced by preincubation with purified L1 or N-CAM fragments or antibodies to them, implying that cell adhesion molecules may couple cell surface events to alterations in the activities of protein tyrosine kinases or phosphatases (Atashi *et al.* 1992). Since intracellular portions of L1/NILE/Ng-CAM and N-CAM are phosphorylated (Salton *et al.* 1983; Mackie *et al.* 1989) and axon fasiculation has been shown to be regulated by these cell adhesion molecules and to be dependent on cytoplasmic protein phosphorylation (Cervello *et al.* 1991), alteration in protein phosphorylation is likely to be a mechanism of regulating neurite outgrowth and growth cone guidance. It is therefore of interest that recent studies demonstrate developmentally regulated expression of several tyrosine kinases and of tyrosine phosphatase activity in growth cone preparations made from embryonic brain (Bixby and Jhabvala 1993).

Receptor protein tyrosine phosphatases (RPTPs) are a diverse group of proteins which contain a variety of extracellular cell adhesion molecule (CAM) motifs, including fibronectin type III and immunoglobulin constant region repeats, and a pair of C-terminal intracellular catalytic phosphatase domains (Fischer *et al.* 1991; Saito and Streuli 1991; Charbonneau and Tonks 1992; Pot and Dixon 1992). Binding of the CAM domains of the RPTP to extracellular matrix constituents or to other CAMs could potentially regulate phosphatase

activity, transducing this interaction into an alteration in the state of phosphorylation of key intracellular regulatory proteins. Several RPTPs containing CAM motifs have been shown to be differentially expressed on subsets of axons in the developing *Drosophila* central nervous system, suggesting a possible role in axon outgrowth and guidance (Tian *et al.* 1991; Yang *et al.* 1991). In addition, a novel subfamily of RPTPs that include RPTPγ/HPTPγ and RPTPβ/HPTPζ has been identified (Kaplan *et al.* 1990; Krueger *et al.* 1990; Krueger and Saito 1992; Barnea *et al.* 1993); these contain a distinctive extracellular carbonic anhydrase (CA)-like domain, a fibronectin type III repeat, and two intracellular phosphatase domains. None of the vertebrate receptor tyrosine phosphatases that have been fully characterized to date, however, have been shown to be expressed in a distribution which might suggest a functional interaction with any specific growth factor receptors. Here, through the cloning and characterization of the rat homologue of human RPTPζ/β, I demonstrate that mRNA encoding the rat PTP RPTPζ/β is distributed relatively selectively throughout the rat peripheral and central nervous systems. In addition, RPTPζ/β mRNA is shown to be expressed in cells of both neuronal and glial lineage, *in vitro*. Our characterization of the rat RPTPζ/β cDNAs and the major rat RPTPζ/β transcripts further indicates that significant heterogeneity in the structure of the RPTPζ/β polypeptide(s) is likely to result from alternative patterns of RNA processing. The unique localization of this transmembrane PTP suggests the possibility that RPTPζ/β may modulate signaling transduction in the developing and mature vertebrate central and peripheral nervous systems.

Results

Isolation and direct identification of rat PTP mRNAs that are regulated during the development of the cerebellum

We employed the polymerase chain reaction (PCR) using degenerate oligonucleotide primers to conserved regions within the catalytic domains of most protein tyrosine phosphatases (PTPs) (Hunter 1989) to amplify PTP sequences. cDNA synthesized from postnatal rat cerebellar total RNA was amplified by PCR and the products inserted into plasmids and transformed into *E. coli* (detail see Chapter 2). Transformants were grid plated, transferred to replicate nitrocellulose filters, and hybridized to radiolabeled amplified PTP sequences synthesized from either adult or P6 cerebellar cDNAs. Clones that were labeled differentially, indicating that expression of the corresponding mRNAs was likely to be regulated during cerebellar development, were sequenced and the tissue distribution of the corresponding RNAs determined by RNase protection analysis. In this screen we defined eight unique rat PTP cDNAs including rat LAR (Streuli *et al.* 1988), rat LAR2 (B. Goldstein, Genbank; also called PTP NE-3 (Walton *et al.* 1993)), rat PTPXA and two clones, one closely related to human HPTPΔ and the other to human HPTPγ, each of which was developmentally regulated during maturation of the cerebellum, abundant in the embryonic and postnatal CNS, and widely expressed outside the nervous system (data not shown).

One clone, 2-3-14, was found to be 85% homologous at the nucleotide level to the phosphatase domain of human PTPζ (Krueger

et al. 1990; Krueger and Saito 1992), and corresponded to an RNA which was distributed exclusively in the rat nervous system (Fig. 1). Human PTPζ, independently isolated and called RPTPβ (Kaplan *et al.* 1990), is a transmembrane tyrosine phosphatase with a unique carbonic anhydrase (CA)-like extracellular domain. Because the catalytic domains of most tyrosine phosphatases are at least 30-50% identical in amino acid sequence, we isolated ten overlapping cDNA clones that spanned the length of one of the three major rat PTP transcripts in order to better define the relationship of our rat PTP to human PTPζ/RPTPβ (Fig. 2). The amino acid (aa) sequence deduced from two overlapping rat PTP cDNAs (hereon referred to as rat RPTPζ/β) was highly homologous to the human PTPζ (88.7% aa identity, 97.3% homology with conservative substitutions), and apparently is the rat homologue of the short human RPTPζ/β variant recently described (Levy *et al.* 1993). The short rat RPTPζ/β variant is predicted to encode a 1457 aa polypeptide. The longest open reading frame is initiated with an AUG codon in a favorable context, having a G in position -3 (Kozak 1991); an in-frame termination codon was noted 99 nucleotides upstream of the initiator codon. The nucleotide sequences of the rat and human RPTPζ/β cDNAs both 5' of the predicted initiator codon and 3' of the poly-A addition site, were found to be much less homologous than those encoding the deduced RPTPζ/β polypeptide, further implying that RPTPζ/β is translated as predicted. A consensus AAUAAA polyadenylation signal was found 16 bp 5' to the poly-A addition site, and two AUUUA consensus destabilization sequences were found ~250 bp 5' of the poly-A addition site. Additional cDNA clones corresponding to the long form

of rat RPTPζ/β were also isolated, and regions within the ~2.5 kb insert domain, which distinguishes the long from the short form, were sequenced. The polypeptide predicted from the ~300 bp sequence of the insert probe (see Fig. 2B) was found to be highly homologous to the corresponding portion of the human long form (73.7% identity, 95% homology with conservative substitutions). A small 8 amino acid insertion was found in the extracellular domain of rat RPTPζ/β and a 7 amino acid deletion was identified between the putative membrane spanning region and phosphatase domain I of rat RPTPζ/β, when compared to the amino acid sequence predicted from the human PTPζ clone and that deduced from additional related rat RPTPζ/β cDNA clones, suggesting the possibility that alternative splicing may lead to additional heterogeneity among the three major RPTPζ/β transcripts (see Chapter 4). The amino acid sequences predicted by the rat and human RPTPζ/β clones, within the individual structural domains of the RPTPζ/β polypeptide, were even more highly conserved than those within the insert region: the phosphatase catalytic domains I and II are 95.5% and 97.4% identical at the amino acid level (99.6% and 100% homology with conservative substitutions), respectively, while the N-terminal carbonic anhydrase (CA)-like domains are 93.3% identical (99.2% homology with conservative substitutions), and the fibronectin type III (FN-III) repeats are 93.7% identical (100% homology with conservative substitutions) (see Fig. 3) at the amino acid level. A possible conservation of function of these individual domains and the macromolecule as a whole during mammalian evolution is therefore suggested. Alignment of related sequences with the CA-like domain

of rat RPTPζ/β demonstrates similarity to other tyrosine phosphatases and to two carbonic anhydrases, human CA isozyme VI and mouse CA I, while phosphatase domain I of the rat RPTPζ/β is highly homologous to mouse and human RPTPγ, a related PTP which also contains a CA-like domain, and to rat LRP, *Drosophila* LAR, and mouse LRP (Fig. 3).

Characterization and tissue distribution of the major rat RPTPζ/β transcripts

To better characterize the mRNA encoding the RPTPζ/β macromolecule which lacks phosphatase domain I, I screened the rat brain cDNA library with a fragment from the 2.5 kb insert region. Clones that contained the insert but not phosphatase domain I were sequenced and compared to the cDNA sequences corresponding to the long and short variant RPTPζ/β phosphatases (Fig. 4). Four unique cDNA clones corresponded to an mRNA which contained sequences that were identical to those encoding the extracellular domain of the large RPTPζ/β phosphatase variant but which diverged at the potential splice junction that defines the difference between the long and short phosphatases (see schematic diagram, Fig. 4A). An in-frame translation stop codon two bases 3' to this potential splice junction was noted (Fig. 4B). Thus the truncated protein predicted to be translated from the 9.0 kb transcript contains the extracellular polypeptide of the long RPTPζ/β but lacks the membrane spanning region and phosphatase domains I and II. Recent cloning of the rat brain chondroitin sulfate proteoglycan 3F8 (Maurel *et al.* 1994) and comparison of the 3F8 (phosphacan)

sequence to those of the three variant rat RPTPζ/β transcripts presented here reveals that the truncated rat RPTPζ/β and 3F8 cDNA sequences are identical. Sequence we obtained from the 3' untranslated region (UTR) of cDNAs corresponding to the truncated RPTPζ/β variant extends the 3F8 sequence by 30 bases, includes a consensus AATAAA polyadenylation site 15 bases 5' to the poly-A tail, and is different from the 3' UTR of the long and short RPTPζ/β phosphatase cDNAs (data not shown).

By northern analysis, an RPTPζ/β cDNA probe hybridized to three major RNAs (Fig. 5A, lanes 1-3). The RPTPζ/β Domain I (DI) probe hybridized only to RNAs encoding the two major catalytic isoforms of RPTPζ/β that differ in the extracellular domain (Fig. 5A, lanes 4-6). Northern analysis further demonstrated that a probe (CA) complementary to mRNA encoding the carbonic anhydrase extracellular domain recognized mRNAs encoding both RPTPζ/β catalytic isoforms (10.7 and 7.2 kb), and the non-catalytic isoform, phosphacan (9.0 kb) (Fig. 5A, lanes 7-9). A probe (Pcan) complementary to the 3' untranslated region of phosphacan mRNA specifically hybridized with only the 9.0 kb RNA (Fig. 5A, lane 11), while a probe (In) complementary to RNA encoding the large extracellular insert domain shared by phosphacan and the large catalytic isoform of RPTPζ/β hybridized with both the 9.0 and 10.7 kb RNAs (Fig. 5A, lane 10).

I employed three unique antisense [^{32}P]-labeled RNA probes (described in Fig. 2B), complementary to portions of either the phosphatase domain I, the CA-like domain, or the 2.5 kb insert region, to investigate whether the three major RPTPζ/β transcripts

might be differentially expressed *in vivo*. Based on the northern analysis described in Fig. 5, the CA-like domain probe will hybridize with each of the three major RPTPζ/β transcripts, the phosphatase domain I probe with the 10.7 and 7.2 kb RNAs, and the insert region probe with the 10.7 and 9.0 kb RNAs. Using RNase protection analysis, the phosphatase domain I probe was found to protect RNAs in the nervous system, both in the CNS (E16, E17, P2, and P10 brain, and E16 spinal cord) and PNS (P10 superior cervical ganglia), and in the P10 adrenal, but not in a number of other embryonic, postnatal, and adult non-neural tissues (Fig. 6, panel A; see also Fig. 1). The probes complementary to the CA-like and insert regions also protected a similar set of RNAs isolated from nervous tissues (Fig. 6, panels B-E). In panels C and E, protected fragments of 170 and 200 bp were detected in addition to the major protected band of 300 bp, in samples of brain and spinal cord RNA analyzed with the insert region probe but not in control assays, suggesting that these additional bands were the result of distinct patterns of RPTPζ/β RNA degradation or sequence heterogeneity within the RPTPζ/β insert region, perhaps as a result of alternative splicing. Interestingly, fragments protected by the insert and CA-like domain probes were also barely detectable in adult rat lung, adrenal, kidney, and cardiac muscle, suggesting that the 9.0 kb RPTPζ/β transcript (the only transcript which contains both the CA-like and insert regions but does not contain phosphatase domain I) may be expressed at very low levels in these tissues. Alternatively, RNase protection assays in which the CA-like and insert probes are employed may detect lower levels of RPTPζ/β mRNA than those in which the phosphatase domain

I probe is utilized. These data indicate that the expression of each of the three RPTPζ/β transcripts is limited primarily to the nervous system and neural crest derived postnatal adrenal, but that extremely small but detectable amounts of one or more of the RPTPζ/β transcripts is likely to be produced in several non-nervous tissues.

Detection of RPTPζ/β mRNA in cells of neuronal and non-neuronal lineage

To further characterize which tissues and cell types within the central and peripheral nervous systems express RPTPζ/β, RNase protection analysis and the RNA probe complementary to the RPTPζ/β phosphatase domain I were employed. In Fig. 7, RPTPζ/β mRNA was found to be abundantly expressed in the developing spinal cord and brain, and at lower levels in the adult sciatic nerve. To investigate which cell types within sciatic nerve might express RPTPζ/β, RNA isolated from primary cultures of sciatic nerve-derived Schwann cells (Einheber *et al.* 1993) was similarly analyzed and RPTPζ/β RNA was detected. Treatment of these primary Schwann cell cultures with glial growth factor (GGF) and forskolin for approximately 2 weeks results in a more elongated phenotype, an increase in the production of myelin, and a down-regulation of many gene products that are not directly involved in myelination (Jessen and Mirsky 1991; Einheber *et al.* 1993). A small decrease in RPTPζ/β RNA levels was reproducibly seen in these growth factor and forskolin treated cultures. I then measured RPTPζ/β RNA levels in several different transformed cell lines, including murine neuroblastoma N2A and

neuroblastoma/glioma hybrid n115, and rat PC12 and C6 glioma cells. The highest RPTPζ/β RNA levels were found in C6 glioma cells (Fig. 7), while in PC12 cells, RPTPζ/β RNA was detected in PC12 cells treated with nerve growth factor (NGF) for 10-14 days, but not in untreated PC12 cells nor in those treated for 1 hour (Fig. 7), 3 hours, 5 hours, 24 hours, 48 hours, 72 hours, 5 days, or 7 days with NGF (data not shown). PC12 cells grown in the absence of NGF resemble adrenal chromaffin cells, while PC12 cells treated with NGF for 10-14 days share many properties with sympathetic neurons. Taken together, these data suggest that RPTPζ/β is likely to be expressed in both non-neuronal and neuronal lineages, *in vivo*, in the nervous system.

Since RPTPζ/β mRNA was found in C6 glioma cells, I examined whether RPTPζ/β mRNA is expressed by astrocytes in primary cultures of embryonic rat midbrain (Casper *et al.* 1991). In these cultures, treatment with either epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) has been shown to cause glial proliferation (Leutz and Schachner 1981; Pruss *et al.* 1982; Simpson *et al.* 1982; Pettmann *et al.* 1986), such that >90% of the cells in the cultures treated for 7-14 days with either of these growth factors are or will become astrocytes based on positive staining for the glial fibrillary acidic protein (GFAP). Equal numbers of plates of control, EGF- or bFGF-treated cultures were harvested, and the RNA isolated and analyzed for RPTPζ/β; for comparison, the neuronal mRNA VGF (Van Den Pol *et al.* 1989; Salton *et al.* 1991) and the widely expressed mRNA cyclophilin (Danielson *et al.* 1988) were also quantified. Treatment of midbrain cultures with bFGF for 14 days

(Fig. 8, panel A) resulted in a 7-fold increase in cyclophilin RNA levels, paralleling the increase in cell number that occurs in response to bFGF or EGF. A similar 7-fold increase in RPTPζ/β RNA levels was measured in the 14 day bFGF-treated cultures in comparison to untreated controls, suggesting that the proliferating glia most likely express RPTPζ/β RNA. RPTPζ/β mRNA levels in midbrain cultures treated with either bFGF or EGF for 8 days were similar to those found in the 14 day bFGF-treated cultures. Comparable levels of cyclophilin and RPTPζ/β RNAs were also found in midbrain cultures that were treated for 9 days with EGF, trypsinized and replated in the presence of EGF, and harvested after an additional 7-10 days *in vitro*, further enriching the cultures in astroglia (data not shown). In contrast, expression of the neuronal mRNA VGF did not change significantly in response to either NGF or EGF treatment for 2 or 8 days, while cyclophilin levels were found to have increased after 8 days of EGF treatment, in parallel with the proliferation of astrocytes (Fig. 8, panel B). These data suggested that RPTPζ/β mRNA is expressed by primary astrocytes derived from embryonic rat midbrain. In addition, I have also directly detected the expression of all five identified RPTPζ/β transcripts in primary mixed glial cultures purified from early postnatal rat cerebral cortex (Fig. 13B and data not shown).

Discussion

Results in this chapter demonstrate that the RPTPζ/β gene is unique among those encoding vertebrate transmembrane tyrosine phosphatases on the basis of its relatively selective expression in the developing and adult central and peripheral nervous systems. The coding sequence of RPTPζ/β has been highly conserved during mammalian evolution, as demonstrated by comparison of the rat and human cDNA sequences. The high homology between the carbonic anhydrase (CA)-like, fibronectin type III (FN-III), and phosphatase domains of the rat and human cDNAs, and the detection of both long and short RPTPζ/β variants in both the rat and human, suggest that these regions may be critical to the function of the RPTPζ/β phosphatase. In addition, characterization of the rat RPTPζ/β transcripts by northern analysis suggests that the 9.0 kb RNA encodes a protein which does not possess the active phosphatase domain I but does possess the CA-like and insert domains, and which, therefore, may subserve a somewhat different function than the 'long' and 'short' RPTPζ/β phosphatases (discussed in detail in Chapter 4).

Molecular modeling of the extracellular CA-like domain of RPTPγ and ζ/β suggest that this region is unlikely to catalyze the reversible hydration of CO_2 to HCO_3^- as does carbonic anhydrase, but might be capable of binding a small ligand up to several amino acids in length (Barnea *et al.* 1993). Transduction of an environmental signal into intracellular tyrosine dephosphorylation might also involve binding to the FN-III repeat, a domain identified in a number of cell adhesion

molecules (CAMs). The recent demonstration that RPTPμ, containing an Ig-like domain and four FN-III repeats, binds in homophilic fashion when expressed in Sf9 cells, indicates that the CAM domains of at least one phosphatase function to facilitate cell-cell adhesion (Brady-Kalnay *et al.* 1993; Gebbink *et al.* 1993). It is thus possible that RPTPζ/β may transduce peptide signals, and/or contact with surface molecules on other cells. In fact, RPTPζ/β is the first and only RPTP to date whose heterophilic ligands have been identified (detail see Chapter 1).

Although a number of truncated variants of several trans-membrane tyrosine kinase receptors, lacking all or part of the catalytic domain, have been characterized {see for example (Klein *et al.* 1990; Middlemas *et al.* 1991)}, and a tyrosine phosphatase with an alternatively spliced exon within the catalytic domain has been described (Matthews *et al.* 1990), to my knowledge this is the first tyrosine phosphatase to be identified for which a major variant transcript lacking the catalytic domain has been characterized. The presence of an 8 amino acid insertion and a 7 amino acid deletion in the amino acid sequence deduced from one of our rat RPTPζ/β cDNA clones, in comparison to the human PTPζ sequence and additional rat RPTPζ/β cDNAs, were also noted. This structural variation may reflect further alternative splicing of RPTPζ/β transcripts, generating microheterogeneity within the three major RNA transcripts detected by northern analysis, and potentially resulting in an increase in the functional diversity of this family of RPTPs (detail see Chapter 4). It is interesting to note that tissue-specific alternative splicing generates two isoforms of the TrkA tyrosine kinase receptor which

differ by virtue of a 6-amino acid insertion in their extracellular domains (Barker *et al.* 1993). In addition, alternative splicing of the RPTP rat LAR has recently been reported (Zhang and Longo 1995). A 12 bp exon that is inserted just 3' of sequences encoding the rat LAR transmembrane domain was identified, similar in position to the 21 bp deletion we identified in rat RPTPζ/β.

In the current study, I have shown that the mRNA encoding RPTPζ/β is expressed relatively selectively in both the developing central and peripheral nervous systems. Although the expression patterns of a number of invertebrate tyrosine phosphatases have been shown to be nervous system specific (Tian *et al.* 1991; Yang *et al.* 1991), characterization of many vertebrate tyrosine phosphatases has resulted in the identification of only three PTPs that are relatively selectively produced in the nervous system, RPTPζ/β, NE-3 (LAR2) and STEP. The mRNA encoding the intracellular PTP STEP is expressed most abundantly in the central nervous system, within distinct neuronal classes in the basal ganglia, and at much lower levels in the kidney and adrenal (LaForgia *et al.* 1991; Lombroso *et al.* 1991; Lombroso *et al.* 1993). Studies of the receptor tyrosine phosphatase NE-3 (Walton *et al.* 1993) suggest that one of the mRNAs encoding this PTP is expressed almost exclusively in the rat brain and olfactory neuroepithelium. In our preliminary characterization, using RNase protection analysis, of the distribution of one of our PCR-amplified PTP domain cDNAs, later found to be identical in sequence to rat LAR2 (also called NE-3), we found that LAR2 mRNA is expressed almost as abundantly in the adult testis and adrenal as in the adult nervous system, with lower levels

detected in the kidney and spleen (data not shown). Our RNase protection studies, however, would not have been able to discriminate among the splice variants of NE-3 that contain identical PTP domain sequences. Recent studies of the human and mouse RPTPζ/β have demonstrated, by northern analysis, relatively selective expression of RPTPζ/β RNA in the adult brain in comparison to several non-neural tissues (Levy *et al.* 1993), findings that my current study corroborates and extends. By *in situ* hybridization we have previously shown that RPTPζ/β RNA is expressed almost exclusively (Li *et al.* 1993; Li *et al.* 1994; Li *et al.* 1996; Snider *et al.* 1996) in the developing PNS and CNS, in cells derived from the neural crest and the neuroepithelium. Furthermore, it remains a distinct possibility that the low level hybridization to RPTPζ/β RNA in non-neural tissues may in fact be to cells of neural crest or neuroepithelial origin, since the appearance of this RPTPζ/β signal parallels neural cell migration into these tissues. Clearly, the distributions of the STEP, NE-3 and RPTPζ/β mRNAs are distinct, which coupled with the relative abundance of each within the nervous system, suggests that these macromolecules may perform unique functions in the developing and mature nervous system.

We have presented a variety of experimental evidence which suggests that RPTPζ/β mRNA is likely to be expressed, *in vivo*, by both mature glial and neuronal cell types, and their immature precursors. Examination of primary cultures and transformed cell lines reveals that both neuronal and non-neuronal cells express RPTPζ/β mRNA *in vitro*. We find abundant production of RPTPζ/β mRNA by C6 glioma cells and cultured primary astroglia, and low

level synthesis by NGF-treated PC12 cells and primary Schwann cell cultures. Our previous *in situ* hybridization studies (Li *et al.* 1993; Li *et al.* 1994; Li *et al.* 1996; Snider *et al.* 1996) localize RPTPζ/β mRNA in the PNS and CNS are consistent with this. Specifically, we find intense RPTPζ/β expression in the ventricular zone of the embryonic cerebrum and spinal cord (E11.5-E19.5) which contains primarily mitotically active multipotential neuronal and glial precursors and differentiating neurons initiating migration into outer layers. In addition, cells of neuronal lineage are shown to express RPTPζ/β mRNA (Li *et al.* 1996; Snider *et al.* 1996). For example, RPTPζ/β mRNA is expressed in developing neurons in the cortex, retina, cranial ganglia, and peripheral ganglia, and in mature neuronal populations such as the cerebellar Purkinje cells and dentate granule cells (Li *et al.* 1996; Snider *et al.* 1996). Moreover, RPTPζ/β mRNA is expressed at high levels in the subventricular zone of the forebrain from E17.5 through adulthood. This second zone of actively proliferating cells gives rise primarily to glia after birth, and continues to do so even in the adult.

Conclusions

Molecular cloning of the rat homologue of RPTPζ/β demonstrates the high degree of sequence conservation of this gene during mammalian evolution, especially in the CA-like, FN III-like and PTP catalytic domains, as evident by comparison of the rat and human cDNA sequences.

The mRNAs that encode RPTPζ/β are relatively selectively expressed in the developing and mature CNS and PNS. This rather restricted expression pattern in the nervous system makes RPTPζ/β one of few such neural PTPs. In addition, detection of RPTPζ/β mRNA in cell cultures of neuronal and non-neuronal lineage suggests that the encoded protein is likely to be expressed, *in vivo*, by both mature glial and neuronal cell types, and their immature precursors.

Chapter 4: Alternative mRNA Splicing of RPTPζ/β Gene

Introduction

Alternative mRNA splicing is one of the major cellular post-transcriptional mechanisms for regulating gene expression and generating protein diversity (Smith *et al.* 1989). It has the potential to greatly increase the coding capacity of a single gene or a small multi-gene family. Alternative mRNA splicing significantly contributes to the diversity of protein tyrosine phosphatases isoforms. For many cytosolic protein tyrosine phosphatase (PTPase), their C-terminal accessory domains, which are important for subcellular localization, are often regulated by alternative mRNA splicing. For example, two forms of the C-terminal domain are generated by alternative mRNA splicing of the *Drosophila* PTPase DPTP-61F, and the splice selection at this site determines the subcellular localization: one isoform is found on a cytosolic membrane, whereas the other one is found in the nucleus (McLaughlin and Dixon 1993). However, the two DPTP-61F isoforms have similar PTPase activity *in vitro*. For the receptor-like protein tyrosine phosphatases (RPTPs), alternative mRNA splicing occurs both in their extracellular and intracellular domains. For example, RPTP CD45 plays a crucial role in the regulation of lymphocyte activation by coupling activation signals from antigen receptors to intracellular signaling pathways (Thomas 1994). Multiple CD45 isoforms, generated by regulated alternative mRNA splicing in at

least three exons, differ in the length and glycosylation of their extracellular domains. These isoforms are expressed in a cell type-specific pattern on functional subpopulations of lymphocytes. The pattern of exon usage is controlled both in lymphocyte activation and differentiation, suggesting that there are distinct interactions for each variable exon (Thomas 1994). In the nervous system, several abundantly expressed PTPase have been shown to be regulated by alternative mRNA splicing. Two *Drosophila* RPTPs DPTP99A and DPTP10D, that are selectively expressed on CNS axons in the *Drosophila* embryo have been shown to produce complex sets of transcripts that differ in their intracellular C-terminal tails, owing to the alternative utilization of exons and polyadenylation sites (Tian *et al.* 1991; Yang *et al.* 1991). Recently, analysis of loss-of-function mutants of these DPTPs demonstrates that they are essential for motor axon guidance in the developing *Drosophila* nervous system (Desai *et al.* 1996; Krueger *et al.* 1996). In the mammalian nervous system, the leukocyte common antigen-related (LAR) RPTP has been demonstrated to have a complex array of nervous system-preferential extracellular and intracellular alternative splicing which includes deletions of specific FNIII domains and insertions of novel mini exons into intracellular and juxtamembrane regions (Zhang and Longo 1995). These splicing events were shown to be regulated during development and by denervation, NGF-induced PC12 cell differentiation and cell confluence.

Our data from molecular cloning of the rat RPTPζ/β gene shows that there are several different rat RPTPζ/β tyrosine phosphatase transcripts(see Chapter 3). These include the three major messages

detected by northern blot, 10.7, 9.0, and 7.2 kb in length, which are presumably generated by alternative mRNA splicing from the same RPTPζ/β gene. When compared to the amino acid sequence predicted from the human RPTPζ/β (Krueger and Saito 1992; Levy *et al.* 1993) and that deduced from additional related rat RPTPζ/β cDNA clones, two additional minor differences were found within the short 7.2 kb rat RPTPζ/β transcript. One is a small 8 amino acid insertion (KEVSADLS) found in the extracellular spacer domain and the other one is a 7 amino acid deletion (TLKEFYQ) identified between the putative membrane spanning region and phosphatase domain I. Thus alternative mRNA splicing may lead to additional heterogeneity among the three major RPTPζ/β transcripts. This issue became more important after the recent finding that the cloned rat brain chondroitin proteoglycan 3F8 (Maurel *et al.* 1994) (phosphacan) has identical cDNA sequence with our 9.0 kb truncated rat RPTPζ/β cDNA. In order to determine whether these related transcripts are indeed generated by alternative mRNA splicing and encoded by the same gene, genomic sequence analysis around putative splicing exon/intron junction and genomic Southern analysis have been employed.

Results

Three major RPTPζ/β transcripts are generated through alternative RNA splicing and are transcribed from a single gene

To define whether the variant RPTPζ/β mRNAs resulted from alternative splicing of RNA transcribed from a single gene, we screened a rat genomic library using several different rat cDNA RPTPζ/β clones and isolated five unique genomic clones. Subsequent analysis revealed that one of these (5-2-1) contained sequences which corresponded to the splice junction at the 3' end of the insert region (see Fig. 4A). Genomic and phosphacan cDNA RPTPζ/β sequences were identical at this junction, suggesting that an alternative exon is retained in the 9.0 kb transcript (encoding phosphacan), but is spliced out of mRNAs encoding the long and short RPTPζ/β phosphatases. This alternative exon does not appear to be spliced back into sequences corresponding to the long and short phosphatase mRNAs because the 3' UTR of the 9.0 kb transcript differs from that of the RPTPζ/β phosphatases. By analyzing genomic sequence, we also determined the genomic junction at the 5' end of the 2.5 kb insert. Genomic and the full length cDNA RPTPζ/β sequences were also identical at this junction, suggesting that the 2.5 kb cDNA insert is an alternative exon that is normally retained in the full length RPTPζ/β transcript, but is spliced out of the short RPTPζ/β transcript (Fig. 9A). Detailed genomic sequence analysis (Fig. 9B) at putative alternative splicing junctions revealed that the intron/exon junction sequences matched well with that of the consensus

mammalian RNA splice donor and acceptor sites (Smith *et al.* 1989). Thus, three major RPTPζ/β transcripts are generated through alternative RNA splicing: the short transcript was generated by the alternative usage of the 5' splice donor site at the beginning of the 2.5 kb insert region, leading to the splicing out of the 2.5 kb insert, whereas the phosphacan transcript was generated by retention of the alternative exon immediately following the 2.5 kb insert.

In order to confirm the theory that three major RPTPζ/β transcripts are encoded by the same gene and generated through alternative mRNA splicing, rat genomic Southern blots were probed using ^{32}P-αdCTP labeled cDNA fragments (Fig. 5B and 10A) from various regions of the coding sequence for the gene (Fig. 10B). Probe Pcan (specific for the phosphacan transcript) hybridized to the same single Bgl II digested fragment of 4.5 kb on the blot as did probe TM (specific for the two transmembrane transcripts). This result demonstrated that the phosphacan transcript and the two transmembrane transcripts are encoded by the same gene. In addition, probe Insert that can detect only the full length and phosphacan transcripts hybridized to the same single EcoR I digested fragment of 3.4 kb on the blot as did the 5' Insert probe, which can detect all three transcripts. This result demonstrated that the short transcript and the other two transcripts are encoded by the same gene. Furthermore, probe 5' Insert (Fig. 10B, lane 4) and probe CA (Fig. 10B, lane 5) that hybridize to a region that is shared by all three RPTPζ/β transcripts detected a single fragment by Southern blotting, also suggesting that all three RPTPζ/β mRNAs are transcribed from one single RPTPζ/β gene. Therefore, alternative splicing of RPTPζ/β

transcripts results in the production of two variant transmembrane phosphatases and the related phosphacan, allowing several functionally diverse but structurally related macromolecules to be synthesized from a single gene.

To simultaneously measure mRNA transcripts that encode the three major RPTPζ/β isoforms, I developed a novel βpan probe to be employed with a quantitative and extremely sensitive RNase protection assay (Fig. 11A). The βpan probe is designed to span the 3' end of the 2.5 kb insert region and the transmembrane segment, thus allowing each of the three RPTPζ/β transcripts to be simultaneously detected on the gel as distinct protected fragments. By using this assay system, I have surveyed the temporal developmental profile of the three transcripts in the CNS. The expression of these RPTPζ/β transcripts were found to be highly regulated during CNS development (Fig. 11B). At early development stages of embryonic day 13 (E13), all three RPTPζ/β transcripts were expressed at low levels in the rat brain and spinal cord. The level of expression of the three transcripts increased late in embryogenesis (E18), a time point that reflects the high degree of neurogenesis and neural migration that occurs in the CNS. The peak level of expression was reached at early postnatal days (P5 and P10), a time period that coincides with the peak of gliagenesis. As the CNS matures into adulthood, the level of RPTPζ/β mRNA expression dramatically decreased (Li *et al.* 1996; Snider *et al.* 1996; and data not shown).

Of the three RPTPζ/β transcripts, phosphacan was the most abundant at all stages of CNS development examined. Unlike in the PNS (detailed in Chapter 5), the short transcript was expressed at

much lower levels than that of the phosphacan in the CNS, with a level slightly higher than that of the full length transcript. Interestingly, the ratios of expression levels between the phosphacan transcript and the other two transcripts increased as the CNS matured, suggesting that the phosphacan isoform may associate closely with more mature neural cells in the CNS.

Generation of additional minor heterogeneity in the juxtamembrane domain of rat RPTPζ/β

In addition to the three major RPTPζ/β transcripts that I have characterized above, several cDNA clones from our screening showed additional minor sequence heterogeneity when compared to the original full length clone (Fig. 2). In some of these clones, there was a stretch of 21 bp sequence missing in the cDNA region that encoded the juxtamembrane domain (region lies between the single transmembrane domain and the first phosphatase catalytic domain, see Fig. 2) of the phosphatase. As a result of this in frame deletion, a 7 amino acid peptide with the sequence of TLKEFYQ will be missing from the translated products of these cDNA clones. In order to determine whether one or both of the two transmembrane RPTPζ/β phosphatases contain the TLKEFYQ sequence, RT-PCR was performed on cDNA from early postnatal brain mRNA, using pairs of primers that span the juxtamembrane region and are specific for the full length or short transcript. As shown in Fig. 12, the 21 bp sequence (sp21) can be expressed in both the short (primer set 1) and full length (primer set 2) RPTPζ/β transcripts. Moreover, deletion of sp21 also occurs in both RPTPζ/β transmembrane transcripts (primer sets

3 and 4). To further characterize the expression of RPTPζ/β mRNAs encoding the TLKEFYQ variant, I designed the probe sp21 (Fig. 13A) to be employed in the sensitive RNase protection assay, allowing simultaneous detection of RPTPζ/β mRNAs with (sp21+) or without (sp21-) the sp21 sequence. Both sp21+ and sp21- mRNAs were found to be widely expressed during development in the nervous system at comparable levels, such as in the embryonic and postnatal rat brain (Fig. 13B) and in primary mixed glial cell cultures. These RT-PCR and RNase protection assay data suggested that this 21 bp mini variant is possibly generated by alternative RNA splicing, and is present in both the full length and short RPTPζ/β transcripts. On the other hand, the 8 amino acid mini-insert in the extracellular spacer domain probably represent a species difference of the RPTPζ/β gene, since RT-PCR and RNase protection assays did not detect any alternative transcripts in this region (data not shown).

Expression of the sp21+ and sp21- mRNAs were also regulated in the distal segments of sciatic nerve following a crush lesion (Fig. 14). At 7 days after the injury, the levels of both sp21+ ($P < 0.01$, unpaired *t*-test) and sp21- ($P < 0.01$, unpaired *t*-test) mRNAs were greatly induced. However, the sp21- mRNA appeared to be induced to a greater extent (an 18-fold induction for sp21+ versus a 7-fold induction for sp21-) than that of the sp21+ mRNA, even though the absolute level of expression for sp21+ was still higher than that of the sp21-. In addition, similar to the regulated expression of the three major RPTPζ/β transcripts (Fig. 20), the level of sp21+ and sp21- mRNAs was reduced in C6 glioma cells treated with dibutyryl cAMP (Fig. 13B). These results further support the existence of the

sp21 variants for RPTPζ/β mRNA. Moreover, differential regulation of sp21+ and sp21- mRNAs suggests that expression of the TLKEFYQ sequence in RPTPζ/β may have functional implications.

Within the seven amino acids TLKEFYQ encoded by sp21 cDNA sequence, the threonine and the surrounding residues match a putative phosphorylation site for casein kinase II (CKII) and protein kinase C (PKC), suggesting that phosphorylation of the intracellular domains of RPTPζ/β might be regulated by the alternative splicing of sp21. In order to test this possibility, we synthesized short peptides that contained the 7 amino acids and surrounding residues. In an *in vitro* phosphorylation assay using casein kinase II (data not shown), the threonine residue failed to be phosphorylated by CKII, as evidenced by the similar number of incorporated ^{32}P-γATP cpms after the reaction between the wildtype peptide and a peptide in which the threonine residue had been mutated to an alanine residue. In addition, CKII efficiently catalyzed phosphorylation of a control peptide.

Discussion

Present data in this chapter demonstrates that three major RPTPζ/β transcripts are generated through alternative mRNA splicing and are encoded by the same gene. This has been achieved by the combination of genomic Southern blotting and genomic DNA sequencing of exon/intron splicing junctions. Alternative mRNA splicing has become a very important cellular mechanism for regulating gene expression and generating protein diversity (Smith *et al.* 1989). It is well known that many of the cell adhesion molecules (CAM) and extracellular matrix (ECM) proteins are subjected to very complex alternative splicing regulation. Numerous alternatively spliced ECM molecules define the specificity of extremely complicated interaction between the ECM and their receptors. Interestingly, many of these alternatively spliced molecules, such as NCAM, Ng-CAM, tenascin and the neurexin-homologue Caspr, have direct or indirect interactions with RPTPζ/β. For example, the glial-derived extracellular matrix protein tenascin (TN-C) undergoes extensive alternative splicing in its fibronectin type III repeats (FNIII) (Erickson 1993). As a result of this alternative splicing, various isoform-specific TN-C domains possess distinct cell binding, repulsive, and neurite outgrowth promoting sites for neurons (Gotz *et al.* 1996). At the same time, RPTPζ/β is also a component of the ECM network, by virtue of its expression as a chondroitin sulfate proteoglycan (CSPG). As I have shown in the current study, RPTPζ/β also undergoes highly regulated alternative mRNA splicing during neural development. For the three

alternatively spliced RPTPζ/β isoforms, their sequence differences suggest that they may subserve different functions in the PNS and CNS. First, the generation of the soluble phosphacan isoform, which lacks the transmembrane and intracellular phosphatase catalytic domains, results in the synthesis of a molecule in the extracellular space that potentially regulates the ligand-receptor interaction of the other two transmembrane catalytically-active isoforms. Phosphacan may act as a dominant-negative competitor of the catalytic isoforms by sequestering their ligands, since the three isoforms colocalize with each other in many cells of the nervous system (Li *et al.* 1996; Snider *et al.* 1996). Interestingly, similar inhibitory mechanism has been proposed to explain the action of the alternatively spliced and non-transmembrane isoforms of NCAM (Walsh and Doherty 1996), which happens to be putative heterophilic ligands for RPTPζ/β. These NCAMs that lack a normal cytoplasmic domain were found to ablate neurite outgrowth in response to the transmembrane NCAM isoforms (Saffell *et al.* 1995). Similar non-catalytic isoforms of neurotrophin receptors, for example, trkB, have also been hypothesized to have such a dominant-negative action on their catalytic counterparts (Beck *et al.* 1993). On the other hand, it is also possible that the role of the soluble phosphacan played is a positive one. They could present the catalytically-active RPTPζ/β isoforms with a higher local concentration of their ligands by clustering, so as to enhance their signal transduction capabilities.

Second, the short RPTPζ/β transcript, with the missing 2.5 kb insert, may generate very important functional diversity in the extracellular region of the phosphatase. In contrast to the full length

and phosphacan isoforms that are mostly expressed as CSPGs, the short RPTPζ/β isoform is detected primarily without glycosaminoglycan (GAG) (Sakurai *et al.* 1996). This difference is very likely due to the fact that three out of the total four potential consensus chondroitin sulfate-attachment sites for RPTPζ/β are located within the 2.5 kb insert region (Maurel *et al.* 1994), which is spliced out of the short transcript. At least two of the putative ligands identified for RPTPζ/β to date, axonin-1/TAG-1 (Milev *et al.* 1996) and pleiotrophin/HB-GAM (Maeda *et al.* 1996), bind to RPTPζ/β in a chondroitinase-sensitive fashion, which means that they primarily bind to the chondroitin sulfate GAG chain. Although the exact binding sites for the two molecules on RPTPζ/β are still unknown, they are not likely to have a high affinity binding with the short isoform and their primary binding sites may be localized in the 2.5 kb insert region. In addition, contactin has been shown to bind to the carbonic anhydrase (CA)-like domain of RPTPζ/β (shared by all three isoforms) and to induce cell adhesion and neurite outgrowth of primary neurons (Peles *et al.* 1995). On the other hand, NCAM, Ng-CAM, and tenascin have been shown to bind to RPTPζ/β isoforms independent of chondroitin sulfate GAG chains, and their binding is inhibitory for neural adhesion and neurite outgrowth (Grumet *et al.* 1993; Grumet *et al.* 1994; Milev *et al.* 1994). The exact sites of interaction for these ligands are still controversial (Milev *et al.* 1995; Sakurai *et al.* 1997), but apparently they bind to extracellular domains shared by all three RPTPζ/β isoforms. Thus, the short RPTPζ/β isoform generated by alternative mRNA splicing has a different glycosylation profile than the other two isoforms. It may

also fail to bind to or to have different affinities with some RPTPζ/β ligands. Therefore, by regulating their ability to interact with various heterophilic ligands through alternative mRNA splicing, the three RPTPζ/β isoforms may serve different functions in specific temporal and spatial cellular contexts.

The sp21 mini variant in the juxtamembrane domain is possibly generated through alternative RNA splicing, as shown by our RT-PCR and RNase protection assay data. I have tried to determine the genomic DNA sequence surrounding the sp21 cDNA sequence by genomic library screening and PCR, but large intron(s) within this region prevented me from obtaining genomic DNA sequence surrounding the putative exon/intron juncture.

Recently, Bilwes and colleagues (Bilwes *et al.* 1996) have reported the first crystal structure of the intracellular domains for a receptor-type protein tyrosine phosphatase, RPTP-α. Their results suggested that an amino-terminal helix-turn-helix segment in the juxtamembrane domain of RPTP-α plays an essential role in the ligand-mediated dimerization and active-site blockage of RPTP. Furthermore, it is likely that this dimerization and active-site blockage may be a general mechanism physiologically important for downregulating the catalytic activity of RPTPs because of the sequence conservation of this helix-turn-helix motif in many other RPTPs, including RPTPζ/β (Bilwes *et al.* 1996). The 7 amino acid region that are deleted in some RPTPζ/β isoforms locates right in the carboxyl end of the second helix segment. In the dimer form of the RPTP-α crystal structure, several amino acid side chains from this second helix segment interact directly with residues that form the

active site in the opposing monomer and block the binding between active site and the substrate. This stereochemical arrangement provides the structural basis for inactivation of phosphatase activity by dimerization. Thus, deletion of a 7 amino acid sequence in the second helix segment of RPTPζ/β is very likely to affect the ability of these isoforms to dimerize upon ligand binding, which would result in subsequent downregulation of catalytic activity. Therefore, those RPTPζ/β isoforms with the 7 amino acid deletion may be constitutively active upon ligand binding, and they might serve different functional roles from the other isoforms in signal transduction of the receptor during neural development and regeneration. Alternative mRNA splicing might also regulate the sub-cellular distribution of the two groups of RPTPζ/β isoforms, serving as an important mechanism of regulating RPTPζ/β function in specific cellular context.

In addition, RPTP-α has been shown (Tracy *et al.* 1995) to be phosphorylated by protein kinase C (PKC) on two serine residues near the dimer interface in the juxtamembrane domain. This phosphorylation maybe responsible for the induction of a rapid, transient increase in RPTP-α catalytic activity by treatment of phorbol ester (a direct activator of PKC) in RPTP-α transfected cells (den-Hertog *et al.* 1995). Therefore, serine/threonine phosphorylation may be another possible mechanism in the regulation of RPTP dimerization. Although the threonine residue within the 7 amino acid deletion of RPTPζ/β failed to be phosphorylated by casein kinase II *in vitro*, it will be interesting to

further test whether PKC can phosphorylate it, because sequence around this residue also matches a PKC phosphorylation site.

Moreover, receptor protein tyrosine phosphatase RPTPγ, which shares the same RPTP sub-family with RPTPζ/β because of its extracellular carbonic anhydrase like domain (Barnea *et al.* 1993), also has an isoform with 29 amino acid deletion in its juxtamembrane domain, although the deletion lies outside of the helix-turn-helix segment (Shintani *et al.* 1997). Interestingly, the RPTPγ isoform with the 29 amino acid deletion has been shown to be the major isoform in many murine and human tumor cell line (Sorio *et al.* 1995), whereas in various normal tissue this is only a minor form being expressed (Shintani *et al.* 1997). Furthermore, as a tumor suppressor gene candidate (LaForgia *et al.* 1991), intragenic deletion of the RPTPγ gene has been reported in an tumorgenic cell line (Wary *et al.* 1993). Therefore, RPTPγ isoform with the 29 amino acid deletion may be correlated with the immortal cell growth of certain tumor cell lines, and it may be also a constitutively active RPTP with dimerization deficiency. Similar variants with mini deletion in their juxtamembrane domains have also been reported in another RPTP sub-family, the LAR/PTPσ/PTPδ sub-family (Pulido *et al.* 1995). Although all of these variants are not directly located within the helix-turn-helix segments, they may still be able to influence the dimerization of the receptors. Therefore, alternative mRNA splicing in the juxtamembrane domain of RPTPs maybe a general mechanism for regulation of RPTP dimerization and downregulation of catalytic activity.

In summary, my current study has determined that multiple isoforms of RPTPζ/β are generated by alternative mRNA splicing from a single gene rather than encoded by multiple closely related genes through gene duplication during molecular evolution: First, extensive cDNA sequencing consistently generated identical sequence data from multiple overlapping RPTPζ/β cDNA clones, except at putative sites of alternative splicing. These results strongly argue against the existence of closely related genes that are responsible for the generation of multiple RPTPζ/β isoforms. Second, results from various RPTPζ/β mRNA analysis, including Northern blot, RNase protection assay, and RT-PCR, also point to the existence of a single RPTPζ/β gene. Third, determination of RPTPζ/β genomic sequence at putative exon/intron junctures further supports the alternative mRNA splicing of this gene. Fourth, results from genomic and phage Southern blotting, using RPTPζ/β cDNA probes from various regions of different RPTPζ/β transcripts, clearly demonstrated that different RPTPζ/β transcripts are encoded by a single gene. In particular, hybridization patterns of DNA fragments digested within both intron and exon sequences are consistent with the existence of only a single RPTPζ/β gene. Taking this evidence together, I have shown here that alternative splicing of mRNA encoding the extracellular and intracellular domains of RPTPζ/β will generate at least five different protein isoforms. Each of these highly regulated isoforms will likely serve unique functions in the nervous system, suggesting that by alternative mRNA splicing, products of the RPTPζ/β gene play very important roles during the development and regeneration of the mammalian nervous system.

Conclusions

Genomic analysis clearly demonstrates that the three major RPTPζ/β transcripts are generated through alternative mRNA splicing and are encoded by the same gene. The three RPTPζ/β mRNAs are co-expressed in most regions of nervous system. However, through their expression as distinct glyco-isoforms and interaction with different heterophilic ligands, the three RPTPζ/β isoforms are likely to subserve different functions in the developing and mature CNS and PNS.

The identification and characterization of the novel sp21 variant RPTPζ/β mRNAs indicate that additional heterogeneity of rat RPTPζ/β exists in the juxtamembrane domain of both transmembrane isoforms of RPTPζ/β. The location of sp21 in the juxtamembrane domain is thought to be crucial for the ligand-mediated dimerization and active-site blockage of RPTPs. Therefore, regulation of RPTPζ/β dimerization by alternative splicing of sp21 may serve as an important mechanism for controlling enzymatic activity of the phosphatase.

Chapter 5: Regulation of RPTPζ/β mRNAs Following Sciatic Nerve Injury

Introduction

One of the most remarkable properties of the adult mammalian peripheral nervous system (PNS), in contrast to the central nervous system (CNS), is its capability to regenerate injured axons and to achieve gradual functional recovery (Fawcett and Keynes 1990). Lesion of peripheral nerve induce the development of a supportive environment for axonal regeneration. This environment consists of Schwann cells and their basal laminae, degenerating axon and myelin, epineurial fibroblasts, and macrophages (Fawcett and Keynes 1990; Bunge and Griffin 1992). Successful regeneration first requires that the nerve segment distal to the lesion site undergoes the morphological changes of Wallerian degeneration (Fawcett and Keynes 1990; Bunge and Griffin 1992), in which the axon segments and myelin degenerate and are phagocytosed by infiltrated macrophages. Coincident with Wallerian degeneration, normally quiescent Schwann cells in the distal nerve segment dedifferentiate and start to proliferate. They form arrays of cells, namely the bands of Büngner, within the remaining endoneurial tubes. Growth cones of the regenerating axons elongate along these bands of Büngner. Subsequently, Schwann cells stop proliferating and start the process of differentiation and remyelination. Eventually this leads to a gradual regaining of nerve functions.

Schwann cells in the distal nerve segment are critical for the success of axonal regeneration, supported experimentally by the severe reduction of axonal growth when live Schwann cells are eliminated from the injury site (Hall 1986). Furthermore, axonal regeneration and reinnervation are achieved when primary Schwann cells are grafted into a lesion site in the CNS (Richardson *et al.* 1980; Benfey and Aguayo 1982). These data clearly demonstrates the indispensable role of Schwann cells in promoting nerve regeneration. When Schwann cells start to dedifferentiate and proliferate after nerve injury, their cellular phenotype shifts rapidly from a myelinated state to a premyelinated state (Bunge and Griffin 1992), which is very similar to their status before myelination during development. The specific changes include marked decreases of synthesis of major myelin proteins (Trapp *et al.* 1988) and lipids (White *et al.* 1989), and increased expression of those proteins that are important for axonal growth, including neurotrophic factors (Heumann *et al.* 1987a) and their receptors (Heumann *et al.* 1987b; Taniuchi *et al.* 1988) and neural cell adhesion molecules (Martini 1994). Most of the early changes in Schwann cell phenotype seem to be dependent upon the axon-Schwann cell interactions, as many of these properties revert when axons are regenerated. In contrast, expression of major myelin proteins increases as the axon regenerates and remyelination is initiated (White *et al.* 1989; Snipes *et al.* 1992). Therefore, elucidation of axon-Schwann cell signal transduction pathways during the degeneration and regeneration process of peripheral nerve is of great interest.

As one of the major receptor tyrosine phosphatases that is expressed primarily in the mammalian CNS and PNS, RPTPζ/β has a unique temporal and spatial distribution pattern during the development and maturation of the nervous system (Canoll *et al.* 1993; Engel *et al.* 1996; Meyer-Puttlitz *et al.* 1996; Snider *et al.* 1996). The overall level of RPTPζ/β expression was relatively high during the period of active development in the nervous system, particularly abundant in the developing fiber tracts and all the ventricular zones throughout the brain, where neuronal and glial progenitor cells are actively dividing. There is a marked decrease of RPTPζ/β expression as the nervous system matures. In the adult nervous system, although the overall level of expression is fairly low, high level of expression persists in the subependymal zones, where newly generated neurons and glial cells continue to migrate out of the area. Thus, in the nervous system RPTPζ/β isoforms, especially the two transmembrane transcripts, appear to be preferentially expressed in areas in which a high level of proliferation occurs. It is well known that, cellular responses of recovery from neural injury in the adult often recapitulates many developmental events. Therefore, the developmental expression pattern of RPTPζ/β suggested that this molecule might play a role in the cellular response to neural injury.

In addition, isoforms of RPTPζ/β can be expressed as chondroitin sulfate proteoglycans, macromolecules that have often been implicated in the response to neural injury (Mckeon *et al.* 1991; Silver 1994; Small *et al.* 1996). Furthermore, RPTPζ/β has also been shown to interact with a group of diverse heterophilic ligands, including neural adhesion molecules such as NCAM, Ng-CAM,

contactin, TAG-1 and Nr-CAM, the extracellular matrix protein tenascin, and the neurotrophic growth factor pleiotrophin, all of which have either directly been implicated in mediating cellular responses to various neural injuries or have been shown to regulate neuronal migration, axonal guidance and pathfinding. Through these interactions, various RPTPζ/β isoforms have been demonstrated to affect neural adhesion, neurite outgrowth and neuronal differentiation. For example, the short isoform of RPTPζ/β can induce neurite outgrowth and promote differentiation of cortical neurons through its interaction with contactin and Nr-CAM (Sakurai *et al.* 1997). These studies, therefore, further support the involvement of RPTPζ/β in neural injury responses.

Recently, our laboratory reported (Snider *et al.* 1996) that RPTPζ/β mRNAs are induced in regions of compensatory axonal sprouting in response to a hippocampal deafferentation lesion, and in regions of glial scarring after a nonspecific stab wound lesion in the brain. In order to further characterize potential involvement of RPTPζ/β in other lesion paradigms, especially those capable of regeneration in the PNS, I have determined the pattern of RPTPζ/β mRNA expression following a regeneration-capable sciatic nerve crush injury.

Results

Expression of RPTPζ/β mRNAs in intact sciatic nerve, lumbar DRG and spinal cord

To simultaneously measure mRNA transcripts that encode the three major RPTPζ/β isoforms, I developed a novel βpan probe to be employed with a quantitative and extremely sensitive RNase protection assay (see Fig. 11). The three major RPTPζ/β transcripts were found to be constitutively expressed at relatively high level in adult rat L4-L6 segments of spinal cord, comparable to the level of expression detected in the brain (Fig. 15 and data not shown). The expression in the L4-L6 DRGs was somewhat lower than that in the spinal cord (Fig. 15). However, extremely low levels of expression were found in the intact adult rat sciatic nerve (Fig. 15 and Fig. 7). Similar results were obtained in RNA samples from sham operated rats (Fig. 15).

Detailed analysis of relative abundance of the three transcripts revealed that there was a striking difference between CNS tissues such as the spinal cord and PNS tissues, for example sciatic nerve and DRG (Fig. 16). As measured by the ratio between the short and phosphacan transcripts, the short transcript is the most abundant of the three transcripts in the peripheral nervous system (sciatic nerve and DRG), with an average short/phosphacan ratio of 1.5 and 1.2 respectively. However, in the CNS such as the spinal cord and brain (Fig. 16 and 11B), the level of the phosphacan transcript is the highest, as evidenced by a short/phosphacan ratio of 0.6 in spinal

cord. All these difference in ratios is highly significant statistically (Fig. 16, all $P < 0.0001$, paired t-test).

Throughout the CNS and PNS, the full length RPTPζ/β transcript is consistently the least abundant among the three major RPTPζ/β mRNAs (Fig. 11B and Fig. 18B). Analysis of its relative abundance to the other two transcripts (data not shown) confirmed that the short transcript is expressed at a higher level than the phosphacan transcripts in the PNS, whereas in the CNS expression of the phosphacan transcript is higher than the short transcripts.

Induction of RPTPζ/β mRNAs in the sciatic nerve following crush injury

In order to study the functional role of RPTPζ/β during degeneration and regeneration of the peripheral nervous system, I have determined the expression and regulation of RPTPζ/β mRNAs in the sciatic nerve following an experimental crush lesion. Because the endoneurial tubes of the crushed nerve remain mostly intact, maximal axonal regeneration can be facilitated. Total RNAs from segments of lesioned or sham-operated sciatic nerve distal or proximal to the lesion site were analyzed using the RNase protection assay. Time points between 1 and 28 days post-lesion were analyzed. The expression of the RPTPζ/β transcripts in the sciatic nerve of non-operated control and sham-operated rats was not significantly different (data not shown).

In the proximal segments of sciatic nerve, very low level of RPTPζ/β mRNA expression was not altered during the period which

extended from 1 to 28 days after crush injury (Fig. 15 and data not shown).

In the distal segment of the nerve, one day after the crush, no significant change was detected in RPTPζ/β mRNA levels as compared to those in sham-operated or non-operated control nerve segments (data not shown). However, 4 days after the injury, expression of RPTPζ/β mRNAs was dramatically induced in the crushed distal nerve segments (all three transcripts $P < 0.01$, ANOVA) (Fig. 17). Peak expression was reached seven days after injury (Fig. 15 and 17), with more than ten fold induction compared to the sham-operated control (all three transcripts $P < 0.0001$, ANOVA). Although the level of expression gradually decreased from the peak levels detected 7 days after injury, expression was still significantly greater than sham-operated controls 14 , 21 and 28 days after the lesion (all $P < 0.05$, ANOVA). Ten weeks after the lesion, expression was not significantly different from the low control levels (data not shown).

Following the crush lesion, all three RPTPζ/β mRNAs were up-regulated in the distal nerve segment, showing an extremely similar temporal profile (Fig. 17). However, there was a significant difference in terms of the extent of induction among the three transcripts: The phosphacan transcript was induced to a greater extent than the other two in the crushed distal nerve segments (Fig. 18). Because of inter-assay variability in the absolute amounts (pg/5 μg total RNA) of the three transcripts, and the apparent regulation of cyclophilin mRNA levels (internal control) by the lesion paradigm, I have expressed amounts as the ratio of one isoform relative to another at that time point (determined by quantification of each of

the three bands within a single lane of a 5% non-denaturing gel using the PhosphorImager). This is shown at 7 days after crush injury by the average decrease of short/phosphacan ratio (Fig. 18A) from about 1.5 to 1.0 ($P < 0.001$, unpaired t-test), and the increase of phosphacan/long ratio (Fig. 18B) from about 4.0 to 5.9 ($P < 0.05$, ANOVA). Similar statistically significant results were also obtained by comparing these ratios within each time point from 4 to 28 days after lesion (data not shown). Determination of the short/long ratios at these time points (Fig. 18B and data not shown) indicated that the short/long ratio did not change significantly after injury in the distal nerve segments, suggesting that although all three isoforms were induced, phosphacan mRNA levels increased the most.

These results demonstrated that expression of all three major RPTPζ/β transcripts was greatly induced in the distal segments of sciatic nerve following a crush lesion. Timecourse study of the induction suggested that the regulation of RPTPζ/β mRNA expression correlated well with that of the degenerative and regenerative responses by the distal segments of the injured nerve.

Expression of RPTPζ/β mRNAs in lumbar dorsal root ganglia (DRG) and spinal cord following sciatic nerve crush injury

RPTPζ/β mRNA expression in lumbar spinal cord segments (Fig. 19A) and dorsal root ganglia L4-L6 (Fig. 19B), whose axons travel within the sciatic nerve, showed no significant changes between day 1 and Day 28 following sciatic nerve crush lesion (Fig. 19 and data

not shown). These data has been expressed as a percentage of the corresponding sham-operated control within the same time points.

These results suggested that the regeneration-capable sciatic nerve crush lesion did not cause a significant change in the RPTPζ/β mRNA level in the L4-L6 DRG or spinal cord segments, whose sensory and motor axons travel through the sciatic nerve.

Regulation of RPTPζ/β mRNA expression in primary Schwann cell culture and C6 glioma cell lines

In order to determine the cellular localization of RPTPζ/β mRNA in the sciatic nerve and to study its regulatory role in glial cell proliferation and differentiation, I have examined the expression of RPTPζ/β mRNAs in the primary rat Schwann cell culture and C6 glioma cell line.

In primary Schwann cell cultures low level of RPTPζ/β mRNA expression was detected, which further decreased following glial growth factor (GGF) and forskolin treatment for two weeks (Fig. 7). Forskolin is a reversible adenyl cyclase inhibitor, and its functions is to increase the level of intracellular cAMP. It is known that the combined treatment of GGF and forskolin mimics many effects of axonal contact on the premyelinated Schwann cells during development and regeneration (Jessen *et al.* 1991; Morgan *et al.* 1991). GGF is a Schwann cell mitogen, but under conditions of contact inhibition, it can promote the differentiation of Schwann cells (Jessen and Mirsky 1991). In the presence of GGF and forskolin, primary Schwann cells develop a more elongated phenotype, and

many gene products that are not directly involved in myelination are down-regulated (Morgan *et al.* 1991).

In the rat C6 glioma cell line, treatment with cAMP analogs such as dibutyryl cAMP leads to an increase in intracellular cAMP, causing C6 cells to stop proliferating and to send out many long and thin processes from their somas. Thus, the C6 cell line has been used widely as an *in vitro* model for glial cell differentiation. Very high levels of RPTPζ/β mRNA expression was found in the C6 cells, with the short transcript being the most abundant isoform, as was found in sciatic nerve and DRG. Expression of RPTPζ/β mRNAs was greatly reduced after 1 mM dibutyryl cAMP treatment for 24 hours (Fig. 20), and the levels of each of three transcripts were reduced to a similar extent (Fig. 20).

These results demonstrate that RPTPζ/β mRNA expression is down regulated in differentiating primary Schwann cells and C6 glioma cells. These results indicate that RPTPζ/β transcripts are localized and regulated in Schwann cells *in vitro*, suggesting that RPTPζ/β transcripts in the sciatic nerve are most likely to be localized in the Schwann cells.

Discussion

In the present study, I have shown that RPTPζ/β mRNAs are greatly induced following sciatic crush lesion in the distal segments of the nerve. The time course of this induction correlated well with that of the sciatic Schwann cell proliferation and differentiation (remyelination) response after the same lesion paradigm (Fawcett and Keynes 1990; Bunge and Griffin 1992; Carroll *et al.* 1997). In addition, I found that there was a significant difference of RPTPζ/β expression between the PNS and CNS in terms of the relative abundance of the three alternative RPTPζ/β transcripts. The short RPTPζ/β transcript, which has been shown to be able to induce neurite outgrowth and promote neuronal differentiation (Sakurai *et al.* 1997), is the most abundant of the three transcripts in the peripheral nervous system. On the other hand, the phosphacan transcript, which is often being associated with inhibition of neuronal adhesion and neurite outgrowth (Grumet *et al.* 1994; Milev *et al.* 1994), is the most abundant RPTPζ/β transcripts in the CNS. This finding suggests that various isoforms of RPTPζ/β may serve diverse functions in different temporal and spatial context *in vivo*. Since synthesis is differentially regulated following injury and in the PNS compared to the CNS, these differences could account in part for the strikingly different regeneration capabilities of the PNS and CNS.

The current data shows that the induction of the RPTPζ/β mRNA after lesion only occurs in the distal nerve segments, and no significant change has been detected in the proximal nerve segment, lumbar L4-L6 DRGs and spinal cord. These results implied that the

involvement of RPTPζ/β in peripheral degeneration and regeneration is mostly restricted locally at the lesion site where axonal regrowth occurs. Thus, it is critical to determine the timecourse of RPTPζ/β mRNA expression in the lesioned distal nerve segments, and compare it to that of the cellular responses by the nerve. This comparison revealed that the temporal expression pattern of the RPTPζ/β mRNAs following the sciatic crush lesion correlated very well with the proliferation and subsequent remyelination profile of the sciatic nerve Schwann cells after the same lesion. As previously reported in rat (Carroll *et al.* 1997), sciatic Schwann cells in the distal nerve segment start to proliferate three day after the crush lesion, which is very close to the first time point that induction of RPTPζ/β mRNAs was observed in the distal nerve segment. The rate of Schwann cell proliferation reach a peak at about one week post-injury (Carroll *et al.* 1997), matching the peak of induction observed for RPTPζ/β mRNAs. From 1 week to around 4 weeks, the rate of proliferation starts to taper off to a lower but still detectable level (Carroll *et al.* 1997). This is very similar to the time course of induction for RPTPζ/β mRNAs during the same time period after the lesion: expression declined from the peak but was still detected at a level significantly higher than that in the control animals.

Therefore, the cellular source of the RPTPζ/β transcripts in the lesioned distal sciatic nerve segment is mostly likely to be the proliferating Schwann cells. In the proximal segment of crushed sciatic nerve, where no Schwann cell proliferation occurs after lesion, no induction of RPTPζ/β transcripts was observed. No studies have reported RPTPζ/β expression in any non-neural cell types, including

the other two cell types in the lesioned distal nerve segment: proliferating epineurial fibroblasts and infiltrative macrophages. In addition, the number of infiltrated macrophages peaks at about two days after lesion (Clemence *et al.* 1989), prior to the observed induction of RPTPζ/β mRNAs. Furthermore, we have shown expression of RPTPζ/β mRNAs in proliferating primary Schwann cells, and that the expression was down-regulated upon differentiation of these Schwann cells (Fig. 7). These are *in vitro* manipulation analogous to the *in vivo* injury response process by the sciatic Schwann cells, shifting from dedifferentiation (proliferation) to remyelination (differentiation). Taken together this evidence strongly suggested that the Schwann cells are the source of the induced RPTPζ/β mRNA expression in the crushed distal sciatic nerve segments. As an internal control in the RNase protection assay, the cyclophilin mRNA also induced in the distal nerve segment after the lesion, presumably reflecting the increase of cell number due to Schwann cell proliferation. The extent of induction of RPTPζ/β mRNAs is greater than that of cyclophilin (data not shown), suggesting that increased mRNA per cell is a major determinant in the induction of RPTPζ/β expression observed and each proliferating Schwann cells are likely to express more RPTPζ/β mRNAs. Interestingly, some of the identified ligands of RPTPζ/β, such as NCAM and L1/Ng-CAM, have been demonstrated to be expressed on the surface of both regenerating sciatic axons and Schwann cells (Martini 1994). Therefore, RPTPζ/β may participate in the peripheral nerve degeneration and regeneration processes by regulating the

axon-Schwann cell interactions through its various isoforms and ligands.

The finding that the short RPTPζ/β transcript was the most abundant isoform in the PNS (both in DRGs and sciatic nerves), while in the CNS (both in the brain and spinal cord) phosphacan transcript was the most abundant, is of great interest. Using *in situ* hybridization, RNase protection assay and Northern blot analysis, our lab and others have previously determined the temporal and spatial expression pattern of the three RPTPζ/β transcripts in the developing and mature nervous system (Canoll *et al.* 1996; Engel *et al.* 1996; Snider *et al.* 1996). To our surprise, the three mRNAs co-localized in almost all the regions of the nervous system surveyed except in a few areas of high neural cell proliferation, such as the subependymal layer of the brain ventricles, where the mRNAs encoding the transmembrane isoforms of the phosphatase seemed to be expressed at higher levels than those encoding the phosphacan isoform. It has also been reported (Canoll *et al.* 1996) that the three RPTPζ/β transcripts are also differentially expressed in glial cell cultures, with the O2A progenitors expressing high levels of transcripts encoding phosphacan and full length phosphatase, and the type 1 astrocyte progenitors predominantly expressing the short transcript. However, when these progenitors are differentiated, expression of the transmembrane isoforms is greatly reduced relative to that of phosphacan. Differentiated astrocytes and oligodendrocytes in the CNS are known to be inhibitory for axonal regeneration. In addition, the full length and phosphacan isoforms are believed to be expressed primarily as chondroitin sulfate proteoglycans (CSPG) (Shitara *et al.*

1994; Sakurai *et al.* 1997), a family of proteins mostly regarded as inhibitory for axonal growth (Brodkey *et al.* 1993; Silver 1994; Small *et al.* 1996). Furthermore, phosphacan has been shown to bind to neurons and inhibit their neural adhesion and neurite outgrowth capability (Grumet *et al.* 1994; Milev *et al.* 1994). In contrast, the short RPTPζ/β isoform has been demonstrated to be able to induce neurite outgrowth, through interaction with contactin and Nr-CAM (Sakurai *et al.* 1997), and not to be detected as a CSPG (Sakurai *et al.* 1996). It is therefore of interest that the relative level of mRNA encoding the short RPTPζ/β isoform is highest in the regeneration-competent PNS compared to the CNS. In addition, even in the regenerated PNS, where a greater increase of phosphacan transcript induction was observed, the short/phosphacan ratio is still significantly higher than that in the CNS (Fig. 18). Based on these previous findings, our current data suggested that mature glial cells in the CNS and PNS may preferentially express different RPTPζ/β isoforms to interact heterophilically with different ligands on the cell surfaces, so as to either inhibit or promote axonal regeneration.

Previously, we have demonstrated that expression of RPTPζ/β mRNAs is also induced in regions of axonal sprouting and glial scarring in response to CNS lesions (Snider *et al.* 1996). Thus, induction of RPTPζ/β may be part of a general mechanism by which glial cells responded to neural injury. The expression of different amounts of the RPTPζ/β isoforms might contribute in part to the different capabilities of the CNS and PNS to regenerate. To directly support this hypothesis, future studies are necessary to determine the relative abundance of RPTPζ/β isoforms induced in lesion

paradigms of the CNS. In addition, antibodies against different RPTPζ/β isoforms should be included in antibody blocking experiment in different lesion paradigms, in order to access the functional importance of these isoforms for axonal regeneration.

Conclusions

The dramatic upregulation of RPTPζ/β mRNA expression in the distal segment of sciatic nerve following a crush injury indicates a role for RPTPζ/β in the cellular processes of degeneration and regeneration in PNS. The time course of this induction correlates well with the proliferation and differentiation (remyelination) responses of Schwann cells to injury in this paradigm. The cellular source of the RPTPζ/β expression in the distal segment of lesioned sciatic nerve is therefore most likely to be the proliferating Schwann cells. RPTPζ/β may consequently participate in the peripheral nerve degeneration and regeneration processes by regulating the axon-Schwann cell interactions through its various isoforms and heterophilic ligands.

Furthermore, a significant difference was revealed in the relative abundance of RPTPζ/β isoforms expressed in the PNS and CNS. The short RPTPζ/β transcript, which has been shown to be able to induce neurite outgrowth and promote neuronal differentiation, is the most abundant of the three transcripts in the PNS. On the other hand, the phosphacan transcript, which is often associated with inhibition of neuronal adhesion and neurite outgrowth, is the most abundant RPTPζ/β transcripts in the CNS. These unique differences could account in part for the strikingly different regeneration capabilities of the PNS and CNS, and regulation of various isoforms of RPTPζ/β expression in glial cells may be part of a general mechanism for neural injury responses.

Figures

Figure 1: Tissue distribution of RPTPζ/β mRNA. RNA was isolated from a number of embryonic (E), postnatal (P) and adult (Ad) tissues as described in Chapter 2, and the relative levels of RPTPζ/β RNA in 10 μg samples of total RNA determined by RNase protection analysis using [^{32}P]-labeled antisense probes to the RPTPζ/β phosphatase domain I (solid arrow) and to cyclophilin (open arrow). Assays were run in duplicate and protected fragments were resolved on nondenaturing 5% polyacrylamide gels and visualized by autoradiography. Ad, adult; Cer, cerebellum; Cbl, cerebrum.

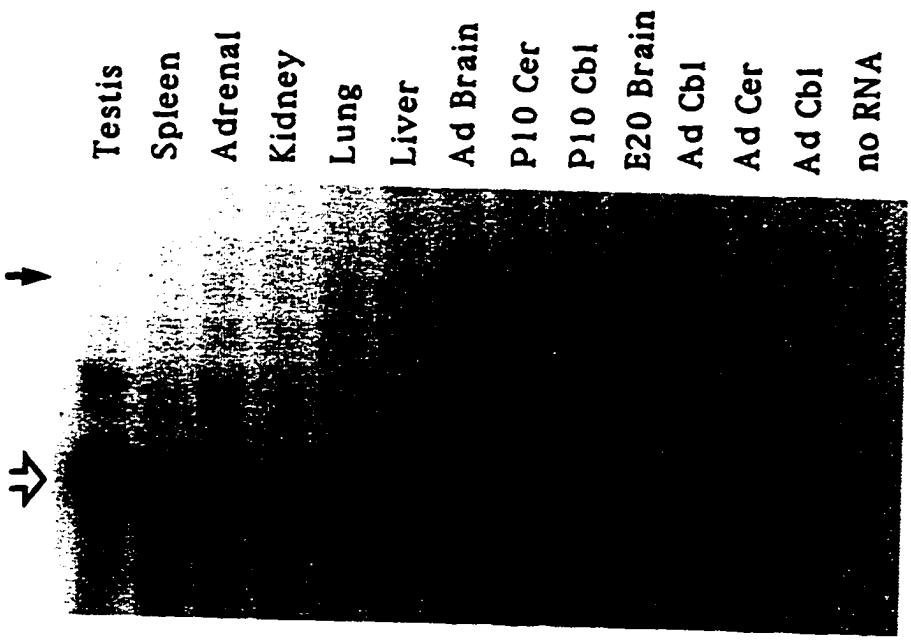
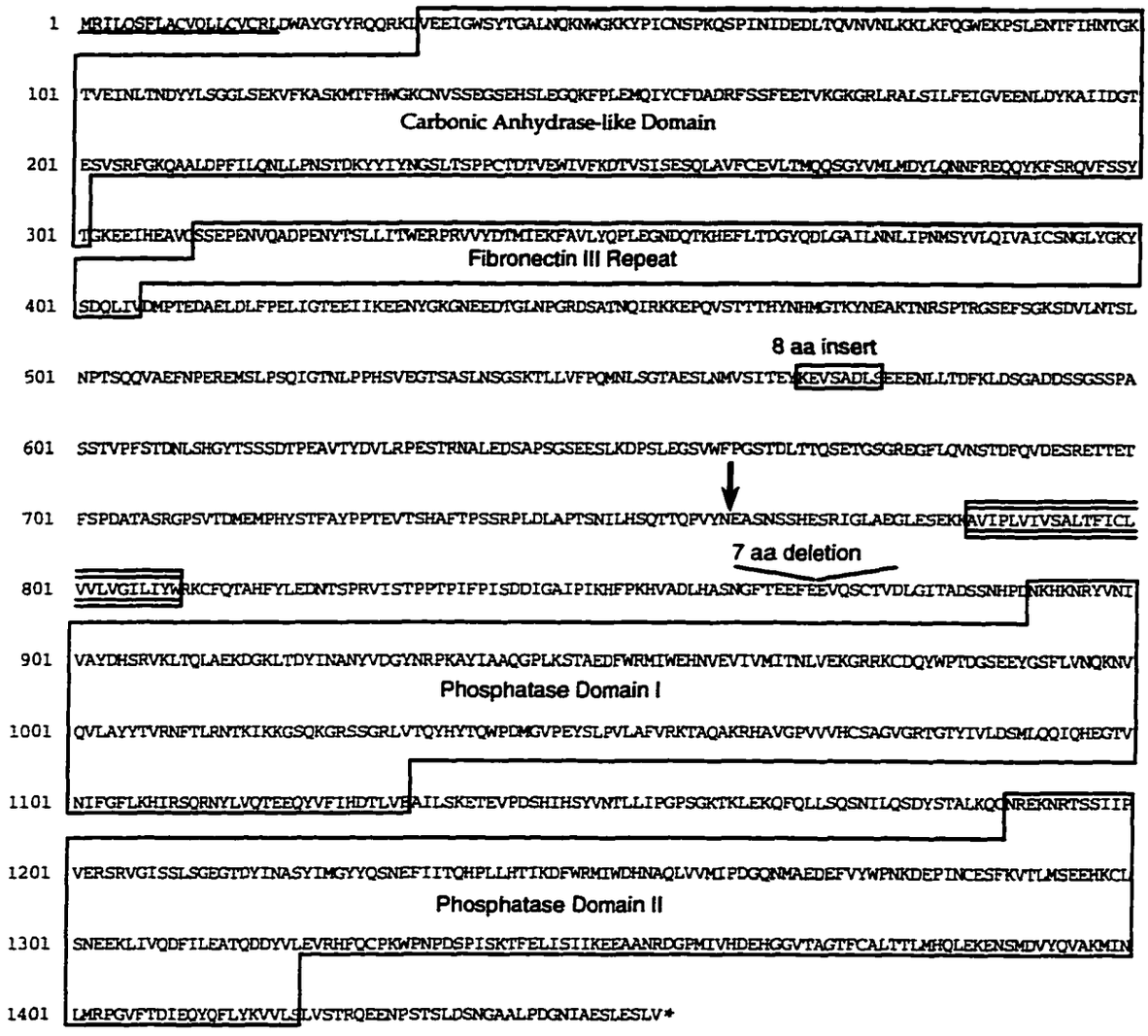


Figure 2: Amino acid sequence predicted by the rat RPTPζ/β cDNA clones. In panel A, the amino acid sequence deduced from the rat RPTPζ/β nucleic acid sequence is shown. The putative N-terminal signal sequence has been underlined. The carbonic anhydrase (CA)-like, FN-III, predicted transmembrane (doublebox), and phosphatase domains I and II are indicated. The position of the large extracellular insert region, distinguishing large and small forms of RPTPζ/β, is indicated by the arrow, and the positions of the 7 amino acid deletion and 8 amino acid insertion are also shown. In panel B, the regions of the RPTPζ/β cDNAs that were used to construct riboprobe vectors, containing either the CA-like, insert, or phosphatase I domains of the indicated sizes, are shown; the positions of these sequences within both the RPTPζ/β cDNA and the corresponding predicted RPTPζ/β polypeptide are also shown. These linearized vectors were employed to synthesize sense and anti-sense RNA, labeled with [^{32}P] or [^{35}S], for use in RNase protection analysis (see Chapter 2).

A



B

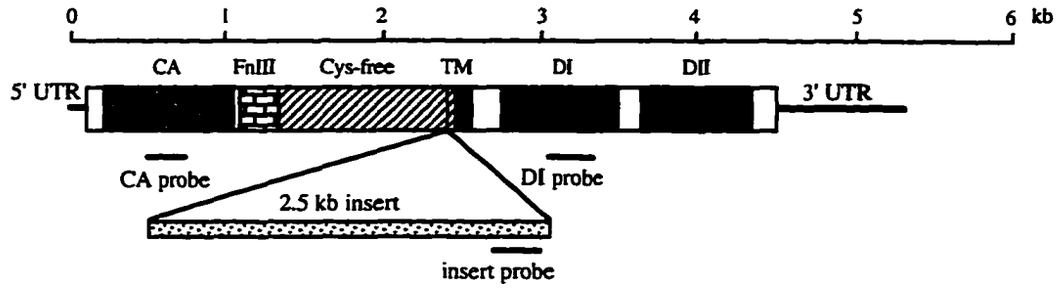


Figure 3: Sequence conservation of the RPTPζ/β phosphatase domain I and carbonic anhydrase (CA)-like region. The CA-like region (panel A) and phosphatase domain I (panel B) of the deduced rat RPTPζ/β amino acid sequence were aligned with related amino acid sequences in the database, using TFASTA and PILEUP programs (GCG). Positions of amino acid identity occurring in at least 4 of the aligned sequences have been identified by boxing the corresponding residues. In panel A, the CA-like domain of rat RPTPζ/β has been aligned to human PTPζ, mouse PTPγ, human PTPγ, human CA VI, and mouse CA I. In panel B, rat RPTPζ/β phosphatase domain I has been aligned with human PTPζ, human PTPγ, rat LRP, *Drosophila* LAR, and mouse LRP. PCR amplification of PTP sequences was carried out using two fully degenerate oligonucleotide primers that correspond to the amino acid residues indicated by the asterisks.

A

```
        1                                                                    70
rRPTPβ  LVEEIGWSYT.GALNQKNWGKKYPICNSPKQSPINIDEDLTQVNVNLKKLKFQGWEKPSLENTFIHNTGK
hPTPζ   LVEEIGWSYT.GALNQKNWGKKYPTCNSPKQSPINIDEDLTQVNVNLKKLKFQGWDKTSLENTFIHNTGK
mRPTPγ  ASGDPYWAYS.GAYGPEHWVTSSVSCGGSHQSPIDILDHHARVGDEYQELQLDGFDNESSNKTWMKNTGK
hRPTPγ  ASGDPYWAYS.GAYGPEHWVTSSVSCGGSHQSPIDILDHHARVGDEYQELQLDGFDNESSNKTWMKNTGK
hCA6    AQHVSDWTYSEGALDEAHWPQHYPACGGQRQSPINLQRTKVRYNPSLKGLNMTGYETQAGEFPMVNNGHT
mCAI    .MASADWGYGSEN.GPDQWSKLYPIANGNNQSPIDIKTSEANHDSSLKPLSISYNPATAKEIVNVGHSFH

        71                                                                  140
rRPTPβ  TVEINLTNDYYLSGGLSEKVFKASKMTFHWGKCNVSSEGSEHSLEGQKFPLEMQIYCFDADRFSSFEETV
hPTPζ   TVEINLTNDYRVSGGVSEMVFKASKITFHWGKCNMSSDGSEHSLEGQKFPLEMQIYCFDADRFSSFEEAV
mRPTPγ  TVAILLKDDYFVSGAGLPGRFKAEKVEFHWGHSN.GSAGSEHSVNGRRFPVEMQIFFYNPDDFDSFQTAI
hRPTPγ  TVAILLKDDYFVSGAGLPGRFKAEKVEFHWGHSN.GSAGSEHSVNGRRFPVEMQIFFYNPDDFDSFQTAI
hCA6    VQIGLPSTMRMTVADGIV..YIAQQMHFHWGGASSEISGSEHTVDGIRHVIEIHIVHYNS.KYKTYDIAQ
mCAI    VIFDDSSNQSVLKGGPLADSYRLTQFHFHWGN..SNDHGSEHTVDGTRYSGELHLVHWNSAKYSSASEAI

        141                                                                 210
rRPTPβ  KGKGRLRALSILFEIGV.EENLDYKAIIDGTESVSRFGKQAALDPFILQNLLPNSTDKYYIYNGSLTSPP
hPTPζ   KGKGKLRALSILFEVGT.EENLDFKAIIDGVESVSRFGKQAALDPFILLNLLPNSTDKYYIYNGSLTSPP
mRPTPγ  SENRIIGAMAIFFQVSP.RDNSALDPIIHGLKGVVHHEKETFLDPFILRDLLPASLGSYYRYTGSLTTPP
hRPTPγ  SENRIIGAMAIFFQVSP.RDNSALDPIIHGLKGVVHHEKETFLDPFILRDLLPASLGSYYRYTGSLTTPP
hCA6    DAPDGLAVLAAFVEVKNYPENTYYSNFISHLANIKYPGQRTTLTGLDVQDMLPRNLQHYYTYHGSLTTPP
mCAI    SKADGLAILGVLMKVG..PANPSLQKVLDALNSVKTKGKRAPFTNFDPSSLLPSSLD.YWTYFGSLTHPP

        211                                                             278
rRPTPβ  CTDTVEWIVFKDTVSISESQLAVFCEVLTMQQSGYVMLMDYLQNNFREQQYKFSRQVFSSYT......
hPTPζ   CTDTVDWIVFKDTVSISESQLAVFCEVLTMQQSGYVMLMDYLQNNFREQQYKFSRQVFSSYT......
mRPTPγ  CSEIVEWIVFRRPVPISYHQLEAFYSIFTTEQQDHVKSVEYLRNNFRPQQALNDRVVSKSAV......
hRPTPγ  CSEIVEWIVFRRPVPISYHQLEAFYSIFTTEQQDHVKSVEYLRNNFRPQQALNDRVVSKSAV......
hCA6    CTENVHWFVLADFVKLSR...TQVWKLENSLLDHRNKTI...HNDYRRTQPLKHRVVESNFPNQEYTL
mCAI    LHESVTWVICKDSISLSPEQLAQLRGLLSSAEGESAVPV...LSNHRPPQPLKGRTVRASF.......
```

B

Figure 4: Organization of three major RPTPζ/β variant transcripts. In panel A, the proteins predicted to be translated from the various rat RPTPζ/β cDNA sequences are shown schematically. Note that the sequence that is downstream of the putative splice site (indicated by the arrow in Figure 2), and that is retained in the cDNA clones corresponding to the truncated RPTPζ/β variant (phosphacan), contains a translation stop two bases 3' to the putative splice site. In panel B, partial nucleic acid and predicted amino acid sequences of the rat RPTPζ/β cDNA clones, corresponding to the three major RPTPζ/β transcripts, which bracket the putative splice junction are aligned.

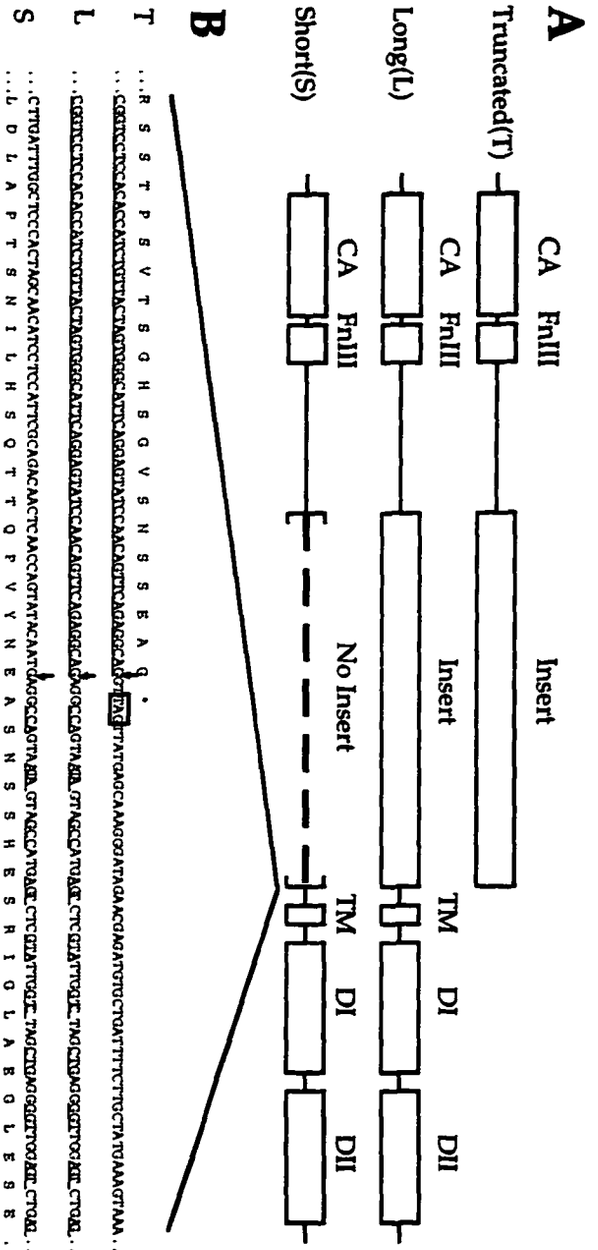
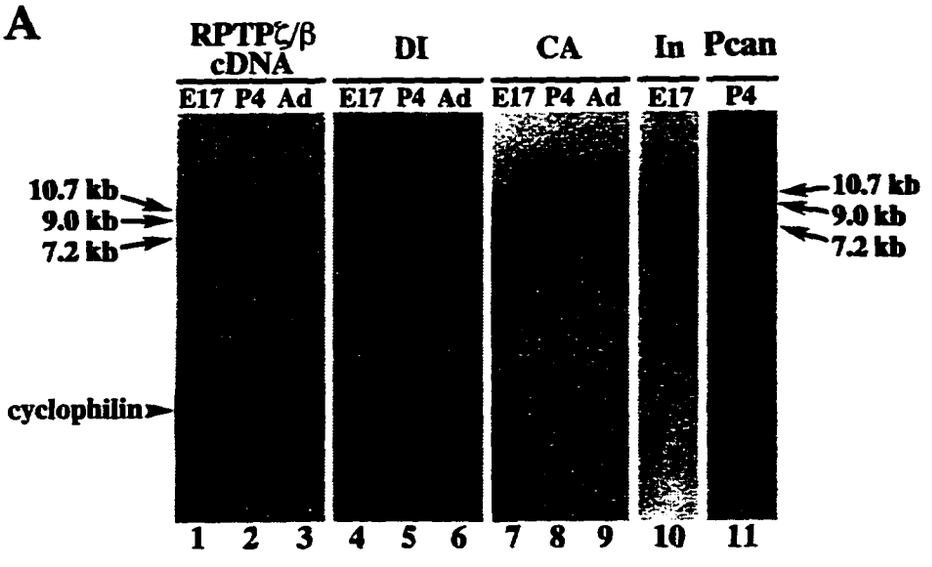


Figure 5: Northern analysis of RPTPζ/β mRNAs. In panel A, samples containing 1 or 2 μg poly-A (+) RNA, isolated from embryonic day 17 (E17) (lanes 1, 4, 7, and 10), postnatal day 4 (P4) (lanes 2, 5, 8, and 11) and adult brain (lanes 3, 6, and 9), were subjected to northern analysis and replicate blots were hybridized with [^{32}P]-labeled probes as follows: lanes 1-3 with cDNA fragments of RPTPζ/β that did not include sequences encoding the phosphatase domains and C-terminus, and cyclophilin (a control for RNA loading; arrowhead), lanes 4-6 with a probe to RPTPζ/β phosphatase domain I (DI; 310 bp), lanes 7-9 with a probe to the RPTPζ/β carbonic anhydrase-like domain (CA; 213 bp), lane 10 with a probe to a region within the ~2.5 kb RPTPζ/β insert (In; 298 bp), and lane 11 with a probe to the 3' untranslated region of phosphacan (Pcan; 302 bp). The regions within the phosphacan and RPTPζ/β mRNAs to which the specific probes hybridized are indicated in the diagram shown in panel B. Positions of additional probes (5' Insert and TM) used in genomic Southern analysis (Fig. 10B) are also shown. Blots were washed to high stringency and autoradiographed. Exposure times were 2 days (lanes 1-3), 7 days (lanes 4-6), 3 days (lanes 7-9) and 18 hours (lanes 10 and 11). Transcript sizes, indicated by the arrows on the left and right of panel A were determined by comparison to co-electrophoresed RNA standards.



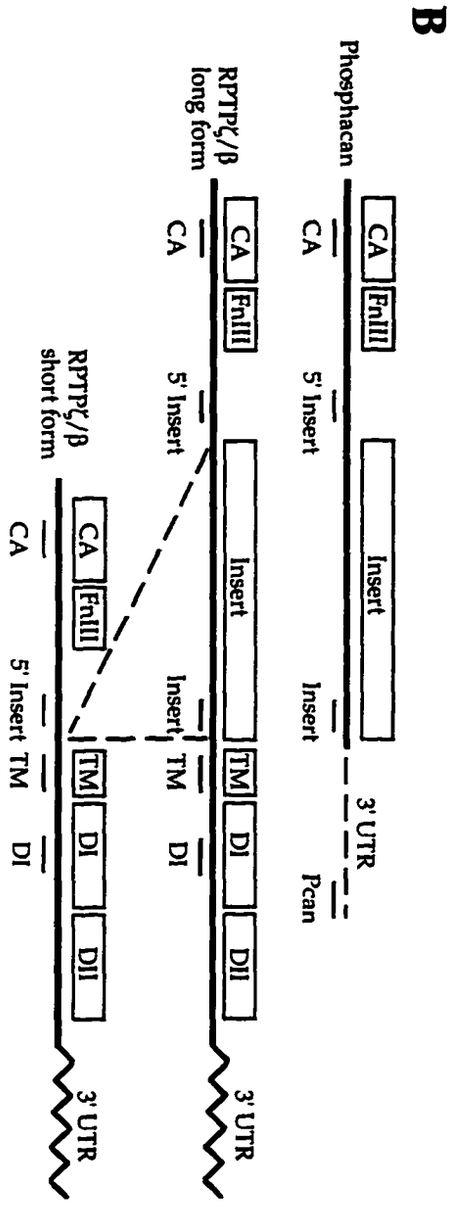


Figure 6: Tissue distribution of variant RPTPζ/β transcripts determined through the use of specific probes for the phosphatase I (panel A), carbonic anhydrase-like (panels B and D), and insert domains (panels C and E). In panels A-C, RNase protection analysis was used to quantify RPTPζ/β (solid arrow) and cyclophilin (open arrow) RNA levels in 10 μg samples of total RNA isolated from the embryonic, postnatal, and adult tissues noted above panel A, with the following exceptions: the SCG RNA used in panels A and B was isolated from P10, while that in panel C was from adult; and in panel C, only 2 μg of total RNA from P10 adrenal was used. In panel D, lanes 1-6, 10 μg samples of RNA isolated from adult brain, kidney, liver, adrenal, lung, and spleen were analyzed using the RPTPζ/β CA-like and cyclophilin probes. In panel E, lanes 1-6, 10 μg samples of RNA isolated from adult brain, C6 glioma, kidney, liver, adrenal and lung were analyzed using the RPTPζ/β insert and cyclophilin probes. All assays were performed in duplicate or triplicate, as described in Chapter 2.

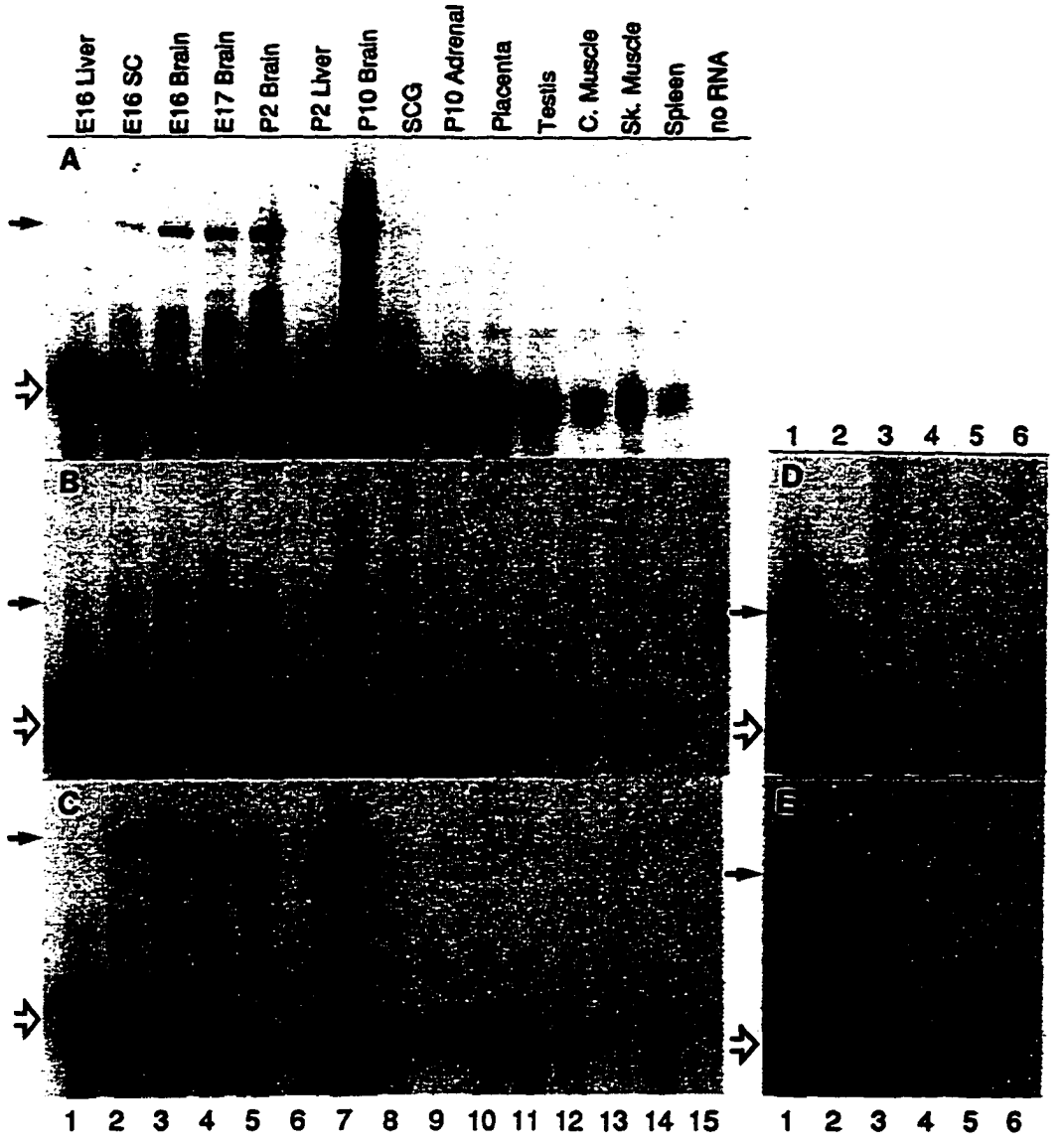


Figure 7: Distribution of RPTPζ/β mRNA in cells of neuronal and non-neuronal origin. RNase protection analysis was used to quantify RPTPζ/β (solid arrow) and cyclophilin (open arrow) RNA levels. For comparison, 10 μg samples of total RNA from embryonic (E) and postnatal (P) spinal cord (SC), and brain were analyzed in parallel with RNA isolated from sciatic nerve, untreated and NGF-treated PC12 cells, C6 glioma cells, and primary cultured Schwann cells grown in the absence or presence of both glial growth factor (GGF) and 2μM forskolin (F). Assays were run in duplicate and protected fragments were resolved on nondenaturing 5% polyacrylamide gels and visualized by autoradiography.

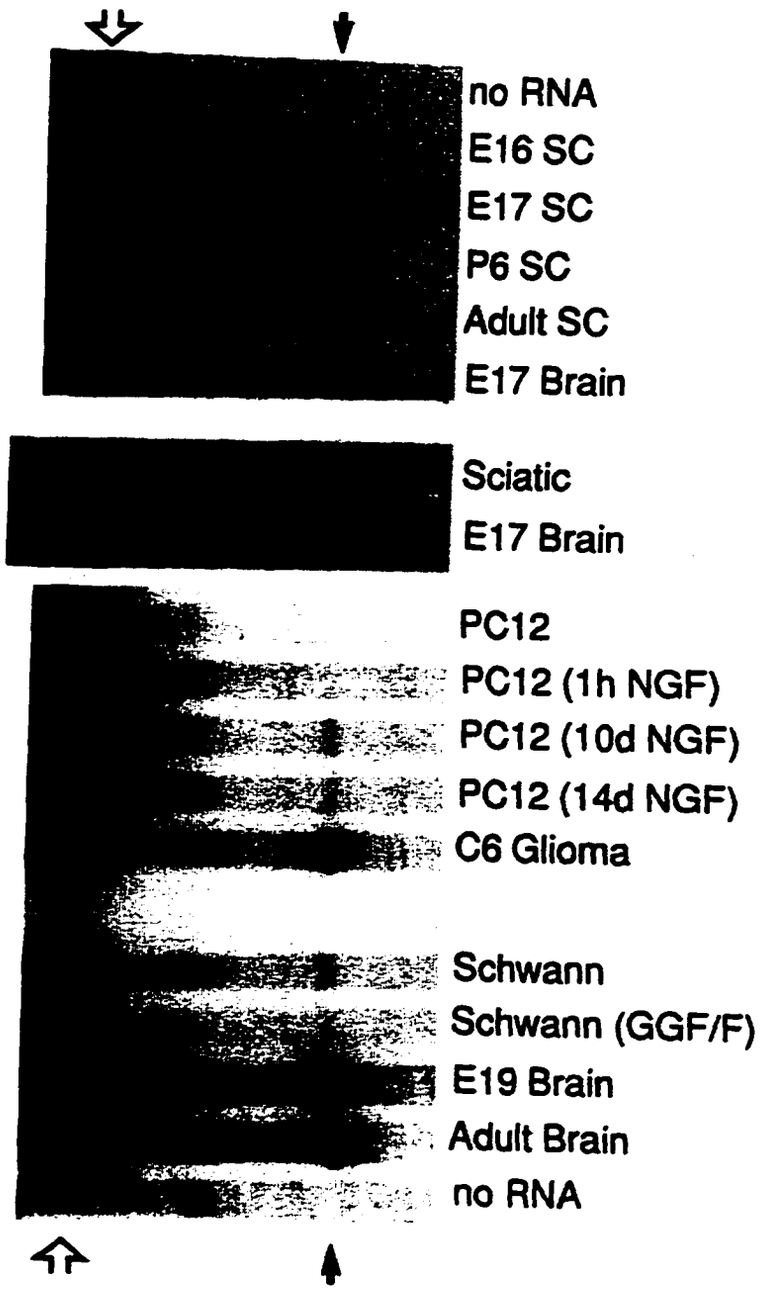


Figure 8: Expression of RPTPζ/β and VGF mRNAs in primary embryonic midbrain cultures. Rat E16 midbrain cultures were grown in defined medium in either the absence (control) or presence of 10 ng/ml EGF, 50 ng/ml NGF, or 30 ng/ml bFGF. In panel A, equal numbers of plates were harvested after either 14 days or 8 days in culture, total RNA was isolated as described in Chapter 2, and RNase protection analysis carried out using the RPTPζ/β phosphatase (solid arrow) and cyclophilin (open arrow) antisense probes on samples containing 2 μg (14 day control), 5 μg (14 days bFGF), 10 μg (8 days bFGF), and 10 μg (8 days EGF) total RNA. In panel B, equal numbers of plates were harvested after either 2 days or 8 days *in vitro*, total RNA isolated, and RNase protection analysis carried out using VGF (solid arrow) and cyclophilin (open arrow) antisense probes and samples containing 4 μg (2 day control), 4 μg (2 days NGF), 4 μg (2 days EGF), 3 μg (8 day control), 3 μg (8 days NGF) and 16 μg (8 days EGF) total RNA.

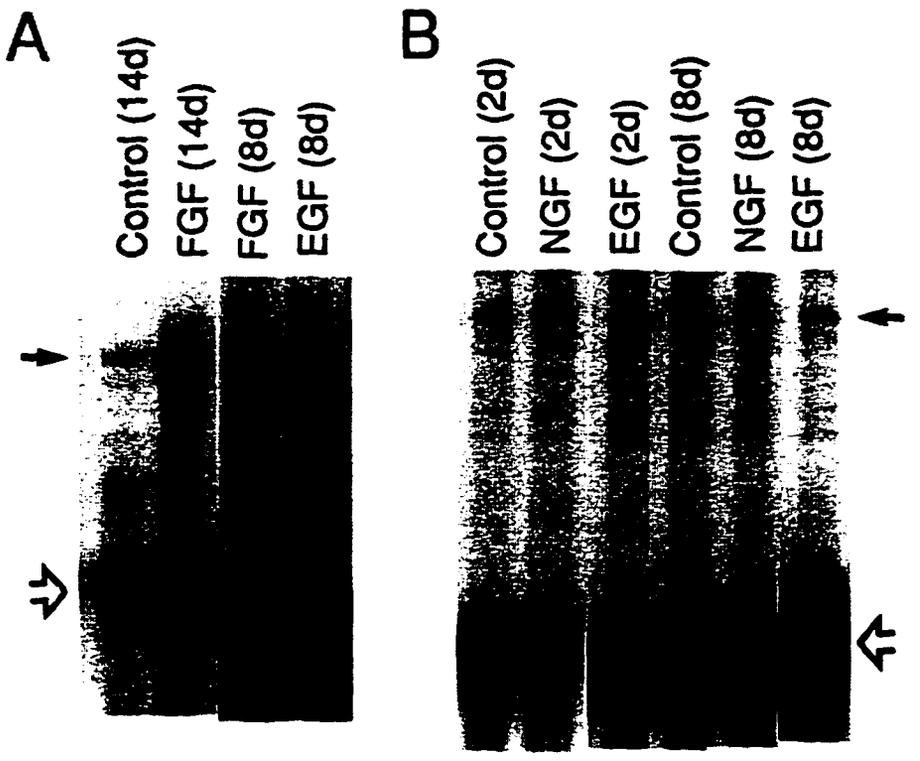
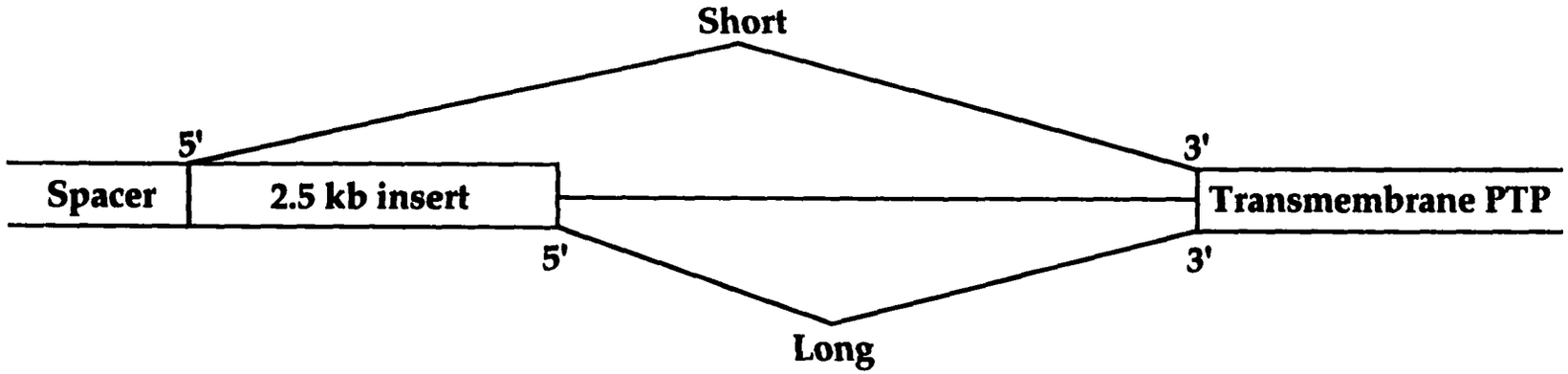


Figure 9: Genomic sequence analysis of RPTPζ/β exon-intron boundaries. In panel A, alternative splicing of RPTPζ/β is shown schematically. Note that the sequence difference between the long (full length) and short RPTPζ/β transcripts is caused by the alternative usage of 5' splice donor site, and the 2.5 kb insert represents an alternatively spliced exon. In the case of the phosphacan transcript, neither 5' donor sites are utilized, resulting in the translation of a truncated, soluble RPTPζ/β isoform. In panel B, sequences of the alternative splice junctions in panel A are shown along with the consensus splice junction sequence. *Upper case*, exon sequence; /, splice junction; *lower case*, intron sequence; y, pyrimidines C or T; M, A or C; r, purines A or G; n, any nucleotide.

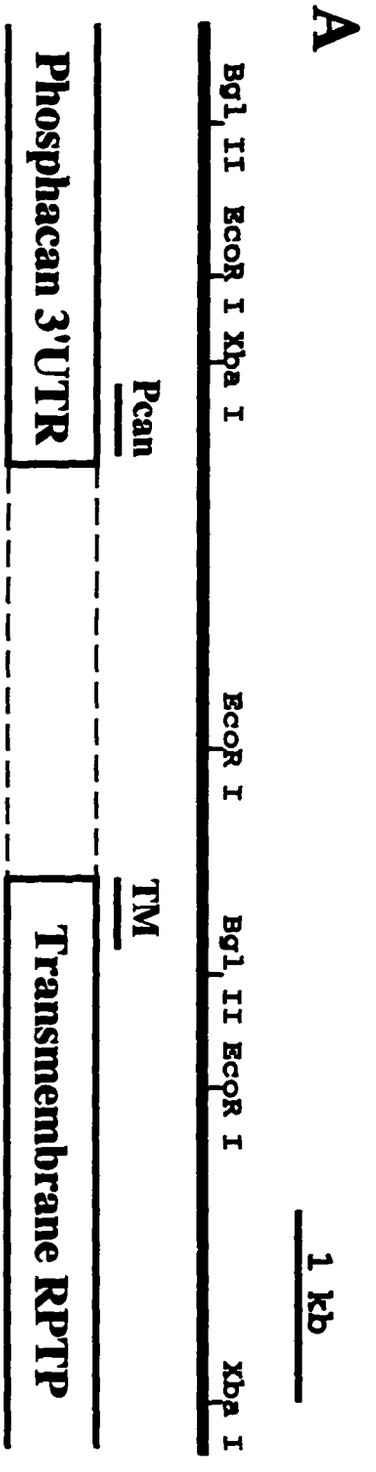
A



B

		Splice donor	Splice acceptor	
Consensus		MAG / **gt**ragt	yyyyyyyyyyyyny**ag** / G	
Long	**5'**	CAG / **gt**tagt	ttttctccgactac**ag** / A	**3'**
Short	**5'**	ATG / **gt**gaga	ttttctccgactac**ag** / A	**3'**

Figure 10: Genomic Southern analysis of the RPTPζ/β gene. In panel A, partial restriction mapping of rat genomic DNA demonstrates the relationship between the phosphacan 3' UTR/polyadenylation signal and sequence encoding the transmembrane region of RPTPζ/β isoforms. The relative positions of probes Pcan and TM used in the Southern blot hybridization are shown. In panel B, rat genomic DNA (10 μg) from liver was digested with Bgl II (lane 1 and 2), EcoR I (lane 3 and 4), and Xho I (lane 5) and subjected to Southern analysis. Blots were hybridized with ^{32}P-labeled cDNA probes as marked on top of each lane: probes Pcan and TM correspond to unique cDNA fragments from the phosphacan and transmembrane RPTPζ/β isoforms, respectively. The Insert probe will detect the full length and phosphacan transcripts. Probes 5' Insert and CA hybridize to regions shared by all three RPTPζ/β transcripts, yet detect only single restriction fragments (e.g. lanes 4 and 5), suggesting that all RPTPζ/β isoforms are encoded by a single gene. The relative position of probes Insert, 5' Insert and CA are shown in Fig. 5B.



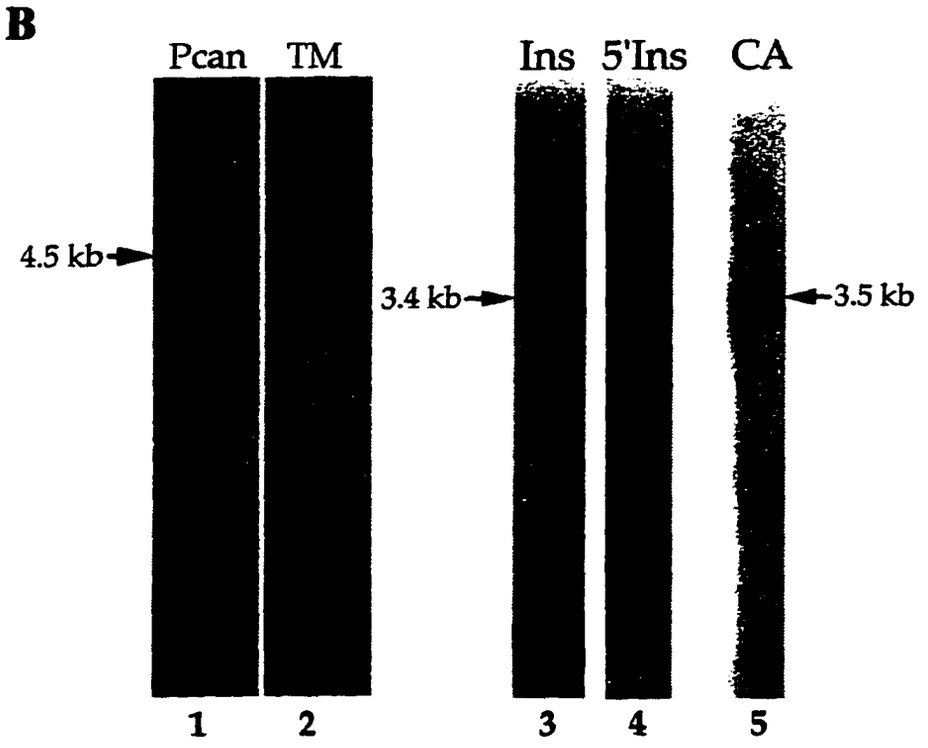
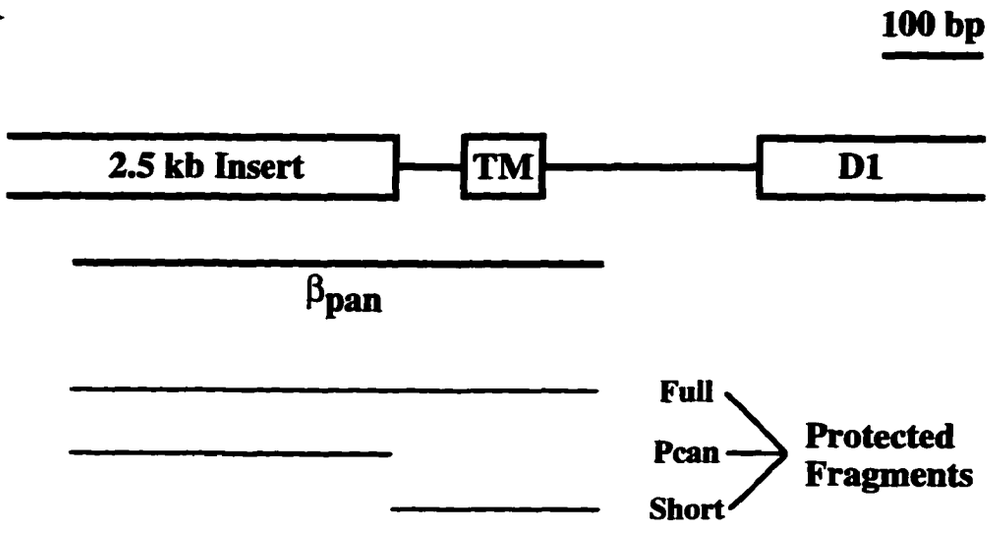


Figure 11: Expression of the three major RPTPζ/β mRNAs is regulated during neural development. In panel A, a schematic cDNA map, showing the relative position of the βpan riboprobe used in RNase protection assay in panel B, is shown. The expected protected fragments for each of the three major RPTPζ/β isoforms are shown at the bottom. In panel B, quantification of the mRNA for the three major RPTPζ/β isoforms during development is shown. RNase protection analysis was performed using the βpan and cyclophilin probes with 10 μg total RNA isolated from the sources as indicated. The level of expression of each transcript is expressed as the ratio of the arbitrary volume unit number from PhosphorImager reading for RPTPζ/β vs. that of cyclophilin, and the mean ratio ± SE from separate samples is shown.

A



B

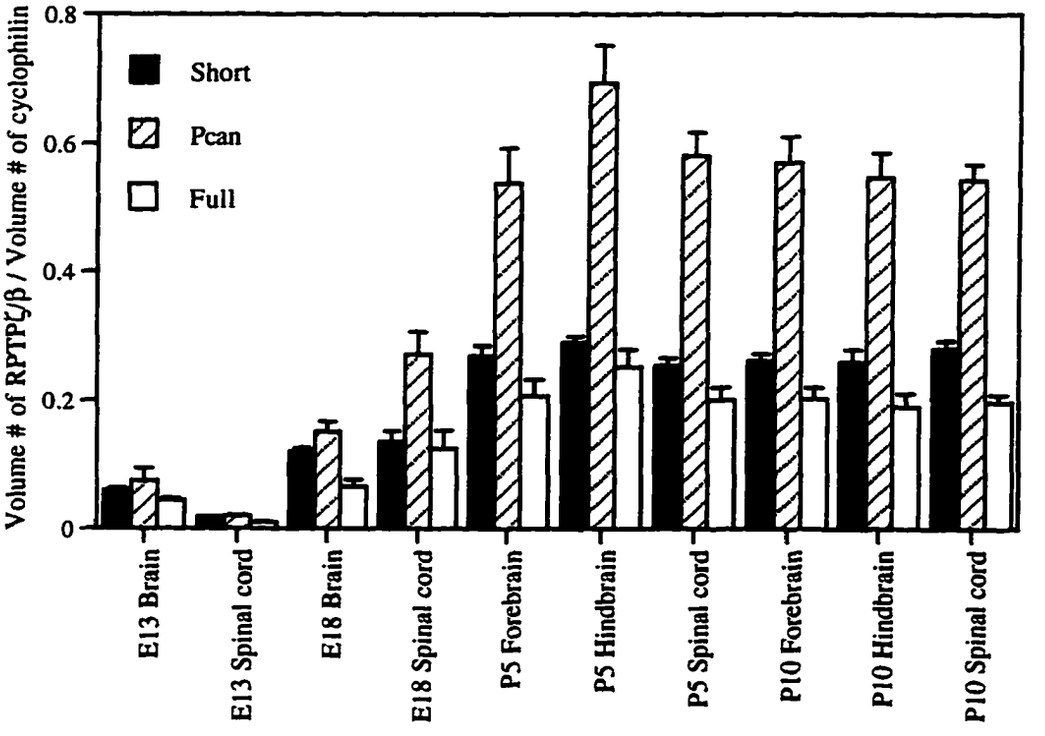


Figure 12: Occurrence of 21 bp deletion in the juxtamembrane regions of both transmembrane RPTPζ/β transcripts. RNAs from early postnatal (P4) rat brain were subjected to RT-PCR analysis as detailed in Chapter 2. Four pairs of primers were used for cDNA amplification: Primer set 1 only amplifies the short transmembrane transcripts with the 21 bp sequence; Primer set 2 only amplifies full length transmembrane transcripts with the 21 bp sequence; Primer set 3 amplifies the short transmembrane transcripts both with and without the 21 bp sequence; Primer set 4 amplifies full length transmembrane transcripts both with and without the 21 bp sequence. -, no cDNA control, +, with cDNA sample, M, 100 bp DNA marker (start from the bottom at 300 bp).

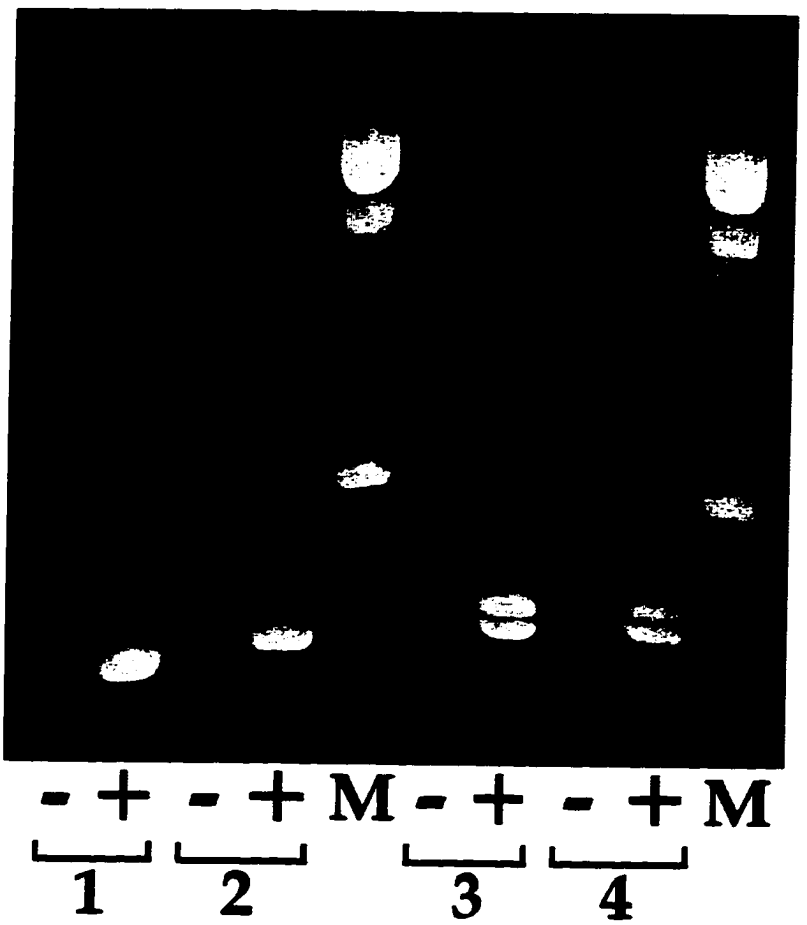
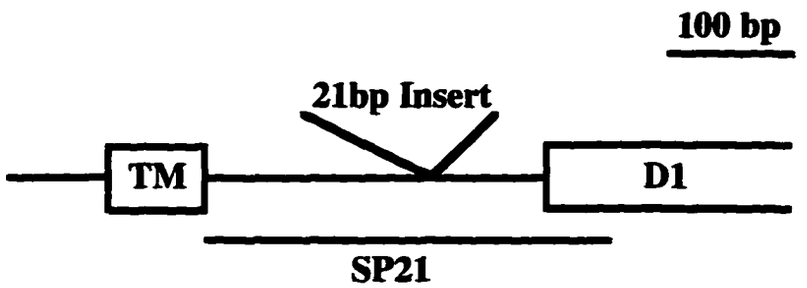


Figure 13: Expression of sp21 variants of RPTPζ/β mRNA in the nervous system. In panel A, a schematic cDNA map shows the relative position of the 21 bp insert. The position of the riboprobe SP21 used for RNase protection assays in panel B is also shown. In panel B, RNase protection analysis was used to quantify mRNA levels of RPTPζ/β isoforms with (sp21+) or without (sp21-) the 21 bp insert, using the SP21 riboprobe as shown in panel A. 10 μg of samples of total RNA, isolated from E13 spinal cord (lane 1), P5 hindbrain (lane 2), P5 spinal cord (lane 3), mixed primary glial culture isolated from P1 cerebral cortex harvested at 14 DIV (lane 4), C6 glioma cells treated with 1 mM db-cAMP for 24 hours (lane 5), and control C6 glioma cells (lane 6) were analyzed. The 330 bp protected fragment corresponds to the isoform without the 21 bp insert (sp21-), and the 185 bp and 145 bp fragments correspond to the isoform with the 21 bp insert (sp21+). Cyclophilin (cyclo) probe was used as an internal control.

A



B

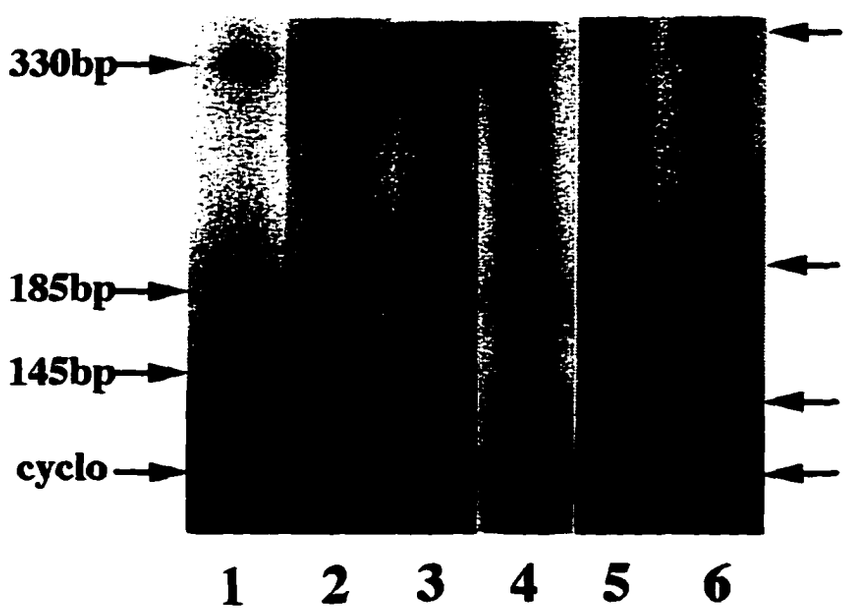


Figure 14: Regulated expression of sp21 variants of RPTPζ/β mRNA following sciatic nerve injury. RNase protection analysis was used to quantify sp21 variant RPTPζ/β mRNA levels using the SP21 riboprobe, in 5 μg samples of total RNA isolated from distal segments of rat sciatic nerves that were harvested seven days after a bilateral crush injury or a bilateral sham operation. Data was quantified using a Molecular Dynamics PhosphorImager and the pg RPTPζ/β mRNA values were determined by comparison to a sense SP21 transcript standard curve. Values significantly different from those in sham-operated animals are marked with asterisks (*, $p<0.01$; unpaired *t*-test). The mean pg RPTPζ/β mRNA ± SE from at least three separate samples is shown.

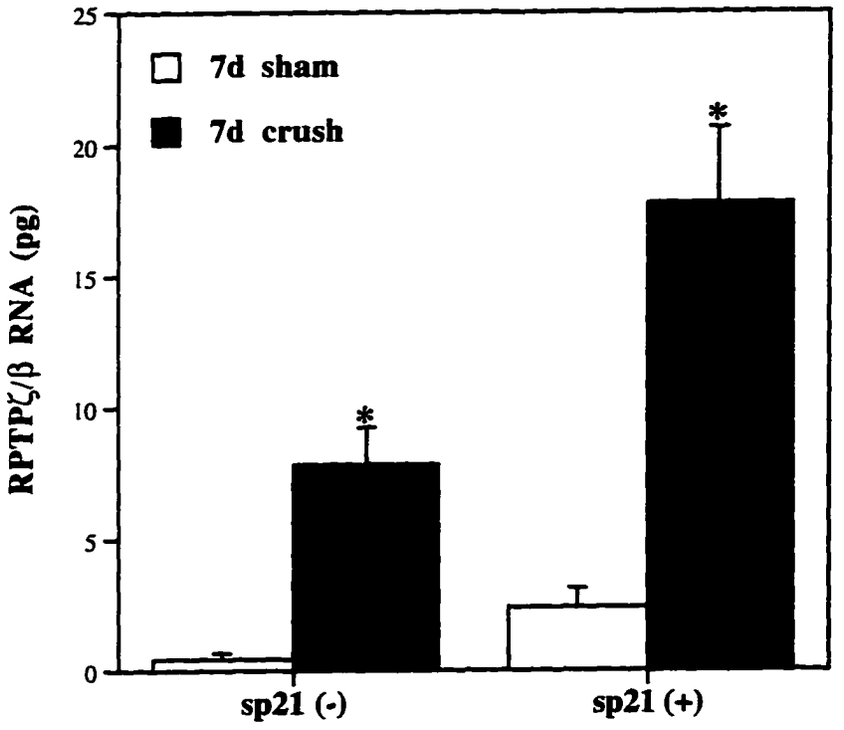


Figure 15: Expression of RPTPζ/β mRNAs in normal and injured PNS and spinal cord. RNase protection analysis was performed used the βpan and cyclophilin probes with 5 μg of total RNA from sources as indicated seven days following either a bilateral crush injury (+) or a bilateral sham operation (-). DN, distal segment of sciatic nerve. PN, proximal segment of sciatic nerve. SC, lumbar 4-6 segments of spinal cord. DRG, lumbar 4-6 dorsal root ganglia. Protected fragments are labeled by arrows: Full, the full length RPTPζ/β transcript; Pcan, phosphacan transcript; Short, the short RPTPζ/β transcript; Cyclo, cyclophilin transcript. Similar results were obtained using non-operated and sham-operated rats.

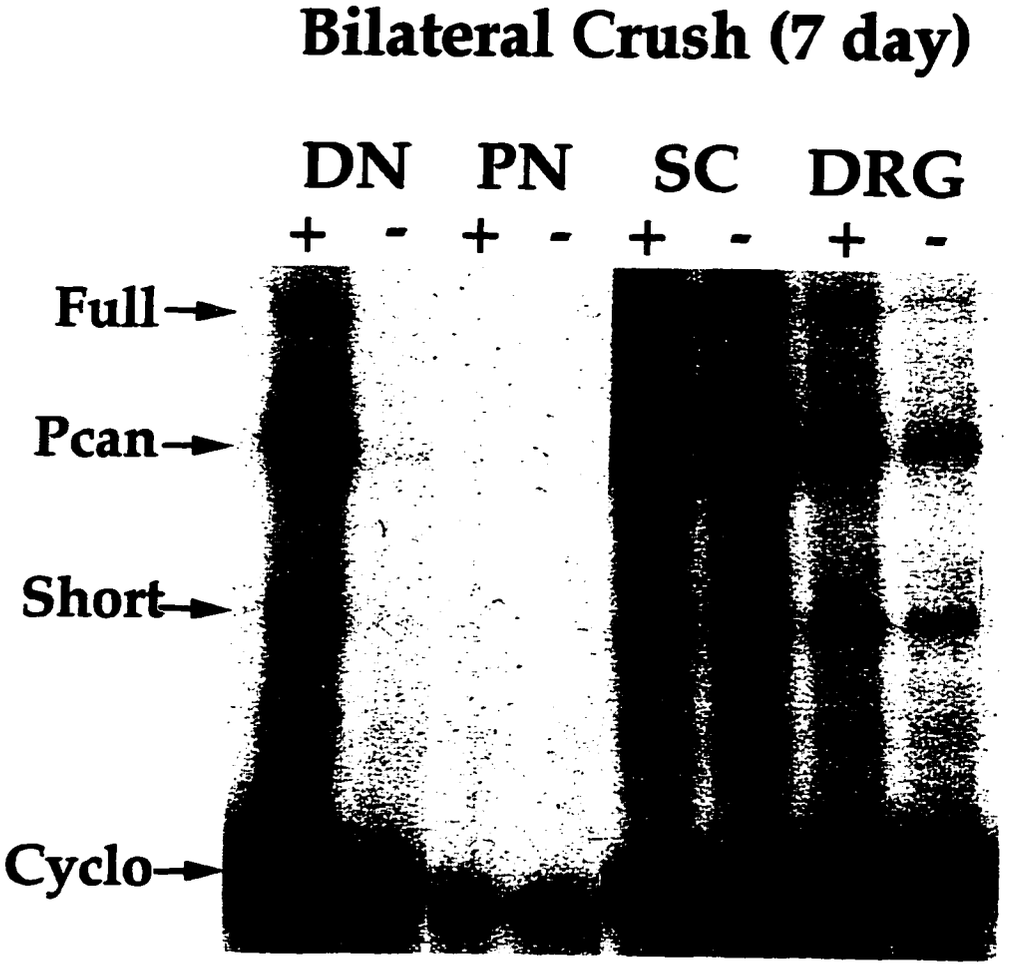


Figure 16: Relative abundance of short and phosphacan RPTPζ/β transcripts in PNS and CNS. Levels of expression (pg values) of the short and phosphacan RPTPζ/β transcripts were quantified by RNase protection assay using the βpan probe and RNA isolated from tissues removed as indicated from non-operated control or sham-operated rats. The ratio of the two transcripts was calculated by dividing the respective pg values determined from the same sample lane on the gel. The mean ratio ± SE from separate samples is shown. SN, sciatic nerve. SC, lumbar 4-6 segments of spinal cord. DRG, lumbar 4-6 dorsal root ganglia.

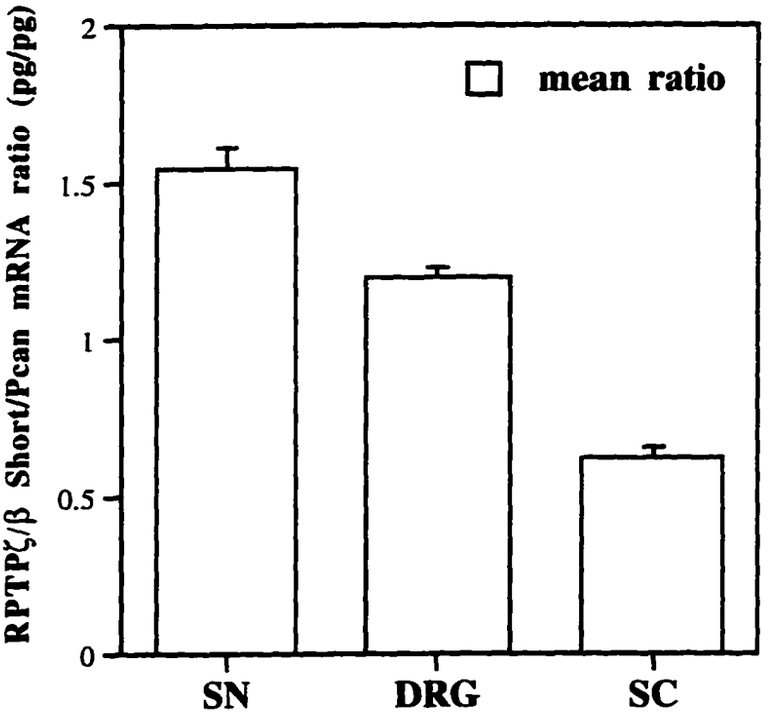


Figure 17: Induction of RPTPζ/β mRNAs in the distal segment of rat sciatic nerve following crush injury. RNase protection assay quantification of long (full length), phosphacan (pcan), and short RPTPζ/β mRNA expression, using the βpan probe in distal segments of sciatic nerve (DN) 4, 7, 14, 21 and 28 days after either bilateral crush injury (open diamonds) or bilateral sciatic nerve sham operations (open squares). The mean pg ± SE from separate nerve segments is plotted for each of the three transcripts at the above time points.

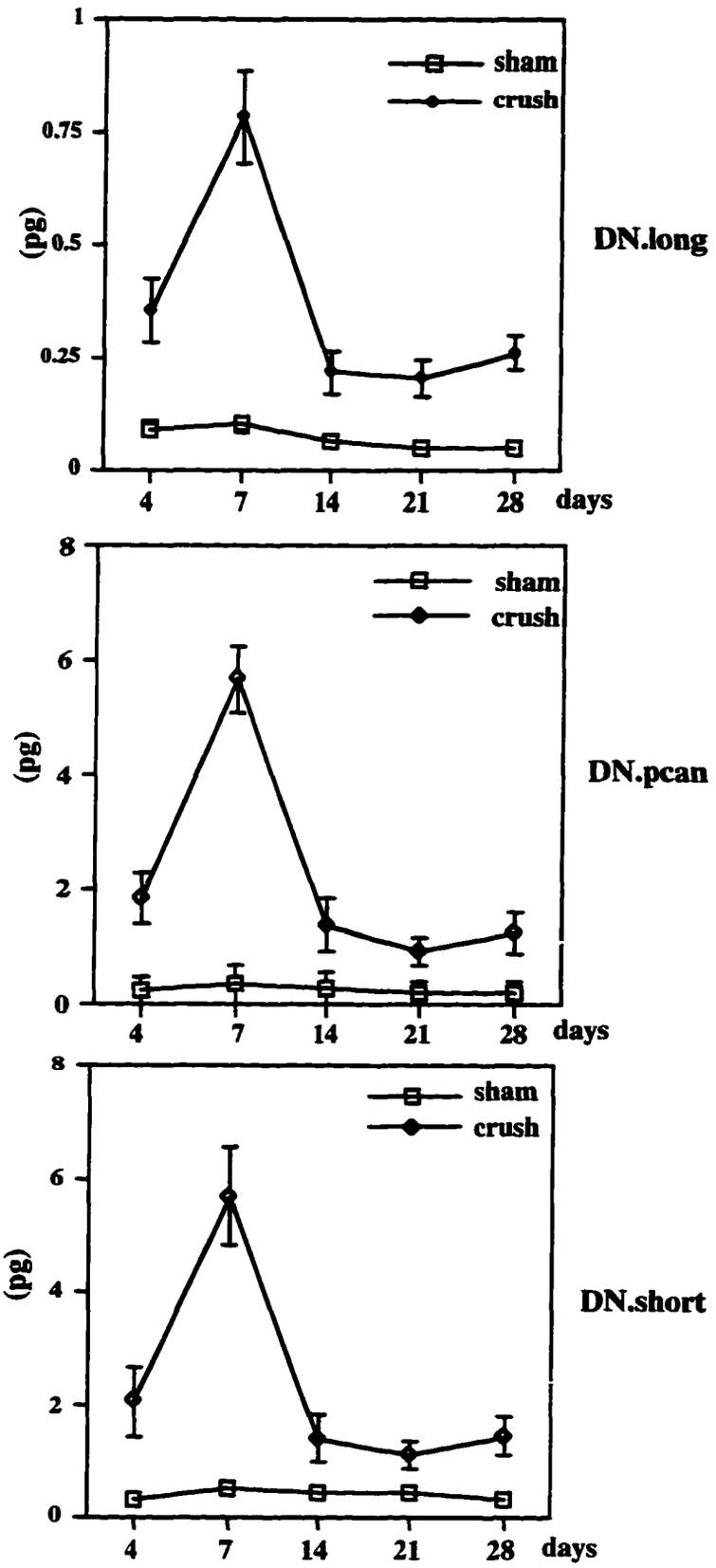
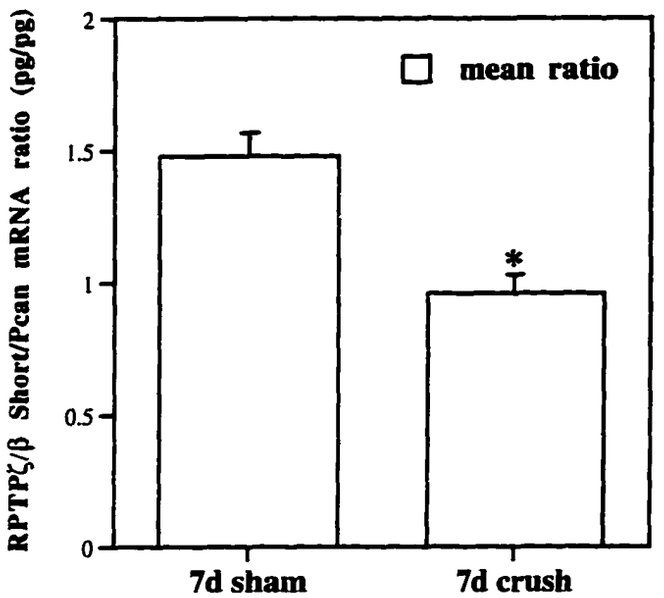


Figure 18: Differential magnitude of induction by the three RPTPζ/β transcripts following sciatic nerve crush injury. Levels of expression (pg values) in distal segments of sciatic nerves for all three RPTPζ/β transcripts were quantified by RNase protection assay as in Fig. 17 on bilaterally crush-injured or sham-operated rats. The short/phosphacan (in panel A) ratio, the pcan/long and short/long ratios (in panel B) were calculated by dividing their respective pg value from the same sample lane on the gel. Values significantly different from those in sham-operated animals are marked with asterisks (*, $p<0.001$ in A, unpaired t-test; $p<0.05$ in B, ANOVA) The mean ratio $\pm$ SE of the seven day time point from separate samples is shown. Similar results were obtained for these ratios at other time points.

A



B

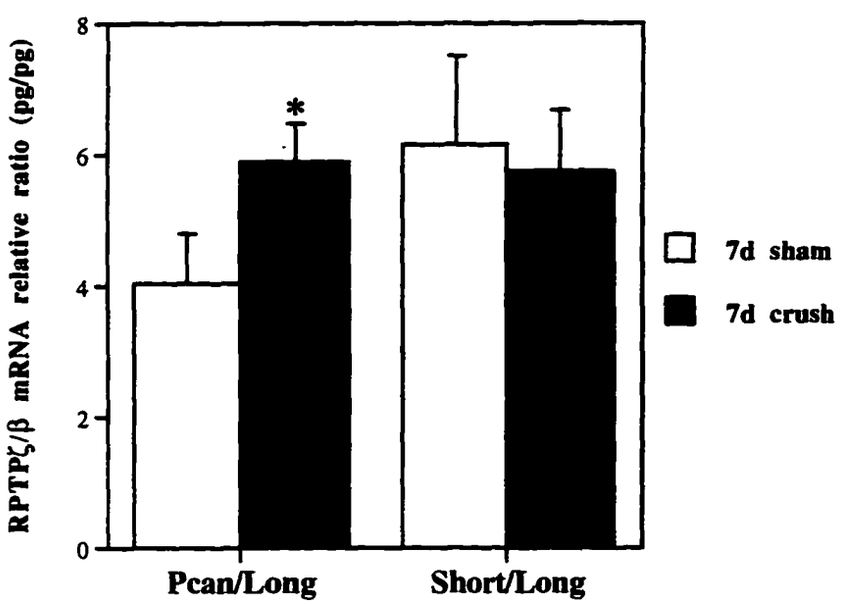
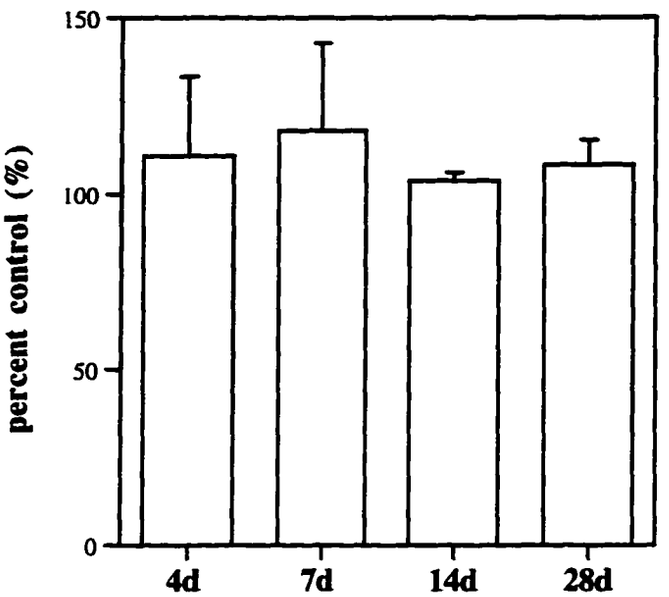


Figure 19: Expression of the short RPTPζ/β transcript in the lumbar DRG and spinal cord after sciatic nerve crush injury. RNase protection analysis was used to quantify short RPTPζ/β mRNA expression using the βpan probe in lumbar 4-6 DRGs (panel A) and lumbar 4-6 segments of spinal cord (panel B) 4, 7, 14, and 28 days after either bilateral or sham sciatic nerve crush injury. The levels of the short transcript in tissues from the injured rats are expressed as percentages of those from the corresponding sham-operated control rats. The mean percentage ± SE from separate samples is plotted for the short RPTPζ/β transcript at the above time points. Similar results were also obtained for the other two RPTP transcripts.

A



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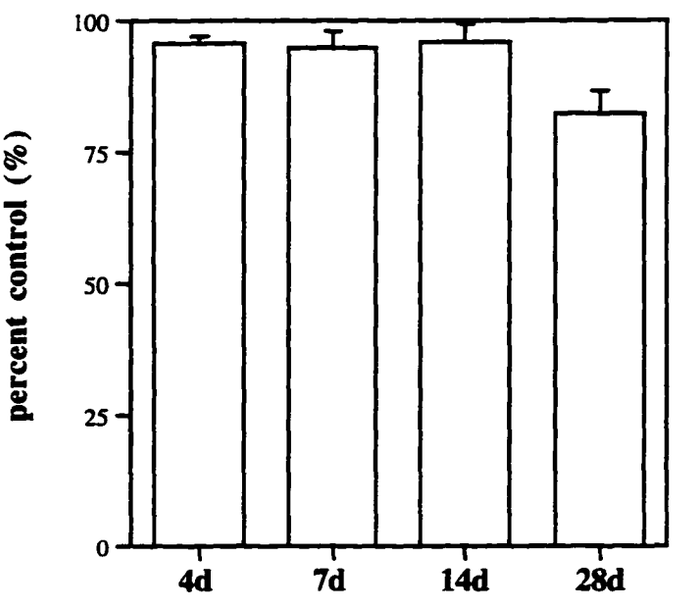
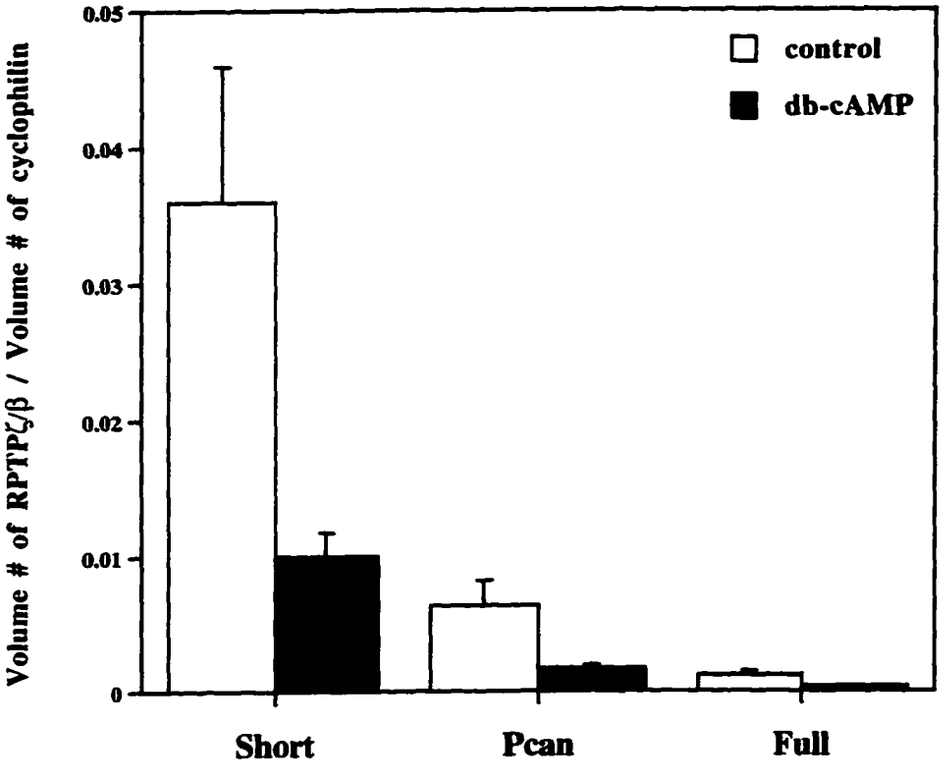


Figure 20: Regulation of RPTPζ/β mRNAs in C6 rat glioma cells. RNase protection analysis was performed using the βpan and cyclophilin probes with 10 μg of total RNA from C6 rat glioma cells that either received 1 mM dibutyryl-cAMP (db-cAMP) or control treatment for 24 hours. The levels of expression of each transcripts are expressed as the ratio of the arbitrary volume unit number reading from the PhosphorImager for RPTPζ/β to that of cyclophilin, and the mean ratios ± SE from separate samples are shown.



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