PHYSIOLOGICAL AND MOLECULAR FUNCTIONS OF THE MURINE RECEPTOR PROTEIN TYROSINE PHOSPHATASE SIGMA (RPTPσ)

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ABSTRACT

The control of cellular tyrosine phosphorylation levels is of great importance in many biological systems. Among the kinases and phosphatases that modulate these levels, the LAR-RPTPs have been suggested to act in several key aspects of neural development, and in a dysfunctional manner in various pathologies from diabetes to cancer. The aim of this thesis is to describe the physiological functions of one of the members of this subfamily of RPTPs, namely RPTPσ. First, we showed that glucose homeostasis is altered in RPTPσ null mice. They are hypoglycemic and more sensitive to exogenous insulin and we proposed that the insulin hypersensitivity observed in RPTPσ-null mice is likely secondary to their neuroendocrine dysplasia and GH/IGF-1 deficiency. In addition to regulating nervous system development, RPTPσ was previously shown to regulate axonal regeneration after injury. In the absence of RPTPσ, axonal regeneration in the sciatic, facial and optical nerves was enhanced following nerve crush. However, myelin-associated growth inhibitory proteins and components of the glial scar such as CSPGs (chondroitin sulfate proteoglycans) have long been known to inhibit axonal regeneration in the CNS, making spinal cord injury irreversible. In collaboration with Dr Samuel David, we unveiled that RPTPσ null mice are able to regenerate their corticospinal tract following spinal cord hemisections as opposed to their WT littermates. We then isolated primary neurons from both sets of animals and found that the absence of RPTPσ promotes the ability of the neurons to adhere to certain inhibitory

substrates. Finally, in order to better understand the physiological role of RPTPσ, we used a yeast substrate-trapping approach, to screen a murine embryonic library for new substrates. This screen identified the RhoGAP p250GAP as a new substrate, suggesting a downstream role for RPTPσ in RhoGTPase signaling. We also identified p130Cas and Fyn as new binding partners. All these proteins have clear functional links to neurite extension. The characterization of RPTPσ and its signaling partners is essential for understanding its role in neurological development and may one day translate into treatments of neural diseases and injuries.

ABRÉGÉ

La phosphorylation sur résidus tyrosine des protéines est un mécanisme clé de la régulation de nombreux processus biologiques. Parmi les protéines tyrosine kinases et phosphatases qui contrôlent ces niveaux, la sous-famille des LAR-RPTPs joue un rôle important dans le développement du système nerveux et a été impliquée dans diverses pathologies comme le diabète et le cancer. La présente thèse définit les fonctions physiologiques d'une des LAR-RPTPs, soit RPTPσ. Premièrement, nous démontrons que les souris modifiées par transgenèse ciblée RPTPσ (-/-) sont hypoglycémiques et plus sensibles à l'insuline exogène. Nous proposons que l'hypersensibilité à l'insuline observée chez ces souris soit fort probablement secondaire à leur dysplasie neuroendocrinienne résultant en une carence en hormone de croissance et IGF-1. Deuxièmement, il a été démontré qu'en l'absence de RPTPσ la régénération axonale du nerf sciatique ou facial était accélérée suite à une axotomie. De plus, l'absence de RPTPσ promeut la repousse axonale des neurones ganglionnaires de la rétine dans l'environnement hostile du nerf optique. Les facteurs inhibiteurs associés à la myéline et à la cicatrice gliale sont maintenant reconnus comme un obstacle majeur à la repousse axonale dans le système nerveux central, faisant des lésions à la moelle épinière une condition irréversible. Avec la collaboration du Dr. Samuel David, nous démontrons ici que les souris RPTPσ (-/-) ont la capacité de régénérer leurs axones de la voie corticospinale suite à une hémisection de la moelle épinière. De plus, les neurones provenant de souris

RPTPσ (-/-) ont une meilleure capacité à adhérer sur un substrat inhibant. Afin de mieux comprendre le rôle physiologique de RPTPσ, nous avons procédé à une approche modifiée du double-hybride dans la levure afin d'identifier deux nouveaux substrats de RPTPσ, soit p250GAP et p130CAS. Nous avons aussi identifié la tyrosine kinase Fyn comme nouveau partenaire de RPTPσ. Ces protéines ont tous un lien fonctionnel avec l'élongation axonale. En conclusion, la caractérisation de RPTPσ et de ses partenaires de signalisation est essentielle à la compréhension de son rôle dans le développement du système nerveux et pourrait éventuellement se traduire par de nouvelles alternatives de traitements des lésions de la moelle épinière ou maladies neurodégénératives.

DEDICATION

À mes parents adorés,

Jean et Jeannine,

pour n'avoir jamais douté que j'irais au bout de ce projet.

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CONTRIBUTION of AUTHORS

This section acknowledges the contribution of authors to the work included in this thesis. In Chapter 2, Dr. Mounib Elchebly initiated the project and provided figure 2.2B and 2.2C. Dr. Noriko Uetani provided technical assistance with mice work and injection of insulin into the hepatic portal vein. Luce Dombrowski and Dr. André Marette provided the essentials of Figure 2.4. Dr. Robert A. Mooney provided the insulin measurements in Table 1. Dr. Alan Cheng provided insightful discussions that contributed to the work. Chapter 3 was done in collaboration with Dr. Samuel David's laboratory. Dr. Elizabeth Fry and I contributed equally to the preparation of this manuscript, although I wrote most of what is presented in Chapter 3. She provided most of Figures 3.3, 3.4 and 3.5, as well as quantification analysis in Figure 3.6. Dr. Noriko Uetani did the infographics in Figure 3.2. Hiba Kazak and Nia Tsatas provided excellent technical help with performing the spinal cord hemisections, and isolation of primary cells. In Chapter 4, Dr. Takanobu Nakazawa, and Dr. Tadashi Yamamoto provided p250GAP related plasmids and Dr. Masahide Noda provided the pBridge-LexA-vSrc plasmid. Dr. Christophe Blanchetot provided technical help with the yeast-two-hybrid assay as well as insightful discussions that contributed to the work.

PUBLICATIONS

Publications arising from work presented in this thesis:

1. Chagnon M.J., Elchebly M., Uetani N., Dombrowski L., Cheng A., Mooney R.A., Marette A., Tremblay M.L. 2006. Altered glucose homeostasis in mice lacking the receptor protein tyrosine phosphatase sigma. Can. J. Physiol. Pharmacol. 84: 755-763; Chapter 2

2. Fry E.*, Chagnon M.J.*, Lopes Vales R., Tremblay M.L.#, David S#. The corticospinal tract regenerate after spinal cord injury in receptor protein tyrosine phosphatase sigma knockout mice. Submitted to Glia. (*Co-first author, #co-corresponding author); Chapter 3

3. Chagnon M.J., Blanchetot C., Noda M., Tremblay M.L. Identification of p250RhoGAP as a new substrate of RPTPσ using a modified yeast-two-hybrid approach. In preparation; Chapter 4.

Review articles:

1. Chagnon M.J., Uetani N., Tremblay M.L. 2004. Functional significance of the LAR receptor protein tyrosine phosphatase family in development and diseases. Biochem Cell Biol. Dec;82(6):664-75

2. Blanchetot C, Chagnon M.J., Dubé N., Hallé M., Tremblay M.L. 2005 Substrate-trapping techniques in the identification of cellular PTP targets. Methods. 2005 Jan;35(1):44-53.

Non-thesis related publications:

1. Uetani N., Chagnon M.J., Kennedy T.E., Iwakura Y., Tremblay M.L. 2006. Mammalian motoneuron axon targeting requires receptor protein tyrosine phosphatases sigma and delta. J Neurosci. May 31;26(22):5872-80.

I developed a method for efficiently genotyping RPTPσ and RPTPδ mice by PCR. I helped maintaining the mice colony and I generated Figure 1 and Table 1 and I provided scientific input into the writing of the manuscript.

2. Uetani N., Bertozzi K., Chagnon M.J., Hendriks W., Tremblay M.L., Bouchard M. 2008. Maturation of ureter-bladder connection and craniofacial development by LAR receptor protein-tyrosine phosphatases. In Press J. Clin. Invest.

I helped maintaining and genotyping the mice colony. I generated Table 1 and I critically revised the manuscript.

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LIST OF ABBREVIATIONS

A, Ala	Alanine
Aa	Amino acid
Abl	Abelson
BSA	Bovine serum albumin
C, Cys	Cysteine
CD (CD45)	Cluster of differentiation
CNS	Central nervous system
Crk	CT10 regulator of kinase
C/S	Cysteine mutated in serine
Csk	C-terminal Src kinase
CSPG	Chondroitin sulphate proteoglycan
D, Asp	Aspartic acid
D/A	Aspartic acid mutated in alanine
DMEM	Dulbecco's modified Eagle's medium
DH	Dbl homology domain
DSPs	Dual-specific phosphatases
E, Glu	Glutamic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor-receptor
ERK 1/2	Extracellular signal-regulated kinase 1/2
ES cells	Embryonic stem cells
EV	Empty vector
FAK	Focal adhesion kinase
FasL	Fas ligand
FN	Fibronectin
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GH	Growth hormone

G, Gly	Glycine
GPI	Glycosylphosphatidylinositol
Grb-2	Growth factor receptor binding protein-2
GST	Gluthathione S-transferase
GTP	Guanosine triphosphate
HSPG	Heparan sulphate proteoglycan
IB	Immunoblot
IG	Immunoglobulin-like
IGF-1	Insulin-like growth factor-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	Immunoprecipitation
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	c-Jun NH2-terminal kinase
KO, (-/-)	Knockout
LAR	Leukocyte common antigen related
MAPK	Mitogen activated protein kinase
mRNA	Messenger RNA
NO	Nitric oxyde
NRPTPs	Non-receptor PTPs
NT	Nucleotide
P, Pro	Proline
p130Cas	Crk-associated substrate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGF-R	Platelet derived growth factor receptor
PEST	Pro, Glu, Ser, Thr amino acid enriched sequence
PFA	Paraformaldehyde

PH	Pleckstrin homology domain
PKC	Protein kinase C
PNS	Peripheral nervous system
PTB	Phosphotyrosine binding
PTKs	Protein tyrosine kinases
PTPs	Protein tyrosine phosphatases
PTP-1B	Protein tyrosine phosphatases 1B
PTP-PEST	Protein tyrosine phosphatase PEST
PVDF	Polyvinylidene difluoride
RPTP	Receptor-like PTP
RTKs	Receptor tyrosine kinases
ROCK	Rho-associated coiled-coil-containing protein kinase
Q, Gln	Glutamine
Q/A	Glutamine mutated in alanine
S, Ser	Serine
SH2	Src homology 2
SH3	Src homology 3
SHP2	SH2 domain-containing protein tyrosine phosphatase 2
TC-PTP	T-cell PTP
T, Thr	Threonine
TNF	Tumour necrosis factor
TRK	Tropomyosin-related kinase
V, Val	Valine
WPD	Tryptophan, proline, aspartic acid
WT	Wild-type
X	Any amino acids
Y, Tyr	Tyrosine

CHAPTER 1: Introduction

1.1 Introduction to tyrosine phosphorylation

The control of cellular phosphorylation levels is now recognized to be of great importance in many biological systems. Development and survival of multicellular organisms entails highly organized and coordinated responses of cells to diverse external stimuli, including soluble factors, extracellular matrix components, and molecules on the surface of adjacent cells. One of the earliest biochemical events detected in response to such stimuli is protein tyrosine phosphorylation, which is involved in relaying information from the cell surface to the nucleus as well as reorganizing the cytoskeleton. Although now widely accepted, the vital contributions of protein tyrosine phosphorylation to these processes has recently expanded.

1.1.1 Historical perspective

In 1955 Edmond H. Fischer and Edwin G. Krebs first described a reversible protein phosphorylation reaction that served as a biological regulatory mechanism (Fischer and Krebs, 1955). Although this landmark discovery eventually earned its authors a Nobel Prize, it took an additional 25 years before phosphorylation on tyrosine residues was widely appreciated. Tony Hunter surprisingly found that v-src, the transforming gene of the Rous sarcoma virus, defined two years earlier, was not only a kinase (Collett and Erikson, 1978), but a tyrosine kinase (Hunter and Sefton, 1980). Shortly thereafter, a series of growth factor receptors were identified as

protein tyrosine kinases. Many of these could act as their own substrates through autophosphorylation on multiple tyrosine residues. The significance of this autophosphorylation event was uncovered in 1986 when Anthony Pawson found that proteins containing a Src homology 2 (SH2) domain are able to bind to tyrosine-phosphorylated receptors (Sadowski et al., 1986). The interaction of this non-catalytic SH2 domain with other kinases and with tyrosine-phosphorylated proteins is now known to be crucial for the organization of the downstream intracellular signalling pathways, which control normal and aberrant cellular functions.

Although interest in tyrosine kinases continued to grow, it was only in 1988 that the first protein tyrosine phosphatase, named PTP1B, was purified and characterized by Nicholas Tonks and colleagues (Tonks et al., 1988a, b). This led to the interesting discovery that cluster of differentiation (CD) 45, often found on the surface of haematopoietic cells, was homologous to PTP1B, whose function was unknown prior to the identification of what would become the prototypical PTP. Other PTPs were subsequently identified based on the presence of a highly conserved phosphatase domain, including, in 1993, a new receptor phosphatase highly expressed in the rat nervous system (Walton et al., 1993; Yan et al., 1993). The following year, the murine homolog of what is today known as RPTPσ was cloned at McGill University in the laboratory of Michel L. Tremblay (Wagner et al., 1994). Furthermore, this group later published the gene-targeted deletion of RPTPσ

in mice (Elchebly et al., 1999a) These knockout mice have proven to be a very valuable tool for studying the function of this enzyme.

1.1.2 The Protein Tyrosine Phosphatase (PTP) family

Although it represents less than 1% of total protein phosphorylation occurring in the cell, tyrosine phosphorylation still represents a major regulator of intracellular signalling. Effective signalling is achieved by the opposing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). The sequencing of the human genome has led to the identification of almost 100 PTKs (reviewed in (Manning et al., 2002)) compared to 107 PTPs. These PTPs can be classified into four protein families (Alonso et al., 2004) based on the amino acid sequences of their catalytic domain. The largest family can be further subdivided into two main subfamilies based on their substrate specificity (Figure 1.1).

The first group contains the dual specificity phosphatases (DSP), comprised of approximately 65 genes able to dephosphorylate their substrates on both tyrosine and serine/threonine residues (reviewed in (Pulido and Hooft van Huijsduijnen, 2008)). The second group contains the 38 tyrosine-specific classical PTPs, which are further divided into the non receptor cytoplasmic PTPs and the transmembrane receptor-like PTPs (RPTPs) (Figure 1.2).

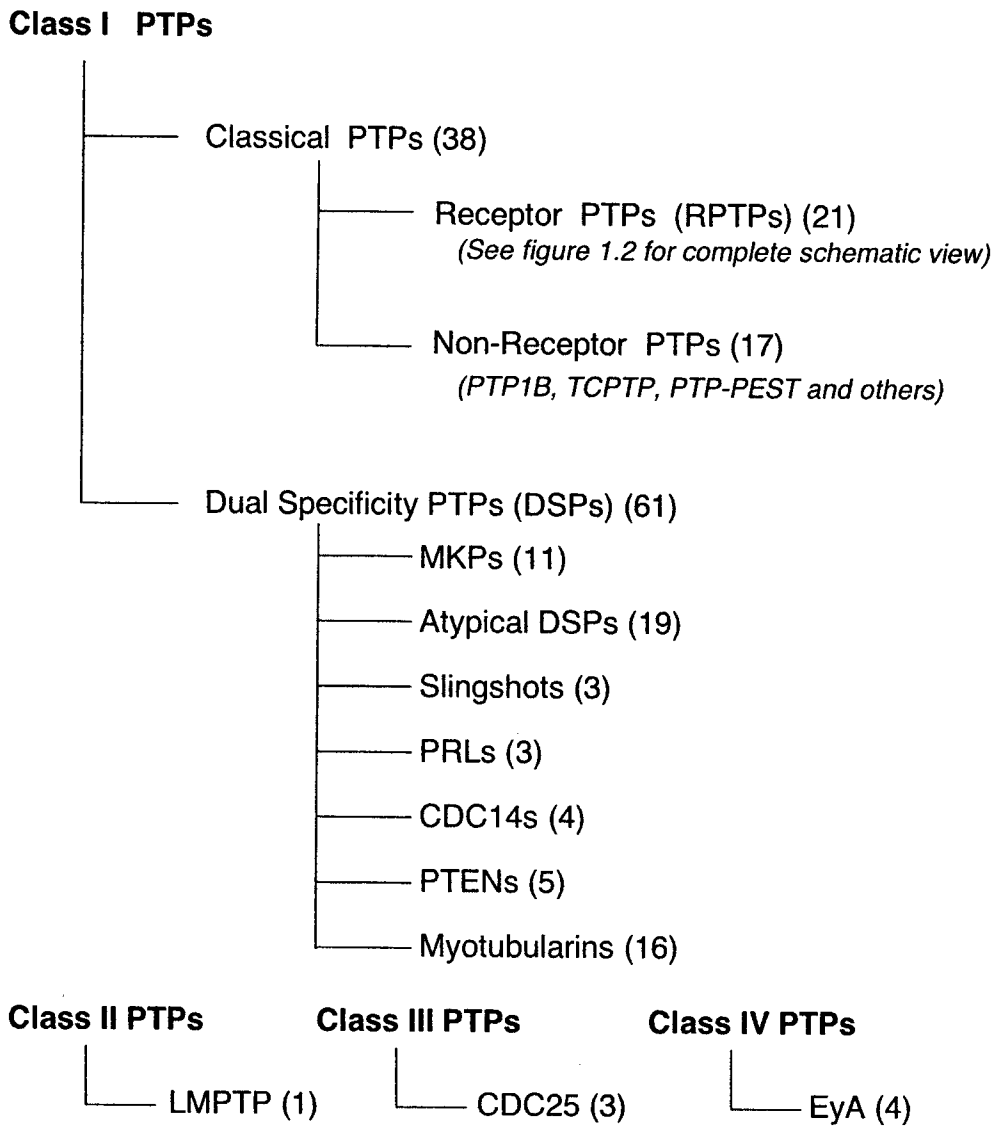

Figure 1.1 Classification of the four different PTP families.

Number in brackets indicates the number of members in each group. Adapted from (Alonso et al., 2004).

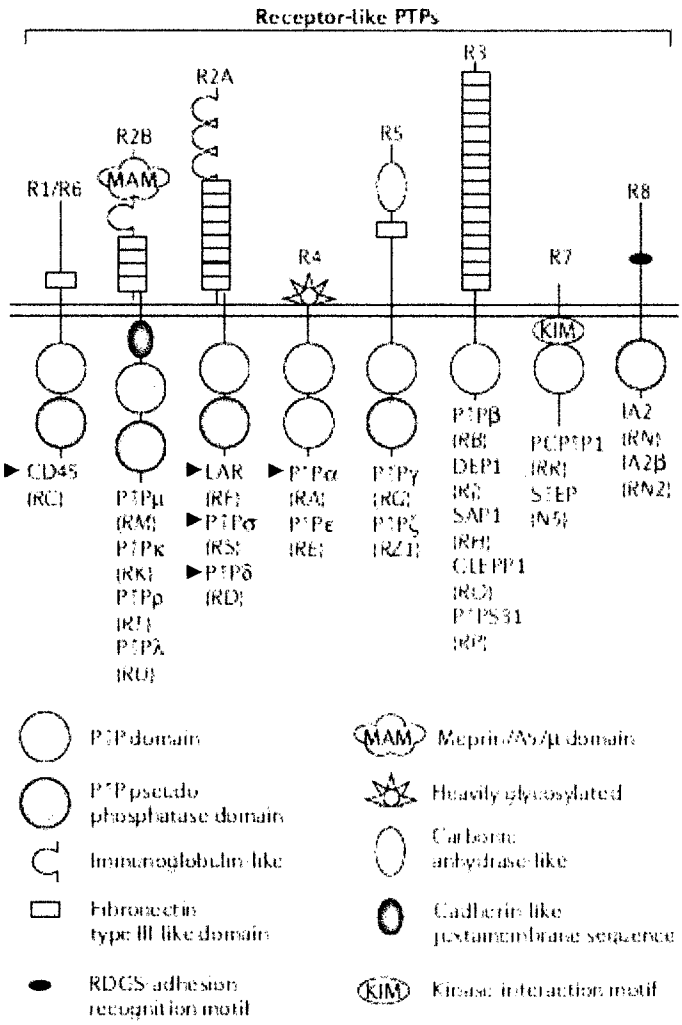

Figure 1.2 The classical RPTPs.

Schematic view of the domain structure of the RPTP families. The gene symbol is included in parentheses for clarifications. Arrowheads point to RPTP that are discussed in more details in the text. Adapted from (Tonks, 2006).

1.1.3 The signature PTP domain

All PTPs contain a conserved catalytic domain characterized by the signature motif HCxxGxxR. Residues in this motif form the phosphatase binding loop. As illustrated in Figure 1.3, the cysteine residue carries out a catalytic nucleophilic attack on the phosphoryl group of its target substrate, transiently accepting the phosphate during catalysis. The conserved arginine is involved in substrate binding as well as the stabilization of the phospho-enzyme complex. Binding of the phosphorylated substrate to the catalytic pocket triggers a major conformational change in the enzyme which traps its substrate in a closed active site. Upon this conformational change the WPD loop is brought into close vicinity of the cysteinyl-phosphate intermediate. The aspartic acid residue acts as a general acid to promote the cleavage of the phosphate from the tyrosine by protonating the oxygen residue of the tyrosyl-leaving group. In a second catalytic step, this same aspartic residue will act together with a conserved glutamine residue to serve as a general base in order to activate a water molecule that will then promote the hydrolysis of the cysteinyl-phosphatase intermediate (reviewed in (Andersen et al., 2001) and (Zhang, 2003)).

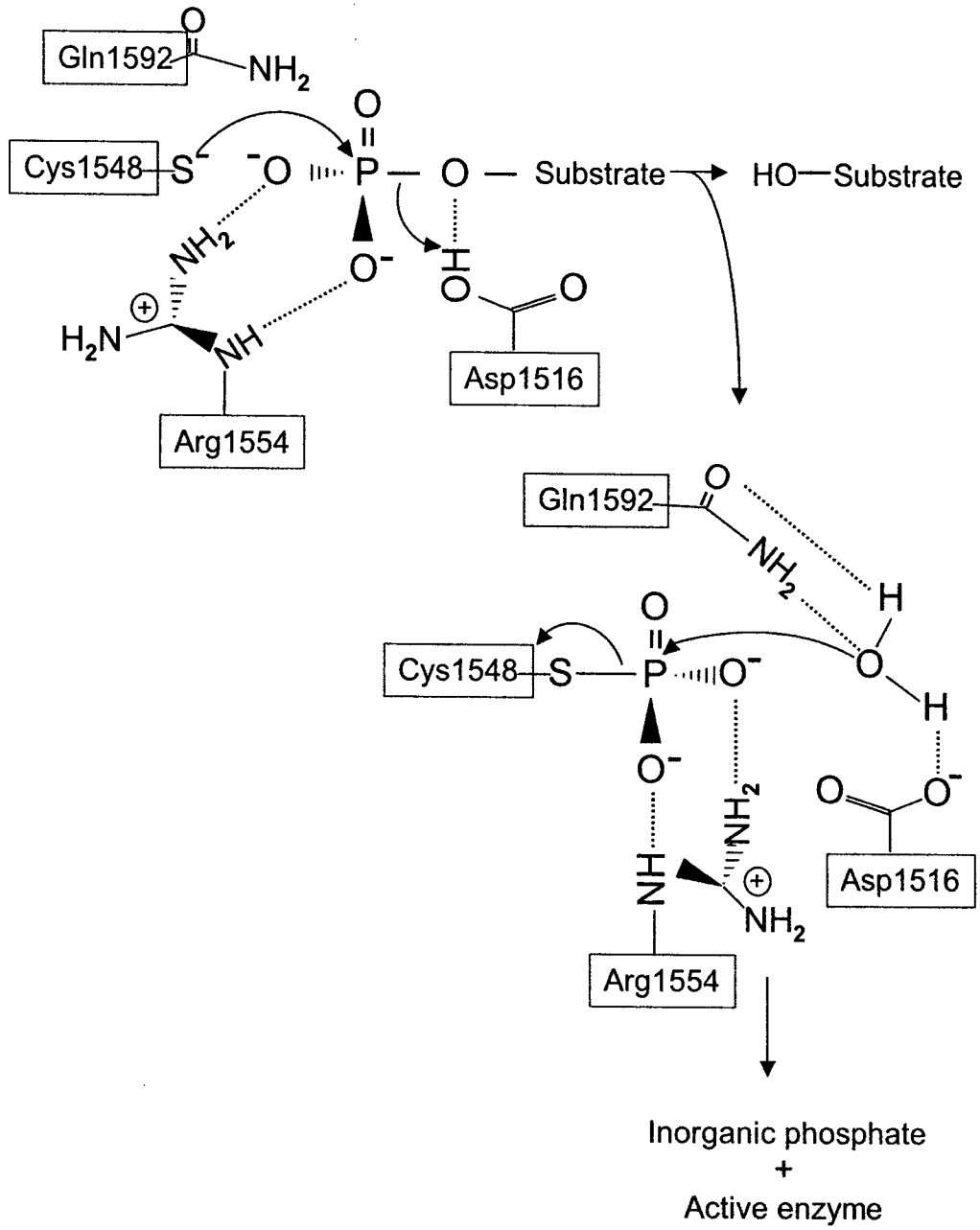

Figure 1.3 Chemical analysis of the two-step RPTPσ-catalyzed dephosphorylation reaction.

The cysteine residue carries out a catalytic nucleophilic attack on the phosphoryl group of its target substrate leading to its dephosphorylation. Adapted from (Zhang, 2003).

1.1.4 The substrate-trapping mutant

Mutation of the invariant residues within the catalytic domain of PTPs causes a dramatic decrease in catalytic activity. For example, mutating the cysteine residue to a serine or alanine ablates all phosphatase activity. Mutational analyses of these catalytically important residues have led to the elaboration of invaluable methods for identifying new substrates of PTPs and allow us to gain insight into their molecular mechanisms. Although cysteine to serine mutants have been utilized extensively in the identification of a number of substrates, the novel approach of mutating the aspartic acid of the WPD loop to alanine results in a dramatic decrease of enzymatic K_{cat} without affecting the K_m (Flint et al., 1997). In other words, the mutated PTP is able to form a stable cysteinyl-phosphate intermediate with the substrate but cannot carry out the catalytic reaction necessary to release it. As a result, these mutants become effective substrate traps, and are the basis of the "substrate-trapping" method (Blanchetot et al., 2005). Although this technique has been widely used in order to identify specific substrates (Garton et al., 1996), it still lacks robustness in the sense that identification of the "trapped" substrate relies on trial and error using commercially available antibodies against proteins of corresponding molecular weight. More sophisticated approaches that do not rely on educated guesswork and antibody availability have been developed, i.e. large-scale substrate-trapping experiments combined with LC/MS/MS protein sequencing. A modified yeast-two-hybrid approach employing substrate-trapping represents an

additional option (discussed in Chapter 4). These approaches with substrate-trapping mutants have led to the identification of a multitude of putative PTP substrates. In order to ensure the physiological significance of these interactions a set of criteria had to be established (Tiganis and Bennett, 2007). Briefly, there should be a direct and stable interaction of the PTP substrate-trapping mutant with the substrate whereas the wild-type PTP enzymes should not be able to stably trap the substrate. Secondly, the endogenous phosphorylation levels of the putative substrate should be modulated by altering the activity of the PTP. Finally, the PTP should be able to directly dephosphorylates the substrate *in vitro*. Although these criteria are guidelines and cannot always be met, they should provide standardization to aid the identification of PTP substrates.

1.2 The LAR-RPTP subfamily

Among the classical RPTPs, it has been suggested that members of the LAR subfamily of RPTPs act in several key aspects of neural development, as well as in a dysfunctional manner in various pathologies ranging from diabetes to cancer. This subfamily represents a dynamic set of enzymes that can be modulated by transcriptional and posttranscriptional events. Their general extracellular domain structure, reminiscent of the immunoglobulin superfamily, alludes to their potential interaction with the vast network of membrane-associated proteins that are responsible for cell-cell and cell-extracellular matrix interactions. The composition,

similarities, and conservation of LAR-RPTPs among various metazoans and their mechanism of action and regulation will be presented below. Recent findings on their action in neuronal growth and regeneration have led us to believe that they will become key targets in the treatment of various human pathologies.

1.3 Structure and post-translational modifications of LAR-RPTPs

The LAR subfamily of RPTPs, also classified as receptor type IIA PTPs (Andersen et al., 2001), is among the most widely studied groups of RPTPs. This subfamily is composed of three vertebrate homologs: LAR, RPTPσ and RPTPδ. In humans, they are encoded by the genes PTPRF, PTPRS and PTPRD, which have been mapped to chromosomes 1p32, 19p13.3, and 9p24 respectively (Hasegawa et al., 1993; Harder et al., 1995; Wagner et al., 1996). This subfamily also includes the invertebrate RPTPs: Dlar, DPTP69D in *Drosophila*, PTP-3 in *Caenorhabditis elegans*, and HmLAR1, HmLAR2 in *Hirudo medicinalis*. These proteins were grouped with the LAR-RPTPs based on their highly similar structure: 66% amino acids identity in the full-length proteins and 84% identity in the catalytic domains (Pulido et al., 1995). The large extracellular domain of LAR-RPTPs is highly reminiscent of the cell adhesion molecule (CAM) structure and generally consists of three immunoglobulin-like (Ig-like) domains and four to eight fibronectin type III (FNIII) domains, depending on alternative splicing (Figure 1.4A) (Pulido et al., 1995). They have a hydrophobic transmembrane stretch followed by two cytosolic

PTP domains, one proximal to the membrane (D1) and the other distal (D2). LAR-RPTPs are expressed as a 200 kDa proprotein spanning the plasma membrane once with an extracellular amino-terminus. The proprotein is cleaved to generate a 150 kDa extracellular domain (E-subunit), non-covalently bound to an 85 kDa intracellular tandem phosphatase domain (P-subunit). This first proteolytic cleavage step is mediated by furin-like endoproteases that recognize a critical penta-arginine sequence at the C-terminus of the extracellular domain. A second, non sequence-specific, calcium-dependant proteolytic cleavage step promotes extracellular domain shedding (Serra-Pages et al., 1994) and internalization of the P-subunit (Aicher et al., 1997) (Figure 1.4B). Notably, the function of the proteolytic cleavage and shedding is still unknown. Similar to all PTPs, the 280 amino acid catalytic domain contains an invariable signature motif ([I/V]HCXAGXXR[S/T]G), including an essential cysteine that catalyzes the nucleophilic attack on the phosphoryl group of its target substrate leading to its dephosphorylation (Guan and Dixon, 1991). The D1 domains have robust catalytic activity, while the D2 domains have weak, if any, catalytic activity against a variety of substrates (Streuli et al., 1990). This suggests that D2 serves a regulatory, rather than a catalytic role (discussed below). The three members of the LAR-RPTPs also share additional post-translational modifications such as glycosylation (Yu et al., 1992) and phosphorylation (Tsujikawa et al., 2002). All these modifications, as well as proteolytic cleavage, may contribute to the regulation of LAR-RPTP activity.

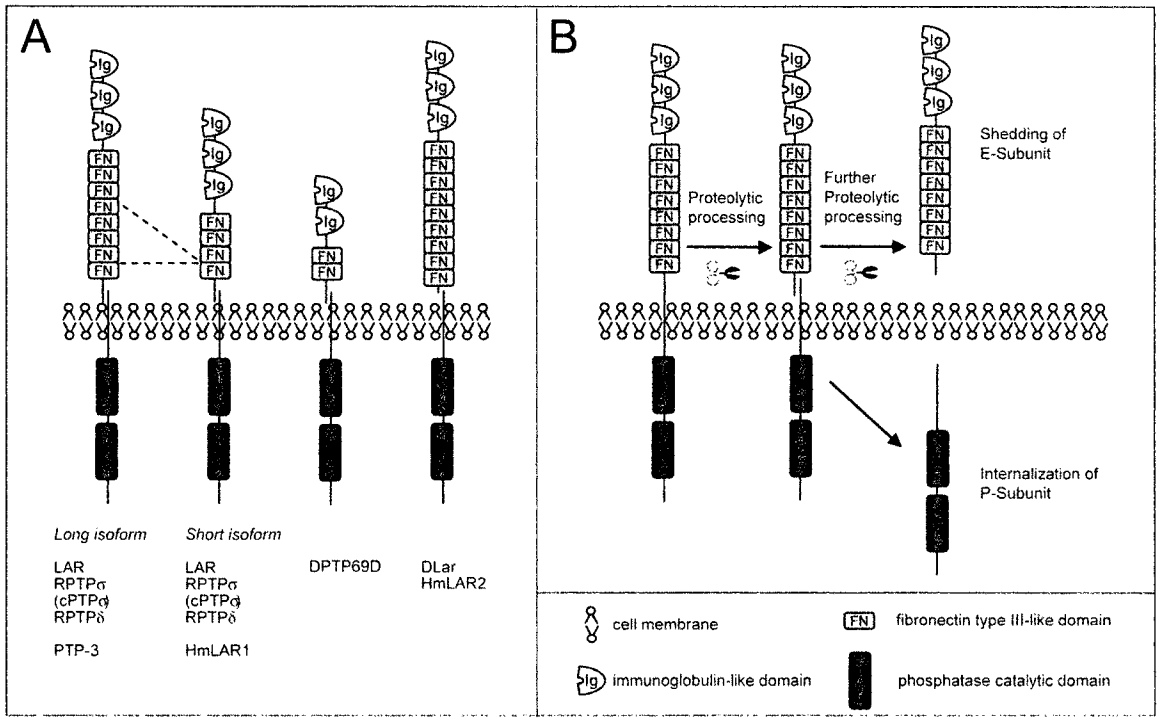

Figure 1.4 Structure and proteolytic cleavage of LAR-RPTPs.

A) Schematic diagram of the major LAR-RPTPs isoforms in vertebrates and invertebrates. Each contains three N-terminal immunoglobulin-like (Ig-like) domains, four to nine fibronectin type III (FNIII) domains and two cytoplasmic PTP domains (D1 and D2).

B) LAR-RPTPs are initially expressed as a ~200 kDa proprotein, which is then proteolytically cleaved by a furin-like endoprotease to generate a 150 kDa E-subunit and a non-covalently attached 85 kDa P-subunit. Further cleavage results in shedding of the E-subunit and internalization of the P-subunit.

1.4 Regulation of LAR-RPTP activity

LAR-RPTP catalytic activity is most likely negatively regulated by dimerization (ligand-induced or not) as are CD45 (Majeti et al., 1998), RPTPα (Blanchetot and den Hertog, 2000a), and RPTPβ (Meng et al., 2000). Crystallographic studies have demonstrated that the catalytically active RPTPα D1 domain can homodimerize. A helix-loop-helix "wedge" structure at the N terminal of D1 inserts itself in the D1 catalytic domain of the other partner, resulting in the co-inactivation of both catalytic domains (Bilwes et al., 1996). Deletion or mutation of the wedge in RPTPα or CD45 leads to constitutively active RPTPs, or at least RPTPs that are not inactivable by dimerization (Jiang et al., 1999; Majeti et al., 2000). These results suggest a fundamental role of the wedge in the regulation of RPTP activity. Since this wedge structure is conserved among all LAR-RPTPs, they may be regulated in a similar manner. Interestingly wedge domain peptides of LAR were shown to bind homophilically and to inhibit LAR function in cells (Xie et al., 2006). This represents a unique PTP inhibition strategy, which overcomes problems of lack of specificity encountered with classical inhibitors directed against the highly conserved PTP catalytic site.

1.4.1 Regulation of dimerization

Although it is possible that the phosphatase activity of LAR-RPTPs is regulated by dimerization, how, in turn, is the dimerization process regulated? The extracellular domains of LAR-RPTPs exhibit a variety of alternative splicing isoforms, and their expression patterns are spatially and temporally regulated (Mizuno et al., 1993; Pulido et al., 1995; Endo et al., 1996; Yang et al., 1999). It is tempting to speculate that each isoform may have its own specific ligand-binding or dimerization affinity. In support of this idea, the laminin-nidogen complex was shown to be a ligand for a specific splice variant of LAR (see below). Furthermore, recent studies demonstrated that extracellular isoforms of CD45 that were differently glycosylated promoted CD45 homodimerization with varying efficiencies. The stronger the dimerization, the more CD45 enzymatic activity was reduced (Xu and Weiss, 2002).

In a very elegant *in vitro* study, RPTPσ was demonstrated to exist in the cell as a homodimer and, more importantly, for the first time it was shown that ectodomain dimerization was necessary for heterophilic ligand binding (Lee et al., 2007). Moreover, the two main isoforms described above were shown to alter ligand specificity largely by changing the rotational conformations of the binding site thus imparting new ligand capabilities. The functionality of ligand binding to the homodimerized form of RPTPσ still remains to be assessed.

Consistent with a ligand induced regulation of RPTPs, a small ectodomain isoform of LAR was able to bind homophilically and to positively regulate it (Yang et al., 2005). Probably this molecule binds to the monomerized form of LAR, thus preventing inactivation of LAR by dimer formation. On the other hand, unlike RPTPα, the LAR intracellular domain does not crystallize as a dimer (Nam et al., 1999). Therefore, LAR phosphatase activity may be regulated differently. Although it cannot yet be ruled out that LAR-RPTPs exist simply as monomers or monomerize upon ligand binding, it should be noted that the full intracellular domain of LAR (D1 and D2) was used in the crystallization studies, while in the case of RPTPα only D1 was used. In the LAR crystal, D1 sits on the top of D2 with a large interface between the two domains, and the presence of D2 prevents the dimerization of D1 by steric hindrance. Recent evidence suggests that the interaction between D1 and D2 is not rigid and can be regulated by external stimuli (Blanchetot et al., 2002). Thus, D2 putatively regulates the activity of D1. Indeed, using an *in vitro* system, the D2 of RPTPδ binds to and reduces the phosphatase activity of RPTPσ D1 by 50% (Wallace et al., 1998). Also, the D2 domains of LAR-RPTPs bind to the D1 domain of RPTPα with different affinities (Blanchetot and den Hertog, 2000b). Thus, the LAR-RPTPs probably regulate and/or are regulated by inter- and intra-molecular interactions mediated by their D2 domains. Again, the crystal structure of the tandem PTP domains of LAR suggested that D2 might have

an enzymatic role in addition to its regulatory role. The tertiary structure of both PTP domains are similar, but two amino acids crucial for catalysis and the proper conformation of a functional PTP catalytic domain are different in D2 (Nam et al., 1999). Predictably, when these two amino acids are mutated to the D1 equivalent, robust PTP activity is observed, suggesting that D2 may act as an active enzyme on specific substrates in a particular cellular context, although this appears unlikely to us. Interestingly, the Dlar full-length inactive mutant (C1638S) was able to rescue the Dlar null phenotype in *Drosophila*. This rescuing ability was lost in mutants missing D2 (Krueger et al., 2003) but not in mutants with an inactive D2 (C1929S). Moreover, the simple replacement of CD45 D2 by LAR D2 affected CD45 activity. All these results strongly suggest that the D2 domains have other functions rather than simply regulating enzymatic activity *in vivo*. Instead of acting on the substrates themselves, it is possible that the D2 recruits and presents substrate(s) to the D1 domains or other signaling partners and/or binds to proteins important for relocalization of the enzyme.

The E-subunits of the LAR-RPTPs are important for the function and regulation of LAR-RPTPs. Again, the catalytically inactive mutant of Dlar was sufficient to rescue the Dlar null phenotype (Krueger et al., 2003). In addition, the Ig-like domains were essential for survival while the FNIII domains were required for oogenesis. Different sections of the Dlar E-subunit are then critical at specific

stages of *Drosophila* development. Further understanding of the regulatory role of the LAR-RPTPs E-subunit by splicing, glycosylation, dimerization and shedding will be instrumental in elucidation of their signaling and physiological roles.

1.4.2 Regulation by reversible oxidation

Reversible oxidation has emerged as a new means of regulation of PTPs (Tonks, 2005). The production of reactive oxygen species (ROS) in response to a wide variety of stimuli such as growth factors, cytokines, and cellular stress are responsible for the oxidation of the highly susceptible invariant cysteine residue found in the PTP motif, thus impairing its nucleophilic function in catalyzing the tyrosine dephosphorylation. In the context of RPTPs, oxidation also provides another level of regulation since the PTP D2 domain of RPTPα was shown to be more reactive to oxidation than D1 (Persson et al., 2004) and may explain how the D2 domain regulates D1 even though it is catalytic inactive. The response to oxidation of RPTPα has been extensively studied (Blanchetot et al., 2002; van der Wijk et al., 2004; Groen et al., 2005) but similarities were also found with LAR. Oxidation of LAR-D2 contributes to its dimerization by forming intermolecular disulphide bonds, inducing conformational changes to the cytoplasmic domain and changing the rotational coupling dependant on D2 (Groen et al., 2008). This may force the LAR dimer into an inactive conformation or affect the binding of extracellular ligands. Since the amino acid sequence similarity of the D2 domain of

all three members of the LAR-RPTPs is higher than the D1 domain (Andersen et al., 2004), it suggests a similar function across the subfamily.

1.5 Signaling pathways regulated by LAR-RPTPs

1.5.1 Extracellular domain interactions of LAR-RPTPs

Like some other RPTPs, LAR-RPTPs are involved in adhesion signaling (reviewed in (Beltran and Bixby, 2003)). The CAM-like extracellular domains of LAR-RPTPs suggest that they act as adhesion molecules and, unlike CAMs, they are in a unique position to transduce extracellular signals into intracellular signaling pathways by means of their PTP domains. RPTPδ was shown to function as a homophilic cellular adhesion molecule, and this interaction promoted both neuronal adhesion and neurite growth (Wang and Bixby, 1999). The same group further described RPTPδ as an attractive guidance molecule (Sun et al., 2000b). Additionally, a small ectodomain isoform of 11 kDa of LAR was shown to bind homophilically to LAR promoting neurite outgrowth (Yang et al., 2003) by activating LAR-induced positive signalling of Src, Trk, and FAK kinases and their downstream signalling partners (Yang et al., 2005). Much evidence supports a role for LAR-RPTPs acting as their own homophilic ligands in order to promote, not only axonal guidance, but neuronal growth as well. The LAR-RPTP extracellular domains are also capable of heterophilic interactions. A specific isoform of LAR missing a region in FN5 due to alternative splicing was shown to bind to the laminin-

nidogen complex. How this interaction regulates LAR function is not known. However, cells plated on laminin-nidogen extended cellular processes that contain high levels of LAR. Importantly, this cytoskeleton rearrangement is blocked by antibody-induced cross-linking of LAR. This suggests a role for LAR dimerization in laminin-induced cell morphological changes (O'Grady et al., 1998). Furthermore, Stoker's group presented the first evidence that cPTPσ (formerly known as Crypα) had isoform-specific ligands in the retinotectal pathway (Haj et al., 1999). By promoting ligand/receptor interactions, they observed intraretinal axon growth (Ledig et al., 1999a) through the remodeling of the growth cone lamelipodia (Mueller et al., 2000). They also identified Heparan Sulfate Proteoglycans (HSPG [mainly agrin and collagen XIII]) as the first class of RPTPσ heterotypic ligands (Aricescu et al., 2002). Using molecular modeling and biochemical analyses, they identified a Heparin/HSPG binding site in the first Ig-like domain of cPTPσ. Since HSPG are known regulators of axonal extension (Chang et al., 1997; Kim et al., 2003; Baerwald-De La Torre et al., 2004) and because of their overlapping expression pattern with cPTPσ in the developing retina, HSPG are physiologically relevant ligands. The HSPGs syndecan and dallylike were shown to interact functionally with Dlar during motor axon guidance and synaptogenesis (Fox and Zinn, 2005; Johnson et al., 2006). They appear to act on a common signalling pathway and compete with each other for binding to Dlar binding in order to modulate its PTP activity. A similar role in mammals remains to be assessed.

Recently, a new class of ligand of the cPTPσ short isoform, not related to HSPG, was found in myotubes (Sajnani-Perez et al., 2003) and identified as nucleolin (Alete et al., 2006). Interestingly, the cPTPσ short isoform is highly expressed in developing motor neurons suggesting a function in targeting motor neurons to the appropriate muscle.

It is noteworthy that α-latrotoxin, a black widow spider toxin, binds to the extracellular domain of RPTPσ and stimulates neuronal exocytosis (Krasnoperov et al., 2002). The intracellular domain of RPTPσ is not necessary for this exocytotic stimulation, suggesting that RPTPσ also acts as a ligand for other extracellular foreign molecules.

These findings suggest that LAR-RPTPs can no longer be considered orphan receptors but receptors for homophilic and heterophilic ligands. The ligands identified thus far confirm the role of the LAR-RPTPs as receptors for cell-cell as well as cell-extracellular matrix associated cues, as was expected by their CAM-like structures. Additional work is needed to determine the regulatory and functional roles of these ligand/receptor interactions, and their link to the cytoskeleton.

1.5.2 Intracellular signaling pathways elicited by LAR-RPTPs

The structure of LAR-RPTPs suggests that their E-subunits sense the cell extracellular environment and transduce signals intracellularly through their PTP activity. So far, the intracellular signaling mechanisms have not been extensively defined but evidence points toward transmission of adhesive signalling crucial for a variety of cellular event such as formation and maturation of neuronal synapses. Moreover extensive work with *Drosophila* have lead to a major role towards the remodeling of the actin cytoskeleton (summarized in Figure 1.5).

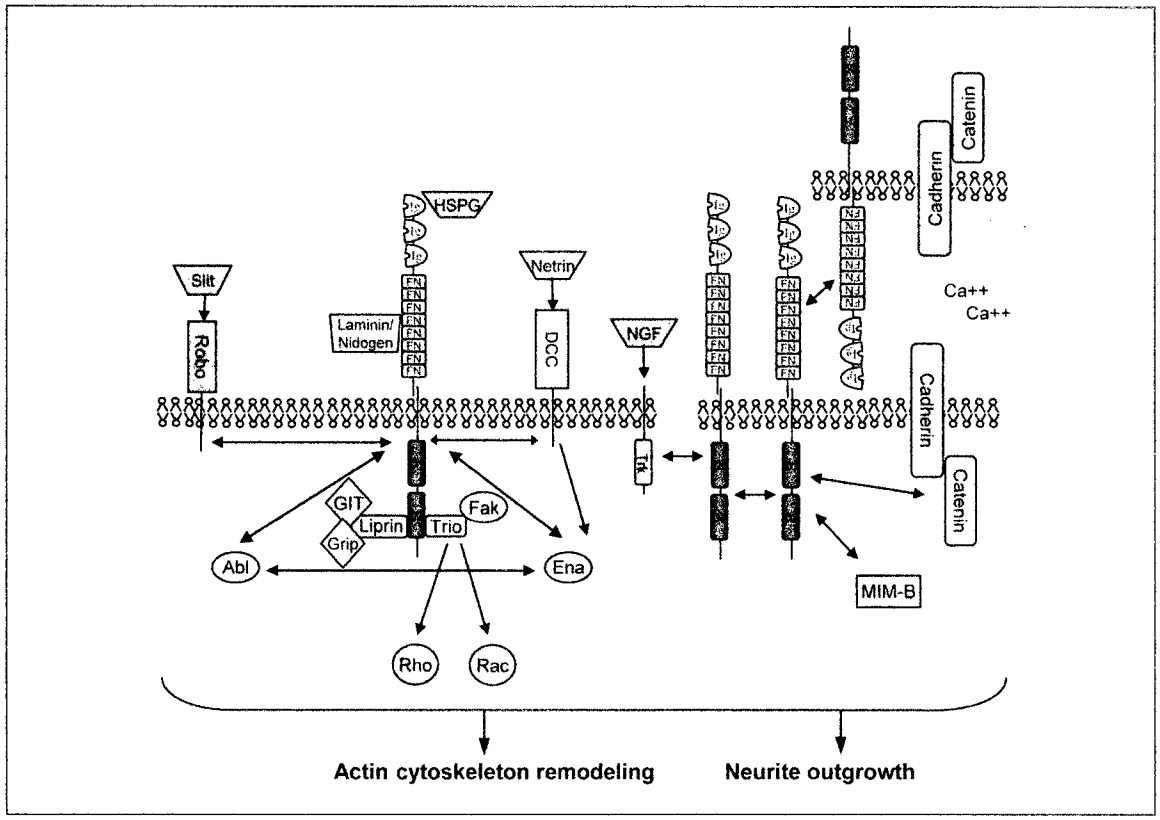

Figure 1.5 LAR-RPTPs mediated cellular signaling.

Possible interactions between LAR-RPTPs and effectors of actin cytoskeleton remodeling. Double-headed arrows indicate putative interactions and arrows indicate a positive effect.

1.5.2.1 Liprins

The D2 domains of all three members of the LAR-family interact with liprin-α (LAR-interacting proteins), a family of proteins that contains SAM and coiled-coil domains responsible for protein-protein interactions (Pulido et al., 1995). The LAR-liprin complexes localize to focal adhesions, further suggesting a role for LAR-RPTPs in adhesion signaling (Serra-Pages et al., 1995).

Upon ligand binding, the integrin family of receptors aggregates at focal complexes and mediates intracellular signaling, causing actin remodeling of the cytoskeleton into stress fibers (reviewed in (DeMali et al., 2003)). During this process, focal adhesion kinase (FAK) is activated, and PTPs are most probably involved in the dynamic regulation of this process. Liprins, however, are not tyrosine phosphorylated and are unlikely to be substrates of LAR-RPTPs. Perhaps the liprins recruit the LAR-RPTPs within the vicinity of their substrates. The functional role of this interaction is seemingly more obvious in the developing nervous system. Recent evidence has suggested a role for liprins in maintaining the integrity of the pre-synaptic zone assembly in *C.elegans* (Zhen and Jin, 1999) and *Drosophila* (Kaufmann et al., 2002). Liprin-α1 was also identified as a major binding partner of the glutamate receptor interacting protein (GRIP) (Wyszynski et al., 2002) and GIT1 (Ko et al., 2003) in neurons. Together these proteins have the capacity to form a large scaffolding protein complex associated with AMPA receptors at the post-

synaptic zone. Thus, recruitment of LAR-RPTPs into a complex with GRIP and GIT1 by liprins implies a role for the LAR-RPTPs in AMPA receptor trafficking, synaptic zone assembly, and/or maintenance of synaptic transmission. In fact, LAR-RPTPs are concentrated at excitory synapses in hippocampal neurons and are postulated to control synapse morphogenesis by targeting the cadherin/β-catenin complex to the GRIP-liprin-AMPA receptor protein complex (Dunah et al., 2005).

1.5.2.2 Trio

The signal transduction pathways downstream of the LAR-RPTPs, which lead to changes in axonal extension and guidance are mainly unknown, but ultimately involve cytoskeleton remodeling and therefore require RhoGTPases. One of these proteins, Trio, was found as a binding partner of LAR using a yeast-two-hybrid approach (Debant et al., 1996). This large multidomain protein was shown to have two guanine-nucleotide exchange factor domains specific for Rac1 and RhoA, respectively, as well as a serine/threonine kinase domain. Most of the functional data on Trio was obtained from work on *Drosophila*, where Trio was shown to regulate axonal guidance (Bateman et al., 2000). Interestingly, Trio also interacts with, and is tyrosine phosphorylated by, FAK (Medley et al., 2003). This suggests that LAR, once recruited to the focal adhesions by liprins, could be in a prime position to first regulate tyrosine phosphorylation by counteracting FAK kinase activity, and then activate the RhoGTPases by modulation of Trio. Another

link between the cytoskeleton and LAR-RPTPs is the actin binding protein MIM-B, which was recently identified as a binding partner of RPTPδ (Woodings et al., 2003).

1.5.2.3 Abl /Ena

Abl and its substrate Ena, two proteins implicated in axon growth and guidance, also interact with Dlar and act as substrates of one another *in vitro* (Wills et al., 1999). Abl and Ena possess antagonistic functions to Dlar in *Drosophila* and together are thought to act as a phosphotyrosine-dependent switch that controls axonal guidance. In addition, RNAi of HmLAR1 led to hyperphosphorylation of Ena further suggesting that Ena is an *in vivo* substrate of LAR in leech neurons (Biswas et al., 2002). Recently, it was shown that clr-1 in *C.elegans* inhibited netrin-mediated attraction, possibly by dephosphorylating the netrin receptor UNC-40/DCC or its effector Unc-34/Ena (Chang et al., 2004). Clr-1 does not have a clear homolog in mammals, but its extracellular domain is highly reminiscent of LAR-RPTPs. A growing body of evidence (reviewed in (Lanier and Gertler, 2000)) implicates Abl and its signaling partners in cell adhesion signaling and actin polymerization, resulting in regulation of growth cone motility during axonogenesis. Therefore, LAR-RPTPs are ideal intermediates to transduce adhesive cues regulating axon growth and guidance via actin remodeling.

1.5.2.4 Cadherin/β-catenin complex

LAR-RPTPs not only transduce signals from cell-extracellular matrix interactions, but from cell-cell interactions as well. LAR associates with and dephosphorylates β-catenin in PC12 cells *in vitro* (Kypta et al., 1996) and also localizes with γ-catenins in adherens junctions (Muller et al., 1999). LAR expression in the MCF7 breast cancer cell line and the rat Ln3 mammary adenocarcinoma cell line increases with cell density (Symons et al., 2002). This regulation of expression is lost if E-cadherin complexes are disrupted. LAR also prevents epithelial cell migration dependent on β-catenin phosphorylation (Muller et al., 1999). These data support a role for LAR in cell density-dependent contact inhibition and retention of epithelial integrity by regulation of the tyrosine phosphorylation of cadherin/catenin complexes. RPTPσ mRNA expression is also upregulated in dense cell cultures (Celler et al., 1995) and E-cadherin and β-catenin were found as *in vivo* substrates in the colon (Muise et al., 2007). In this context, RPTPσ would help maintain the integrity of adherens junctions demonstrating, again, that LAR-RPTPs regulate cell-cell interactions and density-dependent growth arrest.

As mentioned above, LAR-RPTPs were shown to dephosphorylate β-catenin *in vitro* and LAR-RPTP-mediated dephosphorylation of β-catenin promoted its accumulation at the synapse as well as the cell-adhesion functions of the cadherin/β-catenin complex at the synapse (Dunah et al., 2005). RPTPσ was the first RPTP shown to directly dephosphorylate not only β-catenin but N-cadherin as well, a

significant advancement towards the understanding of the role of RPTPσ (Siu et al., 2007). Dephosphorylation of N-cadherin in DRG neurons results in increased adhesiveness due to remodelling of the actin cytoskeleton and leads to a reduced rate of axon outgrowth, as observed in the context of active RPTPσ.

1.5.2.5 Protein tyrosine kinases as LAR-RPTP substrates

Many PTPs have been shown to interact with and modulate different PTK signaling pathways and LAR-RPTPs are no exception. LAR and the insulin receptor (IR) co-immunoprecipitate in CHO cells when overexpressed (Tsujikawa et al., 2001). Both D1 and D2 are important for this interaction, and the catalytically inactive LAR C/S mutant binds more tightly to the IR than the wild-type form. Autophosphorylation levels of the IR are increased in cells transfected with the C/S mutant, suggesting that the IR could be a LAR substrate. Furthermore, data from mice overexpressing LAR in insulin-target tissues also suggest a role for LAR in insulin signaling. Insulin-induced IR and IRS-1 phosphorylation in LAR overexpressing mice is unaffected, but IRS-2 phosphorylation is decreased (Zabolotny et al., 2001). PI3-kinase activity associated with IRS-1 and IRS-2 is also reduced, indicating that LAR may not act directly on the IR, as suggested by *in vitro* studies, but at the level of IR substrates (Figure 1.6). Moreover, the protein tyrosine kinases EGFR (Suarez Pestana et al., 1999), HGFR (Kulas et al., 1996), RET (Qiao et al., 2001), LCK and FYN (Tsujikawa et al., 2002) have been reported to be

substrates of LAR-RPTPs. However, the physiological significance of these interactions has not yet been assessed.

The Trk family of PTKs are receptors for neurotrophin growth factors known to promote axon outgrowth (reviewed in (Gillespie, 2003)). LAR has been shown to regulate signalling of TrkA and TrkB and was also found in a complex with them (Xie et al., 2006; Yang et al., 2006). Downregulation of LAR led to augmentation of TrkA signalling and neurite outgrowth (Tisi et al., 2000), while LAR activity could activate TrkB signalling through activation of Src (Yang et al., 2006). So, depending on the context, LAR could positively or negatively regulate Trk signalling. RPTPσ, on the other hand, was found in a stable complex with TrkA and TrkC while being able to dephosphorylate all three members of the Trk family. This interaction was functionally significant as overexpression of RPTPσ in chick DRG neurons limited NGF-induced neurite outgrowth (Faux et al., 2007).

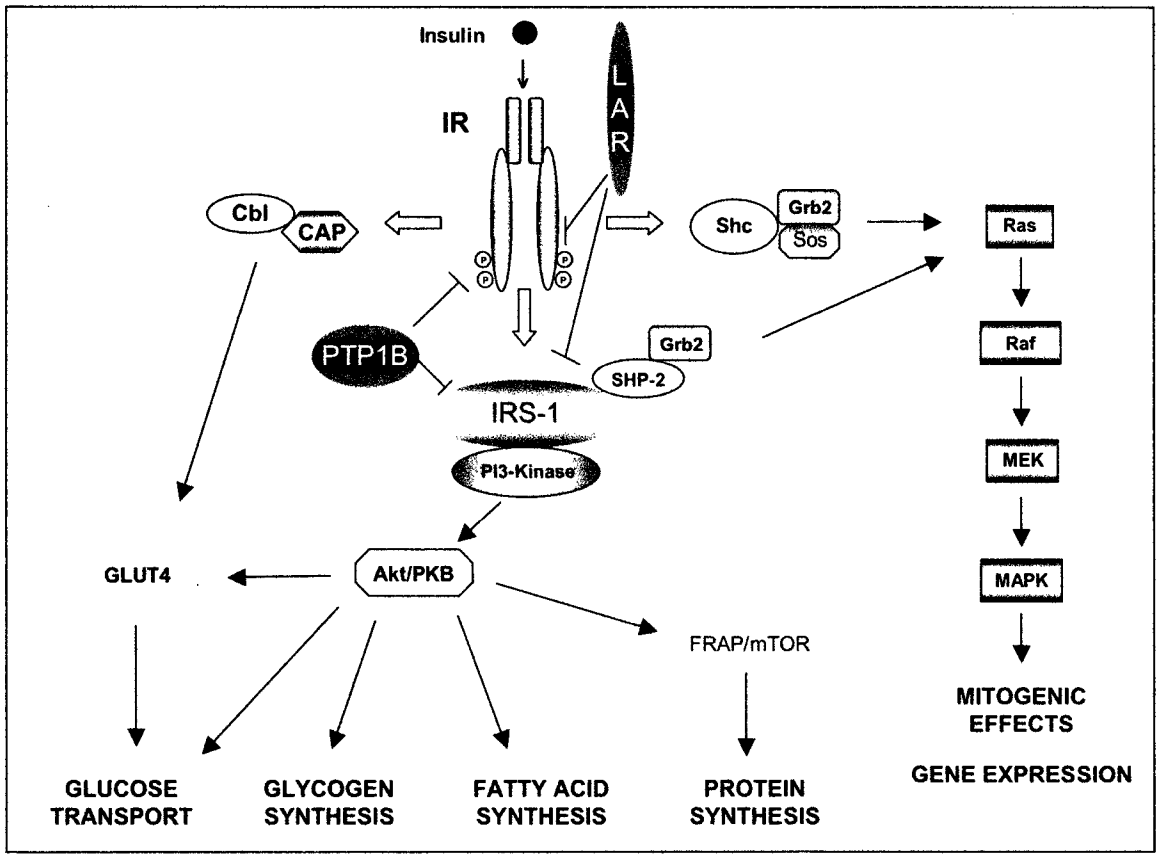

Figure 1.6 Schematic view of the major insulin signaling pathways.

The activated insulin receptor phosphorylates IRS proteins, SHC, CAP and other substrates onto tyrosine residues. These phosphorylated substrates bind to various downstream signaling effectors, transmitting the metabolic and mitogenic response of insulin. PTPs such as LAR and PTP1B have been shown to regulate these pathways. Adapted from (Cheng et al., 2002).

1.6 Expression and physiological functions of LAR-RPTPs

From invertebrate to vertebrate, the majority of the LAR subfamily of RPTPs are expressed in the developing nervous system (Tian et al., 1991; Desai et al., 1994; Stoker et al., 1995; Sommer et al., 1997; Schaapveld et al., 1998), and immunohistochemical studies have revealed that they are localized to axons and growth cones in *Drosophila* (Tian et al., 1991; Clandinin et al., 2001; Maurel-Zaffran et al., 2001), leech (Gershon et al., 1998b), chick (Stoker et al., 1995) and mammals (Thompson et al., 2003). These data suggest that the physiological role of LAR-RPTPs in the nervous system is evolutionally conserved. This is discussed in more detail for each organism.

1.6.1 *Drosophila Melanogaster*

In *Drosophila*, four out of five RPTPs (DLAR, DPTP69D, DPTP10D and DPTP99A) are expressed specifically in the developing nervous system (Tian et al., 1991; Yang et al., 1991; Desai et al., 1994). Specific antibodies against DLAR, DPTP69D and DPTP99A demonstrate expression of these RPTPs in the central nervous system (CNS) nerve roots, developing motor nerves and the visual system (Tian et al., 1991; Desai et al., 1994; Clandinin et al., 2001; Maurel-Zaffran et al., 2001). In mutant embryos lacking either DLAR or DPTP69D, segmental nerve b (SNb) motor axons stop growing before reaching their muscle targets or follow incorrect pathways that bypass the muscles, suggesting that there are functional

redundancies between DLAR and DPTP69D (Desai et al., 1996; Krueger et al., 1996). Interestingly, further genetic studies on DLAR, DPTP69D, and DPTP99A, using either double or triple combination mutants, have revealed that these RPTPs function as competitors or cooperators depending on the cellular context at the different SNb nerve branch points (Desai et al., 1997; Desai and Purdy, 2003).

During the development of the visual system in *Drosophila*, DLAR and DPTP69D are required for layer-specific targeting of photoreceptor axons (Garrity et al., 1999; Newsome et al., 2000; Clandinin et al., 2001; Maurel-Zaffran et al., 2001) (summarized in Figure 1.7). The fruitfly has a compound eye comprised of roughly 750 omatidia, each of which contains eight photoreceptor neurons (R cells), R1 to R8. Within each cluster, the R8 cell extends its axon towards the optic lobe as a pioneer and terminates in the medulla. The R1-R6 and the R7 axons follow this pathway, fasciculating tightly with the R8 axon. The R1-R6 axons then terminate in the lamina, and the R7 axon terminates in the medulla beyond the region within the optic lobe targeted by R8 (reviewed in (Tayler and Garrity, 2003)). Two studies using loss-of-function mutants revealed that DPTP69D is involved in lamina target specificity of the R1-R6 axons, and also in the correct targeting of the R7 axon. The R1-R6 axons frequently fail to terminate in the lamina, and instead follow the R8 axon into the medulla in DPTP69D mutant embryos (Garrity et al., 1999). Furthermore, in DPTP69D mutants, the R7 axons fail to reach their targets,

stopping at the same level as the R8 axons, indicating DPTP69D plays a permissive role rather than an instructive role in axon targeting (Newsome et al., 2000) (reviewed in (Stoker, 2001)). In Dlar loss-of-function mutants, R1-R6 axons stop at the lamina correctly but often fail to choose correct target neurons and R7 axons incorrectly retract to the R8 target layer (Clandinin et al., 2001; Maurel-Zaffran et al., 2001). This R7 targeting defect can be fully rescued by transgenic expression of Dlar in the R7 neurons, but is only partially rescued if Dlar is expressed in R8, suggesting that Dlar plays a role as either a receptor in R7 neurons or as a ligand in R8 neurons.

It has also been shown that DPTP69D and DPTP10D are required for repulsion of growth cones from the midline of the embryonic CNS by positively regulating Slit/Robo repulsive signaling in the embryonic CNS (Sun et al., 2000a). As well, Dlar, together with liprins, are involved in normal synaptic morphogenesis at the neuromuscular junction (NMJ) (Kaufmann et al., 2002).

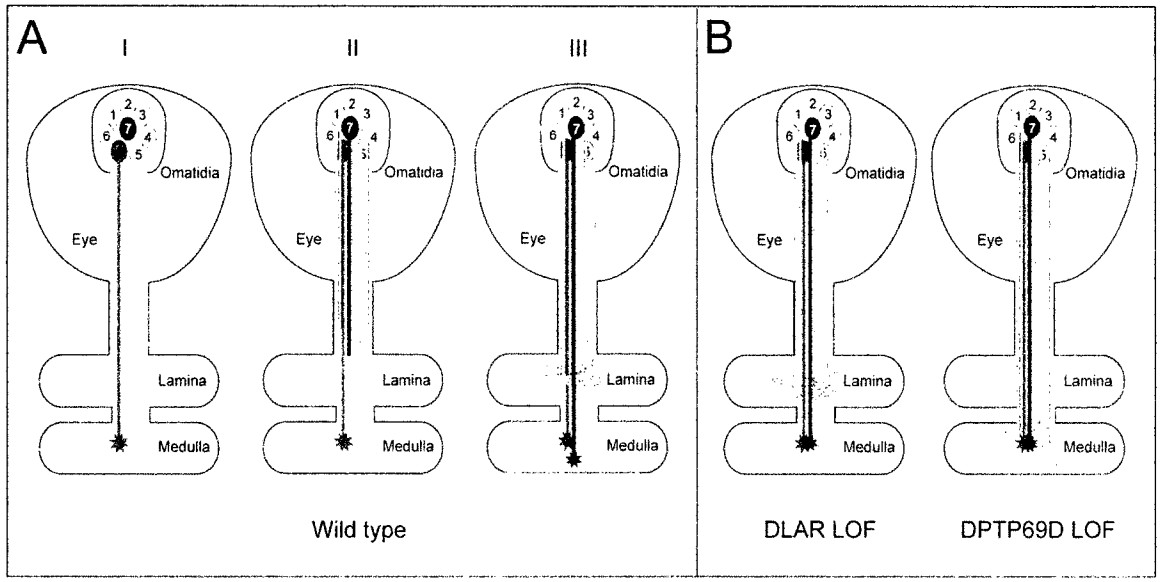

Figure 1.7 Targeting defects of photoreceptor axon guidance in RPTP loss-of-function *Drosophila.*

(A) Schematic diagrams of photoreceptor axon guidance in wild type *Drosophila.* Firstly, the R8 cell extends pioneer axon and terminates in the medulla (I). Secondly, the R1-R6 and the R7 axons follow this pathway (II). Finally, the R1-R6 axons terminate in the lamina and the R7 axon terminates in the medulla beyond the R8 targeted place (III).

(B) Typical photoreceptor guidance defects in DLAR loss-of-function (DLAR LOF) and DPTP69D loss-of-function (DPTP69D LOF) mutants are represented. In DLAR LOF mutants, although the R1-R6 axons stop in the lamina correctly, they terminate on incorrect target cells. In the DPTP69D LOF mutant, the R1-R6 and R7 axons fail to terminate in the correct place, following R8 axons and terminating nearby.

1.6.2 *Hirudo Medicinalis*

Following the work on Dlar, other studies have revealed that leech HmLAR2 is required for guidance and extension of neuron-like comb cells. This requires the ectodomain of HmLAR2, which transduces extracellular guidance cues (Gershon et al., 1998a). Further evidence suggests that HMLAR2 acts on actin cytoskeleton through an ENA/Vasp substrate (Biswas et al., 2002). Taken together, these data suggest that HmLAR2 regulates the morphology of the growth cone by controlling actin polymerization and focal adhesion complexes(Baker et al., 2008).

1.6.3 *Caenorhabditis Elegans*

In *C.elegans*, Clr-1, a RPTP structurally similar to LAR-RPTPs, is a key regulator of anterior ventral microtubule (AVM) axon guidance. Loss-of-function mutations in Clr-1 resulted in enhanced netrin-dependent axon attraction and suppressed ventral guidance defects in slt-1/Slit mutants (Chang et al., 2004). On the other hand, it was shown that PTP-3 is involved in epidermal and early neural morphogenesis, although axon guidance in selected neurons appears normal. Further genetic analysis revealed that the loss-of-function of PTP-3 synergistically enhances the phenotypes of the PTK Eph receptor VAB-1 and ephrin EFN-4 mutants (Chin-Sang et al., 2002; Harrington et al., 2002).

1.6.4 Chick

High levels of the cPTPσ protein are localized in chick retinal axons extending from the eye to the tectum, primitive inner plexiform, tectobulbar axons and non-retinal fibres of the tectal stratum opticum (Stoker et al., 1995; Ledig et al., 1999b). Consistent with this expression pattern, it has been shown that cPTPσ is required for intraretinal axon growth (Ledig et al., 1999a) and topographic mapping of retinal axons in the chick visual system (Rashid-Doubell et al., 2002). Moreover, double-stranded RNA interference mediated knockdown of RPTPσ and RPTPδ as well as PTPRO (a type III RPTP), have provided a first set of *in vivo* evidence for a RPTP role in axon growth and motor axon guidance (Stepanek et al., 2005).

1.6.5 Mammalian expression and function

In mammals, LAR is predominantly expressed in basal lamina-associated epithelial tissues and is less specific to neuronal tissues (Schaapveld et al., 1998). In contrast, RPTPσ and RPTPδ are more highly expressed in the developing nervous system and exhibit a parallel expression pattern (Yan et al., 1993; Mizuno et al., 1994; Sommer et al., 1997; Schaapveld et al., 1998) (summarized in Figure 1.8). In the developing murine nervous system, LAR expression is highly restricted to the subventricular zone. The level of expression is low in the cortical layer within the CNS, but high in peripheral nervous system (PNS) nuclei such as dorsal root ganglia (DRG) (Schaapveld et al., 1998). RPTPσ mRNA is predominantly expressed in the

CNS and PNS during embryogenesis with maximal expression at embryonic day 16 (E16), a period when important axonal outgrowth occurs. Specific expression of RPTPσ was also found in the olfactory epithelium and in Rathke's pouch (the embryonic tissue leading to pituitary gland development). Other sites of expression include the germinal cell layer lining the lateral ventricles of the developing brain, the neural fold, the DRG, cranial nerve ganglia and retina (Yan et al., 1993; Schaapveld et al., 1998). RPTPδ mRNA expression is also largely restricted to the developing mouse CNS and more precisely to the cortex, diencephalon, pituitary grand, olfactory bulb, olivary nucleus, retina and inner ear. In the embryonic spinal cord, RPTPδ expression is restricted to the roof plate and to differentiated neurons in the motor columns, suggesting a likely function in motor axon guidance in vertebrates (Schaapveld et al., 1997; Sommer et al., 1997). *In situ* hybridization analysis revealed that RPTPδ expression in the adult brain is localized to the pyramidal cell layer of the hippocampal CA2–CA3 region, thalamic reticular nucleus and piriform cortex (Mizuno et al., 1993; Sommer et al., 1997; Schaapveld et al., 1998).

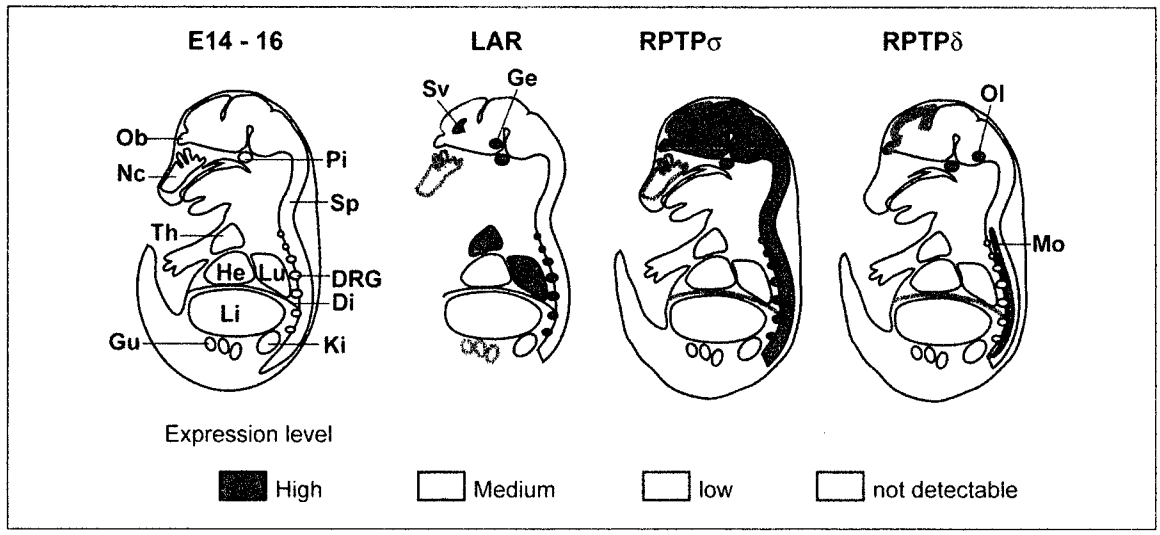

Figure 1.8 Expression patterns of LAR-RPTPs during mouse embryogenesis.

A summary of the LAR, RPTPσ and RPTPδ expression patterns from mice embryonic age E14 to E16 is shown. Ob, olfactory bulb; Nc, nasal cavity; Th, thymus; He, heart; Lu, lung; Li, liver; Gu, gut; Pi, pituitary gland; Sp, spinal cord; DRG, dorsal root ganglia; Di, diaphragm; Ki, kidney; Sv, subventricular layer; Ol, olivary nucleus; Ge, geniculate ganglion; Mo, motor column.

1.6.5.1 Single LAR-RPTP KO phenotype

Mice lacking LAR grow normally, and yet show defects in mammary gland development during the gestation period (Schaapveld et al., 1997) and minor defects in cholinergic innervations of the hippocampal dentate gyrus (Yeo et al., 1997; Van Lieshout et al., 2001) leading to spatial learning impairment and hyperactivity (Kolkman et al., 2004). Mice lacking either RPTPσ or RPTPδ display more striking nervous system phenotypes as compared to LAR deficient mice. RPTPσ deficient mice exhibit severe growth retardation and semi-lethality after birth with abnormal pituitary development and neurological defects including: motor dysfunction, defective proprioception, hippocampal dysgenesis, and a thinner corpus callosum and cerebral cortex (Elchebly et al., 1999a; Wallace et al., 1999; Meathrel et al., 2002). RPTPδ deficient mice also exhibit early growth retardation and neonatal mortality likely due to insufficient food-intake caused by motor dysfunction. These mutant mice exhibit spatial learning impairment, due to the significantly enhanced magnitude of hippocampal long-term potentiation (LTP) (Uetani et al., 2000).

Interestingly, recent data has revealed that LAR and RPTPσ are involved in peripheral nerve regeneration. The absence of LAR delays axonal regeneration *in vivo* following injury to the sciatic nerve, a mixed sensory and motor nerve (Xie et al., 2001; Van der Zee et al., 2003). The upregulation of LAR protein expression in DRG neurons after sciatic nerve injury suggested a role for LAR in promoting

rather than inhibiting neurite outgrowth in sensory neurons (Xie et al., 2001). On the other hand, it was reported that axonal regeneration in the sciatic and facial nerve is enhanced following nerve crush, in the absence of RPTPσ (McLean et al., 2002; Thompson et al., 2003). Most interestingly, deletion of RPTPσ also promoted axonal regrowth of retinal ganglion cells following optical nerve lesion into the non-permissive environment of the central nervous system (Sapieha et al., 2005). In contrast to the effect of LAR on both sensory and motor neurons, RPTPσ seems to impair axon outgrowth, suggesting that LAR and RPTPσ may have the opposing function in different types of neurons and in different cellular contexts as seen in *Drosophila*. As mentioned above, the *in vitro* experiments indicate that a soluble form of the RPTPδ extracellular domain can stimulate both the growth and steering of CNS neurites (Wang and Bixby, 1999; Sun et al., 2000b); however, it has not been reported whether RPTPδ promotes or impairs nerve regeneration in mice.

1.6.5.2 Double KO phenotype

Axon outgrowth and guidance defects in the single knockout mice are not nearly as severe as those observed when Dlar is mutated in *Drosophila*, suggesting that in mammals loss of one LAR-RPTP subfamily member may be functionally complemented by the other two. Downregulation of RPTPσ, RPTPδ and PTPRO in the chick first suggested complementation of RPTPs in vertebrates, but a clear role for these enzymes in mammalian development was only obtained after generation of

double mutant mice. When RPTPσ/RPTPδ double KO mice were generated their phenotype was seen to be much more severe than single knockout mice. RPTPσ/RPTPδ double-mutant E18.5 embryos are paralyzed, unable to draw a breath and die right after caesarean section. Detailed analysis of the phrenic nerve revealed that although the motoraxons emerge from the spinal cord they are unable to properly target to the diaphragm (Uetani et al., 2006) showing an essential role for these enzymes during development of the mammalian nervous system.

1.7 Potential targets in human diseases

1.7.1 LAR-RPTPs in cancer

Compelling evidence has implicated associated members of the PTP family with various congenital diseases (reviewed in (Tautz et al., 2006)) and led to the recognition that PTPs control many physiological processes. For example, some LAR-RPTP chromosomal loci are amplified (Harder et al., 1995; Andersen et al., 2004) or mutated (Wang et al., 2004) in different human cancers, ie. small cell lung or colon cancer. LAR expression is significantly increased in thyroid carcinomas (Konishi et al., 2003) and breast cancer (Yang et al., 1999). Although more research is needed, expression of LAR may correlate with the prognosis of breast cancer metastases (Levea et al., 2000). As discussed above, LAR associates with the cadherin-catenin complex, which maintains epithelial integrity and inhibits tumor formation in nude mice (Muller et al., 1999). Accordingly, RPTPσ was shown to

reduce colony formation in a soft agar assay (Suarez Pestana et al., 1999). Clearly this subfamily of PTPs plays a role in neoplastic transformation. More work is necessary in order to determine whether the increased expression of LAR-RPTPs in different tumors is beneficial, by counteracting an oncogenic kinase activity, or detrimental, due to unregulated activity that could contribute to the tumorigenic transformation. The later situation raises the possibility that specific inhibitors of RPTPs could be designed for cancer treatment.

1.7.2 RPTPσ and inflammatory bowel disease

Ulcerative colitis is a major type of inflammatory bowel disease (IBD) affecting mainly the colon and the rectum. RPTPσ is expressed in the colon and RPTPσ KO mice display features associated with IBD such as cachexia, and mild colitis (Wallace et al., 1999; Batt et al., 2002). Indeed RPTPσ KO mice were shown to develop severe colitis when treated with known inducers of experimental model colitis. Furthermore three SNPs found in the human PTPRS gene are associated with ulcerative colitis (Muise et al., 2007). These SNPs result in novel alternative splicing of the long isoform of RPTPσ, resulting in the loss of the third Ig-like domain, which could affect ligand binding and RPTPσ regulation. These observations indicate that polymorphisms in the human PTPRS gene are associated with ulcerative colitis and will provide better understanding of this disease.

1.7.3 LAR-RPTPs in metabolic disease

The function of LAR in insulin signaling has been recently reviewed extensively (Cheng et al., 2002; Mooney and LeVea, 2003). LAR possibly downregulates signaling downstream of the IR (Figure 1.6). The most recent *in vivo* data showing that LAR regulates glucose homeostasis and signaling comes from the study of transgenic mice overexpressing LAR in skeletal muscle (MCK-hLAR mice) (Zabolotny et al., 2001). These mice maintain normal glucose levels, but they exhibit a 2.5-fold increase in fasting insulin levels and had reduced glucose uptake in skeletal muscle compared to controls. Both observations are signs of insulin resistance. Overexpression of PTP1B in skeletal muscle also caused a similar impairment in insulin action (Zabolotny et al., 2004). However, overexpression of both PTPs did not yield an additive effect, suggesting that there are some redundancies in the effects of overexpression of these proteins on insulin resistance *in vivo*. Although PTP1B has become the main PTP target for diabetes and obesity treatment, as seen by the enormous number of reports in the literature, its effects seem to be tissue-specific. In adipose tissue in particular, IR phosphorylation is unchanged in PTP1B-deficient mice (Elchebly et al., 1999b). Interestingly LAR and RPTPσ are highly expressed in adipose tissue (Norris et al., 1997) and could be prime drug targets for tissue-specific regulation of insulin resistance and reduction of adiposity.

1.7.4 LAR-RPTPs in nerve regeneration

The recent findings that RPTPσ inhibits the rate of axonal extension and nerve regeneration in the peripheral (McLean et al., 2002; Thompson et al., 2003) and central (Sapieha et al., 2005) nervous system of mice has generated interest in this subfamily of enzymes as possible new targets for the treatment of neurodegenerative diseases and nervous system repair. This enzyme, which is known to be involved in nervous system development, could probably have a more prevalent role in this therapeutic area than in cancer or diabetes considering its expression pattern. Future work will ensure that the downregulation of this RPTP will not amplify tumorigenic or unbalance metabolic signaling. Another challenge for drug development will be to generate specific compounds that selectively inhibit RPTPσ but not the highly similar subfamily members LAR and RPTPδ, which have opposite effects on regeneration (Xie et al., 2001; Van der Zee et al., 2003) and neurite outgrowth (Johnson et al., 2001). LAR-RPTPs represent great potential for the discovery of new nervous system treatments, an area of therapeutics that has provided very few answers thus far.

1.8 Thesis overview

The control of cellular tyrosine phosphorylation levels is of great importance in many biological systems. Among the kinases and phosphatases that modulate these levels, the LAR-RPTPs are definite players in several key aspects of neural

development, and in a dysfunctional manner in various pathologies from diabetes to cancer. This thesis focuses on the research that I have been conducting in the laboratory of Dr. Michel L. Tremblay in order to describe the physiological functions of one member of this subfamily, namely RPTPσ.

Several PTPs expressed in insulin sensitive tissues have been suggested to be key regulators of the insulin receptor (IR) signaling pathway. Among these, RPTPσ became a prime candidate for further study since it is expressed in relatively high levels in insulin-target tissues. Chapter 2 describes the role of RPTPσ in the maintenance of glucose homeostasis using the RPTPσ knockout mouse model.

In the absence of RPTPσ, axonal regeneration in the sciatic, facial and optical nerve is enhanced following nerve crush. However, myelin-associated growth inhibitory proteins and components of the glial scar such as CSPGs (chondroitin sulfate proteoglycans) have long been known to inhibit axonal regeneration in the CNS, making spinal cord injury irreversible. The aim of Chapter 3 is to investigate whether RPTPσ plays a role in regulation of axonal outgrowth in the inhibitory environment of the CNS following spinal cord injury.

Identification of a new set of RPTPσ substrate(s) will be the key to understanding its physiological role. In Chapter 4, we used a modified yeast-two-

hybrid approach, to screen a murine embryonic library for new substrates of RPTPσ. The new substrate and binding partners identified will be described.

The characterization of RPTPσ and its signaling partners is essential for understanding its role *in vivo* and may one day translate into treatment of neural diseases and injuries. This is discussed further in the thesis summary and future perspectives sections.

CHAPTER 2: Altered Glucose Homeostasis in Mice Lacking the Receptor Protein Tyrosine Phosphatase Sigma

2.1 Abstract

Several protein tyrosine phosphatases (PTPs) expressed in insulin sensitive tissues are proposed to attenuate insulin action and could act as key regulators of the insulin receptor (IR) signaling pathway. Among these PTPs, RPTPσ is expressed in relatively high levels in insulin-target tissues. We show that RPTPσ knockout (-/-) mice have reduced plasma glucose and insulin concentrations in the fasted state compared to their wild-type siblings. The knockout animals were also more sensitive to exogenous insulin as assayed by insulin tolerance test. Despite increased whole-body insulin sensitivity, tyrosine phosphorylation of the IR was not increased in muscle of RPTPσ (-/-) animals, as would be expected in insulin-sensitive animals. Instead, the levels of IR tyrosine phosphorylation and PI3-kinase activity were reduced in the muscle of knockout animals stimulated with insulin *in vivo*. However, insulin-stimulated Akt serine phosphorylation was essentially identical between both groups of mice. Accordingly, muscles isolated from RPTPσ (-/-) mice did not have a significant increase in glucose uptake in response to insulin, suggesting that RPTPσ did not play a direct role in this process. Taken together, our results suggest an indirect modulation of the IR signaling pathways by RPTPσ. Since low dose injection of GH normalized the response to exogenous insulin in RPTPσ (-/-) mice, we propose that the insulin hypersensitivity observed in RPTPσ (-/-) mice is secondary to their neuroendocrine dysplasia and GH/IGF-1 deficiency.

2.2 Introduction

Insulin binds to its cell surface receptor and induces a variety of metabolic and mitogenic responses. Upon insulin binding to the α-subunit of the insulin receptor (IR), the β-subunits of the IR undergo tyrosine autophosphorylation, which leads to increased IR tyrosine kinase activity towards its intracellular substrates, notably Shc and the insulin receptor substrate (IRS) protein family (Saltiel and Kahn, 2001; White, 2002). Tyrosine phosphorylated IRS proteins serve as large scaffolding proteins by binding to several SH2 (Src-homology 2) effector proteins such as PI3-kinase, Shp-2, and Grb2. Subsequently, these proteins mediate the metabolic and mitogenic effects of insulin.

An important regulatory step in insulin signal transduction cascade is the dephosphorylation of tyrosine-phosphorylated residues from several effector proteins by protein tyrosine phosphatases (PTPs), which likely participate in attenuating insulin signaling (Cheng et al., 2002). Of these PTPs, PTP1B has emerged as the most potent negative regulator of insulin signaling. We and others, have generated PTP1B deficient mice, which showed increased insulin sensitivity and protection against obesity induced by a high fat diet (Elchebly et al., 1999b; Klaman et al., 2000). This phenotype correlates with an increased IR tyrosine phosphorylation in liver and muscle, but not in the adipose tissue of these knockout

mice, suggesting that other PTPs might have specific preferences for this receptor in different insulin-sensitive tissues.

LAR, which belongs to the LAR-RPTPs family that also includes RPTPσ and RPTPδ, associates and dephosphorylates the IR *in vitro* (Ahmad and Goldstein, 1997; Mooney and LeVea, 2003). The localization of this RPTP at the plasma membrane made it a logical candidate for dephosphorylating the IR. Studies with LAR-deficient mice, however, were inconclusive in confirming a role for LAR in negative modulation of the IR signaling (Ren et al., 1998). Insulin-stimulated tyrosine phosphorylation of the IR and basal PI3-kinase activity were only modestly increased with reduced LAR expression. In contrast, insulin-stimulated PI3-kinase activity was reduced in these mice in comparison to control. It should be noted that LAR has a role in neuronal development and the lack of proper neuronal development in the knockout mice has made the study of glucose homeostasis in these animals more complex. To overcome this issue, transgenic mice specifically over-expressing LAR in muscle were generated (Zabolotny et al., 2001). These mice are insulin resistant and maintain normal glucose levels at higher plasma insulin levels. Glucose uptake is also reduced in skeletal muscles. Insulin-induced tyrosine phosphorylation of the IR and IRS-1 are normal in muscle of these mice, but IRS-2 phosphorylation is decreased along with IRS-1 and IRS-2 associated PI3-kinase activity. These results indicate that LAR could negatively modulate the insulin

signaling through dephosphorylation of IRS-2, although other proteins might be affected.

Since the intracellular domain of LAR is 85% identical to that of RPTPσ at the amino acid level (Pulido et al., 1995), it is possible that both could act on a common set of substrates. Moreover, RPTPσ is expressed at higher levels than LAR in the insulin-sensitive adipose tissue and muscle (Norris et al., 1997). These observations suggest that RPTPσ could play a role in glucose homeostasis.

The generation of RPTPσ (-/-) mice has demonstrated the importance of this RPTP in neuroendocrine development, neuronal axon growth and regeneration (Elchebly et al., 1999a; Wallace et al., 1999; McLean et al., 2002; Thompson et al., 2003). In the present study, we utilized adult RPTPσ (-/-) mice to further explore whether this enzyme plays a role in glucose homeostasis and insulin signaling. We determined that these mice maintain lower blood glucose levels in the fasted state despite having lower insulin levels. They are also more responsive to exogenous insulin administration. Examination of insulin action in the major insulin-responsive tissues did not show any marked increased in tyrosine phosphorylation of the IR or activation of its associated downstream signaling molecules or muscle glucose uptake. Since the sensitivity to exogenous insulin observed in RPTPσ-deficient mice could be rescued by injection of low dose of GH, we conclude that the phenotypic

changes in glucose homeostasis in the RPTPσ-deficient mice are mainly related to the neuroendocrine function of this PTP.

2.3 Materials and methods

2.3.1 Animal husbandry and genotyping

RPTPσ (-/-) mice were generated as previously described (Elchebly et al., 1999a) and genotyped by PCR (Thompson et al., 2003). WT and RPTPσ (-/-) mice on a Balb/c background (11 to 15 weeks old) were used. Animals were housed under 12-hours light/dark cycle and had free access to water and regular animal chow. All animal experiments described in this manuscript followed the Canadian Council of Animal Care guidelines.

2.3.2 Glucose and insulin measurements

Blood from fed or overnight-fasted animals was collected from the tail or saphenous vein for the measurement of the glucose and insulin concentrations. Glucose levels were determined using an Accu-check glucometer (Roche). Serum insulin concentrations were determined as previously described (Elchebly et al., 1999b).

2.3.3 Insulin and glucose tolerance tests

Insulin tolerance tests were performed by intraperitoneal injection of insulin (Humulin, Eli Lilly) at 0.75 U/kg after the mice had been fasted for six hours. Glucose tolerance tests were performed by intraperitoneal injection of 1g/kg of glucose after the mice had been fasted overnight. In both cases blood was withdrawn at 0, 30, 60, and 120 minutes following injection and glucose concentrations were measured.

2.3.4 *In vivo* insulin stimulation

Experiments were carried out on mice fasted overnight and anesthetized with 20 µl/g of 2.5% Avertin. Insulin (10 mU/g) was injected through the hepatic portal vein. Hind limb muscles, adipose tissue (reproductive fat pad) or liver were removed at the indicated time after insulin injection and immediately frozen in liquid nitrogen. Tissues were homogenized in Ripa buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate, 1 mM Na3VO4, 50 mM NaF, and a cocktail of protease inhibitors (Complete, Roche)). Protein concentrations were determined using a protein assay kit (BioRad).

2.3.5 Immunoprecipitation

Muscle lysates (1 mg of protein) were immunoprecipitated overnight at 4°C with 3 µl of anti-IR β-subunit rabbit polyclonal antibody (Santa Cruz). The resulting immune complexes were recovered by the addition of 30 µl of protein A-agarose

(Invitrogen) for 90 minutes at 4°C. The precipitates were washed 3 times with homogenization buffer. The final protein A-agarose pellets were resuspended in SDS sample buffer and boiled for 5 minutes.

2.3.6 Immunoblotting

Proteins from immunoprecipitation or 20 μl of total tissue lysates were separated by SDS/PAGE on 8% gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). Membranes were then incubated with anti-phospho IR (1162/1163) (Biosource), anti-phospho-Akt (Cell Signaling), anti-insulin receptor β-subunit, anti-Akt (Cell Signaling), anti-IRS1 (Cell Signaling), anti-p85 (Cell Signaling), anti-Actin (Sigma) or anti-phosphotyrosine (4G10, Upstate) according to manufacturer's instructions.

2.3.7 Phosphatidylinositol 3-kinase assay

Phosphotyrosine-associated PI3-kinase activity was assessed by immunoprecipitation of muscle lysates with anti-phosphotyrosine monoclonal antibody (PY20, Transduction Laboratories). After washing the immunoprecipitates, the pellets were resuspended in kinase buffer (4 μg of phosphatidyl inositol (Sigma), 10 μM ATP, 5 mM MgCl2, and 10μCi of γ-32P-ATP) and incubated for 20 minutes at 30°C. The kinase reaction was stopped by the addition of 100 μl of 1 N HCl and 200 μl of a 1:1 mixture of chloroform and

methanol. The lipids were extracted and applied to silica thin-layer chromatography (TLC) plates (EM sciences) with CHCl3/CH3OH/H2O/NH4OH (60:47:11:2) as the mobile phase. The radioactivity in phosphatidyl inositol spots was then quantified by Phosphor Imager (Molecular Dynamics).

2.3.8 Glucose Uptake in Isolated Muscle

In vitro glucose uptake experiments were performed in isolated extensor digitorum longus (EDL) and soleus muscles. Glucose transport was measured using the glucose analogue 2-[3H]-2-deoxy-D-glucose as previously described (Kapur et al., 1997).

2.3.9 Serum IGF-1 quantification

IGF-1 serum concentrations were measured by radioimmunoassay using the DSL-2900 Rat IGF-1 RIA kit (Diagnostic Systems Laboratories). Analyses were performed according to the manufacturer's protocol.

2.3.10 GH injection

Human rGH (National Hormone and Peptide Program) was injected subcutaneously into the mice twice daily for 6 days at a concentration of 0.5 μg/g. On the seventh day, insulin tolerance tests were performed as described above.

2.4.11 Statistical methods

The data are presented as means ± SEM for the indicated number in each group. Unpaired Student's t tests were used for comparison between groups.

2.4 Results

2.4.1 Phenotype and blood levels of glucose and insulin

As previously described, fifteen week old RPTPσ (-/-) mice had a 17% body weight reduction relative to their WT littermates (Elchebly et al., 1999a; Wallace et al., 1999). RPTPσ (-/-) mice had smaller reproductive fat pad mass and this effect persisted even after adjusting to body weight (Table 1). In contrast, muscle and liver ratios to body mass were not significantly changed (data not shown). RPTPσ (-/-) mice had similar food consumption to WT mice while adjusted to body weight (Table 1) suggesting that the decreased size observed is more due to a growth defect than a disturbance in the food intake monitoring.

We next determined whether glucose homeostasis was affected by the absence of RPTPσ. We measured blood glucose and insulin concentrations in the fed and fasted states of RPTPσ (-/-) and WT male mice (Table 1). Under fed conditions, RPTPσ (-/-) mice had unchanged plasma glucose concentrations but tended to have lower plasma insulin than WT mice. Under fasted conditions the plasma glucose and insulin levels were decreased in RPTPσ-deficient mice by 18%

and 40%, respectively, in comparison to their WT counterpart. The lower glucose /insulin ratio, particularly under fasted condition, suggest that RPTPσ (-/-) mice are more sensitive to insulin.

Table 2.1: Metabolic parameters in WT and RPTPσ (-/-) male mice.

	Wild-type	RPTPσ (-/-)
Body weight (g)	30.6 ± 0.8 (12)	25.5 ± 1.1 (7)*
Reproductive fat pad mass (g)	0.718 ± 0.06 (12)	0.427 ± 0.082 (7)**
Fat pad mass/Body weight (%)	2.3 ± 0.2 (12)	1.6 ± 0.3 (7)***
Food intake (5 days)/Body weight (%)	60.5 ± 0.03 (6)	60.8 ± 0.02 (4)
Fasting glucose (mg/dl)	106.7 ± 4.7 (6)	86.6 ± 3.1 (10)**
Fasting insulin (ng/ml)	0.77 ± 0.12 (6)	0.45 ± 0.03 (8)***
Fed glucose (mg/dl)	113.0 ± 10.6 (6)	103.6 ± 5.0 (11)
Fed insulin (ng/dl)	2.64 ± 0.77 (9)	1.72 ± 0.48 (8)

Data are presented as mean ± SEM, P: * < 0.001, ** < 0.01, *** < 0.05

The value in () indicates the number of mice analyzed.

2.4.2 RPTPσ (-/-) mice are hypersensitive to exogenous insulin

To better characterize the effects of RPTPσ deficiency on glucose homeostasis, we performed insulin tolerance tests on males fasted for 6 hours. After a single intraperitoneal bolus injection of 0.75 U/Kg of insulin, glucose clearance occurred more efficiently in RPTPσ (-/-) mice (Figure 2.1A). The glucose concentration reached the lowest level at sixty minutes post-injection in both WT littermate controls and RPTPσ (-/-) animals. However, RPTPσ-deficient mice displayed greater hypoglycemia after 30 minutes through 120 minutes. Similar results were also obtained with female mice (data not shown). The hypoglycemia observed in RPTPσ (-/-) mice demonstrates that these animals are hypersensitive to exogenous insulin.

2.4.3 RPTPσ (-/-) mice clear glucose normally

To further examine the effects of the absence of RPTPσ on glucose homeostasis, glucose tolerance tests were performed on male mice. Following overnight fasting, the baseline glucose levels were lower in RPTPσ (-/-) mice as shown in Table 1. Despite a lower baseline glucose concentration, the ability of RPTPσ-deficient mice to clear glucose was very similar to WT controls (Figure 2.1B). Intraperitoneal glucose administration resulted in an increase in the blood glucose concentration with maximum levels at thirty minutes. Thereafter, the plasma glucose returned to normal levels in both groups of animals. A similar

response was also observed in female mice (data not shown). RPTPσ (-/-) mice appear to respond normally to glucose loading, suggesting that endogenous insulin secretion is adequate.

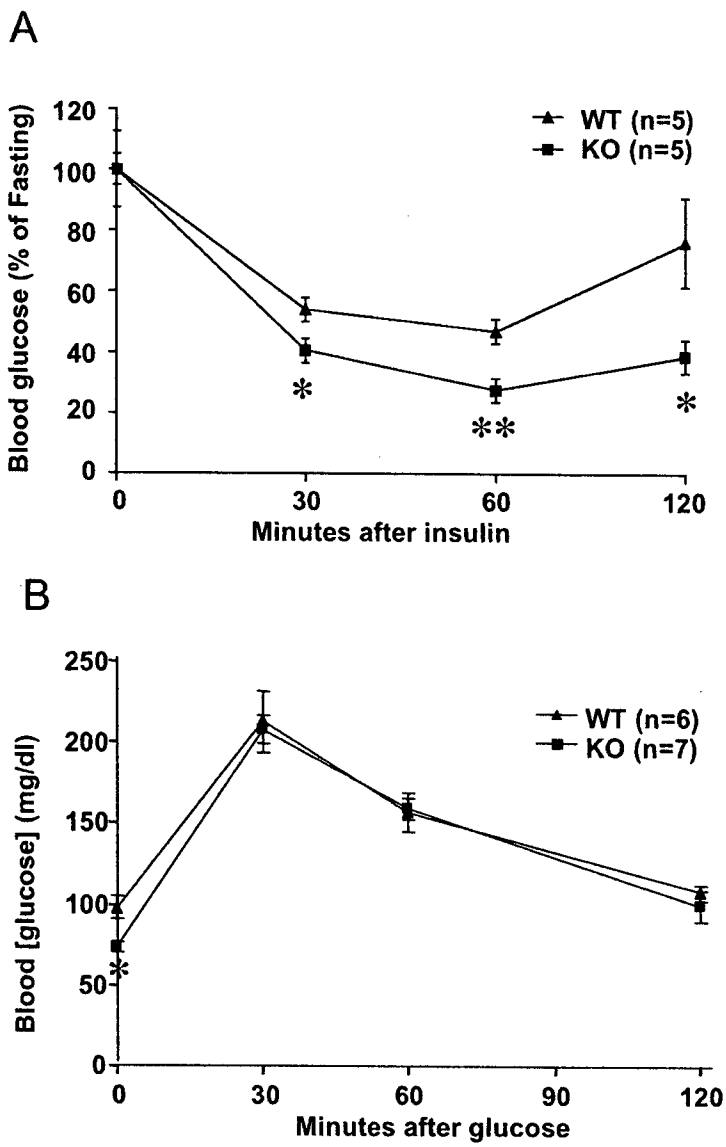

Figure 2.1 Insulin and Glucose tolerance tests in WT and RPTPσ (-/-) mice.

A) Insulin tolerance tests were performed following a six hour fast by intraperitoneal injection of 0.75 U/kg of insulin to WT (triangles, n=5) and RPTPσ (-/-) (squares, n=5) male mice. B) Glucose tolerance tests were performed after an overnight fast by intraperitoneal injection of 1 g/kg of glucose to WT (triangles, n=6) and RPTPσ (-/-) (squares, n=7) male mice. Values represent the mean ± SEM. * $P < 0.05$, ** $P < 0.01$.

2.4.4 Signaling events following *in vivo* insulin stimulation

To gain insight into the molecular mechanisms underlying the increase insulin sensitivity observed in RPTPσ (-/-) mice, the early signaling events following insulin stimulation were studied. To determine if the greater sensitivity to insulin was associated with an increase in the IR signal transduction cascade, we measured the muscle IR phosphorylation in response to a bolus of insulin (10 mU/g) injected into the hepatic portal vein. Protein extracts from muscles were first analyzed by a combination of immunoprecipitation and western blotting using anti-IR β-subunit and anti-phosphotyrosine monoclonal antibodies. Insulin stimulation in the muscle of WT animals caused a marked increase in the tyrosine phosphorylation of the IR reaching a further increase at five minutes post-injection (Figure 2.2A). In comparison to WT, the muscle IR β-subunit phosphorylation of RPTPσ (-/-) mice was decreased by 30-40% at one minute post injection (Figure 2.2B). Thereafter, the IR β-subunit phosphorylation increased to reach levels equivalent to the WT response. A greater decrease in IR phosphorylation in response to insulin was observed in the RPTPσ-deficient mice when a phospho-specific antibody against the IR β-subunit was used (Figure 2.2C). This antibody recognizes the IR that is phosphorylated at tyrosine residues 1162/1163, important sites for the activation of the IR. No significant difference in the level of expression of the IR was observed.

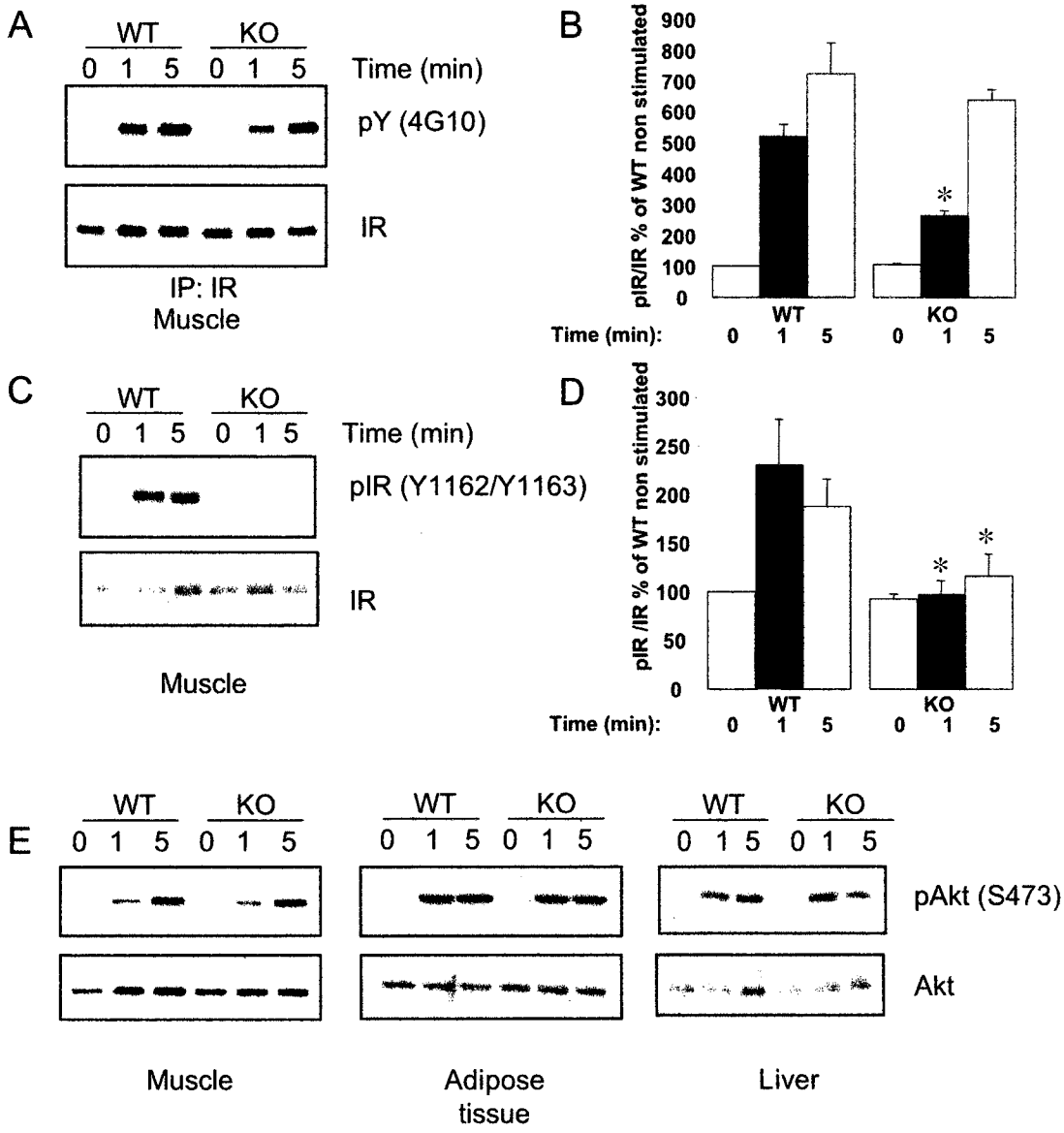

Figure 2.2 Early signaling events following *in vivo* insulin stimulation in WT and RPTPσ (-/-) male mice.

Mice were fasted overnight, anesthetized, and 10mU / kg of insulin was injected into the hepatic portal vein. Muscle tissue was removed at the indicated time following insulin injection or control PBS injection (time = 0). A) The β-subunit of IR was immunoprecipitated as described under Materials and Methods and immunoblotted with anti-phosphotyrosine (4G10) antibody (upper panel) and reprobed anti-IR (lower panel). B) Quantification of panel A using the NIH Image software. The

results are representative of at least three mice in each group. Results are expressed as percentages of WT, non stimulated. Values represent the mean ± SEM. * $P < 0.05$. C) Total muscle protein extracts were immunoblotted using polyclonal antibody against Tyr 1162/1163-phosphorylated IR (upper panel). D) Quantification of panel C using the NIH Image software. The results are representative of at least three mice in each group. Results are expressed as percentages of WT, non stimulated. Values represent the mean ± SEM. * $P < 0.05$. E) Total muscle, adipose tissue, or liver protein extracts were immunoblotted using polyclonal antibody against Ser 473-phosphorylated Akt (upper panel). Membranes were reprobed with anti-IR (lower panel) or anti-Akt (C, lower panel). Results are representative of 4-6 mice per groups.

Downstream of the insulin receptor, Akt kinase is an important mediator of the metabolic effects of insulin. We analyzed Akt serine (473)-phosphorylation in response to the same bolus of insulin in both groups of animals. The levels of expression of Akt, and the increase in its serine (473)-phosphorylation in response to insulin were similar in RPTPσ (-/-) and WT control mice (Figure 2.2E) for all three insulin responsive tissues, namely muscle, adipose tissue and liver.

Since PI3-kinase plays a central role in Akt activation in response to insulin stimulation, we attempted to clarify the discrepancy in the kinetics of IR phosphorylation and Akt phosphorylation that we observed. Therefore, phosphotyrosine-associated PI3-kinase activity was assessed in muscles from WT and RPTPσ (-/-) animals before and after insulin treatment. We first measured the level of expression of IRS1 and p85, the regulatory subunit of PI 3-kinase and observed no change in muscle of RPTPσ (-/-) mice (Figure 2.3A). Tyrosine-phosphorylated proteins were immunoprecipitated and used to *in vitro* phosphorylate phosphatidyl inositol and the production of phosphorylated lipids was measured. Under fasting conditions, both WT and RPTPσ (-/-) mice had very low levels of PI 3-kinase activity (Figure 2.3B), as measured by the low amounts of phosphorylated lipids. Upon insulin stimulation, muscle from WT mice had a marked increase in PI 3-kinase activity that reached its maximum at one minute post-injection (Figure 2.3C). In contrast, insulin injection did not produce the early

PI 3-kinase activation peak in muscle from mice lacking RPTPσ (Figure 2.3B, lane 5). In these mice, insulin injection produced a blunted initial activation of PI3-kinase with PI3P formation equivalent to about 65% of the WT initial peak. The reduced PI 3-kinase activation is consistent with the reduced IR phosphorylation observed in RPTPσ (-/-) mice.

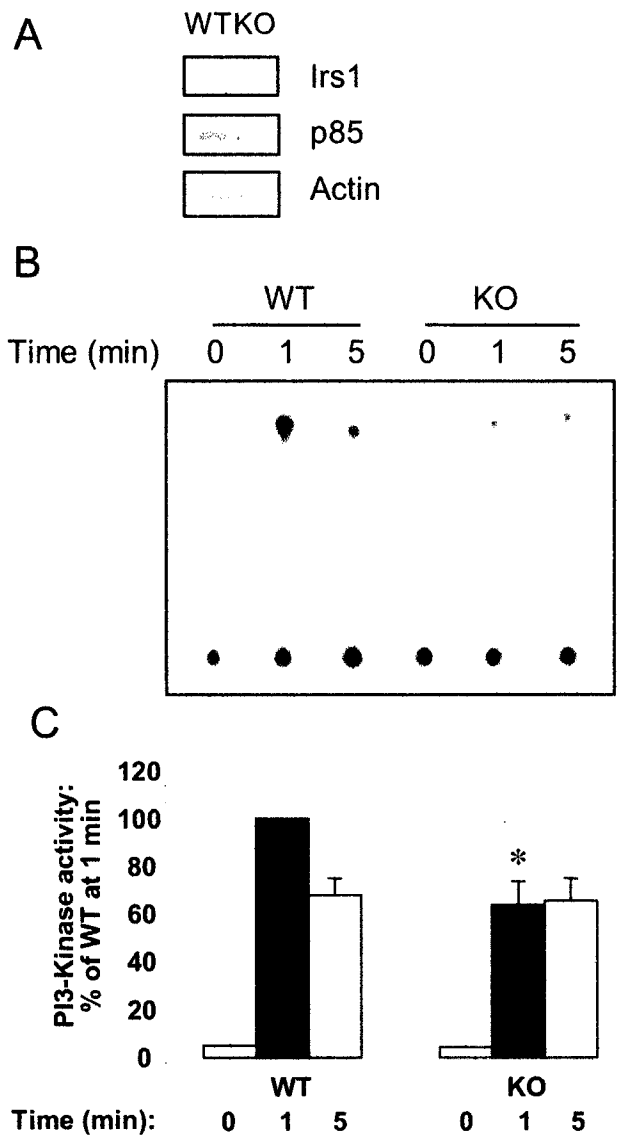

Figure 2.3 Insulin induces blunted PI3-kinase activation in muscle of RPTPσ (-/-) male mice.

A) Total muscle protein extracts were immunoblotted using anti-IRS1 (upper panel), anti-p85 (middle panel), and anti-Actin (bottom panel). B) P13-kinase activity was assessed after a bolus injection of 10 mU/ kg of insulin. Tyrosine-phosphorylated proteins were immunoprepipitated using anti-phophotyrosine antibody (PY-20) from 1 mg of muscle lysates. The precipitates were assayed for PI3-kinase activities.

The 32P-labeled lipid products were separated by TLC. B) The radioactivity in PI3P spots was quantified by Phosphor Imager. The results are representative of at least three mice in each group. Results are expressed as percentages of control at one minute post insulin injection. Values represent the mean ± SEM. * $P < 0.05$.

2.4.5 Normal biological response to *ex vivo* insulin stimulation of muscles

In response to insulin, skeletal muscle is the primary site of glucose disposal. In order to examine the insulin response of muscle independently of the systemic neuronal and metabolic controls in RPTPσ (-/-) mice, we measured the glucose transport in isolated EDL and soleus muscles following insulin treatment. Insulin stimulated glucose transport was measured by determining the uptake of the glucose analogue 2-[3H]-deoxy-glucose in those tissues. Increasing the insulin concentration in the media led to a 1.5 to 2-fold increase in glucose transport in EDL (Figure 2.4A) and soleus muscles (Figure 2.4B) of both WT and RPTPσ (-/-) mice. Even though the glucose transport in response to a maximal dose of insulin tends to be higher in tissues lacking RPTPσ, this was not statistically significant. Thus, as observed for Akt, insulin action on glucose uptake is preserved, but not augmented in muscles isolated from RPTPσ (-/-) mice and stimulated *ex vivo*.

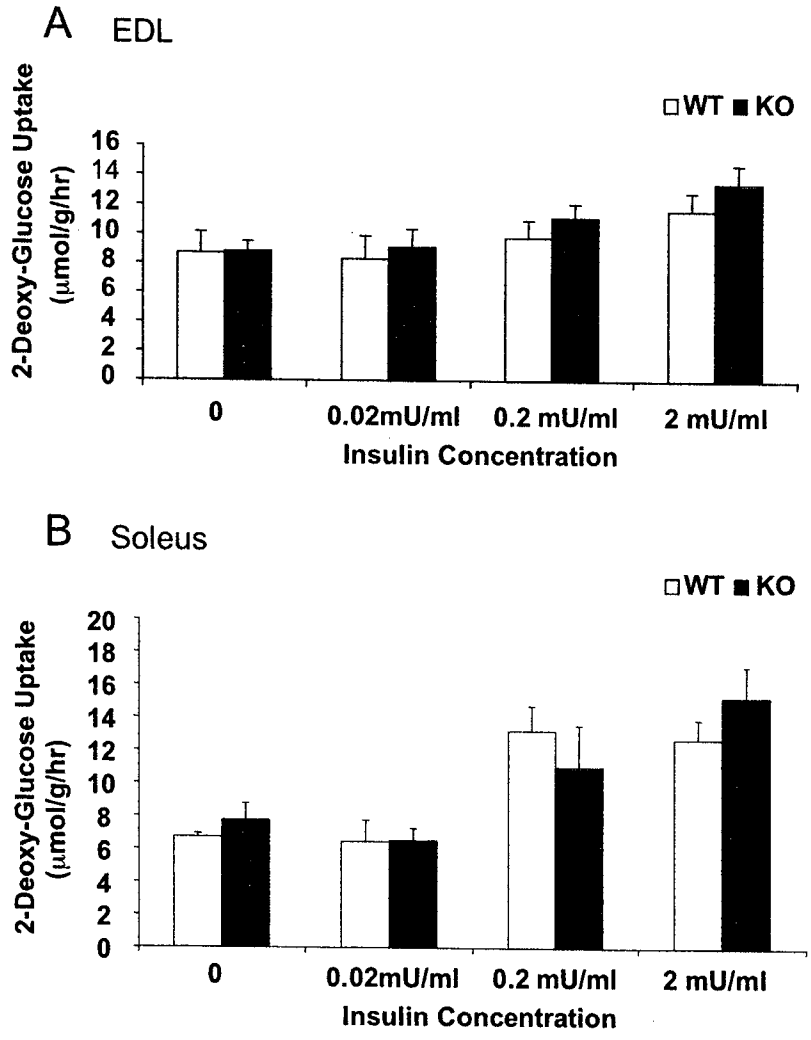

Figure 2.4 Normal response to *ex vivo* insulin stimulation in EDL and soleus muscles of RPTPσ (-/-) male mice.

Isolated EDL (A) or soleus (B) muscles were incubated with the indicated amounts of insulin. Glucose transport was measured using the glucose analogue 2-[3H]-2-deoxy-D-glucose. Results represent the mean ± SEM of four to six different muscles in each group.

2.4.6 Reduced serum IGF-1 concentration in RPTPσ (-/-) mice

Since RPTPσ (-/-) mice displayed a neuroendocrine dysplasia (Elchebly et al., 1999a; Batt et al., 2002), in particular anatomic and histologic abnormalities of the pituitary, we hypothesized that pituitary associated functional defects might explain the higher insulin sensitivity that we observed in these mice. Plasma T4 and corticosterone levels in both males and females were not different in the RPTPσ (-/-) and WT mice (data not shown).

In all mammals, GH is released episodically (pulsatile secretion). In an attempt to avoid individual variations related to this process of secretion, and because IGF-1 expression and secretion from the liver are directly related to the plasma GH level, we determined the plasma levels of IGF-1 rather than GH in our mice. IGF-1 concentrations were decreased in both RPTPσ (-/-) males and females by 25% ($P < 0.02$) and 40% ($P < 0.01$), respectively (Figure 2.5). These findings suggest an involvement of the GH-IGF-1 axis in the glucose homeostasis abnormalities observed in RPTPσ (-/-) mice.

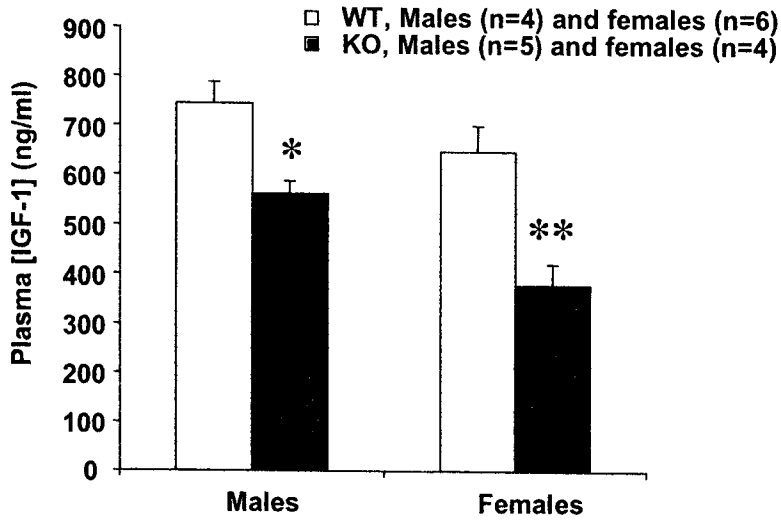

Figure 2.5 Serum IGF-1 levels are reduced in RPTPσ (-/-) male and female mice.

IGF-1 serum concentrations were measured by radioimmunoassay. Results are expressed as mean ± SEM. Four to six male and female mice were used in each group of WT (light bars) or RPTPσ (-/-) (dark bars). * $P < 0.02$, ** $P < 0.01$.

2.4.7 Injection of rGH into RPTPσ (-/-) mice normalizes their response to exogenous insulin

In order to determine if the enhanced sensitivity to exogenous insulin seen in RPTPσ (-/-) mice was secondary to their low levels of circulating GH/IGF-1, we injected these mice with rGH (0.5μg/g) twice daily for 6 days. Control mice were injected with an equivalent volume of PBS. On the seventh day we performed insulin tolerance tests on the different groups of mice. As described above the RPTPσ (-/-) mice injected with PBS were more sensitive to exogenous insulin compared to the WT mice injected with PBS (Figure 2.6). However the RPTPσ (-/-) that were treated with the rGH were not as sensitive to insulin and had a response similar to the WT, which is clearly significant after 2 hours. Thus injection of rGH rescued the glucose homeostasis phenotype observed in RPTPσ (-/-) mice. It is important to note that the amount of rGH used in our study did not have any detectable effects on the WT group of mice in comparison to the PBS-injected WT mice (Figure 2.6). These results confirm our hypothesis that the glucose homeostasis abnormalities observed in these mice is secondary to their reduced serum levels of GH/IGF-1.

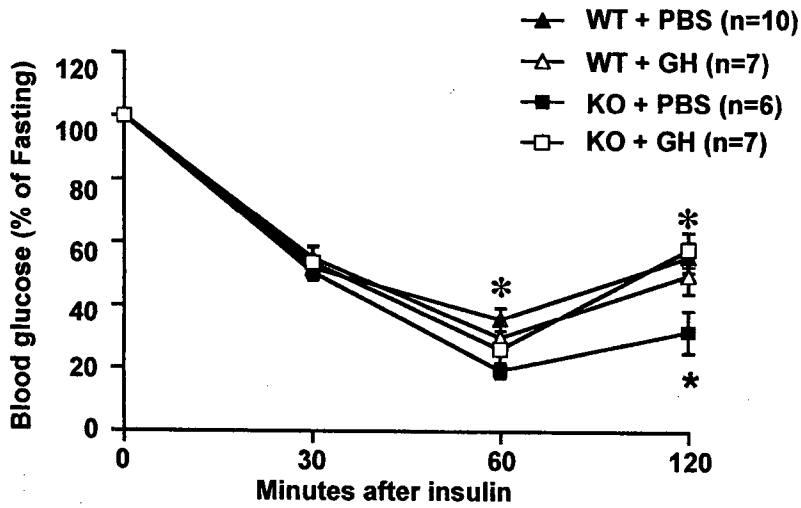

Figure 2.6 Insulin tolerance tests in WT and RPTPσ (-/-) mice injected with rGH.

WT and RPTPσ (-/-) mice were injected subcutaneously with 0.5μg/g of rGH, or an equivalent volume of PBS for 6 days twice daily. Insulin tolerance tests were performed on the seventh day following a six hour fast by intraperitoneal injection of 0.75 U/kg of insulin to WT+PBS (black triangles, n=10), WT +rGH (white triangles, n=7), RPTPσ (-/-) +PBS (black squares, n=7), and RPTPσ (-/-) + rGH (white squares, n=6) male mice. Values represent the mean ± SEM. * $P < 0.05$ (WT + PBS vs RPTPσ (-/-) + PBS), † $P < 0.05$ (RPTPσ (-/-) + PBS vs RPTPσ (-/-) + rGH).

2.5 Discussion

In mammals, insulin is the most potent anabolic hormone responsible for the maintenance of plasma glucose homeostasis. The metabolic effects of insulin occur by inhibiting the hepatic glucose output and promoting glucose uptake into muscle and adipose tissues. At the molecular level, the metabolic effects of insulin are mediated by enhancing IR tyrosine kinase activity, which subsequently leads to activation of the IRS/PI3K/Akt cascade.

In humans, resistance to insulin action in muscle is considered the main pathophysiological process involved in the development of non-insulin dependent diabetes (type 2) and obesity, however, the molecular mechanism leading to the development of insulin resistant state is still poorly understood. Previous work, using gene targeting and gene over-expression approaches, has led to the discovery that PTP1B (Elchebly et al., 1999b; Klaman et al., 2000; Zabolotny et al., 2004) and LAR (Ren et al., 1998; Zabolotny et al., 2001) are major players involved in insulin signaling pathways. PTPs, expressed in insulin-sensitive tissues have thus emerged as prominent negative regulators of insulin action (Cheng et al., 2002; Asante-Appiah and Kennedy, 2003). Hence, the identification of these and other PTPs will provide invaluable insight towards understanding the molecular basis of human insulin resistance development, and will reveal new targets for the treatment of obesity and type 2 diabetes-associated insulin resistance.

Among the PTPs that might be involved in glucose homeostasis, RPTPσ is clearly a potential candidate as it is expressed in insulin-sensitive tissues (Norris et al., 1997), however, its physiological role in insulin signaling and glucose homeostasis had not been previously investigated. The aim of the present study was to determine whether adult mice carrying a homozygous mutation in the RPTPσ gene, exhibited abnormalities in glucose homeostasis. Our previous studies have shown that mice lacking RPTPσ are smaller and display neuroendocrine dysplasia (Elchebly et al., 1999a). We now demonstrate that RPTPσ (-/-) mice have a reduced mass of adipose tissue, consistent with increased insulin sensitivity. Increased whole-body insulin sensitivity was confirmed with exogenous insulin stimulation. Thus, these findings suggest a mechanism whereby an altered metabolic environment produced by RPTPσ deletion can affect glucose homeostasis and whole-body insulin sensitivity.

To determine how the absence of RPTPσ affects glucose homeostasis, we investigated the proximal elements of the insulin signaling pathways. If RPTPσ acts on this signal transduction cascade, its absence should induce an enhancement and/or prolongation of the time course of IR phosphorylation, as we have previously observed in PTP1B (-/-) mice (Elchebly et al., 1999b). Yet, despite a higher insulin sensitivity observed in fasted states and after exogenous insulin injection, our results demonstrate that RPTPσ deletion does not produce an enhancement of the muscle IR-phosphorylation levels. In contrast, we found that deletion of RPTPσ is

associated with reduced IR-tyrosine phosphorylation, and blunted PI 3-kinase activation in response to insulin. This blunted level of PI 3-kinase activation was nevertheless sufficient to fully activate Akt. The mechanism by which this occurs, remains unclear, but this may represent an important safeguard mechanism to prevent hypoglycemia beyond a critical level. Therefore, although the RPTPσ (-/-) mice are insulin sensitive, a direct role for this PTP in the proximal modulation of the IR signaling cascade was not observed in either of the peripheral insulin responsive tissues.

Consistent with the results obtained at the molecular level, muscles isolated from RPTPσ (-/-) animals had a normal rate of glucose transport in response to insulin. Therefore, our findings confirm that RPTPσ is not a physiological PTP that negatively modulates the IR signal transduction cascade in muscles. Rather, they suggest an indirect role for this PTP on IR signaling, whereby it exerts a physiological control on whole animal glucose homeostasis.

Glucose homeostasis is a complex process in which all organisms provide appropriate responses to changes in the cell-external environment to maintain relatively constant concentration of blood glucose (Haymond and Sunehag, 1999; Gerich, 2000). This complex mechanism reflects a constant balance between insulin action and the action of several counter-regulatory hormones. Since these hormones

are released in response to acute hypoglycemia triggered via glucose sensors situated in both the hypothalamus and the porto-hepatic area, we suggest that the apparent enhanced insulin sensitivity that we observed in RPTPσ (-/-) mice could be a secondary effect of the GH deficiency of these mice (Elchebly et al., 1999a; Wallace et al., 1999; Batt et al., 2002). We examined several pituitary axes that may play a counter-regulatory role in glucose homeostasis and that may be affected by a RPTPσ loss-of-function. Of these axes, GH/IGF-1 is a particularly important one to investigate, since the insulin antagonistic effects of GH are very well documented (Dominici and Turyn, 2002).

Our results demonstrated that RPTPσ (-/-) mice had significantly lower plasma IGF-1 concentration. These results agree with the recent findings showing that RPTPσ (-/-) neonates had a marked reduction in circulating GH (Batt et al., 2002). To test whether the GH deficit was responsible for the increased insulin response in RPTPσ (-/-) mice we injected these mice with rGH. Short GH therapy normalized the response of these mice to exogenous insulin as measured by insulin tolerance test. Although the amount injected did not change the tolerance to insulin in the WT group. We can then conclude that the phenotypic alterations in glucose homeostasis observed in these RPTPσ (-/-) mice is caused mainly by their GH deficit.

Other models of mice having reduced levels of GH secretion such as the Ames dwarf mice, or impaired GH signaling as in the Laron mice (GHR (-/-)) show similar increased whole body insulin sensitivities (Dominici et al., 2002; Liu et al., 2004). Furthermore, despite the increase insulin sensitivity, the Ames dwarf mice also have decreased insulin responses in skeletal muscles, as observed in the present study. However, in contrast to RPTPσ (-/-) mice where no detectable changes are observed at the level of Akt phosphorylation, the IR signaling in the liver of the dwarf mice is augmented upon insulin stimulation. The effects seen in RPTPσ (-/-) mice are probably less dramatic since they only have a moderate reduction in GH and IGF-1 production as opposed to the dwarf mice that totally lack these growth factors (Dominici et al., 2002). In humans, GH administration causes hyperinsulinaemia and impairs the ability of insulin to stimulate glucose uptake and to suppress hepatic glucose production. GH also enhances fat utilization by stimulating lipolysis and fat oxidation. These metabolic effects engender increased adiposity in GH deficiency and a reduction in the fat mass associated with acromegaly. In contrast, we observed that a very small number of RPTPσ (-/-) animals show increased adiposity, but on average have a significantly lower mass of adipose tissue as reported in the present paper. GH deficiency certainly accounts for the increased insulin sensitivity observed in RPTPσ (-/-) mice, but we cannot exclude the possibility that other counter-regulatory hormones might also play a role in this phenomenon. In addition, we observed that RPTPσ (-/-) mice have a higher

metabolic rate (data not shown), which correlates with their lean phenotype. It will be interesting to determine by the generation of mice carrying tissue-specific gene deletions, if RPTPσ is involved in the central regulation of whole body homeostasis.

In conclusion, our *in vivo* data indicate that RPTPσ plays an indirect role in the regulation of whole-body insulin sensitivity and glucose homeostasis. Our findings support the conclusion that RPTPσ deletion does not directly alter insulin signaling in insulin sensitive tissues. Since the absence of this enzyme can alter glucose homeostasis and enhances insulin sensitivity it is possible that inhibitors of RPTPσ might become important therapeutic tools for the treatment of insulin resistance associated, or not, with GH over-secretion. Further analysis of this animal model will provide valuable insights into the molecular mechanisms controlling the proper development of the hypothalamo-pituitary axis. Finally, identification of either cellular substrates or ligands for RPTPσ will be crucial steps towards our understanding of the signaling transduction cascade controlled by this PTP and its primary physiological role.

2.6 Acknowledgements

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CHAPTER 3: The corticospinal tract regenerates after spinal cord injury in receptor protein tyrosine phosphatase sigma (RPTPσ) knockout mice

3.1 Abstract

Myelin-associated growth inhibitory proteins and components of the glial scar such as chondroitin sulfate proteoglycans (CSPGs) are known to inhibit axonal regeneration in the central nervous system (CNS). Damage to the spinal cord is therefore irreversible. Here we provide evidence of robust, long distance regeneration of corticospinal tract axons after spinal hemisection in RPTPσ (-/-) mice. In comparison, wild-type control littermates show no regeneration after identical spinal injuries. In addition to the regenerative ability of RPTPσ (-/-) corticospinal axons, primary cortical neurons isolated from RPTPσ (-/-) P9 mice showed a significantly greater attachment to CSPG inhibitory substrate than wild-type control neurons, while no difference in attachment between the two groups was observed on myelin substrate. RPTPσ may therefore be a new class of molecules with an important role in the regenerative capacity of the CNS.

3.2 Introduction

Neuronal axons in the adult mammalian central nervous system (CNS) have a limited capacity to regenerate after injury. These neurons remain permanently damaged and are unable to recapitulate the developmental process of axonal extension as well as connection with appropriate neurological targets. The resulting loss of motor and sensory function is highly debilitating, particularly after spinal cord injuries. Scientific endeavor has thus long been compelled to identify and counteract the molecules responsible for inhibiting successful spontaneous CNS axon regrowth and long distance regeneration. To date, the axon growth-inhibitory roles of proteins associated with degenerating myelin (Nogo, myelin-associated glycoprotein, oligodendrocyte myelin protein) and the glial scar (chondroitin sulphate proteoglycans; CSPGs) at the injury site are well characterized (David and Lacroix, 2003; Filbin, 2003; Silver and Miller, 2004; Gonzenbach and Schwab, 2008). Multiple studies have been designed to overcome these inhibitory elements and promote neuronal regeneration after spinal cord injury. Neutralizing therapies included autoimmunization of mice with myelin homogenate (Huang et al., 1999), application of antibodies against Nogo-A (Schnell and Schwab, 1990; Brosamle et al., 2000) and usage of antagonist peptide to Nogo-66 receptor (NEP-1) to the injury site directly or systemically (GrandPre et al., 2002). While these therapies that target the known inhibitory proteins have been shown to improve post-injury conditions and can produce extensive regeneration with functional recovery, their effects are of a

limited nature. It is clear that other additional regeneration-inhibitory elements are present in the CNS.

Members of the type IIa subfamily of receptor protein tyrosine phosphatases (RPTPs) play an essential role in appropriate axonal extension and guidance during neural network development (Johnson and Van Vactor, 2003; Stepanek et al., 2005; Uetani et al., 2006). RPTPσ is one of three Type IIa RPTP vertebrate members highly abundant in both the developing and mature mammalian nervous system. It can function as an intrinsic "brake" to axonal extension in *in vitro* neuronal cultures (Ledig et al., 1999a; Thompson et al., 2003). Given this important developmental "growth arresting" role of RPTPσ, this enzyme may also have an inhibitory influence on the ability of axons to regenerate after injury. We hypothesize that RPTPσ could be a potential target for improving the long distance regeneration capacity of the CNS. In support of this idea, a number of previous studies with RPTPσ deficient (-/-) mice have reported enhanced regeneration after injury to certain axonal pathways in both the peripheral nervous system (PNS) and CNS as compared to wild type counterparts.

McClean et al. (McLean et al., 2002) reported that sciatic nerve crush and transection injuries sustained in RPTPσ (-/-) mice produce enhanced and more rapid regeneration of peripheral nerve fibers than in wild-type counterparts.

Moreover, Thompson et al. (Thompson et al., 2003) demonstrated that the crushed facial nerve in mice has an accelerated return of function in the absence of RPTPσ. Additionally within the CNS, the retinocollicular system of adult RPTPσ (-/-) mice showed significantly more regenerative axonal outgrowth after optic nerve crush as compared to wild-type controls (Sapieha et al., 2005). The growth inhibitory effect of RPTPσ on neurons is thought to be an intrinsic mechanism as cortical neurons from E15 RPTPσ (-/-) mice are able to extend longer neurites than wild-type cells over the same 36-hour period (Thompson et al., 2003). Together, these results suggest that RPTPσ may also have a potential axon growth-inhibitory role in more complex CNS regions such as the spinal cord.

Here we investigate the ability of corticospinal axons to regenerate after dorsal spinal hemisection injuries in adult RPTPσ (-/-) mice and provide evidence of long distance regeneration through the lesion site. We therefore propose that RPTPσ contributes to the limited regeneration nature of the spinal cord and is a potential target for improving the regenerative capacity of the CNS.

3.3 Material and methods

3.3.1 Animals

RPTPσ (-/-) and wild-type mice were generated from a Balb/c background as previously described (Elchebly et al., 1999a). All surgeries were performed on 8-10

week old female mice with the approval of the McGill University Animal Care committee and following Canadian Council on Animal Care guidelines. The mice were genotyped by PCR analysis on tissue lysates using allele specific primers as described (Uetani et al., 2006).

3.3.2 *In situ* hybridization

Animals were anesthetized and perfused transcardially with PBS and 4% paraformaldehyde for twenty minutes. Sections of the thoracic region of the spinal cord (T8-T10) and motor cortex were dissected and post-fixed in 4% paraformaldehyde overnight, followed by cryopreservation in 30% sucrose overnight. Fifteen-micrometer cryostat sections were mounted on slides. *In situ* hybridization was performed as previously described (Henrique et al., 1995; Thompson et al., 2003). Sense and antisense cRNA probes to murine RPTPσ were labeled with digoxigenin (DIG) RNA labeling mix (Roche). Hybridization was detected using an anti-DIG alkaline phosphatase-coupled antibody (Roche) and visualized with alkaline phosphatase substrate BM purple (Roche).

3.3.3 Western blotting

Tissues were dissected and snap-frozen in liquid nitrogen and then homogenized in TNN buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 50 mM NaF, and a cocktail of protease inhibitors (Complete, Roche)).

Protein concentrations were determined using BCA protein assay kit (Pierce). 20 μg (motor cortex) or 50 μg (spinal cord) of total tissue lysates were separated by SDS/PAGE on 8% gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). Membranes were then incubated with the following antibodies: RPTPσ (17G7.2 (Thompson et al., 2003)), p190 (Transduction Laboratories), Erk 1/2 pT202/pY204, Erk 1/2, p38 pT180/pY182, p38, caspase-3, STAT3 pY705, and STAT3 (Cell Signaling), tubulin (Sigma), NG2 (Chemicon).

3.3.4 Spinal cord surgeries

Mice were surgically anesthetized with ketamine: xylazine: acepromazine (50:5:1 mg/kg) and the lower thoracic spinal cord exposed at T9 by laminectomy. The corticospinal tract was injured at T9 either by a hemisection or contusion injury. The dorsal hemisection of the spinal cord was performed with spring scissors to the level of the central canal (0.5mm), which completely severed the corticospinal tract. For the contusion injury, the spinal column was stabilized with forceps holding the adjacent vertebrae and the Infinite horizons impactor device applied to the exposed dorsal spinal cord with moderate impact injury (50 kdyne, 400-500um displacement of spinal cord).

The regeneration of the corticospinal tract was assessed by cortical injection of the axonal tracer biotinylated dextran amine (BDA). Seven days after spinal

injury, mice were re-anesthetized and two 0.2 μl bilateral injections of 10% BDA were made into the sensory motor cortex with a glass pipette attached to a Hamilton syringe.

The animals were sacrificed two weeks later and transcardially perfused with 4% paraformaldehyde. The spinal cords were dissected out and placed in 30% sucrose in 0.1M phosphate buffer for 36 hours for cryoprotection. 25μm-thick sagittal sections were cut on a cryostat and incubated in 0.6% hydrogen peroxide for 3 hours. After an overnight incubation with streptavidin complex (Vector Laboratories), sections were reacted with the chromagen diaminobenzine to visualize the streptavidin-biotin-dextranamine complex and counterstained with methyl green.

3.3.5 Cerebellar neuron attachment assay

96-well plates were coated with nitrocellulose (40cm^2) dissolved in methanol (50 ml), then pre-incubated overnight with poly-L-lysine (Sigma, 5μg/ml). Chondroitin sulphate proteoglycans (Chemicon, 0.5-2.5 μg) or purified bovine CNS myelin (0.2 μg) in a 2 μl drop was placed in the centre of the wells and incubated for 4 hours at 37°C. Wells were then washed and used for the neurite outgrowth assay. Cerebellar granule neurons were purified from postnatal day 9 RPTPσ (-/-) or wild-type mice by Percoll density gradient centrifugation (Hatten, 1985). Cells were

plated in the wells at a density of 1 x 10^5 in DMEM media supplemented with 1% MEM vitamin solution and 10% fetal calf serum for 24 hours. Cultures were fixed and stained with Coomassie blue. For attachment assays, the number of neurons on four constant sample areas of the substrate droplet, and two areas of the surrounding poly-L-lysine coating were counted on an inverted microscope. To determine the turning behavior of neurites with neuronal cell bodies attached to the boundary between the CSPG substrate and poly-L-lysine, all neurons with neurites on the perimeter of the CSPG substrate droplet were counted for whether or not the neurites grew onto the substrate.

3.3.6 Histochemical staining

For Luxol Fast Blue staining to visualize myelin, sections were washed in PBS, dehydrated in increasing concentrations of ethanol (50-95%) and incubated in 0.1% Luxol Fast Blue (diluted in 95% ethanol and 10% acetic acid) overnight at 37°C. Sections were cooled to room temperature, incubated in 0.05% lithium carbonate and differentiated in 70% ethanol then washed in PBS.

3.4 Results

3.4.1 RPTPσ is endogenously expressed in the adult mouse motor cortex and spinal cord

The expression of RPTPσ protein in the CNS was analyzed by Western blot using a monoclonal antibody (17G7.2) that was raised against the intracellular subunit of RPTPσ. RPTPσ expression was observed in tissue lysates of the adult mouse RPTPσ (+/+) motor cortex and thoracic spinal cord region (T8-T10) as a 75-80 kDa immunoreactive band consistent with the correct molecular weight of the antibody target region of RPTPσ (Figure 3.1A and B, lane +/+). No RPTPσ was detected in lysates from equivalent regions in RPTPσ (-/-) mice (Figure 3.1A and B, lane -/-).

The endogenous expression of RPTPσ mRNA in adult mouse motor cortex and thoracic spinal cord was examined by *in situ* hybridization using a specific DIG-labeled RNA antisense probe. Strong positive staining was detected in presumptive neurons of layers II to VI of the motor cortex including presumptive layer V containing the pyramidal cells from which corticospinal axons extend to the spinal cord (Figure 3.1G). While previous studies have reported reduced cerebral cortical expression of RPTPσ in the postnatal brain in comparison to other structures such as the hippocampus where the levels are the highest (Wang et al., 1995), the present study shows that relatively high expression of RPTPσ is maintained in this region of the mouse cortex.

Positive DIG staining was also observed throughout the grey matter of the thoracic spinal cord in the presumptive neurons of both the dorsal and ventral horns (Figure 3.1I). While it has been previously shown that RPTPσ protein is expressed in developing cortical axons and growth cones (Thompson et al., 2003) and that RPTPσ mRNA is present in presumptive oligodendrocytes (Sapieha et al., 2005), the present study found no detectable signal in the white matter regions of the spinal cord. No staining was detected in either motor cortex or spinal cord sections incubated with control sense probe (Figure 3.1H and J).

We then investigated by Western blot analysis whether spinal cord injury induced a change in the expression of RPTPσ protein at the site of injury (T8-10) or in the motor cortex where the injured axons have their cell bodies. No change was detected in either tissue 24 hours after hemisection injury (Figures 3.1D and 3.1E). Similarly contusion injury also did not cause any changes in RPTPσ protein expression from non-injured levels at 1, 3, 7 or 21 days post injury. This is consistent with previous results which have shown no change in expression of RPTPσ after optic nerve transection (Sapieha et al., 2005) as well as facial nerve crush (Thompson et al., 2003).

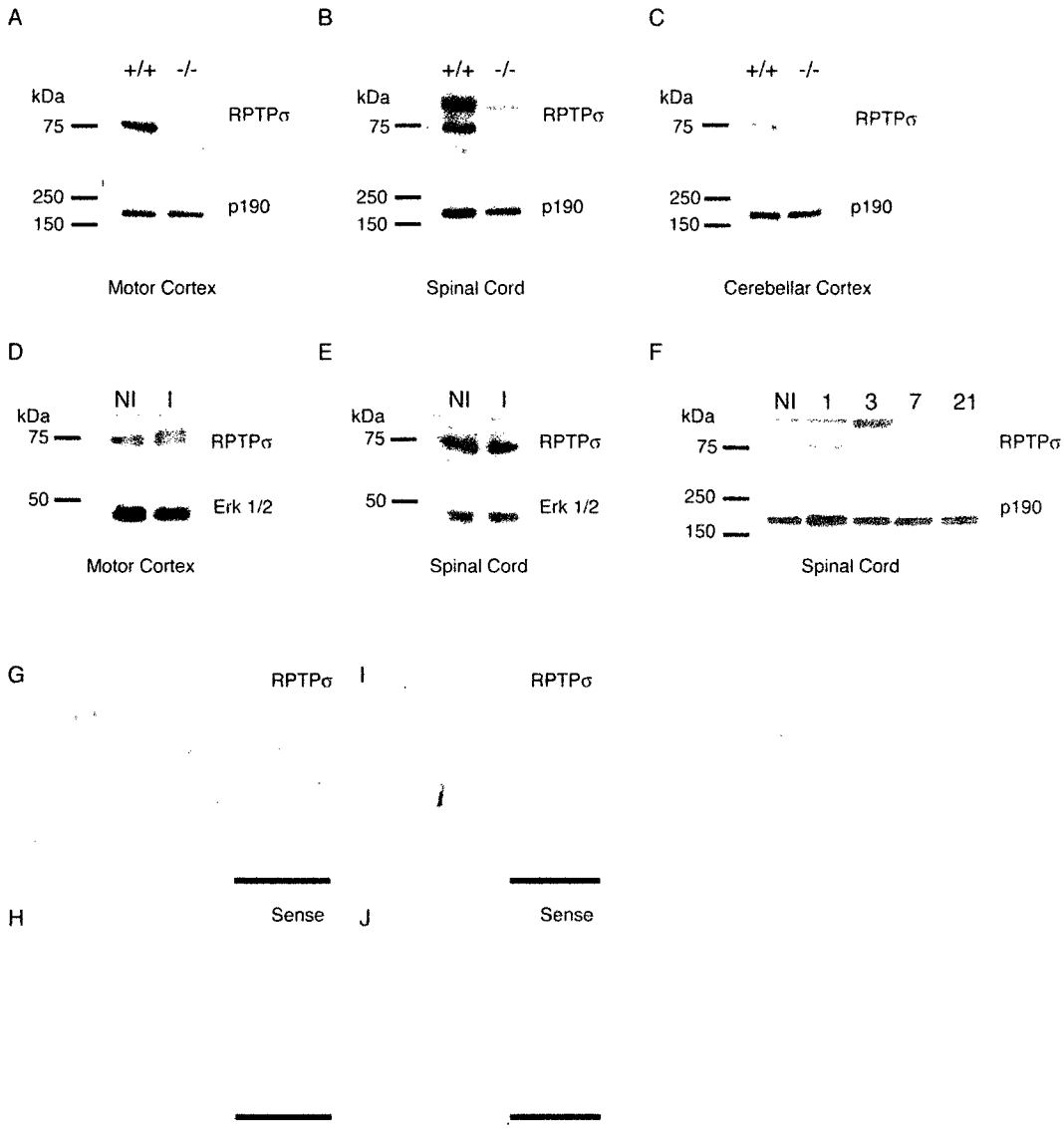


Figure 3.1 Expression of RPTPσ.

Western blot analysis for RPTPσ in tissue homogenates prepared from adult motor cortex (A) and spinal cord (B) and P9 cerebellar cortex (C). RPTPσ expression is detected in wild-type animals while being completely absent from (-/-) animals. (D and E) Twenty-four hours after hemisection (I) the expression of RPTPσ is unchanged as compared to non injured (NI) control in both motor cortex (D) and spinal cord (E). (F) The expression of RPTPσ is consistent in wild-type animals

injured by contusion even after several days. (Non injured: NI, 1 day, 3 days, 7 days, 21 days after injury). (G, H, I, J) RPTPσ mRNA expression detected by *in situ* hybridization using antisense RNA probe on coronal section of the cortex, and transverse section of the spinal cord at the thoracic level. Expression was detected in layers II to VI of the motor cortex (G) as well as in large neurons of the grey matter in the spinal cord (I). (H, J) Sense RPTPσ RNA probe was used as a negative control in the motor cortex (H) and spinal cord (J). Scale bar, 1 mm.

3.4.2 RPTPσ (-/-) mice show robust long distance regeneration of corticospinal tract axons across a dorsal hemisection lesion

Adult RPTPσ (-/-) and wild-type mice were given dorsal spinal cord hemisection at the lower thoracic level 9 to a depth of 500 μm, which effectively severed all axons above the central canal as illustrated in Figure 3.2. The completeness of the hemisection was rigorously assessed to ensure that all animals included in the study had a complete disruption of the dorsal corticospinal tract, eliminating the possibility that any fibers observed distal to the lesion were spared axons from the dorsal funiculus pathway. The criteria for a complete hemisection lesion were: damage to neurons and gliosis above the central canal, disruption of the central canal, and the presence of heme from red blood cells. Samples that did not satisfy each of these criteria were discarded. One week after hemisection, mice were given cortical injections of BDA to assess regeneration of the corticospinal tract. Specifically, the length and number of BDA labeled axons and sprouts distal to the lesion were measured and a semi-quantitative score of regeneration robustness (0 to 4 with 4 being the maximum) was assigned to each spinal cord.

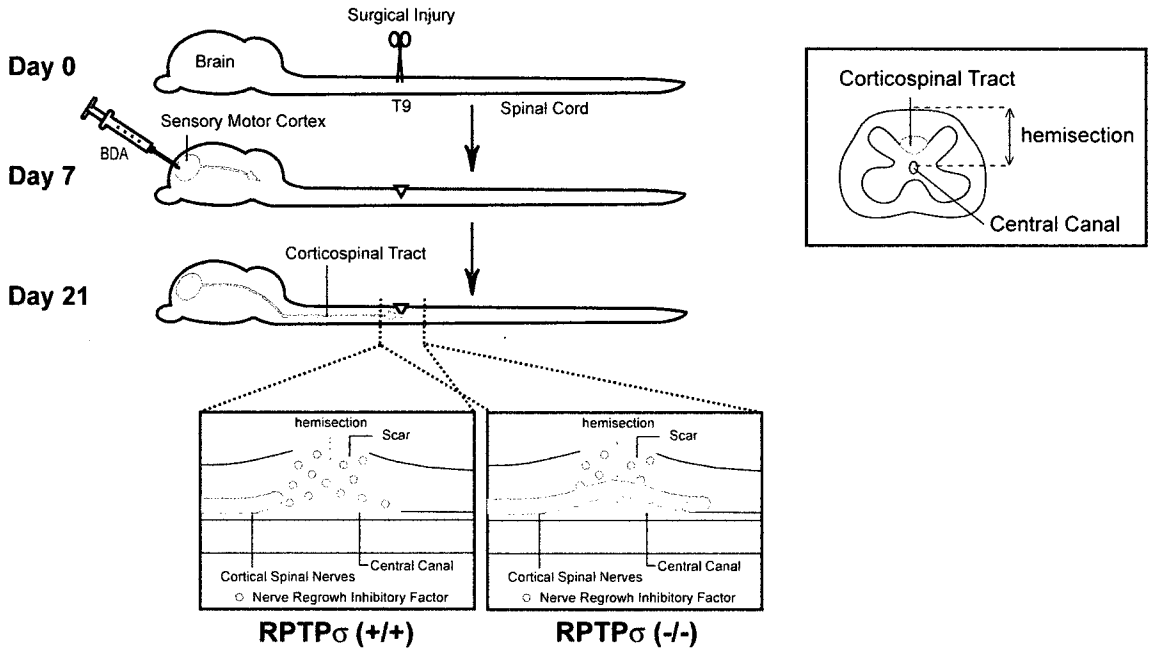


Figure 3.2 Model of spinal cord hemisection.

Schematic model of the spinal cord injury done by dorsal hemisection to the depth of the central canal. This type of lesion completely severs the corticospinal tract. Regenerating axons are detected by BDA anterograde labeling injected into the cortex seven days after injury.

A large proportion of the RPTPσ (-/-) mice (about 85 %; n=9) showed a remarkable degree of robust long distance corticospinal axon regeneration with many BDA labeled corticospinal axons crossing the lesion site (Figure 3.3A-E). Fibers consistently reached 6-12 mm distal to the injury site and grew through both the grey and white matter (Figure 3.3D-E). Labeled axons successfully penetrated through the centre of the lesion site (Figure 3.3B) and were often observed growing around blood vessels in the newly vascularized region (Figure 3.3C). The fibers branched and sprouted in a manner typical of regenerating fibers, emerging through the dorsal grey matter in the first 1mm distal to the hemisection (Figure 3.3B). Further distal from the injury site, these fibers extended through the dorsal white and grey matter above the central canal (Figure 3.3D) often growing in the white/grey matter interface. In other cases the axons penetrated the grey matter above the central canal with numerous sprouts branching ventrally (Figure 3.3E).

In marked contrast wild-type mice (n=6) showed virtually no regeneration beyond the beginning of the lesion site with labeled axons arresting as terminal bulbs at the point of axotomy (Figure 3.3F). Two cases had several fibers extending 100-200um into the proximal damaged lesion area but did not reach the midline of the lesion.

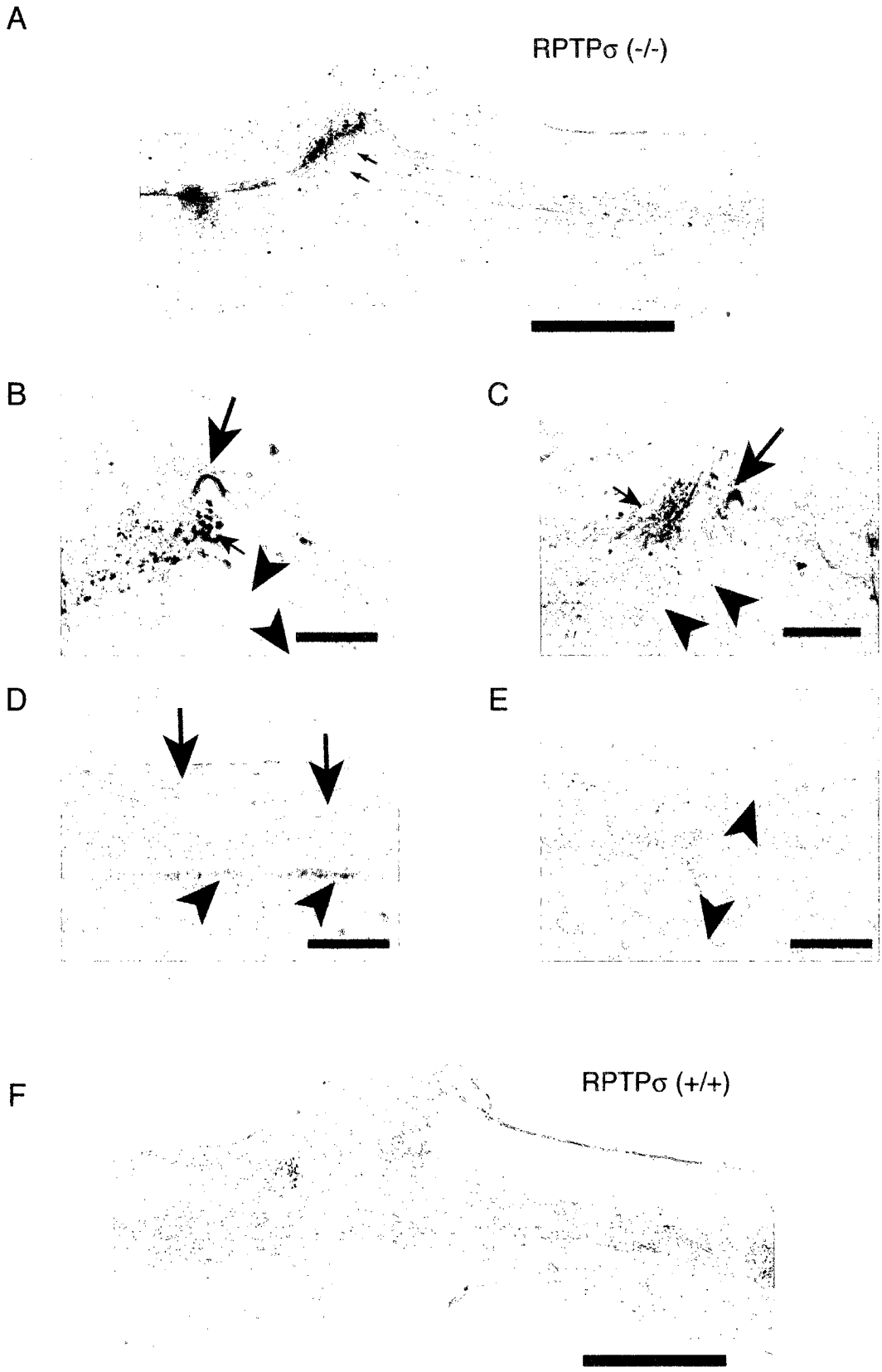

A
RPTPσ (-/-)
B
C
D
E
F
RPTPσ (+/+)

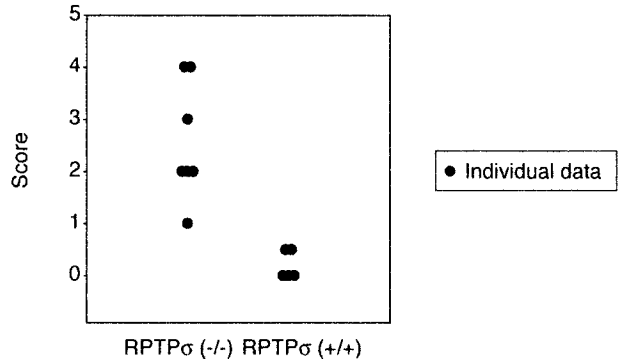
Figure 3.3 Dorsal hemisection in RPTPσ (-/-) and wild-type mice.

(A) Montage showing BDA labeled axons of the corticospinal tract proximal to the lesion (to the left side) in RPTPσ (-/-) mice. Some of these fibers pass through the lesion (arrow) and extend distally for long distances (arrowheads). Damage to the central canal is visible (small arrows). BDA labeled fibers in this and adjacent sections are shown at higher magnification below. Scale bar, 1mm. (B, C): Higher magnification micrograph of the injury site from A (B, Scale bar 100μm) and adjacent section (C, scale bar, 200 μm). Some of the axons end in terminal bulbs (small arrows), while others make a loop around a blood vessel to grow distally through the lesion (arrows). Axon sprouts can also be seen ventral to the tract (arrowheads). (D, E) BDA labeled axons extending through the white matter /grey matter interface (arrows) and the grey matter around the central canal (arrowheads) at 2mm (D, scale bar, 100μm). Axons appear like terminal spouts (arrowheads) in grey matter 6 mm distal to the lesion (E, scale bar 100μm). (F) Montage showing BDA labeled axons of the corticospinal tract proximal to the lesion (to the left side) in wild-type mice but none reaching the lesion site (arrow).

Highly robust axon growth and sprouting was observed in 40% of the RPTPσ (-/-) mice with scores of 3-4 on the semi-quantitative scale (Figure 3.4A) with the majority of animals growing labeled axons a maximum distance above 6mm in both white and grey matter (Figure 3.4B and C). Of these robustly regenerating animals, up to 100 axons and sprouts were observed in each millimeter segment in the distal spinal cord (Figure 3.4D). The mean number of distal fibers for all cases combined being approximately 30 from 0-5 mm distal to the lesion, then dropping to 20 and 13 fibers at 6 mm and 7 mm respectively. While the number of fibers growing further caudal than 6 mm were lower, 66% of the RPTPσ (-/-) mice had at least several fibers beyond this point, some reaching even up to 12 mm in both the white and grey matter (Figures 3.4B and C).

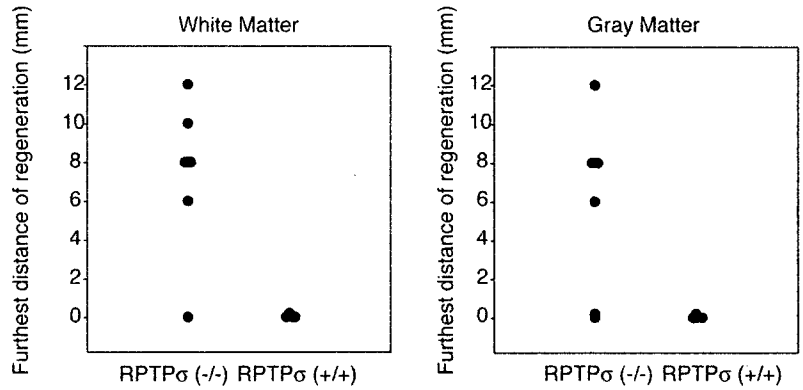
A

Regeneration score after hemisection



B

Maximum distance of regenerating fibres



C

Axons distal to hemisection in RPTPσ (-/-)

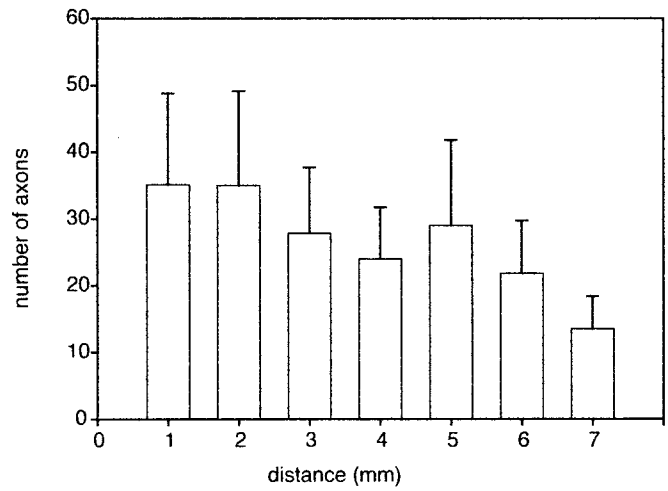


Figure 3.4 Quantification of the regeneration after dorsal hemisection.

(A) Semi-quantitative assessment of the robustness of the regeneration rated on a scale of 0-4, with 4 being the highest and 0 representing no regeneration. (B) Maximum distance to which regenerating fibers extended in the white or gray matter distal to the hemisection lesion. Fibers were consistently seen beyond 6 mm in RPTPσ (-/-) animals while their wild-type counterparts did not extend pass 200μm distal to the lesion. (C) The number of fibers caudal to the injury sites was quantified in RPTPσ (-/-) animals.

3.4.3 RPTPσ (-/-) mice show normal myelin density in the spinal cord

Relative hypomyelination during development in the peripheral nerve of RPTPσ (-/-) mice bred from a C57Bl/6 background has been previously described. This condition matures to larger fiber diameter and myelin thickening in the PNS of adult animals (Wallace et al., 1999). To determine whether the spinal cord of RPTPσ (-/-) mice on a Balb/c background, also exhibited abnormal myelination of fibers, longitudinal spinal cord section from naive RPTPσ (-/-) and wild-type mice were stained with Luxol Fast Blue for the identification of myelin profiles. No difference in myelin density was observed between the two groups (Figure 3.5A and B). There was also no gross difference in the morphology of the spinal cord between the two groups. Sapieha and colleagues (Sapieha et al., 2005) examined the optic nerve morphology in RPTPσ (-/-) mice on the Balb/c background and reported no difference in cellular structure after histological analysis as compared to wild-type mice. This included the number, size and organization of nerve fibers in the optic nerve.

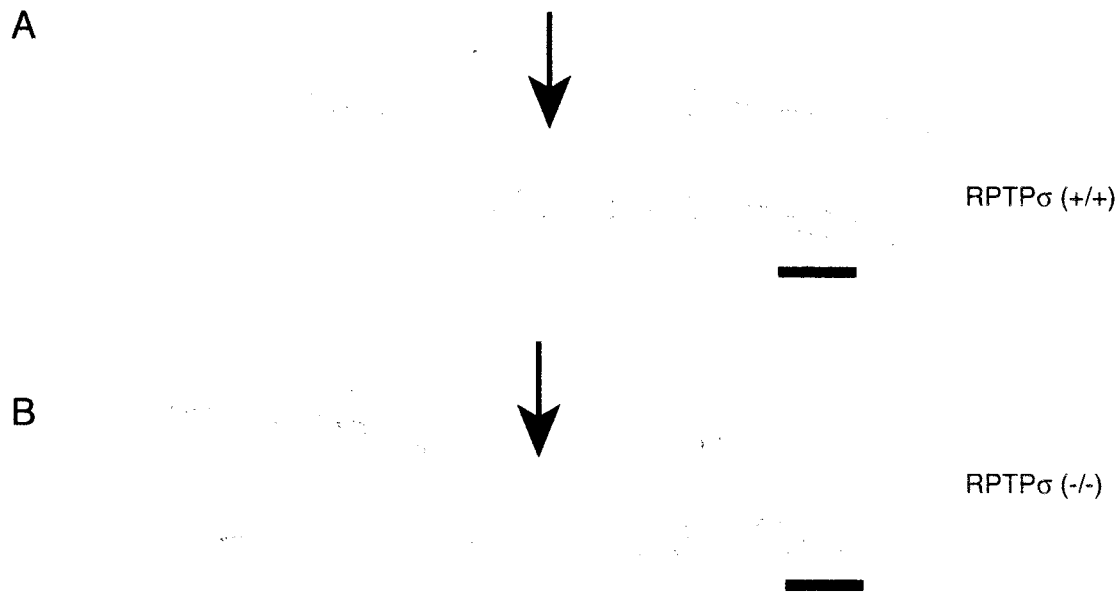


Figure 3.5 Myelination staining in RPTPσ (-/-) and wild-type mice.

(A,B) Previous studies have shown hypomyelination in the sciatic nerve of RPTPσ (-/-) mice. We examined myelination in the spinal cord of wild-type (A) and RPTPσ (-/-) (B) mice by Luxol Fast Blue staining and found no apparent difference. Arrow indicates lesion. Scale bar, 1 mm.

3.4.4 RPTPσ (-/-) cerebellar neurons were not inhibited by CSPG, but were inhibited by myelin substrate *in vitro*

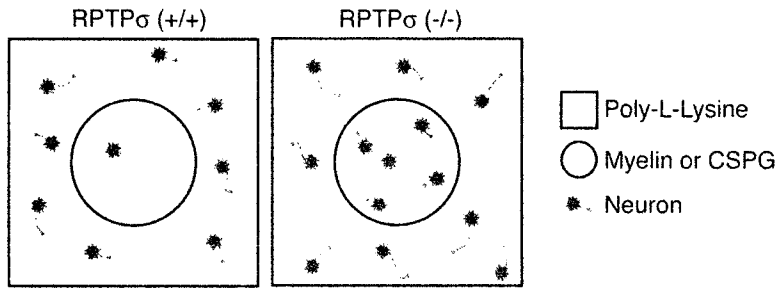
As the inability of CNS axons to regenerate is caused in part by the interaction between the neuron and the axon growth inhibitory elements present at the injury site and region of potential regenerative growth, we tested the ability of the cerebellar granule cells isolated from RPTPσ (-/-) and (+/+) pups (P9) to adhere to inhibitory myelin and CSPG substrates overnight. Expression of RPTPσ protein in the cerebellar cortex was first confirmed by Western blot analysis (Figure 3.1C).

No difference was observed between the two groups of cultured primary neurons in regards to their ability to bind to a droplet of myelin substrate (Figure 3.6B). RPTPσ (-/-) cerebellar granule neurons showed clear attachment inhibition equal to that of neurons isolated from wild-type littermates, with both preferring to attach to the surrounding permissive poly-L-Lysine substrate. This suggests that RPTPσ likely does not interact with myelin proteins.

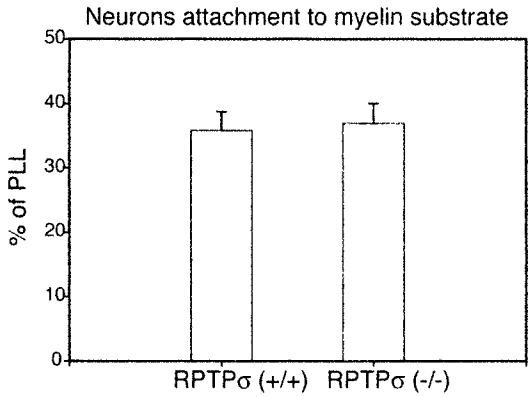
In contrast, there was a very significant dose-dependant increase in the binding of granule cells to CSPG substrate. At 0.5 μg/ml of CSPG the RPTPσ (-/-) neurons showed a 4-fold increase in the number of attached cells as compared to wild-type neurons, while at 0.25 μg/ml there was a 2.5 fold increase in attachment of

RPTPσ (-/-) neurons. This increase in neuronal attachment suggests that the absence of RPTPσ reduces the normal inhibitory effects of CSPG on neurons (Figure 3.6C). Further evidence was seen by observing the growth pattern of neurites with their cells body attached to the very edge of the boundary between the poly-L-Lysine and CSPG substrate. RPTPσ (-/-) neurons extended neurites that were able to cross the boundary and grow onto the inhibitory CSPG substrate. Over 50% of RPTPσ (-/-) neurons grew neurites that were able to cross over the CSPG boundary while less than 10% of (+/+) neurons could do so (Firgure 3.6D). This clearly suggests that neurons lacking RPTPσ have an enhanced ability to cross through the glial scar mostly composed of CSPGs following spinal hemisection injury.

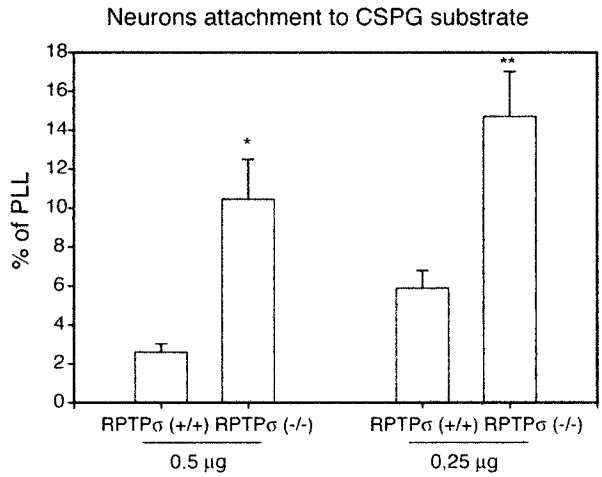
A


RPTPσ (+/+)
RPTPσ (-/-)
Poly-L-Lysine
Myelin or CSPG
Neuron

B


Neurons attachment to myelin substrate
% of PLL
50
40
30
20
10
0
RPTPσ (+/+)
RPTPσ (-/-)

C


Neurons attachment to CSPG substrate
% of PLL
18
16
14
12
10
8
6
4
2
0
*
**
RPTPσ (+/+)
RPTPσ (-/-)
RPTPσ (+/+)
RPTPσ (-/-)
0.5 µg
0,25 µg

D

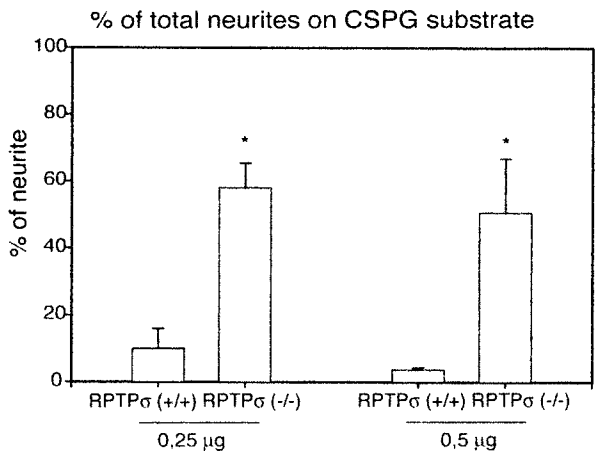

% of total neurites on CSPG substrate
% of neurite
100
80
60
40
20
0
*
*
RPTPσ (+/+)
RPTPσ (-/-)
RPTPσ (+/+)
RPTPσ (-/-)
0,25 µg
0,5 µg

Figure 3.6 *In vitro* neuronal cultures on inhibitory substrate.

(A) Schematic of the neuronal growth experiment on inhibitory surface. The well was first coated with poly-lysine, and a 2 μl drop of myelin or CSPG was gently applied in the middle to act as the inhibitory substrate. 1 X 10^5 cerebellar granule cells were grown on this surface for 24 hours. (B, C) Quantification of the number of cells isolated from RPTPσ wild-type and (-/-) mice adhering to myelin substrate (B) or to two different concentrations of CSPG (C). The numbers are expressed as a percentage of the cells adhering to the poly-lysine permissive substrate (control area). (D) Quantification of the number of neurites crossing from the poly-lysine permissive substrates onto the CSPG inhibitory substrate. The numbers are expressed as a percentage of the total amount of neurites including those that turned away from the inhibitory boundary.

3.4.5 No significant change in the major signaling pathways following dorsal hemisection in RPTPσ (-/-) mice

Since the response of RPTPσ (-/-) neurons to CSPG substrate appeared to be altered we examined the level of protein expression of NG2, which is one major type of CSPG expressed in the spinal cord following injury. The levels of this protein were slightly elevated 24 hours following injury but were similar in both RPTPσ (-/-) and wild-type animals (Figure 3.7A).

We then investigated putative signaling mechanisms involved in axon outgrowth after hemisection injury by Western blot analysis using antibodies that recognize the phosphorylated forms of Erk1/2, p38, and STAT3 or the total amount of protein for caspase-3 (Figure 3.7B). Twenty-four hours after injury, wild-type animals showed a clear increase in the phosphorylated forms of p38 and STAT3, and total caspase-3 protein, while phosphorylated levels of Erk, remained unchanged. When the injury was reproduced in RPTPσ (-/-) mice no significant difference in the activation of the same signaling proteins was observed compared to controls, although the increase in the activation of p38 as well as STAT3 appeared slightly attenuated (Figure 3.7B).

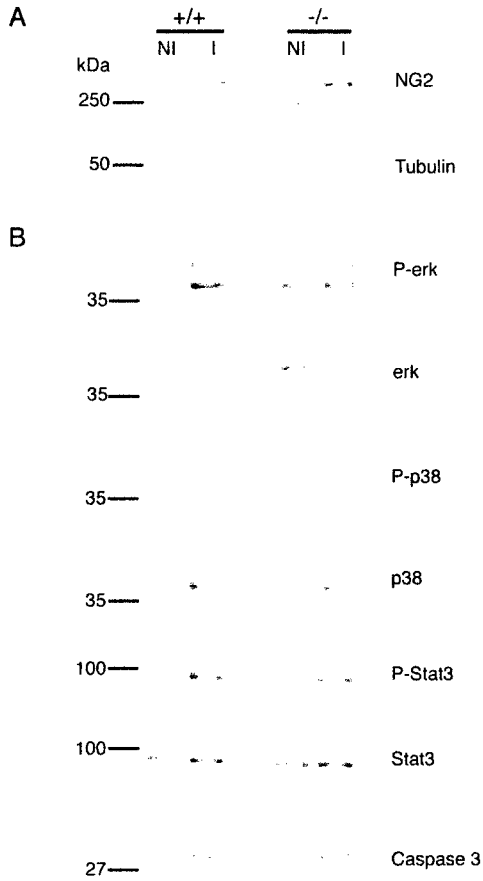


Figure 3.7 Signaling pathways activated twenty-four hours after spinal cord injury.

(A, B) Western blot analysis in spinal cord homogenates before (not inured: NI) and 24 hours (injured: I) after dorsal hemisection performed in wild-type and RPTPσ (-/-) mice. (A) The level of NG2 slightly increases post injury in both wild-type and RPTPσ (-/-) animals (n=3). Levels were normalized against tubulin. (B) Stimulation of p38 and STAT3 was observed after injury in both wild-type and RPTPσ (-/-) (n=2). In both sets of animal however the levels of activated Erk1/2 remained unchanged after injury (n=3). The levels of activated p38, STAT3 and Erk 1/2 were normalized against total amount of these proteins. The amount of protein expression of Caspase-3 increased after injury in a similar fashion in both sets of animals (n=2).

3.5 Discussion

The developing nervous system expresses RPTPσ at the tips of migrating growth cones, where it functions to appropriately arrest the growth of developing axons (Stoker et al., 1995; Thompson et al., 2003). Mice that are deficient for this enzyme show neural-related developmental defects (Elchebly et al., 1999a; Wallace et al., 1999), which have raised much interest in the potential role of RPTPσ in the control of axonal growth following injury.

Indeed RPTPσ has been shown to decrease the rate of axonal extension following injury in the peripheral nerve, which is a permissive environment for regeneration (McLean et al., 2002; Thompson et al., 2003). Moreover the deletion of RPTPσ in mice not only enhanced axonal growth after optical nerve crush but also conferred an ability of axons to regenerate moderately in the inhibitory environment of the CNS (Sapieha et al., 2005).

In the present study, we demonstrate that the CNS of the RPTPσ (-/-) mouse is a novel system in which the spinal cord can spontaneously regenerate after dorsal thoracic hemisection injury. This injury model showed resprouting axons penetrating deeply into inhibitory scar tissue at the lesion site and robust long distance regeneration of severed corticospinal tract (CST) axons was observed caudal to the lesion two weeks post-injury to RPTPσ (-/-) mice. In comparison, wild-type mice did

not show any regeneration of injured axons after identical spinal hemisection. This remarkable amount of highly robust, spontaneous regeneration of severed axons in RPTPσ (-/-) mice suggests a highly pertinent role for RPTPσ in preventing the non-regeneration of CST axons in the injured mouse spinal cord.

While the elevated expression of RPTPσ early in development has been observed to decrease postnatally (Yan et al., 1993; Wang et al., 1995; Wallace et al., 1999), our analysis of RPTPσ expression by western blot and *in situ* hybridization showed that adult cortical and spinal cord neurons still express relatively high amount of RPTPσ. The corticospinal tract was therefore a system of choice for evaluating the role of RPTPσ in axon outgrowth in the adult CNS. The morphology and location of the cells stained by *in situ* hybridization is highly indicative of neuronal cells. However, it is possible that the surrounding glia may contribute to the observed expression of RPTPσ, as glial cells have been previously reported to express RPTPσ in the optic and sciatic nerve of mice (McLean et al., 2002; Sapieha et al., 2005). LAR-RPTPs act as homophilic ligands (Sun et al., 2000b; Yang et al., 2003) and so expression of RPTPσ in the surrounding glia could affect RPTPσ induced signaling in the growing axons. The RPTPσ ectodomain substrate supports the growth of retinal neuritis revealing a non-cell-autonomous role for this enzyme in neurite outgrowth (Sajnani et al., 2005).

The present study showed that expression of RPTPσ remains stable in both the spinal cord and motor cortex at least for up to 21 days after injury. Therefore, RPTPσ would potentially be able to influence the regrowth of axons after injury. This is consistent with previous observations in neuronal populations of retinal ganglion cells and facial motoneurons where the expression of RPTPσ also remained unchanged after injury while still inhibiting axonal regeneration (Thompson et al., 2003; Sapieha et al., 2005). In comparison, RPTPσ expression was increased in dorsal root ganglia and sciatic nerve following injury suggesting that various neuronal cell populations could be differently regulated (Haworth et al., 1998; McLean et al., 2002).

Homologs of the LAR-RPTPs subfamily have been shown to play a role in the projection and guidance of axons across multiple organisms such as *Drosophila melanogaster, Hirudo Medicinalis, Xenopus laevis,* and chick (Krueger et al., 1996; Desai et al., 1997; Gershon et al., 1998a; Johnson et al., 2001; Rashid-Doubell et al., 2002). Similarly, neuronal cells isolated from the embryonic cortex of RPTPσ (-/-) mice were shown to extend longer axons than wild-type controls when cultured on poly-lysine or poly-lysine plus laminin (Thompson et al., 2003). Neurons derived from neural stem cells isolated from RPTPσ (-/-) mice extended neurites twice as long as controls on laminin (Kirkham et al., 2006). These results clearly indicate that removing or inhibiting RPTPσ will promote axonal extension. Indeed signals

intrinsic to the neurons during development are emerging as significant players in the control of axonal regrowth after injury.

Recent identification of N-cadherin as a substrate of RPTPσ shed light on one potential mechanism for RPTPσ-dependent inhibition of axonal growth (Siu et al., 2007) via actin stabilization and increased adhesion. Neurotrophic-induced axon extension could also be regulated by RPTPσ as the Trk family of receptors interact and are dephosphorylated by RPTPσ in overexpression studies with 293T cells, explaining the downregulation of NGF-dependent neurite promoting signaling pathways in primary sensory neurons (Faux et al., 2007). We examined Erk 1/2 activation in response to injury since the Trk family of neurotrophin receptors was previously shown to modulate both MAPK and P13K/ Akt to promote axon outgrowth (Atwal et al., 2000). Moreover, increased basal activation of MAPK was observed in the retina of RPTPσ(-/-) mice (Sapieha et al., 2005). The deletion of RPTPσ in the present study did not affect the Erk 1/2 response to dorsal hemisection as opposed to the observed response in other models. We also examined levels of activated p38 and caspase-3 expression, as they are mediators of neuronal apoptosis following CNS injury (Springer et al., 1999; Wang et al., 2005). As expected their levels were increased after injury but in a similar fashion in both RPTPσ (+/+) and (-/-) spinal cords. This observation suggests that there is no difference in the rate of cell death after injury between RPTPσ (-/-) and wild-type

mice, consistent with observations made in retinal ganglion cells following axotomy (Sapieha et al., 2005). STAT3 is a major mediator of astrogliosis and scar formation (Herrmann et al., 2008) however, its activation response was also comparable in both sets of animal analyzed. RPTPσ could potentially act in a small subset of cells whose mechanistic effect is lost in the heterogeneous spinal cord mixture used for this analysis. Further studies have been initiated in order to further understand the signaling mechanisms by which RPTPσ may regulate axon regeneration in the context of CNS injury.

Multiple lines of evidence suggest that the glial environment of the adult CNS represents a major impediment for spontaneous axonal regeneration. Both myelin-associated inhibitors as well the astroglial scar formed after injury prevent axon outgrowth in adult animals. Embryonic axons are unmyelinated during development as they extend through the nervous system to connect with appropriate targets in response to guidance cues. Developing axons are therefore not exposed to the growth inhibitory effects of myelin-associated proteins. Hypomyelination of the sciatic nerve as well as reduction in the size of the white matter of the spinal cord of RPTPσ (-/-) mice on a C57Bl/6 background were observed (Wallace et al., 1999; Meathrel et al., 2002). These observations were made in young animals and were not seen in the adult RPTPσ (-/-) mice on a Balb/c background (Elchebly et al., 1999a) used in the present study. Strain variations and age differences are likely to account

for this discrepancy. We did not observe any obvious defects in myelination as compared to wild-type mice that could account for the improved regenerating ability observed in RPTPσ (-/-) mice. Furthermore *in vitro* experiments showed that the cerebellar granule cells isolated from P9 RPTPσ (-/-) pups were as equally inhibited as wild-type cells on purified myelin substrate. However the same type of RPTPσ (-/-) cells were clearly less inhibited on a mixture of the extracellular matrix substrate CSPGs, showing much more cellular attachment and neurite extension from more permissive poly-L-lysine onto the CSPG substrate. In spinal cord lysates the expression levels of NG2 in RPTP (-/-) were comparable to wild-type littermates either before of after injury. This suggests both neuronal cell bodies and their neurites in RPTPσ (-/-) mice may have a blunted sensitivity to CSPG despite still retaining a growth inhibitory response to myelin. This may contribute to the ability of corticospinal axons to extend through the inhibitory scar tissue that forms after spinal injury. Interestingly genetic deletion of inhibitors in the glial scar such as GFAP, vimentin (Menet et al., 2003), and EphA4 (Goldshmit et al., 2004) have resulted in better recovery following dorsal hemisection injury than deletion of myelin-associated targets such as Nogo (Simonen et al., 2003; Zheng et al., 2003), NgR (Kim et al., 2004; Zheng et al., 2005), and p75 (Song et al., 2004) suggesting that overcoming the glial scar inhibition could represent a major barrier to adult CNS regeneration *in vivo.* However myelin-associated inhibition is still a major player in the field as targeting Nogo with IN-1 antibody (Brosamle et al., 2000), NgR

with antagonist peptide (GrandPre et al., 2002) or immunizing the mice with myelin (Huang et al., 1999) leads to marked recovery following CST lesions.

Relatively little is known about the neuronal receptors transducing the inhibitory signals of the CSPGs. One specific receptor for NG2 was previously identified on cerebellar granule cells, which signals through Ca++ and cAMP second messengers (Dou and Levine, 1997). We were not able to show direct interaction between RPTPσ and CSPGs (data not shown), which is consistent with previous observations that other proteoglycans such as HSPGs were specific ligands of RPTPσ in the retinal basement membrane (Aricescu et al., 2002). Moreover interaction between RPTPσ and its ligand was shown to promote axon extension (Ledig et al., 1999a), via suppression of its phosphatase activity (Johnson et al., 2001; Rashid-Doubell et al., 2002). Based on this model, CSPG is unlikely to be a direct substrate of RPTPσ. Interactions between CSPG and growth-promoting substrates such as laminin has been previously described (Katoh-Semba et al., 1995; McKeon et al., 1995) and reviewed in (Bovolenta and Fernaud-Espinosa, 2000). It was suggested that CSPGs could prevent axonal elongation by masking such molecules from neurites. Interestingly laminin was found to be a ligand of LAR, a close relative of RPTPσ, and the alternative splicing of a small exon within the fifth FNII repeat required for this interaction is conserved in RPTPσ, suggesting that they may share this common ligand (O'Grady et al., 1998). Therefore, the spatio-temporal

regulation of CSPG may regulate neurite outgrowth through disruption of ligand-dependent inactivation of RPTPσ.

CSPGs act through activation of downstream intracellular signaling pathways such as Rho/ROCK (Monnier et al., 2003) and Rac/PAK (Marler et al., 2005). Dlar, the homolog of LAR-RPTPs in the *D. melanogaster* was previously linked to RhoGTPases signaling via genetic interactions with Trio, a guanine nucleotide exchange factor for Rho and Rac (Bateman et al., 2000). Even though consistent interaction between Trio and mammalian RPTPσ could be observed in yeast-two-hybrid system, neither the interaction with Trio or RPTPσ-dependent downstream signaling of Trio could be demonstrated in mammalian cells (M. J. Chagnon and M.L. Tremblay unpublished observations). Although RPTPσ could still be a candidate regulator of RhoGTPases signaling, we doubt it directly upregulates Rho activity, as an improvement of the adhesion capacity of the RPTPσ (-/-) neuronal cells on the myelin substrate would have been expected. In fact, myelin-associated inhibitors also signal primarily through Rho/ROCK pathways downstream of the co-receptor complex formed by NgR and p75/TROY (Niederost et al., 2002; Winton et al., 2002; Fournier et al., 2003; Yamashita and Tohyama, 2003).

Different mechanisms have evolved to limit axon regeneration in the adult CNS as a possible drawback for the stabilization of the highly complex neuronal

circuitry formed during development. Indeed, LAR-RPTPs expression is fundamental for proper neuronal targeting during development (Stepanek et al., 2005; Uetani et al., 2006), and complementation in this (Uetani et al., 2006) and other processes (Dunah et al., 2005) was observed within the three sub-family members. Improved regeneration in the sciatic nerve of RPTPσ (-/-) was accompanied by multiple errors in directional growth (McLean et al., 2002). We show that downregulation of RPTPσ may be an excellent new target to aid axonal regeneration in the non-permissive environment of the CNS. Further studies are underway to ensure that deletion of RPTPσ will not trigger inappropriate reinnervation. Fine-tuning of the activity mechanisms of RPTPσ and its sub-family members is also required to balance promotion of neurite outgrowth without affecting correct target reinnervation. These issues could be achieved by developing new antibodies or peptides to inactivate RPTPσ either alone or in combination with homologs *in vivo* in a timely and spatial manner. Complete systemic knockout of RPTPσ, such as in the present study may not be ideal for achieving both structural and functional recovery after spinal injury as inactivation of RPTPσ would need to be sufficient to lift the axonal "brake" effect after injury but not cause axons to extend indefinitely. However it has proven to be an excellent system to study and confirm the important role of RPTPσ in complex adult mammalian CNS injuries.

3.6 Acknowledgements

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CHAPTER 4: Identification of new substrates of RPTPσ using a modified yeast-two hybrid approach

4.1 Abstract

A growing body of evidence supports an important role for RPTPσ in the development of the nervous system and nerve regeneration. However, the signaling mechanisms involved in these processes and how they are regulated by RPTPσ remain largely unknown. Since the identification of RPTPσ substrate(s) and binding partners is invaluable to understanding its mechanism of action, we employed a modified yeast-two-hybrid approach, the yeast substrate-trapping system, to screen a murine embryonic library for new substrates of RPTPσ. This strategy involves the mutation of two essential residues (Asp to Ala, and Cys to Ser) in the catalytic domain of RPTPσ. These mutations decrease the catalytic activity of the enzyme and stabilize the interaction between the enzyme and its substrates. These "substrate-trapping mutants" were used as bait in the modified yeast-two hybrid system to screen a library that has been subjected to *in vitro* phosphorylation by an inducible, active protein tyrosine kinase (PTK). Liprin-α4, RPTPδ and Trio were found to interact with RPTPσ in the yeast system in the absence or presence of tyrosine phosphorylation. We also identified the scaffolding molecule p130Cas as well as p250GAP, a highly expressed GAP in the nervous system and a known regulator of the small RhoGTPases, as new substrates of RPTPσ. Indeed p130Cas is hyperphosphorylated in brain lysates isolated form RPTPσ (-/-) as compared to wild-type littermates. In mammalian cells the RPTPσ-D/A mutant is able to trap p250GAP only in the presence of active Fyn. RPTPσ is also able to directly

dephosphorylate p250GAP *in vitro*. We also found that Fyn and RPTPσ can interact with each other independently of tyrosine phosphorylation and that RPTPσ is a substrate of Fyn in mammalian cells.

The substrates and interacting partners identified should allow us to further elucidate the signal transduction mechanisms involving RPTPσ and to explain its role in nervous system development and regeneration.

4.2 Introduction

Protein tyrosine phosphatases (PTPs) maintain the balance of intracellular tyrosine phosphorylation thus controlling a vast array of cellular functions. Improper control of these levels has been linked to a variety of human diseases such as cancer and diabetes (Andersen et al., 2004; Tonks, 2006). Among the 38 classical PTP genes listed in the human genome (Andersen et al., 2001), 21 are transmembrane PTPs, including the LAR subfamily of receptor protein tyrosine phosphatases (LAR-RPTPs).

This subfamily is composed of three vertebrate homologs: LAR (ptprf), RPTPσ (ptprs) and RPTPδ (ptprd) (Chagnon et al., 2004). Similar to most other receptor PTPs, they contain two classical intracellular PTP domains where only the membrane proximal one (D1) was shown to be catalytically active. The uniqueness and diversity among the family primarily resides in the large extracellular domain that is reminiscent of cell adhesion molecules with three immunoglobin-like domains and four to eight fibronectin type III repeats (Beltran and Bixby, 2003). LAR-RPTPs have been suggested to play a key role in neural development and in various pathologies (reviewed in (Stoker, 2001; Johnson and Van Vactor, 2003; Chagnon et al., 2004)). Generation of RPTPσ knockout mice has proven the essential role of RPTPσ in proper nervous system development (Elchebly et al., 1999a; Wallace et al., 1999). Furthermore, overexpression of dominant negative mutants of RPTPσ in

retinal ganglion cells enhanced neurite elongation (Ledig et al., 1999a). Similarly primary cortical neurons isolated from RPTPσ-KO mice spontaneously exhibited increased axonal growth as compared to wild-type controls (Thompson et al., 2003). Most interestingly the removal of RPTPσ in mice affected the regenerative property of nerves following injury in both the peripheral and central nervous system. In the absence of RPTPσ, not only was the regeneration of the facial and sciatic nerve accelerated, but this also promoted the regrowth of the retinal ganglion cells, regrowth where normally no spontaneous regeneration occurs (McLean et al., 2002; Thompson et al., 2003; Sapieha et al., 2005).

Although advances were recently made in this field, relatively little is known about the signaling mechanisms involved in these processes and specifically those regulated by RPTPσ. Similar to other LAR-RPTPs, RPTPσ interacts and colocalizes with liprins at focal adhesions in cultured cell lines (Pulido et al., 1995; Serra-Pages et al., 1998). This interaction contributes to the maintenance of synapse morphogenesis in hippocampal neurons (Dunah et al., 2005). Dlar, the *Drosophila melanogaster* ortholog of all three LAR-RPTPs was linked genetically to the protein kinase Abl and its substrate Ena (Wills et al., 1999) and to the guanine nucleotide exchange factor Trio (Bateman et al., 2000), leading to remodeling of the actin cytoskeleton. Yet experimental support indicating that any of these interactions are mirrored in mammals are still lacking. When overexpressed in HEK-293T cells,

RPTPσ can stably bind to TrkA and TrkC, and is able to dephosphorylate all three members of the Trk family, elucidating in part the role of RPTPσ in NGF-dependent neurite outgrowth (Faux et al., 2007). The negative effect of RPTPσ on neurite outgrowth could also come from interactions with the N-cadherin/β-catenin complex that was recently identified as a substrate using a substrate-trapping approach in RPTPσ knockout mice brain lysates coupled with mass spectroscopy (Siu et al., 2007).

Since the identification of RPTPσ substrate(s) and binding partners is crucial to understanding its mechanism of action, we employed a modified yeast-two-hybrid approach, the yeast substrate-trapping system (Kawachi et al., 2001), to screen a murine embryonic library. To increase the trapping efficiency of the RPTPσ bait, two essential residues (Asp to Ala or Cys to Ser) in the catalytic domain of RPTPσ were mutated. These mutations decrease the catalytic activity of the enzyme and stabilize the interaction between the enzyme and its substrates (Blanchetot et al., 2005). These substrate-trapping mutants were used to screen a library that has been subjected to *in vitro* phosphorylation by an inducible, active protein tyrosine kinase (PTK). In the absence of expression of the PTK, we screened for interactions independent of tyrosine phosphorylation. Using this assay, we identified p250GAP, a highly expressed GAP in the nervous system and a known regulator of the small RhoGTPases, as a new substrate of RPTPσ. We show that active Fyn kinase is

required for the interactions, but can itself bind to RPTPσ. We also find that p130CAS is a new potential substrate and is hyperphosphorylated in the brain of RPTPσ knockout mice. We propose that RPTPσ regulates neurite outgrowth through its interaction with p250GAP and could form a complex with p130CAS and Fyn. The substrates and interacting partners identified should allow us to elucidate the signal transduction mechanisms of RPTPσ and to further explain its role in nervous system development and regeneration.

4.3 Materials and methods

4.3.1 Antibodies and reagents

The following antibodies were used: anti-phosphotyrosine (pTyr) clone 4G10 (Millipore), anti-Myc clone 9E10 (Upstate), anti-Flag clone M2 (Sigma), anti-p130Cas (BD transduction laboratory). Anti-RPTPσ clone 17G7.2 (Thompson et al., 2003) and anti-p250Gap polyclonal antibody (Nakazawa et al., 2003) have been previously described. Rabbit polyclonal anti-Fyn was a kind gift of Dr André Veillette. Ezview red anti-flag clone M2 affinity gel (Sigma) was used to immunoprecipitate Flag-tagged proteins.

4.3.2 Plasmids and constructs

The murine RPTPσ full-length cDNA was obtained by a PCR-based strategy using two partial cDNA constructs. The cDNA sequence corresponding to

nucleotides (nt) 126-3874 (GenBank accession number D28530, obtained from M. Ogata) was combined to cDNA nt 3548-5770 (GenBank accession number X82288) to obtain the full-length sequence. The final PCR product was subcloned into pGEM-T for amplification (pGEM-T vector system I, Promega #A3600). The resulting plasmid was digested with PmeI and EcoRI to release the insert that was then ligated in pcDNA3.1 zeo (+) (Invitrogen) between EcoRI and EcoRV sites. The substrate-trapping mutant RPTPσ-D/A (D1516A) or RPTPσ-C/S (C1548) were prepared by site-directed mutagenesis (QuikChange kit, Stratagene) and verified by sequencing. The generation of the RPTPσ bait for the yeast-two-hybrid screen was made from the sequence corresponding to RPTPσ DIDII domain (nucleotide 3898-5770, GenBank accession number X82288) was PCR amplified incorporating restriction sites and cloned into pBridgeLexA-vsrc (Obtained from Dr. M. Noda) at the EcoRI and BamHI restriction sites. The sequence corresponding to the DI domain (nucleotide 3898-5020, GenBank accession number X82288), the DIDII domain (nucleotides 3898-5770, GenBank accession number X82288) and the DIDIIT (truncated) domain (nucleotides 3898-5401, GenBank accession number X82288) of RPTPσ were PCR amplified and subcloned into pDEST 15 or pDEST 27 using the Gateway recombination technology (Invitrogen) in order to obtain GST-fusion proteins. GST-fusion proteins for PTP-PEST, He-PTP, and MKPX have been previously described (Stuible et al., 2007). Plasmids used for the expression of p250GAP were previously described (Nakazawa et al., 2003) and obtained from Dr.

T. Yamamoto. Fyn, Fyn-Y531F, and Fyn-K299A constructs were a kind gift of Dr. S. Stamms.

4.3.3 Modified yeast-two-hybrid screen

Yeast two-hybrid screening was performed using the *Saccharomyces cerevisiae* strain L40, which harbors the reporter genes *HIS3* and *LacZ* under the control of an upstream LexA-binding site. pBridgeLexA-RPTPσ(WT, C/S or D/A)/v-Src, and the mouse embryonic E17.5 cDNA library, expressed as a fusion protein with GAL4 in the pACT2 vector (Clontech), were transformed in the yeast cells as described previously (Kawachi et al., 2001; Fukada et al., 2005). Approximately 1.08 X 10^6 clones were screened and positive clones were selected on plates lacking leucine, tryptophan, histidine and methionine, and further verified by a β-galactosidase filter-lift assay in the presence or absence of methionine. The clones that showed an increase in blue color development by v-Src induction were selected as candidate substrate molecules. The clones that interacted in both conditions were selected as candidate binding partners.

4.3.4 Cell culture and transfection

COS7 cells were routinely maintained in DMEM containing 10% Fetal Bovine Serum and 1% penicillin/streptomycin (all from Invitrogen). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's

instructions with the indicated constructs. Typically the day before transfection 0.5 X 10^6 cells were plated in a 60mm-dish, and the following day 4 μg of each construct (excepting Fyn where only 0.1 μg was used) were transfected.

4.3.5 Co-immunoprecipitation and immunoblotting

Forty-eight hours post-transfection cells were lysed in ice-cold TNN buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 50 mM NaF, and a cocktail of protease inhibitors (Roche)). When substrate-trapping was performed Na_3VO_4 was not included in the lysis buffer. Lysates were cleared by centrifugation and 10% was kept for total cell lysates analysis. The rest was incubated with Protein A- or G-agarose beads (Invitrogen) and the indicated antibody for 4 hours at 4°C for immunoprecipitation. The precipitated complex was washed three times with ice-cold lysis buffer and bound proteins were resuspendend in 2X Laemmlli sample buffer. Protein samples were separated by SDS–PAGE and transferred to polyvinylidene difluoride membrane (PVDF, Millipore). Western blot analysis was performed under standard conditions using the indicated antibodies. Membranes were probed with horseradish peroxidase-coupled secondary antibodies (Jackson Laboratories) and standard enhanced chemiluminescence reagent (Perkin-Elmer).

4.3.6 Protein purification

GST-PTP fusion proteins were purified from bacterial cultures as previously described (Stuible et al., 2007) with slight modifications for GST-RPTPσ. Briefly a single colony of transformed BL21 cells was grown overnight at 37°C in LB medium that contained ampicillin (100 μg/mL). This culture was then diluted ten-fold, and grown to a cell density of 0.6 OD (A600 nm) before induction with IPTG (1 mM) overnight at 25°C. All further purification steps were done at 4°C. Pelleted bacteria were resuspended in lysis buffer (20 mm HEPES/KOH pH 8.0, 500 mm NaCl, 0.1 mm EDTA, 1% Triton X-100, 1X complete protease inhibitors). Samples were sonicated on ice and centrifuged. The supernatant was then incubated with glutathione Sepharose 4B beads for 1 h with rotation. Beads were washed and resuspended in elution buffer (50 mm Tris/HCl pH 8.0, 10 mm reduced glutathione) and incubated with rotation for 1hr. The purified fusion protein was collected and quickly frozen at -80°C until further use.

4.3.7 *In vitro* phosphatase assay

In vitro phosphatase assay was performed by adding purified PTP enzyme to immunoprecipitated p250GAP that have been previously phosphorylated by constitutively active Fyn in COS7 cells. Assays were performed in 50mM PIPES buffers at pH 6.0 containing 3mM DTT and 0.1 mg/ml BSA. The reaction was performed at 37°C for 20 or 15 minutes.

4.4 Results

4.4.1 Yeast-two-hybrid interaction between p250GAP and RPTPσ

A modified version of the yeast-two-hybrid system where interactions can be monitored for dependence on the tyrosine phosphorylation level of the prey proteins was used to find new substrates of RPTPσ. Schematic representation of the modified yeast-two hybrid assay is presented in figure 4.1A as adapted from Kawachi et al (Kawachi et al., 2001). Essentially two modifications were made: First, v-Src tyrosine kinase was conditionally expressed in yeast in order to phosphorylate all prey proteins on tyrosine residues. Upon deprivation of methionine in the yeast growing media we observed a marked increase in the level of tyrosine-phosphorylated proteins (Figure 4.1B). Secondly, the intracellular domain of RPTPσ containing a substrate-trapping mutation (D1516A) or (C1548S) as well as the WT protein were used as a bait (Figure 4.1C).

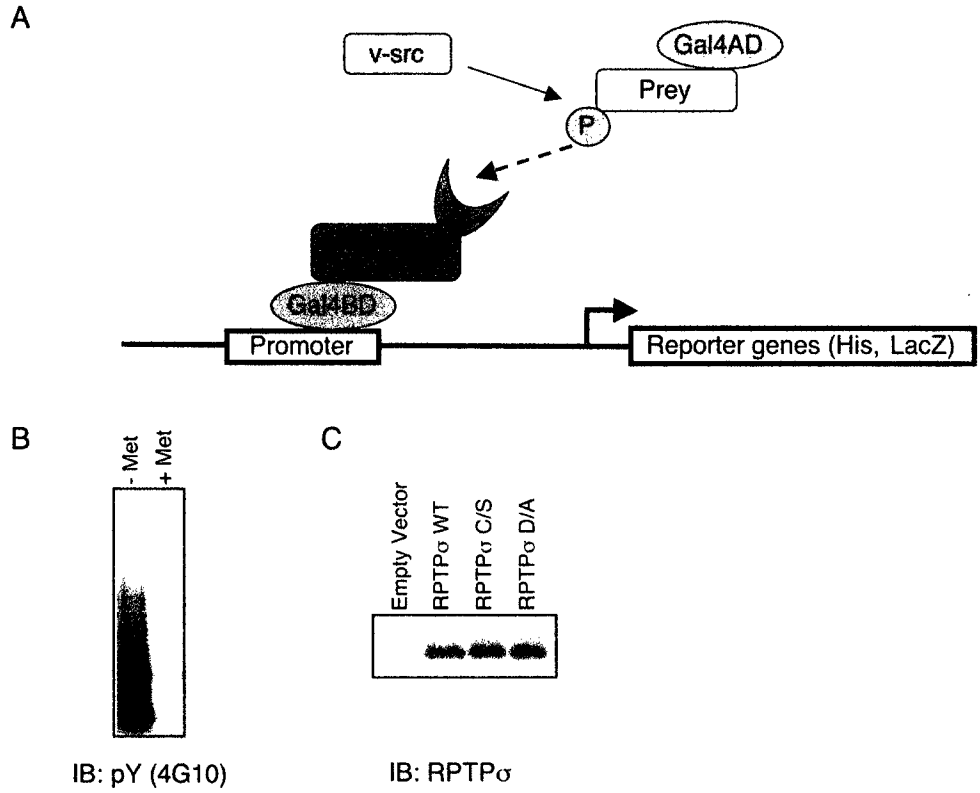


Figure 4.1 Yeast substrate-trapping system.

A) Schematic representation of the modified yeast substrate-trapping assay. In the absence of methionine the prey proteins are phosphorylated by induced v-Src and can be trapped by the RPTPσ-D/A mutant. The complex formation leads to activation of reporter gene and can be screen for growth on media lacking histidine (HIS3) or by an increase in blue color development (LacZ). B) Tyrosine phosphorylation of yeast proteins was detected only when v-Src expression was induced in yeast. C) Protein expression of selected baits in yeast cell lysates used in the assay.

A first screening step was performed where yeast colonies were selected on plates lacking leucine, tryptophan, histidine and methionine (v-Src-induced). Approximately 1.1 X 10^6 clones were screened from a mouse E17.5 embryonic library. The positive clones isolated were further tested by a β-galactosidase filter-lift assay in the presence or absence of methionine (Figure 4.2A). Each positive clone was scored from 0 to 4 based on the intensity of blue coloring, 0 being complete absence of blue tint and 4 being saturated blue color. Clones that developed similar blue color intensity independently of the presence or absence of v-Src were classified as potential partners while the ones that preferentially developed blue color in the presence of v-Src were classified as potential substrates as summarized in Table 1.

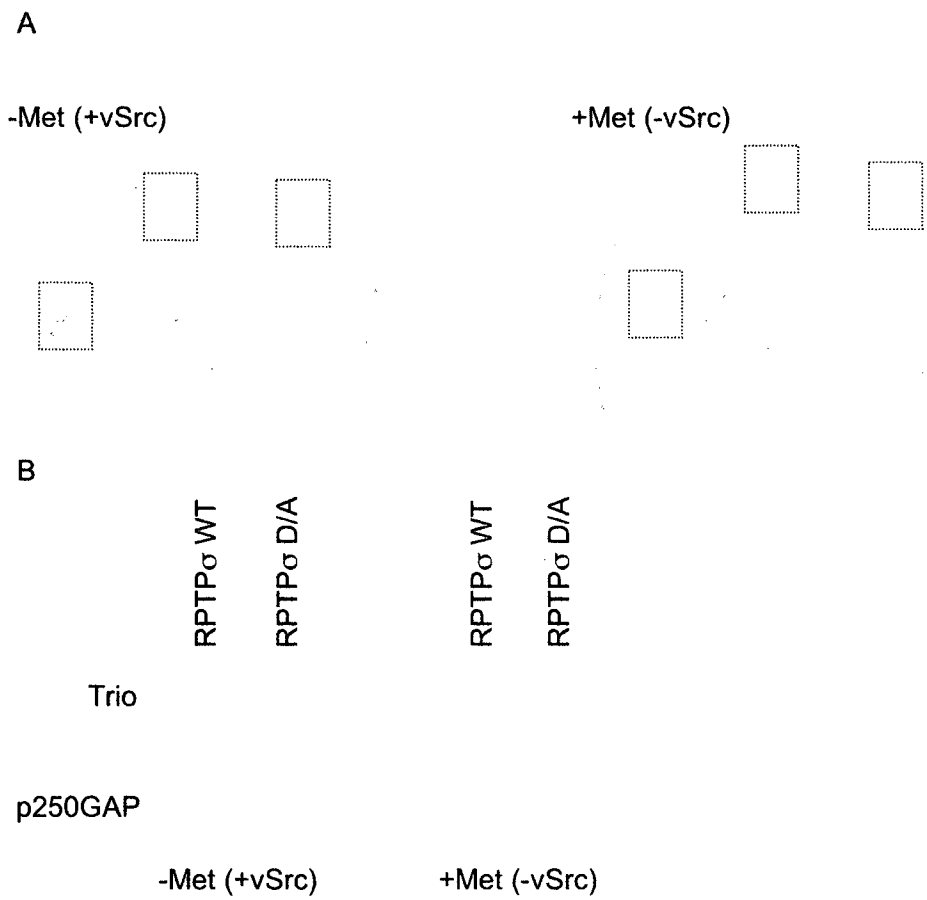


Figure 4.2 Screening for new substrates or binding partner of RPTPσ.

A) β-galactosidase filter-lift assay in the absence and presence of methionine. Dotted squares indicate clones interacting in a tyrosine phosphorylation-dependent manner. RPTPσ-DIDII D/A was used as a bait to screen a mouse embryonic E17.5 library (1.08 X 10^6 clones screened). B) β-galactosidase filter-lift assay in the absence and presence of methionine for specific clones. RPTPσ-WT interaction was compared to RPTP-D/A interaction with the putative substrate.

Table 4.1: List of candidates

Substrates	Known function
p130Cas*	Cell motility, migration
p250GAP	Neurite outgrowth, cell proliferation

Partners	Known function
Liprin alpha4	Cell motility, synapse
RPTP-Delta	Axon guidance, neurite outgrowth
Trio	Axon guidance, neurite outgrowth
p130Cas	Cell motility, migration

In agreement with published reports, liprins and RPTPδ were identified and classified as binding partners of RPTPσ, thus validating our yeast-two-hybrid approach. Multiple clones of the guanine nucleotide exchange factor Trio were also identified. As shown in figure 4.2B, Trio was classified as a binding partner since it interacted with RPTPσ WT and D/A, both in the presence or absence of v-Src. Trio was never shown to interact with RPTPσ but was previously identified as a binding partner of its homolog LAR. Since the intracellular domain of RPTPσ is highly similar to the one of LAR this interaction was somewhat expected. Surprisingly, we were unable to reproduce the interaction of Trio and RPTPσ in mammalian cells (data not shown). In addition, the adaptor protein p130Cas, known to be heavily tyrosine-phosphorylated was found as a potential substrate of RPTPσ. This adaptor p130CAS was previously suggested to be a substrate of LAR (Weng et al., 1999),

PTP-PEST (Garton et al., 1996) and PTP1B (Dube et al., 2004). To determine if this newly found interaction was physiologically relevant we looked at the tyrosine phosphorylation levels of p130Cas in brain lysates of mouse embryos, deficient for either RPTPσ or its homolog RPTPδ as compared to wild-type animals (Figure 4.3). In the absence of RPTPσ we observe a significant increase in the basal tyrosine-phosphorylation level of p130Cas further suggesting that it is a valid *in vivo* substrate. The increase in phosphorylation is also observed in RPTPδ-KO animals, which is consistent with the close identity between the PTP-DI domains of the two homologs.

Of particular interest was the identification of p250GAP, a GTPase-activating protein for cdc42, rho and potentially rac, as a new putative substrate of RPTPσ. The interaction occurred primarily with the RPTPσ substrate-trapping mutant and was dependent on v-Src expression (Figure 4.2B).

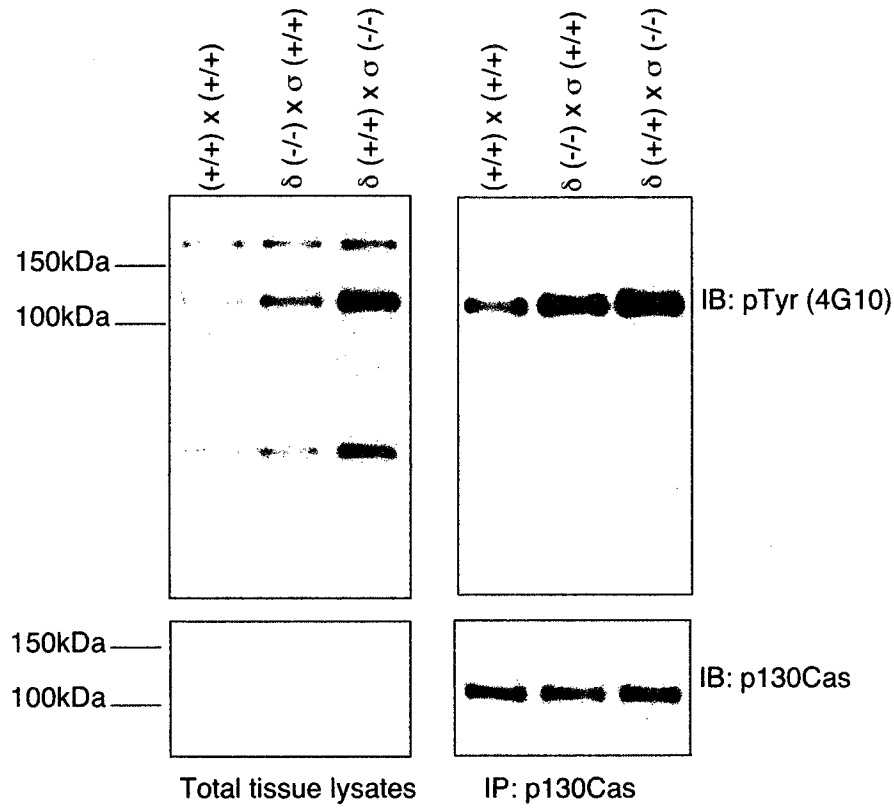


Figure 4.3 p130Cas is hyperphosphorylated in RPTPσ KO brain lysates.

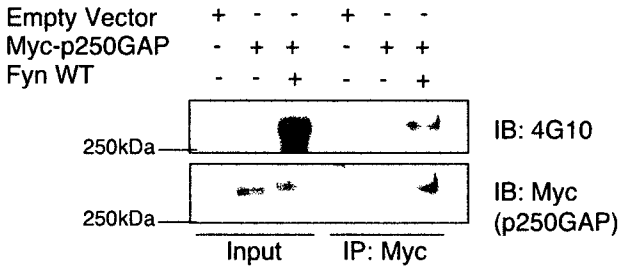
Generation of RPTPσ (Elchebly et al., 1999a) and RPTPδ (Uetani et al., 2000) knockout mice have been previously described. Brain from (+/+), RPTPσ (-/-) , and RPTPδ (-/-) E18.5 embryos were homogenized in (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4, 50 mM NaF, and a cocktail of protease inhibitors). Lysates were immunoprecipitated overnight at 4°C with 3 µl of anti-p130Cas monoclonal antibody and 30 µl of protein G-agarose. Samples were washed three times and ran on SDS-PAGE. The tyrosine phosphorylation level was increased in the absence of RPTPσ and RPTPδ (top panel). All samples expressed equal amount of p130Cas (bottom panel).

4.4.2 RPTPσ and p250GAP interacts in mammalian cells

To validate the interaction of RPTPσ and p250GAP in mammalian cells we transiently transfected Cos7 cells with full-length RPTPσ-WT or D/A together with myc-tagged p250GAP. Since a tyrosine kinase was required to detect the interaction in the yeast system we first showed that Fyn, another member of the Src-kinase family could readily phosphorylate p250GAP in cells (Figure 4.4A). Without the expression of Fyn a very small amount of p250GAP was able to co-immunoprecipitate with RPTPσ-D/A (Figure 4.4B). However, expression of Fyn significantly increased the amount of p250GAP being trapped by RPTPσ D/A while RPTPσ WT as expected did not display trapping capability (Figure 4.4B).

In order to determine if the kinase activity of Fyn was required for the trapping of p250GAP by RPTPσ-D/A we repeated the experiment using Fyn WT, Fyn constitutively activated (CA) or Fyn kinase dead (KD) expression constructs. In this experiment a Flag-tagged p250GAP was used and immunoprecipitated using EZview anti-flag clone M2 affinity gel. We demonstrate that immunoprecipitation of either RPTPσ or p250GAP lead to the co-precipitation of the other partner. In Figure 4.4C, we also show that in the presence of inactive Fyn-KD the interaction between RPTPσ-D/A and p250GAP is significantly reduced as compared to Fyn-WT or activated Fyn-CA.

A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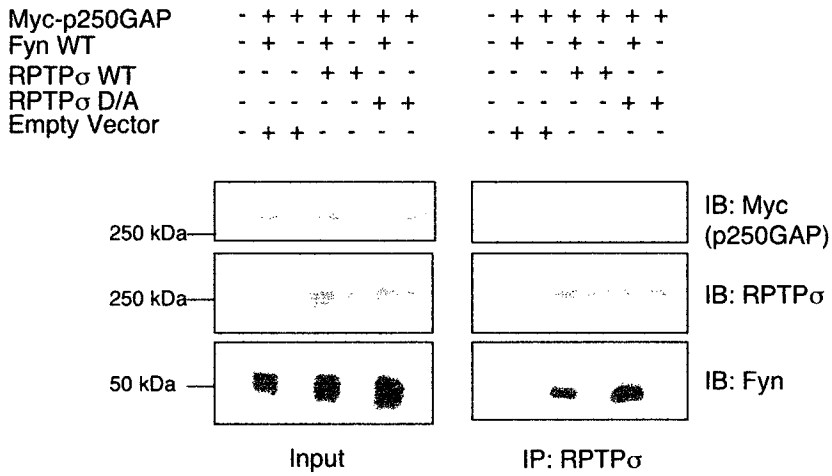
Myc-p250GAP - + + - + +
Fyn WT - - + - - +
250kDa
250kDa
IB: 4G10
IB: Myc
(p250GAP)
Input
IP: Myc

B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Fyn WT - + - + - + - - + - + - + -
RPTPσ WT - - - + + - - - - - + + - -
RPTPσ D/A - - - - - + + - - - - - + +
Empty Vector - + + - - - - - + + - - - -
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IB: Myc
(p250GAP)
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IB: Fyn
Input
IP: RPTPσ

C

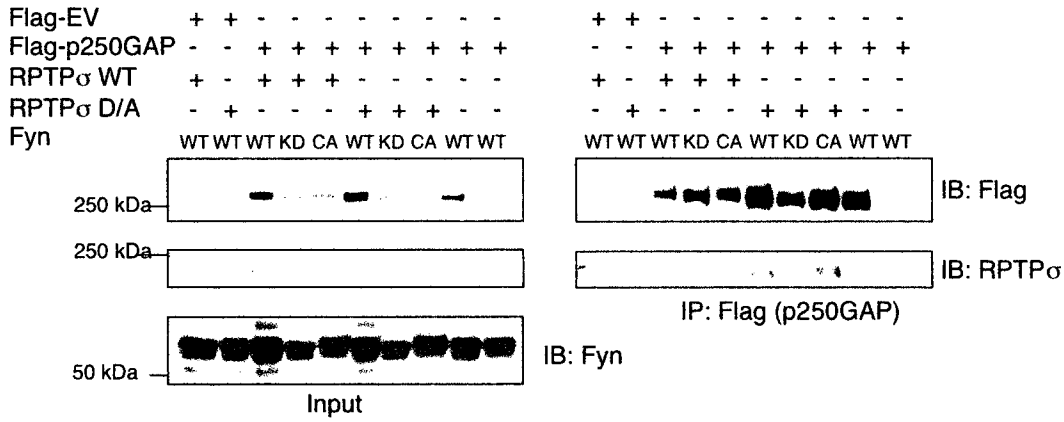

Flag-EV + + - - - - - - - - + + - - - - - - - -
Flag-p250GAP - - + + + + + + + + - - + + + + + + + +
RPTPσ WT + - + + + - - - - - + - + + + - - - - -
RPTPσ D/A - + - - - + + + - - - + - - - + + + - -
Fyn
WT WT WT KD CA WT KD CA WT WT
WT WT WT KD CA WT KD CA WT WT
250 kDa
250 kDa
50 kDa
IB: Flag
IB: RPTPσ
IP: Flag (p250GAP)
IB: Fyn
Input

Figure 4.4 Tyrosine phosphorylation of p250GAP by Fyn is required for trapping of p250GAP by RPTPσ.

A-C) Cos7 cells were transfected with the indicated constructs. Lysates of transfectant were immunoprecipitated with the indicated antibodies for 3 hours at 4°C. Samples were washed three times and separated by SDS-PAGE. The immunoprecipitates and total protein in the cell lysates were subjected to immunoblotting with the indicated antibodies. A) P250GAP is tyrosine phosphorylated by Fyn-WT. B) p250GAP is trapped more efficiently by RPTPσ-D/A than RPTPσ-WT and the trapping is improved in the presence of Fyn-WT. C) Co-Immunoprecipitation of p250GAP and RPTPσ is dependent on the activity of Fyn. WT: wild-type; KD: kinase dead; CA: constitutively active.

4.4.3 RPTPσ interacts and is a substrate of Fyn

We showed that tyrosine phosphorylation by Fyn or Src was required for the trapping of p250GAP by RPTPσ but we also wanted to know if Fyn, which is known to be tyrosine phosphorylated, was a substrate of RPTPσ as well. In figure 4.5 we show that RPTPσ and Fyn consistently interact independently of the activity of phosphatase or the kinase. Interestingly we also observed that RPTPσ was phosphorylated by Fyn. The phosphorylation levels were lower in RPTPσ-WT probably because of auto-dephosphorylation. Other RPTPs have previously been shown to be tyrosine-phosphorylated (Toledano-Katchalski and Elson, 1999; Zheng et al., 2000; Tsujikawa et al., 2002). For example, the phosphorylation of RPTPε by Neu is suggested to help direct the substrate specificity of this phosphatase (Berman-Golan and Elson, 2007). To our knowledge this is the first report that RPTPσ is tyrosine phosphorylated and could bring a new level of regulation to this enzyme.

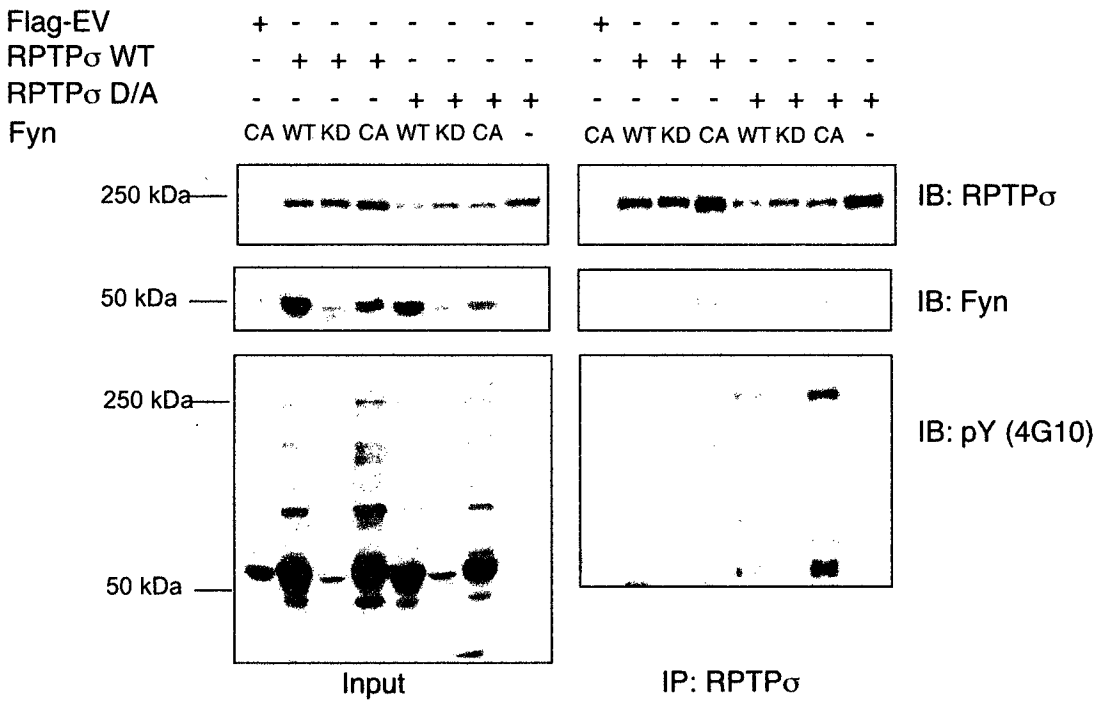


Figure 4.5 Fyn interacts with RPTPσ.

Cos7 cells were transfected with RPTPσ-WT or D/A and Fyn-WT/CA/KD. Twenty-four hours later cells were lysed and immunoprecipitated with the anti-RPTPσ monoclonal antibodies for 3 hours at 4°C. Samples were washed three times and separated by SDS-PAGE. Following Western blotting the membranes were probed with the indicated antibodies. All mutants of Fyn coimmunoprecipitated with RPTPσWT or D/A. Immunoblotting with pTyr antibodies (4G10) revealed that RPTPσ is phosphorylated by Fyn-WT and CA. Wild-type; KD: kinase dead; CA: constitutively active.

4.4.4 The membrane proximal PTP domain of RPTPσ is sufficient to trap p250GAP

As with other members of the type IIa RPTP family, RPTPσ contains two PTP domains, with the membrane proximal one (DI) being catalytically active while the membrane distal one (DII) shows relatively little PTP activity. As seen in figure 4.6, different deletion mutants of RPTPσ (DI alone, DIDII, DIDIIT (DII truncated) were transfected and although the full-length RPTPσ appears to trap a greater amount of p250GAP, the membrane proximal PTP domain (D1) was sufficient to trap p250GAP, which is consistent of p250GAP being a substrate of RPTPσ.

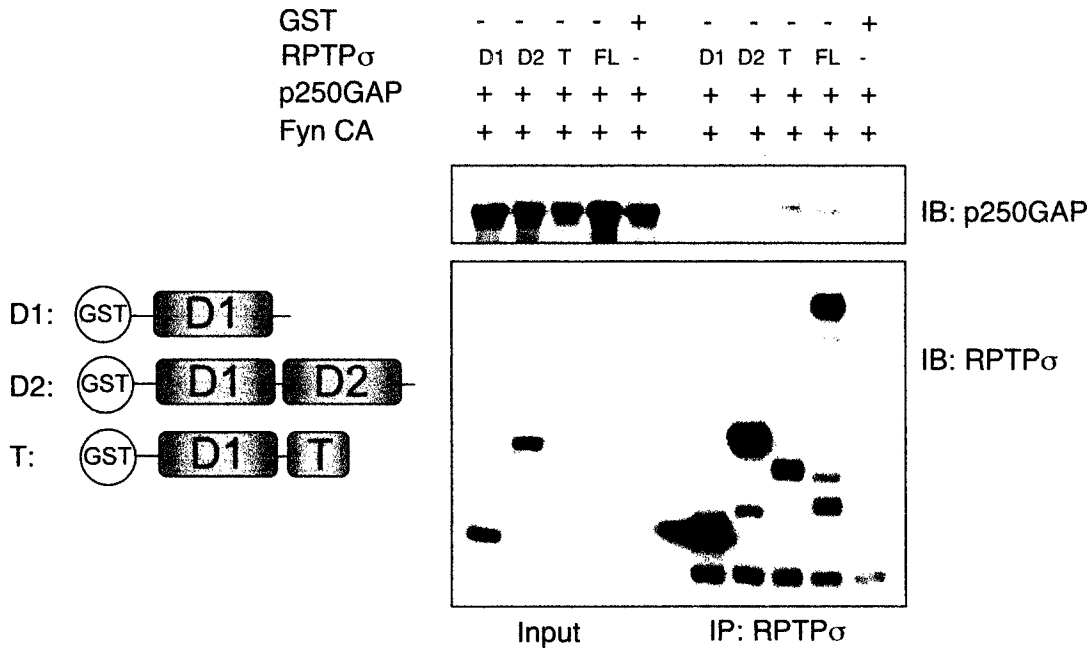


Figure 4.6 RPTPσDI-D/A is sufficient to trap phosphorylated p250GAP.

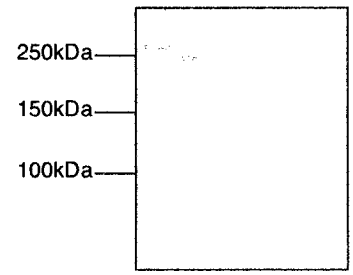
Cos7 cells were transfected with the indicated c-terminal deletion mutants of RPTPσ together with Fyn-CA and p250GAP. Cell lysates were immunoprecipitated with RPTPσ antibodies and the precipitated proteins were immunoblotted for p250GAP. The same membrane was reprobed with anti-RPTPσ to ensure proper expression of each deletion mutants.

4.4.5 Identification of the phosphorylated tyrosine residue in p250GAP

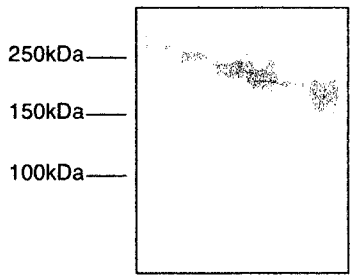
As a first step towards the identification of the specific tyrosine residues targeted by RPTPσ on p250GAP, we identified the region where Fyn tyrosine-phosphorylates p250GAP. We co-transfected various deletion mutants of p250GAP starting from the c-terminal tail with activated Fyn and assessed the tyrosine phosphorylation level of p250GAP (Figure 4.7B). Compared to full-length p250GAP, deletion mutant 1 (amino acids (aa) 1-1516) and 2 (aa 1-1314) were similarly phosphorylated by Fyn; deletion mutant 3 (aa 1-1212) exhibited a slight decrease in phosphorylation. However when the region c-terminal to the residue 1168 and beyond was deleted, virtually all tyrosine phosphorylation was lost on p250GAP (Figure 4.7A). Based on the prediction server NetPhos 2.0 (Blom et al., 1999) two tyrosine residues are most likely to be phosphorylated in the region from aa 1168-1212 which are Y1188 and Y1208. Since it was previously suggested that Fyn interacts with the c-terminal tail of p250GAP through its SH3 (Taniguchi et al., 2003) domain we cannot rule out that losing proper binding for Fyn on p250GAP will affect its ability to be phosphorylated by this kinase. Mutation of these tyrosine residues to phenylalanine to prevent phosphorylation should answer this question in the future.

A

p250GAP FL	+	-	-	-	-	-
p250GAP Del 1	-	+	-	-	-	-
p250GAP Del 2	-	-	+	-	-	-
p250GAP Del 3	-	-	-	+	-	-
p250GAP Del 4	-	-	-	-	+	-
p250GAP Del 5	-	-	-	-	-	+
Fyn CA	+	+	+	+	+	+



IB: pY (4G10)



IB: Flag (p250GAP)

IP: Flag (p250GAP)

B

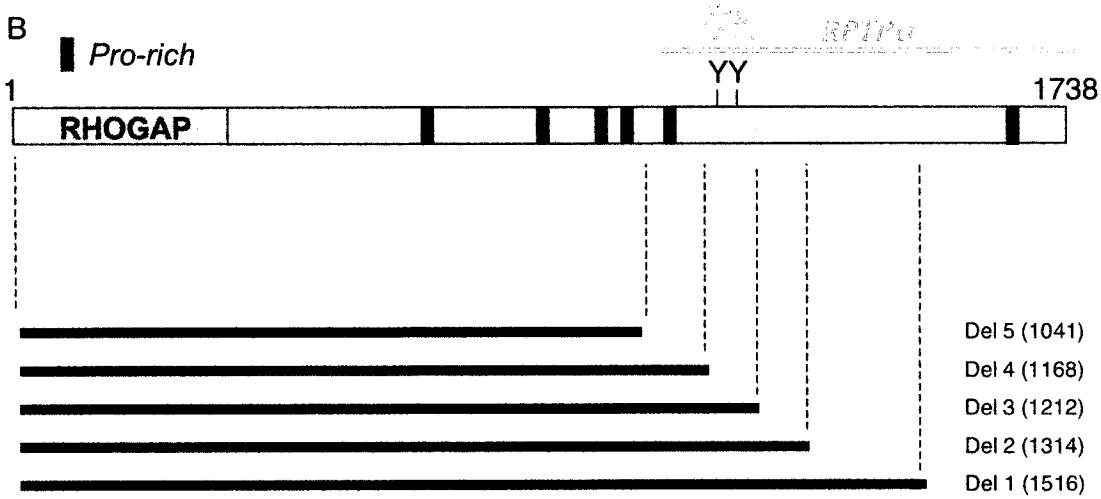


Figure 4.7 Mapping of the tyrosine phosphorylation site on p250GAP.

A) Cos7 cells were transfected with the indicated c-terminal deletion mutants of p250GAP constructs together with Fyn-CA. Flag-p250GAP was immunoprecipitated and subjected to anti-pTyr immunoblotting (Top panel). The same membrane was reprobed with anti-Flag to ensure proper expression of each deletion mutant. B) Schematic representation of the different deletion mutant of p250GAP used in A). The tyrosine residues predicted to be tyrosine phosphorylated by Fyn are indicated, as well as the region of p250Gap identified as an interactor of RPTPσ in the yeast substrate-trapping assay.

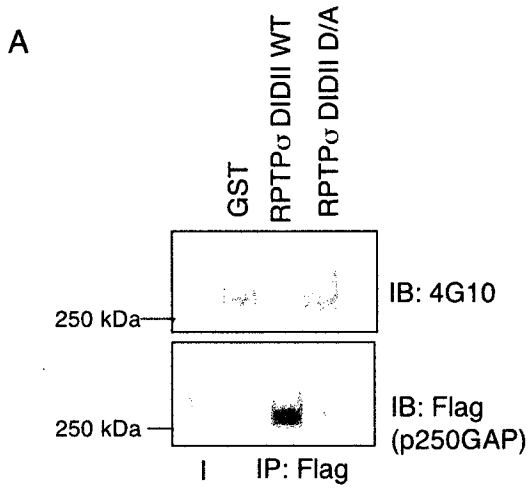
4.4.6 Dephosphorylation of p250GAP by RPTPσ

We next examined the ability of RPTPσ to dephosphorylate p250GAP *in vitro* (Figure 4.8A) and in intact mammalian cells (Figure 4.8C). Flag-p250GAP was first transfected into COS7 cells together with Fyn-WT and was then immunoprecipitated in the absence of sodium vanadate. The intracellular domain of RPTPσ, fused to GST was expressed and purified from BL21 strain of bacteria. An aliquot of the bacterially purified RPTPσ was first tested *in vitro* against pNPP substrate (data not shown) to ensure catalytic activity of the WT protein while the D/A mutant was catalytically dead. The immunoprecipitated Flag-250GAP was then incubated with purified RPTPσ WT or D/A at 37°C for 20 minutes to allow sufficient time for the dephosphorylation to occur. Whereas addition of RPTPσ-WT enzyme completely dephosphorylated p250GAP *in vitro*, the inactive mutant was unable to do so (Figure 4.8A). As controls, we then tested the ability of PTP-PEST, HePTP as well as the dual-specificity PTP MKPX to dephosphorylate p250GAP and determined that active RPTPσ remained the most efficient PTP for dephosphorylating p250GAP (Figure 4.8B). We then looked at the level of tyrosine phosphorylation of p250GAP in the presence of active RPTPσ in cells. Cos7 cells were co-transfected with p250GAP, RPTPσ WT or D/A and Fyn WT. p250GAP was then immunoprecipitated and the total tyrosine phosphorylation levels were detected by Western blotting using anti-phosphotyrosine antibody (4G10) (Figure

4.8C). In the presence of active RPTPσ, p250GAP is clearly less phosphorylated suggesting that it is an *in vivo* substrate of RPTPσ.

Figure 4.8 *In vitro* and *in vivo* dephosphorylation of p250GAP by RPTPσ.

A, B) Cos7 cells were transfected with Flag-p250GAP and Fyn CA. Lysates were immunoprecipitated with Flag-conjugated agarose beads for 2 hours at 4°C. Beads were washed three times. GST-PTPs were expressed and purified from bacteria as previously described. 10 μg (A) or 2 μg (B) of the purified PTP was added to the immunoprecipitate for 20 (A) or 15 (B) min at 37°C. Proteins were eluted in 2X SDS sample buffer. Tyrosine phosphorylation levels were detected using anti-pTyr (4G10). The membranes were reprobed with anti-Flag to ensure consistent loading of p250GAP. C) Cos7 cells were transfected with RPTPσ-WT and D/A and p250GAP, and Fyn-WT. Twenty-four hours later cells were lysed in the presence of sodium vanadate to prevent further dephosphorylation of p250GAP. Lysates were immunoprecipitated and the level of remaining tyrosine phosphorylation detected with anti-pTyr (4G10). The same membrane was reprobed with anti-Flag to ensure consistent expression of p250GAP.


A
GST
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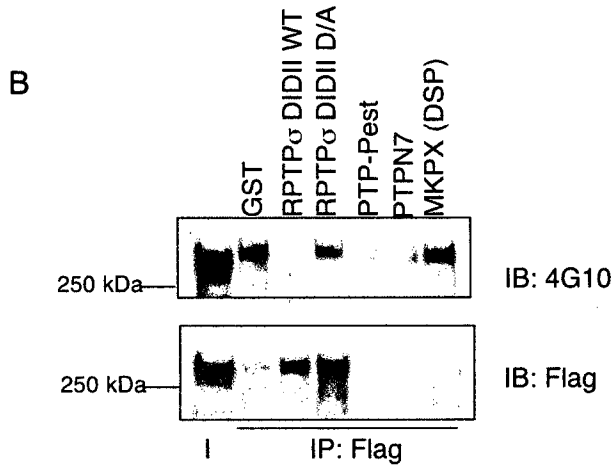
RPTPσ DIDII D/A
250 kDa
IB: 4G10
250 kDa
IB: Flag
(p250GAP)
I
IP: Flag


B
GST
RPTPσ DIDII WT
RPTPσ DIDII D/A
PTP-Pest
PTPN7
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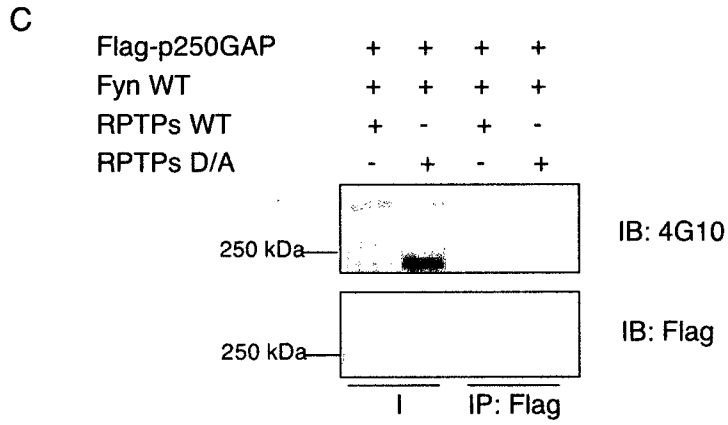
IP: Flag


C
Flag-p250GAP + + + +
Fyn WT + + + +
RPTPs WT + - + -
RPTPs D/A - + - +
250 kDa
IB: 4G10
250 kDa
IB: Flag
I
IP: Flag

4.5 Discussion

Tyrosine phosphorylation is essential for the regulation of a variety of intracellular signaling pathways and its levels are balanced by the concerted actions of kinases and phosphatases. To understand the biological role of phosphatases we need to identify its signaling partners. A yeast-two-hybrid approach has been used in the past to identify new partners of RPTPσ. At the time only the interaction of the membrane proximal PTP domain (DI) of RPTPσ with the membrane distal PTP domain (DII) of its homologue RPTPδ was identified (Wallace et al., 1998). The same result was also found by our approach. In the original screen, the library used was from an adult rat lung cDNA library and an 11-day mouse embryo library, which did not match the physiological RPTPσ expression during development. Moreover a RPTPσ-WT bait was used for screening the library. Since the interaction between a phosphatase and its phosphorylated substrate is very transient because of the high catalytic activity of PTPs, potential substrates may have been missed. In order to circumvent these issues, we employed a modified yeast-two-hybrid approach where we were able to identify protein targets that interacted with the RPTPσ-D/A substrate-trapping mutant known to bind to its substrate with similar affinity but with significantly reduced K_{cat} (Flint et al., 1997; Blanchetot et al., 2005). Moreover the assay was done in the presence or absence of an activated tyrosine kinase in order to present putative phosphorylated substrates to the phosphatase.

Trio is a RhoGTPase exchange factor for rho and rac that was previously identified through yeast-two-hybrid interaction with LAR (Debant et al., 1996), a close relative of RPTPσ. Moreover in *Drosophila* Trio was shown to interact genetically with Dlar in controlling actin remodeling (Bateman et al., 2000). In our yeast-two-hybrid assay we found multiple clones of Trio interacting with RPTPσ independently of tyrosine phosphorylation, but this interaction could not be reproduced in mammalian cells (data not shown). This suggests that at least in mammalian cells RPTPσ would have other substrates and binding partners. In the present study liprin-α4 was also found as a binding partner of RPTPσ. Liprin-α1, 2 and 3 have been known to interact with the LAR-RPTPs family and from their homology binding of liprin-α4 was expected (Pulido et al., 1995; Serra-Pages et al., 1998). Nevertheless, this is the first time that direct binding of liprin-α4 with a member LAR-RPTPs has been demonstrated. P130Cas (crk-associated substrate) is an adaptor and scaffold molecule that plays an essential role in intracellular events underlying cell motility (Defilippi et al., 2006). P130Cas can undergo extensive phosphorylation modification on tyrosine providing binding sites for SH2 and PTB containing effector proteins. In addition it also contains a SH3 domain and a proline rich region that altogether make it an ideal scaffolding molecule. We show that p130CAS is hyperphosphorylated in RPTPσ and RPTPδ (-/-) embryos suggesting that it is a physiological substrate for this sub-family of enzymes. LAR the third member of the family was indeed previously shown to dephosphorylate p130Cas,

which resulted in its destabilization and degradation leading to LAR-induced caspase dependent cell-death (Weng et al., 1999). However some clones of p130Cas were also identified to interact with RPTPσ in the yeast-two-hybrid assay independently of tyrosine phosphorylation, so we cannot rule out that it acts as an adaptor molecule for RPTPσ. Indeed p130CAS had also been previously shown to be a target of PTP-PEST and PTP1B (Garton et al., 1996) but the interaction was mainly through its SH3 domain (Liu et al., 1996; Garton et al., 1997).

Of great interest was the finding that p250GAP is a novel substrate of RPTPσ. P250GAP interacted with the RPTPσ-D/A mutant in the yeast-two-hybrid system as well as in mammalian cells only in the presence of v-Src or Fyn (WT or constitutively active) indicating that tyrosine phosphorylation was required for the interaction. In agreement, RPTPσ-D/A failed to interact with p250GAP in the presence of kinase dead Fyn, further suggesting that phosphorylation, but not the interaction with Fyn *per se* was required. Since RPTPσ-D/A interacted with p250GAP more efficiently that RPTPσ-WT and RPTPσ was able to dephosphorylate p250GAP *in vivo* and *in vitro* it appears that p250GAP is a new substrate of RPTPσ. P250GAP was previously shown to be a substrate of Src-family tyrosine kinases (Taniguchi et al., 2003) but to our knowledge this is the first report showing that a PTP could regulate its tyrosine phosphorylation level.

P250GAP (also known as Grit, RICS, GC-GAP, and p200GAP) (Nakamura et al., 2002; Moon et al., 2003; Nakazawa et al., 2003; Okabe et al., 2003; Zhao et al., 2003) is a Rho family GTPase activating protein (GAP), which promotes the intrinsic GTP-hydrolytic activity to produce a GDP-bound inactive state. The N-terminal GAP domain of p250GAP was shown to have *in vitro* activity towards rho, rac, cdc42, although the identification of its *in vivo* physiological substrates is still debated. Regardless, some reports suggest that it possesses preferential activity towards rhoA and cdc42 over that of Rac1. Beside its GAP domain, p250Gap contains several proline-rich motifs in its C-terminal region as well as several putative phosphorylation sites on serine/threonine as well as tyrosine residues, which could serve as binding sites for SH2 or PTB domain containing proteins.

P250GAP is highly expressed in the adult and developing brain with an expression pattern very similar to that of RPTPσ. Interestingly cerebellar granule cells and cortical neurons isolated from p250GAP (-/-) neurons displayed longer neurites than those isolated form wild-type mice (Nasu-Nishimura et al., 2006), which is very similar to what is observed with RPTPσ (-/-) cortical neurons (Thompson et al., 2003). Cdc42 activity in p250GAP (-/-) neurons was higher than in the wild-type neurons suggesting that p250GAP plays a role in neurite extension by regulating cdc42 activity *in vivo*. Since the ablation of RPTPσ and p250GAP in neurons leads to a similar phenotype they could possibly act in a similar pathway to

regulate neurite outgrowth. We and others showed that Fyn (Taniguchi et al., 2003) and Src (Moon et al., 2003) together with RPTPσ can regulate the tyrosine phosphorylation levels of p250GAP. However no physiological role for its tyrosine phosphorylation have been demonstrated. While, *in vitro* phosphorylation on serine residues by Ca++/calmodulin-dependent protein kinase II of p250GAP was shown to inhibit its activity (Okabe et al., 2003) the role of tyrosine phosphorylation remains elusive. Tyrosine phosphorylation by Fyn regulates the activity of p190RhoGAP (Wolf et al., 2001) and TCGAP (Liu et al., 2006). In the case of p190RhoGAP tyrosine phosphorylation affects its binding affinity to effectors such as p120RasGAP (McGlade et al., 1993) and TFII (Jiang et al., 2005). Further research will determine if p250GAP is regulated in a similar fashion.

The C-terminal region of p250GAP not only interacts with protein kinases but was also shown to exist in different complexes with several signaling partners such as TrkA, N-shc, CrkL/Crk , p130Cas, Gab1/2, Nck, β-catenin/cadherin, NR2B and PSD-95. Interestingly RPTPσ interacts and dephosphorylates all three members of the Trk-family of neurotrophins receptors and suppress NGF-dependent neurite outgrowth (Faux et al., 2007). Similarly the RhoGAP domain of p250GAP as well as the Trk-binding region of p250GAP were able to suppress neurite extension induced by NGF (Nakamura et al., 2002). Remarkably, the full-length p250GAP did not have any significant effect on NGF-induced neurite outgrowth. Thus, p250GAP regulates

neurite outgrowth in a very complex mechanism and its dephosphorylation by RPTPσ or dephosphorylation of TrkA could regulate further recruitment of downstream signaling partners such as N-Shc and Crk/CrkL. In the basal phosphorylated state p130CAS is able to interact with CrkL and p250GAP in mammalian cells. Upon EGF stimulation p130Cas tyrosine phosphorylation levels decrease and the p250GAP/CrkL/p130Cas complex is replaced by the p250GAP/CrkL/EGFR complex (Nakamura et al., 2002). Indeed we show that p130Cas can be dephosphorylated by RPTPσ. Therefore, the interplay between RPTPσ and p250GAP could in part be regulated by protein complexes recruited by the adaptor p130Cas.

The β-catenin binding domain of p250GAP is also important for its neurite outgrowth regulatory activity. Both N-cadherin and β-catenin have been shown to be direct substrates of RPTPσ and that dephosphorylation contributed to remodeling of the actin cytoskeleton and cadherin-dependent adhesiveness resulting in decreased axon outgrowth (Siu et al., 2007). Therefore the interplay between RPTPσ, p250GAP and the β-catenin/cadherin complex could bring another level of regulation in neurite outgrowth.

A role in dentritic spine morphology was also attributed to both p250GAP and RPTPσ dependent on their interaction with the β-catenin/cadherin complex.

P250GAP and β-catenin/N-cadherin associate with the NMDA receptor (N and PSD-95) and regulate spine plasticity through RhoA activation (Nakazawa et al., 2008). In response to NMDA receptor activation, p250GAP was shown to be tyrosine dephosphorylated and to relocalize from the dentritic shafts to the spine. On the other hand all three members of the LAR-RPTPs subfamily together with their binding partner liprin-α control the transport of the β-catenin/cadherin complex to another type of glutamate receptor in this case AMPA receptors to synapses and dentritic spines (Dunah et al., 2005). Altering LAR-RPTPs functions resulted in impaired development and maintenance of excitatory synapses in hippocampal neurons. Although LAR-RPTP/liprin-α complex was shown to interact specifically with AMPA receptor and not NMDA receptors, RPTPσ could still be in a prime position to regulate NMDA-dependent spine remodeling through dephosphorylation of p250GAP. Further work will be required in order to determine how tyrosine-dephosphorylation of p250GAP by RPTPσ results in similar redistribution of p250GAP as observed following NMDA stimulation.

It is now well established that RPTPσ plays an important role in the regulation of neurite outgrowth and development and in synapse morphogenesis. The identification of new signaling partners of RPTPσ such as p130CAS, Fyn and p250GAP will certainly shed light on its mechanism of action. These interactions as well as the previously identified partners N-cadherin/β-catenin, liprins-α and Trk-

family further suggest that RPTPσ plays an important role in remodeling the actin cytoskeleton. This role is conserved from *Drosophila* to mammals although the signaling partners involved are different. Also the existence of additional RPTPσ substrates cannot be excluded as more putative partners not described here were identified by the yeast-two-hybrid screen. Characterization and physiological relevance of these partners await future investigation.

4.6 Acknowledgements

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CHAPTER 5: Discussion and future perspectives

5.1 General discussion

A key mechanism of action for intracellular signaling resides on the ability of proteins to be reversibly phosphorylated by protein kinases and protein phosphatases. This post-translational modification can alter the function or activity of the target protein by inducing conformational changes, creating new docking sites for other proteins or causing its relocation within the cell. In the past years a wide range of signaling pathways regulated by protein tyrosine phosphatases (PTPs) have been elucidated. Moreover, mutations in different PTPs have been linked to specific defects in human pathologies such as cancer (Ostman et al., 2006; Hardy and Tremblay, 2008; Stuible et al., 2008), diabetes (Cheng et al., 2002; Asante-Appiah and Kennedy, 2003) and immune diseases (Mustelin et al., 2005; Pao et al., 2007). Studies using transgenic mouse models have led to the further understanding of the functional role of PTPs in signaling pathways underlying development and diseases (Hendriks et al., 2008). Nowadays, this family of enzymes can no longer be viewed simply as housekeeping genes that counteract kinase action but as true players in the signaling field.

Although LAR-RPTPs were discovered over 15 years ago, much is still unknown about the function and regulation of these enzymes. While diverse putative ligands for LAR-RPTPs have been identified, we are still far from being able to activate or inhibit these PTPs selectively. Similarly, even though Trio has been

identified as one of the most promising intracellular mediators of downstream LAR-RPTPs signaling, it is unclear if it is a common player with all members of this subfamily. Very complex and diverse cellular processes modulate LAR-RPTPs, and their potential role as regulators of nerve growth and regeneration and in modulating glucose metabolism, promises future exciting discoveries. The identification of novel signaling molecules that may control LAR-RPTPs activity encourages further investigation into this promising field of research.

Using RPTPσ deficient mice, the aim of this thesis was to further describe the physiological functions of this particular member of the LAR-RPTPs subfamily in glucose homeostasis and nerve regeneration. We also identified a new set of RPTPσ substrates, which was necessary to elucidate its mechanisms of action, and key to understanding its physiological role.

5.2 RPTPσ is essential for neuroendocrine development

5.2.1 Altered glucose homeostasis in the absence of RPTPσ

Insulin is a potent anabolic hormone whose primary function is to maintain glucose homeostasis. Several protein tyrosine phosphatases (PTPs) expressed in insulin sensitive tissues have been suggested to be key regulators of the insulin receptor (IR) signaling pathway and to attenuate insulin action (Cheng et al., 2002). Among these PTPs, RPTPσ became a candidate for further study since it is

expressed at relatively high levels in insulin-target tissues, such as fat and muscle (Norris et al., 1997). Moreover its close family member LAR was shown to dephosphorylate the IR *in vitro* (Tsujikawa et al., 2001) and overexpression of LAR in muscle caused whole-body insulin resistance (Zabolotny et al., 2001).

We found that the plasma glucose and insulin concentrations in the fasted state are lower in RPTPσ (-/-) mice as compared to wild-type control mice. The homozygous animals are also more sensitive to exogenous insulin. These results suggested that RPTPσ is somehow involved in insulin signaling *in vivo*. However, muscle tissue isolated from RPTPσ (-/-) mice did not show a significant increase in glucose uptake, suggesting that RPTPσ did not play a direct role in this process. Despite whole-body increased insulin sensitivity, at the molecular level IR tyrosine phosphorylation was not increased in muscle of RPTPσ (-/-) animals, as would be expected in insulin-sensitive animals. Taken together, our results suggest an indirect modulation of the IR signaling pathways by RPTPσ.

RPTPσ null mice have pituitary dysplasia and were shown to secrete lower levels of growth hormone (Elchebly et al., 1999a; Batt et al., 2002) leading to decreased IGF-1 production. The lack of growth hormone observed in other models of growth hormone deficient mice also results in decreased blood glucose and insulin levels concurrent with increased insulin sensitivity (Dominici et al., 2002; Yakar et

al., 2004). We therefore propose that the insulin hypersensitivity observed in RPTPσ null mice is secondary to their neuroendocrine dysplasia and GH/IGF-1 deficiency (as summarized in Figure 5.1). Taken together, we think that RPTPσ is not a major player in direct maintenance of glucose homeostasis and regulation of IR signaling.

Tyrosine phosphorylation of the insulin receptor is correlated with its increased activity. A post-receptor defect of insulin signaling is thought to underlie the basis of insulin resistance in Type II diabetes (a disease with increasing rate of occurrence in western societies) (Saltiel, 2001). Consequently the role of PTPs in this pathway has been closely scrutinized over the past several years. A number of PTPs such as LAR, RPTPα, RPTPε, TC-PTP, SHP-2 and SHP-1 were shown to dephosphorylate the IR *in vitro* (reviewed in (Asante-Appiah and Kennedy, 2003) and (Dubois et al., 2006)), although their physiological role in directly regulating IR signaling remains controversial. After years of study, PTP1B remains the best therapeutic PTP target for the treatment of diabetes; antisense-based inhibitors have proven their efficacy and are now in phase II clinical trials (Liu, 2004). However, most of the effects of PTP1B on insulin sensitivity occur in the muscle and liver (Elchebly et al., 1999b; Klaman et al., 2000). Consequently RPTPσ, which compared to other PTPs is relatively highly expressed in adipocytes, might play a more important role in fat tissues (Norris et al., 1997). Unfortunately, RPTPσ (-/-) mice have not proven to be the most efficient model to answer this question since the

systemic neuroendocrine defects of the mice may be masking the metabolic effects in the adipose tissues. One way to overcome this difficulty would be to use tissue specific knockout or *ex vivo* stimulation of adipocytes. This could help us define a specific role for RPTPσ in insulin target tissues.

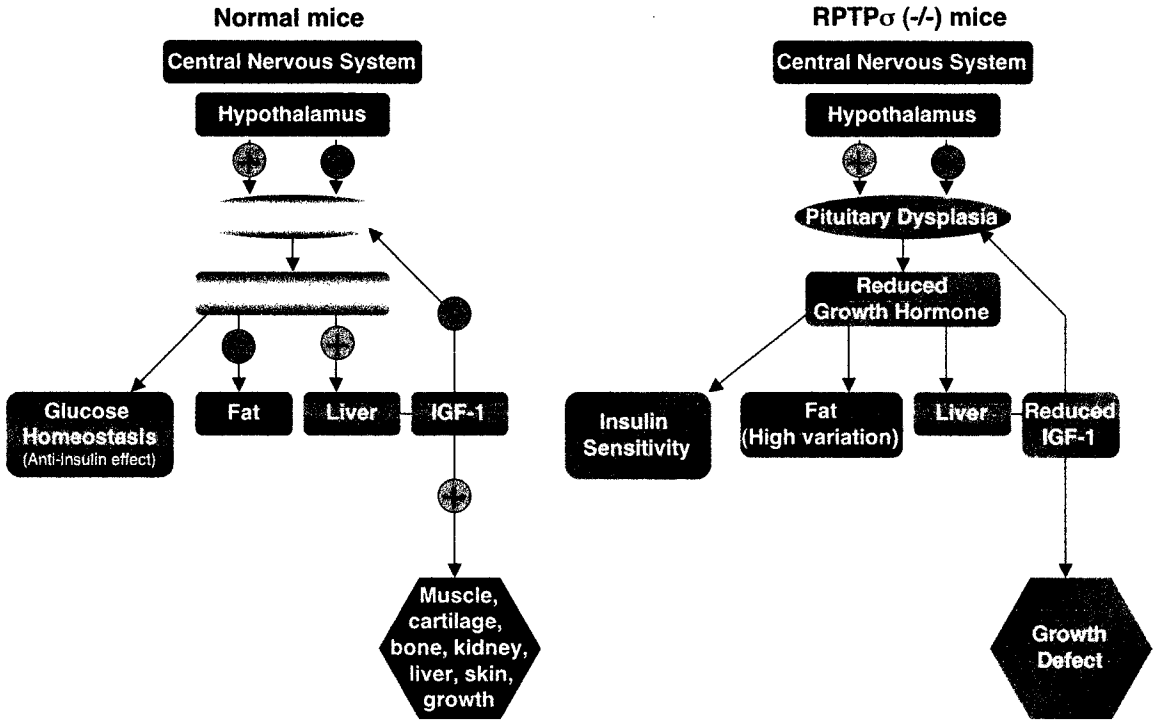

Figure 5.1 Endocrine-mediated changes in glucose homeostasis in RPTPσ (-/-) mice.

We hypothesized that the insulin hypersensitivity observed in RPTPσ (-/-) mice is likely secondary to their neuroendocrine dysplasia and GH/IGF-1 secretion deficiency.

5.2.2 RPTPσ-dependent mechanistic regulation of neuroendocrine development

The increased insulin sensitivity observed in the RPTPσ (-/-) mice is likely secondary to their neuroendocrine dysplasia. Much work will be needed to further define the mechanistic role of this enzyme in the normal development of the pituitary gland and hypothalamus. During development RPTPσ may promote proliferation and detachment as well as proper migration of the cells of Rathke's pouch, giving rise to a properly organized pituitary gland (Elchebly et al., 1999a). As discussed in chapter 4, newly identified substrates of RPTPσ such as cadherin/β-catenin (Muise et al., 2007; Siu et al., 2007) and Fyn (important for cell-cell adhesion), and p130Cas (important for migration) may help explain the role of this enzyme in these processes. Moreover the newly identified interactors may prove important for neuronal migration as well (Lilien and Balsamo, 2005). Our laboratory previously showed that RPTPσ (-/-) mice are hyposmic (Elchebly et al., 1999a) and lacking olfactory responses that are transmitted by neuronal projections into the glomerular complex of the olfactory bulb and relayed to the hippocampus; both these tissues normally express high levels of RPTPσ during development (Walton et al., 1993). These mice are also hypofecund, possibly as a result of their smaller hypothalamus, concomitant with a depletion of luteinizing hormone-releasing hormone (LHRH)-immunoreactive cells in the median eminence. In humans, interruption of LHRH and olfactory neuronal migration are typical characteristics of

Kallmann's syndrome (Crowley and Jameson, 1992). LHRH neurons exhibit axonophilic migration and follow a well-characterized migratory trajectory from the vomeronasal organ (VNO) to the forebrain along the vomeronasal nerve (reviewed in (Wierman et al., 2004)). Therefore this well-described pattern of neuronal migration could be evaluated in RPTPσ (-/-) mice and used as a model for studying the role of RPTPσ in neuronal migration, keeping in mind the new substrates and binding partners that have been discussed in this thesis.

5.3 RPTPσ interferes with nerve regeneration

The recent finding that RPTPσ hinder the rate of axonal extension and nerve regeneration (McLean et al., 2002; Thompson et al., 2003) in murine peripheral nervous system has generated interest for this enzyme as a new target in the treatment of neurodegenerative diseases and nervous system repair. RPTPσ is highly expressed in neurons of both CNS and PNS (Schaapveld et al., 1998), particularly in growth cones where it functions to slow axonal extension (Thompson et al., 2003). In the absence of RPTPσ axonal regeneration in the sciatic, facial and optic nerve is enhanced following nerve crush (McLean et al., 2002; Thompson et al., 2003; Sapieha et al., 2005). On the other hand, myelin-associated growth inhibitory proteins and extracellular matrix components of the glial scar inhibit axonal regeneration in the CNS (David and Lacroix, 2003), making spinal cord injury irreversible in normal animals.

Spinal cord hemisections, which completely damage the axons of the corticospinal tract, were performed on RPTPσ knockout mice. We demonstrated that the CNS of the RPTPσ (-/-) mouse is a novel system in which the spinal cord can spontaneously regenerate after dorsal thoracic hemisection injury. This injury model showed resprouting axons penetrating deeply into scar tissue at the lesion site. Robust long distance regeneration of severed corticospinal tract axons was observed caudal to the lesion three weeks post-injury in RPTPσ (-/-) mice. In comparison, wild-type mice did not show any regeneration of injured axons in our spinal injury models. Furthermore, isolation of primary neurons from RPTPσ (-/-) vs. control mice demonstrated that the absence of RPTPσ enhances the ability of these neurons to adhere to CSPG inhibitory substrate while no improvement was observed on myelin substrate. Therefore RPTPσ represents a new class of putative therapeutic targets contributing to the regenerating capacity of the CNS.

The activity of the RhoA RhoGTPase was shown to be increased following spinal cord injury in wild-type mice, and inhibition of RhoA led to promotion of spinal cord repairs (Dergham et al., 2002). The small RhoGTPases signal to the actin cytoskeleton during various morphological changes, including neurite outgrowth. So far we have been unable to show a direct modulation of RhoA by RPTPσ. In *Drosophila*, Dlar regulates the actin cytoskeleton reorganization through

proteins such as Trio (a guanine-nucleotide exchange factor for RhoA and Rac1) (Debant et al., 1996; Bateman et al., 2000). In this thesis we describe p250GAP as a new substrate of RPTPσ, which preferentially stimulates the GTP hydrolysis of RhoA and Cdc42 (Nakamura et al., 2002; Taniguchi et al., 2003). Taken together, we think that RPTPσ may be in a unique position to transduce extracellular signals to remodeling the cytoskeleton, leading to inhibition of nerve regeneration. RPTPσ may do this either by direct or indirect regulation of RhoGTPases.

Damage to the CNS of higher vertebrates, including humans, often results in devastating and persistent deficits such as paraplegia and quadriplegia. While hemisection injuries are an excellent model by which to study axonal regeneration, they do not reflect the typical spinal injury sustained by humans. A spinal injury model that more closely parallels a clinical situation is the contusion or weight drop injury that compresses and severs axons and spinal tissue by crushing. The contusion injury, like the hemisection, completely severs the CST. However, it also produces much more inflammation, gliosis, widespread tissue damage and cyst formation, which is even less conducive to axonal regeneration given the inhibitory effects of CSPGs, astrocytes, macrophages and other extracellular matrix factors that accumulate after such injuries (Iseda et al., 2008). It will be important to determine the levels to which RPTPσ (-/-) mice are able to functionally regenerate after a contusion injury.

Although efforts are ongoing in order to overcome the barriers to recovery it is clear that no single component is solely responsible for spinal cord regeneration failure. A combination of pharmacological and rehabilitation techniques that will recapitulate basic developmental mechanisms should lead to improved therapies (Thuret et al., 2006). However, even a small improvement in regenerative axonal sprouting and plasticity as observed in RPTPσ (-/-) mice could mediate significant improvements in clinical outcomes, i.e. the recovery of primitive neurological functions as the ability to breathe without a respirator or improved bladder control.

5.4 The role of RPTPσ in cellular signaling

To further define the role of RPTPσ in nervous system development and nerve regeneration we identified p250GAP, p130Cas, and Fyn as signaling partners using the yeast substrate-trapping system (Kawachi et al., 2001). It remains to be determined if tyrosine phosphorylation of p250GAP will influence its activity towards RhoGTPases. Further work will also determine if phosphorylation of RPTPσ by Fyn affects its activity. The newly identified partners of RPTPσ should certainly help uncover the mechanisms by which RPTPσ controls neurite outgrowth in the context of development or regeneration (summarized in Figure 5.2).

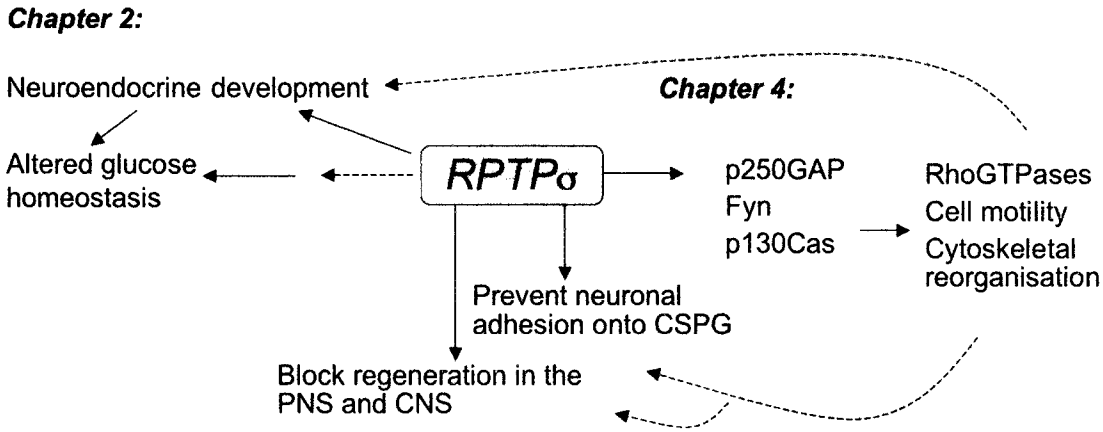

Figure 5.2 Thesis summary.

We think that the newly identified partners of RPTPσ could certainly help uncover the mechanisms by which RPTPσ controls neurite outgrowth in the context of regeneration and development. As well, they could shed light on the mechanisms by which RPTPσ regulates the development of the nervous system in particular the pituitary gland morphogenesis important for glucose homeostasis. The development of new tools to inactivate RPTPσ by dimerization or with interfering peptides could launch us into the validation of a new approach for promoting nerve regeneration.

The yeast-substrate-trapping system has proven to be an efficient alternative to traditional substrate–trapping experiments, which are restricted to the identification of abundant and highly phosphorylated substrates. Although this classical technique has been widely used in order to identify specific substrates (Garton et al., 1996), the method lacks robustness in that identification of the "trapped" substrate relies on trial and error using commercially available antibodies against proteins whose molecular weight corresponds to the size of the trapped substrate. On the other hand, the yeast-substrate trapping system is very flexible, as any PTP can be screened against any type of cDNA library. Moreover, the PTK used in the assay does not have to be restricted to v-Src, and in fact should be carefully selected according to the PTP to be tested. We have adapted this method to search for new substrates of RPTPσ and have extended the application to study other PTPs in the laboratory of Dr. Tremblay. The efficiency of identifying new substrates from such a large pool of candidates has encouraged the search for new alternative substrates for PTP-PEST and LAR to add to previously identified ones. Moreover the same method is being used to identify a first set of substrates for the dual-specificity phosphatase of the PRL family, after classical substrate-tapping experiments failed in this regard.

5.5 The next challenge: Regulating RPTPσ activity

Structural studies based on RPTPα have proposed that RPTPs are able to dimerize resulting in their inactivation (Bilwes et al., 1996). The potential of using dimerization as a mean of shutting down the activity of RPTPσ is challenging but may lead to an exciting control switch in the activity of this protein. Contrary to PTK receptors, RPTPs appear to be active as monomers, and are inactivated upon dimerization (Bilwes et al., 1996; Majeti et al., 1998). The inactivation of RPTPσ by dimerization could launch us into the validation of a new approach for promoting regeneration after nerve injury and stimulating potential regression of some neurodegenerative diseases. Importantly, in the absence of available commercial antibodies a monoclonal antibody directed against the extracellular domain of RPTPσ could be generated. We expect that the antibody could promote the dimerization of the enzyme, which in our predicted model would inactivate them. Based on this idea, it would be essential to develop a screening procedure for RPTPσ, possibly based on the Fluorescence Resonance Energy Transfer (FRET) system (Tramier et al., 2003) or using luciferase complementation imaging (Luker et al., 2004). In this manner we would be able to examine RPTPσ dimerization in real time, and could develop screening approaches for reagents (proteins, antibodies or small molecules agonists/ antagonists) that modulate RPTPσ through its dimerization.

Inducing dimerization of RPTPσ is not the only means by which its activity could be modulated. Interfering peptides may also be used to inhibit the activity of RPTPσ. In the symmetrical RPTP dimer there is an inhibitory helix-turn-helix wedge motif from one PTP domain that occludes the active site of the partner domain. Based on this structure, when a LAR wedge domain peptide was introduced in cells it exhibited homophilic binding to LAR and affected LAR-dependent signaling (Xie et al., 2006) consistent with inhibition of LAR. This new approach is a valid alternative to small molecule inhibitors, which in the case of PTP have proven to be non-specific because of the conserved nature of the PTP catalytic site. The generation of inhibitory peptides or antibodies that distinctively target individual members of the LAR-RPTPs family will be essential to ascertain each of their roles in nervous system development and may eventually serve as potential therapies for nerve regeneration.

CHAPTER 6: Concluding remarks

In this thesis, I show that RPTPσ deficient mice have a defect in maintenance of glucose homeostasis and are hypersensitive to insulin. However, RPTPσ is unlikely to play a direct role in this process and the defects seen are probably secondary to their neuroendocrine deficiency. On the other hand, RPTPσ appears to play a significant role in the prevention of nerve regeneration in the CNS. Further work will allow us to determine if this RPTPσ activity is intrinsic to regenerating neurons or comes from the inhibitory environment making it more permissive. Undoubtedly, the identification of the new set of substrates and/or interacting partners we presented here will shed light on its mechanisms of action and help us understand the physiological role of this enzyme.

RPTPσ has exciting potential to be used as a regulator of nerve growth and regeneration, and perhaps even in modulating glucose metabolism. Challenges remain because of the difficulties in regulating the activity of this enzyme. The identification of novel signaling molecules that may control RPTPσ activity encourages further investigation into this promising field of research, one that is likely to be of great value for the improvement of human health.

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APPENDIX