GENE EXPRESSION IN THE MOUSE CEREBELLAR CORTEX

DISSERTATION

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By

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ABSTRACT

The R2B subtype RPTPs consist of PTP\(\mu\), PTP\(\kappa\), PCP-2, and RPTP\(\rho\). The R2B subtype contains variable extracellular domains, a single transmembrane domain, and an intracellular region composed of two phosphatase domains. The domains found in the extracellular segment of the R2B subtype are commonly found in cell adhesion molecules. The R2B subtype shares a high degree of sequence similarity at the amino acid level, and members are characterized by the presence of an N-terminal MAM domain. This subtype is developmentally regulated in the CNS.

Although the cDNA sequences of these phosphatases are very similar, in the mouse, each of the four genes (PTP\(\mu\), PTP\(\kappa\), PCP-2, and RPTP\(\rho\)) is differentially expressed throughout the body. Within the normal adult mouse cerebellum, in situ hybridization studies have shown that the four R2B RPTP family members have both unique and overlapping mRNA expression patterns. In addition to the normal mouse cerebellum, several mutants exist in which cerebellar morphology is altered in the anterior-posterior plane, including rostral cerebellar malformation (rcm/rcm), Lurcher (Lc/+), and weaver (wv/wv). In situ hybridization, using \(^{35}\)S
and digoxigenin-labeled cRNA probes, was used to determine if the RPTP\(\rho\) expression boundary in the three strains is affected by the reported morphological abnormalities.

The next step was to generate RPTP\(\rho\)-specific polyclonal antibodies that would recognize the native protein in mouse cerebellum, and more specifically to localize the protein within individual cell types. In addition, antibodies available for two other R2B RPTPs (PTP\(\mu\) and PTP\(\kappa\)) were used to localize each protein within specific neuronal types in the mouse cerebellum.

Finally, the aging central nervous system (CNS) is controlled by expression of specific genes; however, the complexity of this system has made it difficult to identify these genes. SAGE (Serial Analysis of Gene Expression), a sequencing-based approach was used to identify and quantify all expressed cerebellar genes in the developing, adult, and aging C57BL/6 mouse cerebellum. This ‘open’ system technology is appropriate for detection and quantification of known genes and novel genes for which no sequence information exists. This analysis identified low abundance genes, which included the R2B subtype RPTPs.
Dedicated to Mihail, Ruxanda, Alexandru, and Paul
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CHAPTER 1

INTRODUCTION

Protein tyrosine phosphorylation is a post-translational modification essential for signal transduction pathways regulating many cellular processes, including growth and differentiation, metabolism, development, gene expression, cell adhesion and migration, and cytoskeletal function (reviewed in Stoker and Dutta, 1998; Tonks and Neel, 2001; Hellberg et al., 2002). Tyrosine phosphorylation is a reversible process maintained by the coordinated and competing actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). PTKs and PTPs phosphorylate and dephosphorylate tyrosine residues (Figure 1.1), respectively; thereby, maintaining cellular phosphotyrosine levels in equilibrium (Tonks and Neel, 2001; Hellberg et al., 2002). Initially, protein tyrosine kinases were considered to be the primary enzymes regulating tyrosine phosphorylation, while the few protein tyrosine phosphatases known were believed to be housekeeping genes or scavengers of phosphotyrosine (Stoker and Dutta, 1998; Tonks and Neel, 2001). In recent years, a number of protein tyrosine phosphatases have been identified and shown to exhibit distinct roles in protein tyrosine dephosphorylation.
Figure 1.1: Protein Tyrosine Phosphorylation and Dephosphorylation. Protein tyrosine kinases transfer a phosphate group from ATP to a tyrosine residue on a target protein (phosphorylation). Protein tyrosine phosphatases reverse this action by removing the phosphate group (dephosphorylation). The outcome of the two reactions is the hydrolysis of ATP to ADP and orthophosphate ($\text{PO}_4^{3-}$).

The first classical protein tyrosine phosphatase, intracellular PTP1B, was purified from human placenta (Tonks et al., 1988a). This 37kDa protein spanned 321 amino acids, and consisted predominantly of a catalytic domain (Tonks et al., 1988a, Tonks et al., 1988b). The PTP1B amino acid sequence was unrelated to the serine/threonine protein phosphatases; but was homologous to the two intracellular domains of CD45 (also known as LCA, leukocyte common antigen, or Ly-5) (Thomas, 1989; George and Parker, 1990). CD45 is a glycoprotein found exclusively in hematopoietic cells, with a role in lymphocyte signaling (Charbonneau et al., 1988). Structurally, CD45 consists of an external segment containing a ligand-binding domain, a single transmembrane domain, and an intracellular segment containing two protein tyrosine phosphatase domains. Although the catalytic functions of CD45 were unknown (Thomas, 1989), structural similarities between PTP1B and CD45 suggested that CD45
might have protein tyrosine phosphatase activity. This was confirmed by studies showing that the cytoplasmic domains of CD45 catalyze the dephosphorylation of phosphotyrosine-containing proteins (Tonks et al., 1988c; Ostergaard et al., 1989; Tonks et al., 1990; Fischer et al., 1991). The extracellular domain of CD45, which is highly glycosylated, was able to bind ligands on either identical or adjacent cells. Thus, CD45 was identified as the first type I receptor protein tyrosine phosphatase, and designated as the prototype of the receptor protein tyrosine phosphatase (RPTP) family (Tonks et al., 1988c; George and Parker, 1990).

1.1 Classification of Protein Tyrosine Phosphatases (PTPs)

Following the discovery of PTP1B, additional tyrosine phosphatases, including many splice variants, were isolated and characterized within vertebrates and invertebrates. The majority of PTPs have been identified in human and rodents, and also are present in *Xenopus* (Johnson and Holt, 2000), *Drosophila melanogaster* (Streuli et al., 1989; Tian et al. 1991), yeast (Ottillie et al., 1991; Guan et al., 1991b; Ota and Varshavsky, 1992), the worm *Caenorhabditis elegans* (Matthews et al., 1991), and a protochordate *Styela plicata* (Matthews et al., 1991). An increasing number of X-ray crystal structures indicate that PTPs are structurally diverse; however a conserved protein tyrosine phosphatase domain is characteristic of all family members (Andersen et al., 2001).
PTPs may be divided into three groups: Dual-specificity phosphatases (DSPs), low-molecular weight PTPs (LMW-PTPs), and the classical PTPs (Andersen et al., 2001; Tonks and Neel, 2001). The DSPs dephosphorylate tyrosine residues, as well as threonine and serine residues (Hooft van Huijsduijnen, 1998; Keyse, 2000). The low-molecular weight PTPs exist with no sequence similarity to the other two groups, except for the presence of a conserved CX_5R motif found in PTPs (Ramponi and Stefani, 1997; Andersen et al., 2001). Classical PTPs have been further divided into non-transmembrane and transmembrane, receptor-like (RPTPs) based on their subcellular location (Tonks and Neel, 2001).

Within the classical PTP category, the non-transmembrane protein tyrosine phosphatases (also known as cytoplasmic, intracellular, or nuclear PTPs) contain a single catalytic phosphatase domain and additional variable noncatalytic sequences at either the N- or C-terminus (Naegele and Lombroso, 1994). Noncatalytic regions are believed to play a regulatory role, either by binding to substrates to control protein-protein interactions, or by targeting the PTP to a particular subcellular location. In some cases, these regions are involved in directly modulating enzyme activity. Nontransmembrane PTPs are classified into nine subtypes (NT1-NT9) based on sequence similarity between the catalytic phosphatase domains (Andersen et al., 2001). Examples of this family include PTP1B, T-cell PTP, SHP1, and SHP2. Many of these enzymes, such as SHP1 and SHP2, contain SH2 domains (src homology domain 2) at the N-terminus, which bind to tyrosine-phosphorylated residues (Matthews et al., 1992); the SH2
domain is present within src-like kinases, as well as in other proteins involved in signaling pathways. Other nontransmembrane family members contain a FERM domain, a PDZ domain, a PEST-like domain, or a combination of these domains, classifying each into distinct subtypes (Andersen et al., 2001).

The transmembrane PTPs, also known as receptor protein tyrosine phosphatases (RPTPs) (Figure 1.2), consist of variable extracellular domains similar to those found in cell adhesion molecules, a single transmembrane domain, and an intracellular region composed of either one or two highly conserved phosphatase domains (Brady-Kalnay and Tonks, 1995; Brady-Kalnay, 2001; Johnson and Van Vactor, 2003). RPTPs were originally classified into five types designated I-V, based on the structure of their extracellular domains (Brady-Kalnay and Tonks, 1995; Bixby, 2000). Identification of novel RPTPs suggested the existence of additional categories (Schaapveld et al., 1997a), and a more recent subtype classification exists consisting of eight subtypes (R1-R8) based on sequence similarity between the PTP catalytic domains (reviewed in Andersen et al., 2001).

Members of the R1/R6 RPTP subtype, such as CD45, are alternatively spliced into multiple isoforms depending on the size or structure of their extracellular domains. CD45 contains a highly glycosylated cysteine-rich segment and a single FNIII repeat within the extracellular region (Brady-Kalnay and Tonks, 1995). CD45 was identified to regulate signal transduction via T-cell and B-cell receptors in lymphocyte adhesion (Trowbridge and Thomas, 1994). This phosphatase is believed to bind proteins that regulate adhesion and
integrin-dependent adhesion pathways. For example, CD45 controls adhesion between the integrin LFA-1 and ICAM-1 or ICAM-3 (intracellular cell adhesion molecule), which are members of the immunoglobulin superfamily (Arroyo et al., 1994; Wagner et al., 1993). Another member of the R1/R6 subtype is chicken PTPλ, which is now considered the avian homolog of CD45 (Andersen et al., 2001).

The R2 subtype, further divided into R2A and R2B, contains large extracellular segments made up of immunoglobulin-like domains (Ig-like) and fibronectin type III (FNIII) repeats (Brady-Kalnay and Tonks, 1995; Brady-Kalnay, 2001). The R2A subtype includes LAR (leukocyte common antigen-related), DLAR, PTPσ, PTPδ, DPTP69D, HmLAR 1 and HmLAR2, and CRYPα, which contain large extracellular domains, similar to those in neural cell adhesion molecules, and consist of 1-3 consecutive Ig-like domains and 2-10 consecutive FNIII repeats. LAR is considered the prototype for receptor protein tyrosine phosphatases containing cell adhesion domains. The R2B subtype, which is comprised of PTPμ, PTPκ, PCP-2, and RPTPρ share a high degree of sequence similarity. Their genes contain an additional motif at the N-terminus known as the MAM (Meprin, A5 and PTPμ) domain. In addition, this subtype contains a subtilisin protease cleavage site (Barr, 1991), and an intracellular juxtamembrane-like domain (Brady-Kalnay and Tonks, 1995).

The R3 subtype, such as DPTP99A, DPTP10D, mOST-PTP, SAP1, and GLEPP1 contain an extracellular domain consisting of various numbers of FN III repeats, and in some cases contain only one intracellular phosphatase domain.
(Stoker and Dutta, 1998; Li and Dixon, 2000). The R4 subtype, such as PTPα and PTPε, contain very short, glycosylated extracellular domains. The R5 subtype RPTPs, such as PTPβ/ζ and PTPγ, have a long extracellular segment containing an N-terminal carbonic anhydrase (CA)-like domain and a single FN III repeat (Stoker and Dutta, 1998). The CA-like domain is thought to act as a substrate for neuronal adhesion and the initiation of neurite outgrowth and differentiation (Tonks and Neel, 1996). PTPβ/ζ also contains a secreted form, referred to as phosphacan, which is a soluble proteoglycan in the brain.

The most recent RPTP subtype, R7, includes PTP-BR7 (also known as PC12 PTP) and PTP-SL, which contains only one cytoplasmic phosphatase domain. These RPTPs show the greatest homology to cytosolic PTPs (Hendriks et al., 1995; Ogata et al., 1995, Sharma and Lombroso, 1995; Andersen et al., 2001). An additional RPTP subtype, R8, includes proteins IA-2 (also known as PTPLP, ICA512, and the mouse homolog PTP35) [Lan et al., 1994; Lu et al., 1994; Kambayashi et al., 1995; Magistrelli et al., 1995], and IA-2β (also known as PTP-NP) [Lu et al., 1996; Chiang et al., 1996; Wasmeier et al., 1996]; both are transmembrane molecules with a RDGS adhesion recognition motif and a single phosphatase domain.
Figure 1.2: Schematic of Receptor-like Protein Tyrosine Phosphatase Classification. The RPTPs consist of variable extracellular domains, a single transmembrane domain, and an intracellular region consisting of either one or two phosphatase domains. This family of enzymes may be divided into eight subtypes (R1-R8) based on sequence similarity among PTP catalytic domains. **TM**=Transmembrane.
1.2 Genomic Organization and Function of the R2B RPTP Subtype:

The R2B RPTPs consist of PTPκ (PTPRK), PTPµ (PTPRM), PCP-2 (PTPRL/U), and RPTPρ (PTPRT). Table 1.1 shows the four human R2B RPTPs, as well as homologs in various vertebrate species. The R2B subtype shares a high degree of amino acid sequence similarity, and members are characterized by the presence of an N-terminal MAM domain. This subtype is developmentally regulated in the central nervous system (Stoker and Dutta, 1998; McAndrew et al., 1998a, b; Fuchs et al., 1998; Besco et al., 2003), and has been identified only in vertebrate species (Walchli et al., 2000). Recently, the genomic organization of the R2B RPTPs was described, showing that both human and mouse genes ranged in size from 88kb to ~1Mb (Besco et al., 2001, 2003; Besco, 2003). In the mouse, the *ptprk* is located on chromosome 10, *ptprm* on 17, *ptprl* on 4, and *ptprt* on 2. In the human, the four genes PTPRK, PTPRM, PTPRL/U, and PTPRT are located on similar regions of chromosomes 6, 18, 1, and 20, respectively (Besco et al., 2003).
Table 1.1 Members of the R2B subtype. Table shows all the R2B family members identified to date in vertebrate species (human, mouse, rat, and *Xenopus*). PTPκ identified in rat represents a soluble version of the entire extracellular domain of PTPκ. Human PTPπ, PTPψ, hPTP-J, and PTPRO share >98% sequence similarity with human PCP-2, and all represent the same gene (shown in the Unigene database found at http://www.ncbi.nlm.nih.gov). PTPf and PTPλ are mouse homologs of PCP-2. RPTPmam4, RPTPrho-1, and RPTPrho-2 are recently identified homologs of mouse PTPρ. RPTPρ and RPTPmam4 are found in the brain, whereas RPTPrho-1 and RPTPrho-2 are found in pancreatic beta cells.
1.2.1 **Extracellular Segment:** The presence of an N-terminal MAM domain makes the R2B subtype unique among the RPTPs. The MAM (Meprin, A5 and PTPµ) domain is a structure first identified in three proteins: meprin A and B, the *Xenopus* A5 glycoprotein, and PTPµ. This domain is comprised of 170 amino acids including two conserved sequence motifs, *tChtFahxxtt* and *ttGhhxhD-hxh* (*t*=turn or polar residues, *h*=hydrophobic residues, *a*=aromatic residues, and *x*=any amino acid residue) and four conserved cysteine residues (Beckmann and Bork, 1993). Meprins are glycoproteins, with metalloendopeptidase activity, and exist as homodimers or heterodimers (Jiang et al., 1992). The *Xenopus* A5 antigen is a transmembrane protein that is predominantly expressed in the visual centers within the brain and is developmentally regulated (Takagi et al., 1991).

In addition to the MAM domain, the R2B RPTPs contain a single immunoglobulin-like (Ig-like) domain and four fibronectin type III (FNIII) repeats; these domains are characteristic of cell adhesion molecules (Gebbink et al., 1991; Brady-Kalnay, 2001). The Ig-like domain is found in a variety of proteins, including cell adhesion or extracellular matrix molecules (Williams and Barclay, 1988; Reichardt and Tomaselli, 1991), and has been identified as the homophilic binding site of cell adhesion molecules, such as neural cell adhesion molecule (N-CAM) (Rao et al., 1992). Members of the NCAM family, include N-CAM, Ng-CAM, fasciclin II, neuroglian, and contactin; this family demonstrates homophilic interactions by binding identical molecules on the surface of adjacent cells (Rutishauser and Jessell, 1988; Edelman and Crossin, 1991). The FNIII repeat
contains a highly conserved hydrophobic region, consisting of 90-100 amino acids, which was originally observed in the extracellular matrix protein fibronectin (Potts and Campbell, 1994); however in most cases its function is unknown.

1.2.2 R2B RPTPs mediate cell adhesion via domains in the extracellular segment: The R2B subtype contains a MAM, an Ig-like, and four FN III domains within the extracellular segment. The Ig-like and FN III domains are structurally similar to those of cell adhesion molecules. Both cadherins and neural cell adhesion molecule (NCAM) have been shown to bind via a homophilic mechanism (Edelman et al. 1987; Takeichi, 1991; Rao et al., 1992). In other words, one molecule on the cell surface binds to an identical molecule on an adjacent cell. Homology between the Ig-like domain and FN III repeats of the cell adhesion molecules and the R2B RPTPs suggested a role for these phosphatases in homophilic binding (Brady-Kalnay et al., 1993; Sap et al., 1994). The first members of the R2B subtype, PTP$_\mu$ and PTP$_\kappa$, were shown to mediate cell-cell adhesion by homophilic binding via the extracellular segment (Brady-Kalnay et al., 1993; Sap et al., 1994; Brady-Kalnay and Tonks, 1994b; Gebbink et al., 1994). PTP$_\mu$ (Brady-Kalnay et al., 1993; Gebbink et al., 1993) and PTP$_\kappa$ (Sap et al., 1994) were expressed independently in nonadherent insect cells, using a baculovirus expression system, to demonstrate that only PTP$_\mu$- or PTP$_\kappa$-expressing cells formed stable cell aggregates. Results suggested that these molecules interact in a homophilic manner. In addition, results from these aggregation studies indicated that cell adhesion mediated by either PTP$_\mu$ or
PTPκ does not require phosphatase activity or proteolytic cleavage, and is calcium-independent (Brady-Kalnay et al., 1993; Gebbink et al., 1993; Sap et al., 1994). Further studies using mink lung (MvLu) epithelial cells, a cell line endogenously expressing PTPμ, showed that on the cell surface PTPμ expression increased with cell density. Overall, PTPμ mediated homophilic binding and was regulated by cell-cell contact (Brady-Kalnay et al., 1993; Gebbink et al., 1995).

Although the above studies suggested that the MAM domain was the homophilic binding site for these R2B proteins, *in vitro* binding assays using various fragments of the PTPμ extracellular segment identified the Ig-like domain to be adequate for homophilic binding (Brady-Kalnay and Tonks, 1994b). The MAM domain and FN III repeats did not promote aggregation in these assays, suggesting that these domains were not directly involved in homophilic binding. *In vitro* studies suggested that during cell-cell aggregation, the MAM domain was involved in sorting PTPμ from homologous molecules such as PTPκ (Brady-Kalnay et al., 1993; Brady-Kalnay and Tonks, 1994b).

Although these two molecules are structurally similar, chimeric studies using a PTPμ molecule containing the MAM domain of PTPκ demonstrated that PTPμ and PTPκ do not interact in a heterophilic manner (Zondag et al., 1995). In addition, a mutant PTPμ construct, lacking the N-terminal MAM domain, was used to show lack of cell-cell aggregation in cell culture (Zondag et al., 1995). This study suggested that the MAM domain, which was believed to play a direct role in aggregation mediated by PTPμ, contained the PTPμ homophilic binding
site. The different results obtained by these two groups may be explained by differences in the types of assays used. Overall, these studies suggest that the ligand for this RPTP is another PTPµ molecule on an adjacent cell, and the extracellular segment is involved in this homophilic interaction.

**1.2.3 Proteolytic cleavage: shedding of the extracellular segment of R2**

RPTPs: A potential proteolytic furin-like cleavage site characterizes several R2 RPTPs, including LAR, PTPκ, PTPµ, and RPTPρ. This region consists of a cluster of basic amino acids in the extracellular segment, within the last FNIII repeat. LAR, a member of the R2A subtype, was the first RPTP shown to be proteolytically cleaved at the basic sequence \( \text{RRRRR} \), generating a complex of two non-covalently associated subunits (Streuli et al., 1992). These subunits consist of a 150kDa E subunit (contains most the Extracellular segment), and a smaller 85kDa P subunit (consists mostly of the transmembrane and intracellular segments, including both PTP domains) (Figure 1.3). The tight association between these two subunits was shown to exist only in growing cells. Once cells were confluent the E subunit was released, whereas the P subunit was degraded. The reason for this type of proteolytic cleavage is not yet known, but it is thought to regulate adhesive properties of specific cell-cell contacts (Johnson and Van Vactor, 2003). In addition, cell culture studies using HeLa cells demonstrated that the E subunit of LAR is released from the cell surface during high cell density (Streuli et al., 1992; O'Grady et al., 1994).
The R2B RPTPs also contain similar basic amino acids within the fourth FN III repeat, which includes PTP<sub>κ</sub> (RTKR) (Jiang et al., 1993), PTP<sub>µ</sub> (RPRRTKK) (Brady-Kalnay and Tonks, 1994a), PTPf (RXKR/RR) (Yoneya et al., 1997), PTP<sub>λ</sub> (RLRR) (Cheng et al., 1997), and RPTP<sub>ρ</sub> (KSRR) (McAndrew et al., 1998a). Site-directed mutagenesis studies confirmed that the RTKR sequence is the endoprotease cleavage site in human PTP<sub>κ</sub> (Fuchs et al., 1996).

Studies were conducted with PTP<sub>µ</sub> in mink lung epithelial (MvLu) cells, showing that the E-subunit was also released from the surface of cells at high density (Brady-Kalnay and Tonks, 1994b). PTP<sub>µ</sub> was immunoprecipitated with a polyclonal C-terminal anti-peptide antibody 37; results identified the full-length glycosylated protein at 195kDa and proteolytically cleaved products at 100kDa (doublet). Next, the immunoprecipitates were treated with Endo F to remove N-linked carbohydrates; results produced a full-length unglycosylated product at 160kDa, and two cleaved products at about 68kDa and 93kDa. In conclusion, the cleaved products consisted of an N-terminal fragment containing the extracellular segment, and a C-terminal segment consisting of a small region of the extracellular segment, the transmembrane domain, and the intracellular domains. Therefore, PTP<sub>µ</sub> was shown to be proteolytically cleaved into two non-covalently associated subunits; both cleaved and uncleaved products were present on the cell surface.
Figure 1.3: Post-translational proteolytic cleavage of the R2 RPTPs. LAR, an R2A RPTP, and several R2B RPTPs (which include PTPκ, PTPµ, RPTPρ, and two mouse forms of PCP-2, PTPf and PTPλ) contain potential proteolytic furin-like cleavage sites consisting of a cluster of basic amino acids in their extracellular segments. The E subunit (ranging from 71-150kDa) contains most of the extracellular segment, whereas the P subunit (ranging from 85-100kDa) consists mostly of the transmembrane and intracellular segment.
Cell culture studies showed both N- and C-terminal PTP$_{\mu}$ (Brady-Kalnay and Tonks, 1994b) and PTP$_{\kappa}$ cleavage products (Jiang et al., 1993) were each approximately 100kDa, which remained tightly associated following proteolytic cleavage. Interestingly, proteolytic cleavage was not observed for human PCP-2 in either endogenously expressing cells (WRL-68 cells) or transfected COS-7 cells. Both immunoprecipitation and Western analysis, using antibodies against the juxtamembrane domain or against the C-terminus, did not detect any cleavage products (Yan et al., 2002). In contrast, a furin-like cleavage site was observed in mouse PTP$_{\lambda}$. Immunoprecipitation and Western blotting of neonatal mouse brain extracts, with an affinity purified antibody directed against a GST-fusion protein of the PTP$_{\lambda}$ extracellular domain, produced a glycosylated full-length form (~190kDa) and two smaller cleavage fragments (~115kDa & ~70kDa) (Cheng et al., 1997). It was hypothesized that the lack of a furin-like cleavage site in human PCP-2, which is highly conserved in the other R2B members, lead to an absence in proteolytic cleavage. This may suggest that different molecular mechanisms for processing exist within the R2B subtype.

1.2.4 Intracellular Segment: The intracellular segment of the R2B RPTPs consists of two consecutive phosphatase domains and an extended juxtamembrane region, which consists of ~130 amino acid residues, compared to 70-90 amino acids in other RPTPs. Compared to the other domains within the R2B subtype, the juxtamembrane region contains the greatest variability in exon size and number (Besco, 2003; Besco et al., 2003). This region is homologous
to the intracellular domain of the cadherins, suggesting a role in the regulation of cell adhesion (Tonks et al., 1992; Brady-Kalnay and Tonks, 1994b, c; Brady-Kalnay et al., 1995; Yan et al., 2002). Within cadherins, this segment interacts with catenins to mediate association with the actin cytoskeleton. The R2B RPTP juxtamembrane region is thought to be involved in substrate recognition and specificity.

The R2B subtype contains two phosphatase domains consisting of a membrane-proximal (N-terminal or D1) PTP domain, which is catalytically active, and a membrane-distal (C-terminal or D2) inactive PTP domain. The catalytically active D1 phosphatase domain is highly conserved in sequence and structure, and is thought to be a common evolutionary origin for the RPTPs (Besco et al., 2003; Charbonneau and Tonks, 1992; Barford et al., 1994; Barford et al., 1995; Bilwes et al., 1996). This D1 phosphatase domain contains a conserved 11 amino acid active site motif, often referred to as the CX₅R motif or “PTP loop” [(I/V)HCxAGxxR(S/T)G] (x=amino acid residue) (Streuli et al., 1989; Zhang et al., 1994; Andersen et al., 2001). Specific cysteine and arginine residues, located within the active site motif, were identified to contribute to the catalytic activity of the phosphatase domain (Streuli et al., 1989; Pot et al., 1991; Zongchao, 1997; Denu and Dixon, 1998). Another catalytically important residue is an aspartic acid (Asp), located approximately 30-40 residues from the active site cysteine on a flexible loop (Li and Dixon, 2000; Tonks and Neel, 2001). Substrate binding induces a conformational change upon the flexible loop within the active site. Upon substrate binding, the active site forms a closed conformational loop,
known as the ‘WPD loop’, around the tyrosine-phosphorylated residue. In the closed state, the conserved Asp residue in the loop is moved within the active site, thereby decreasing the stability of the enzyme-substrate complex (Li and Dixon, 2000; Tonks and Neel, 2001). The membrane-distal PTP (D2) domain, which is highly conserved in sequence and structure among the R2B subtype, does not contain the essential residues for catalysis (Hooft van Huijsduijnen, 1998; Nam et al., 1999).

### 1.2.5 R2B RPTP crystal structure and dimerization compared to other subtypes

Three-dimensional structures of protein tyrosine phosphatases have provided clues about their enzymatic mechanisms that distinguish between phosphorylated tyrosine residues and phosphorylated serine/threonine residues on a substrate. Specifically, identification of these structures has led to the understanding of the interactions that stabilize the active site and form the substrate complex (Zhang, 2002). The PTP active site is a deep pocket, which is specific for phosphorylated tyrosine residues. Phosphorylated serine and threonine residues are too short to access the catalytic cysteine residue within the active site (Barford et al., 1994; Stuckey et al., 1994). Thus, the depth of the PTP active site pocket appears to regulate specificity for phosphorylated tyrosine residues.

The first crystal structure for an RPTP was identified for mouse PTPα, which displayed a stable dimer or multimer quaternary structure (Bilwes et al., 1996; Tertoolen et al., 2001). Recently, crystal structures of R2 RPTPs have been
identified, including the first phosphatase domain of PTP\(\mu\) (Hoffmann et al., 1997) and both phosphatase domains of LAR (Nam et al., 1999). Within the tertiary structure, a high degree of amino acid identity existed between crystal structures of RPTP phosphatase domains (Bilwes et al., 1996; Hoffman et al., 1997; Jiang et al., 1999). However, comparative analysis of the RPTP quaternary crystal structures indicated that PTP\(\alpha\) crystallized as a dimer, whereas PTP\(\mu\) and LAR appeared to crystallize as monomers (Nam et al., 1999).

Structural studies showed that dimerization of PTP\(\alpha\) potentially blocked access to the D1 phosphatase domain active site, suggesting a dimerization mechanism for inactivation of RPTP activity (Bilwes et al., 1996). A study using a chimeric receptor, containing the intracellular region of CD45 and the extracellular region of the EGF receptor, demonstrated that the D1 phosphatase domain formed a dimer inhibiting phosphatase activity by forming an ‘inhibitory wedge’ (Majeti et al., 1998). It was suggested that the wedge region of one monomer, which was structurally constrained, inserted itself into the active site of the other monomer; thereby, causing a conformational change and making the phosphatase domain inaccessible to potential substrates. Based on sequence alignments, it was proposed that the structurally constrained wedge region was a conserved protein motif present in the RPTP family. As more RPTP ligands were identified, a model was proposed stating that ligand binding affected RPTP activity through oligomerization. This potential model implied that on the cell surface, RPTPs existed as inactive dimers; and upon ligand binding, the dimer
dissociated, resulting in activation of phosphatase activity. Therefore, ligand binding induced RPTPs to form dimmers; whereas the C-terminal domains within the dimers inhibited the catalytic activity of the N-terminal domains (Neel and Tonks, 1997).

On the other hand, the proposed dimerization-induced inhibition model proposed for PTPα did not apply for LAR and PTPμ. The crystal structure of the two LAR phosphatase domains indicated that these domains were on opposite sides of the protein, making this type of inhibition unlikely (Nam et al., 1999). In the case of PTPμ, the catalytic site and N-terminal helix-turn-helix region did not form protein-protein interactions (Hoffmann et al., 1997). PTPα crystallized as a dimer, whereas PTPμ and LAR appeared to crystallize as monomers (Nam et al., 1999). Due to differences between the quaternary crystal structures of these RPTP molecules, the dimerization-induced inhibition model proposed for PTPα may not be a mechanism used by all RPTPs.

1.2.6 The R2B RPTP intracellular segment associates with the cadherin-catenin complex: In addition to homophilic binding, the R2B subtype members PTPκ (Fuchs et al., 1996), PTPλ (Cheng et al., 1997), PTPμ (Brady-Kalnay et al., 1995; Brady-Kalnay et al., 1998; Hiscox and Jiang, 1998), PCP-2 (Yan et al., 2002), and RPTPρ (Besco, 2003) have been shown to associate with the cadherin-catenin complex in various tissues and cell lines. Members of the cadherin-catenin family are key molecules involved in cell-cell adhesion, connecting adjacent cells. Cadherins are Ca^{2+}-dependent transmembrane
proteins that interact with catenins via their cytoplasmic domains; thereby, associating with the actin cytoskeleton (Gumbiner, 2000). Within neurons, cadherins are thought to function as adhesion molecules at the synapse (Uchida, et al., 1996; Kohmura, et al., 1998), or are involved in neurite outgrowth (Colman, 1997).

It has been suggested that protein tyrosine phosphorylation controls adhesive properties of cadherin, through the association of the R2B RPTPs with the cadherin-catenin complex. Studies using the human mammary carcinoma cell line (SK-BR-3) showed that endogenously expressed PTP\(\kappa\) associates with \(\beta\)-catenin and \(\gamma\)-catenin at adherens junctions, which suggested that \(\beta\)-catenin may be a potential substrate for human PTP\(\kappa\) (Fuchs et al., 1996).

Immunoprecipitation studies of MvLu lysates with antibodies to PTP\(\mu\), demonstrated that the PTP\(\mu\) intracellular segment associates with the cadherin-catenin complex; this association was also shown to occur in rat heart, lung and brain tissues (Brady-Kalnay et al., 1995). Additional in vitro binding studies demonstrated that PTP\(\mu\) binds directly to the intracellular domain of E-cadherin, but not to \(\alpha\)-catenin or \(\beta\)-catenin (Brady-Kalnay et al., 1995). However, when Zondag et al., 1996 performed a similar study using a variety of antibodies against PTP\(\mu\), cadherins, and catenins; it was concluded that PTP\(\mu\) did not interact with the cadherin-catenin complex. The latter study used the same antibodies against PTP\(\mu\), as those used in the earlier study (Brady-Kalnay et al., 1995), but the results showed precipitation of cadherins in a nonspecific manner,
which appeared to be independent of \( \mathrm{PTP}\mu \). Zondag et al., 1996 concluded that the RPTP antibody (BK2) cross-reacted with cadherins, which resulted in the interactions seen in the Brady-Kalnay study. Based on the results of the Zondag group, \( \mathrm{PTP}\mu \) did not interact with the cadherin-catenin complex; therefore it was unlikely that \( \mathrm{PTP}\mu \) directly regulated cadherin-catenin function (Zondag et al., 1996). Zondag and colleagues later showed that \( \mathrm{PTP}\mu \) overexpression in COS cells resulted in binding of the juxtamembrane domain and the second phosphatase domain of \( \mathrm{PTP}\mu \) to \( p120\)cas, a catenin family member that interacts with cadherins (Zondag et al., 2000).

In a more recent study by Brady-Kalnay et al., 1998, the association of \( \mathrm{PTP}\mu \) was further characterized. Contrary to Zondag’s argument, it was demonstrated that the \( \mathrm{PTP}\mu \) antibody (BK2) did not cross-react with cadherin, and in addition, cadherin was immunoprecipitated with \( \mathrm{PTP}\mu \) by three epitope-specific \( \mathrm{PTP}\mu \) antibodies (Brady-Kalnay et al., 1998). Rat lung extracts were used to show the interaction of \( \mathrm{PTP}\mu \) with E-cadherin, N-cadherin, and R-cadherin. The interaction between \( \mathrm{PTP}\mu \) and E-cadherin was also shown using WC5 cells, a cell line expressing a mutant form of v-Src. Evidence showed that E-cadherin contained 38 residues within the C-terminal region of the intracellular segment which were required for association with \( \mathrm{PTP}\mu \) (Brady-Kalnay et al., 1998). Therefore, this work confirmed observations in previous studies that \( \mathrm{PTP}\mu \) and E-cadherin associated \textit{in vitro}, as well as \textit{in vivo}. 
PCP-2 was identified in human pancreatic adenocarcinoma cells and the protein was shown to co-localize with β-catenin and E-cadherin at areas of cell-cell contact (Wang et al., 1996). A recent study reported that PCP-2 was endogenously expressed at the cell surface, and similar to findings with PTPμ (Gebbink et al., 1995) and PTPκ (Fuchs et al., 1996), elevated levels of mRNA and protein expression increased with cell density (Yan et al., 2002). In addition, an in vivo binding assay showed evidence that a region within the PCP-2 juxtamembrane domain directly interacted with its substrate β-catenin. This interaction was independent of the catalytic activity of PCP-2 and whether β-catenin was tyrosine phosphorylated (Yan et al., 2002). Overall, it was concluded from this study that PCP-2 and β-catenin interaction was involved in cell-cell contact.

Recently, studies have shown that RPTPρ also associates with the cadherin-catenin complex (Besco, 2003). Glutathione S-transferase (GST) pulldown assays and immunoprecipitation phosphatase assays were used to determine interactions between RPTPρ and proteins. The GST-pulldown assays showed that RPTPρ interacted with several proteins in mouse brain homogenates and MIN-6 cell lysates, a cell line endogenously-expressing RPTPρ. These proteins included N- and E-cadherin, cadherin-5, pp120, α-actinin, and α-, β-, and γ-catenin (Besco, 2003); each of these proteins are known molecular components of adherens junctions. RPTPρ also interacted with desmoglein, a desmosomal junction protein. An immunoprecipitation phosphatase assay was used to verify
these results. Results from this assay confirmed that E-cadherin was a likely substrate for RPTPρ, whereas pp120 was a potential substrate, although the latter may interact with other phosphatases.

1.2.7 R2B RPTP role in axon growth and guidance:

Axon growth and guidance require signals generated through protein tyrosine phosphorylation in the growth cone. RPTPs were first shown to be involved in axon guidance and synapse formation in *Drosophila* (Tian et al., 1991; Desai et al., 1994; Krueger et al., 1996). The completed *Drosophila* genome contains eight RPTP genes (Morrison et al., 2000), of which four of these genes are required in axonal development and guidance. Four *Drosophila* RPTPs, DPTP99A, DPTP69D, DLAR, and DPTP10D, were localized to specific embryonic CNS axons and were found to be required for guidance of specific axons (Desai et al., 1996, Krueger et al., 1996). Initially, roles for RPTPs in vertebrate axon guidance were less known, as is the case in mice with mutations in LAR, PTPκ, PTPα, and PTPε. These mice developed normally and had no obvious neural defects (Skarnes et al., 1995). However, more recent studies have shown that vertebrate RPTPs are involved in axon outgrowth and guidance (Stoker, 2001). Cell culture studies with PTPμ and PTPκ showed that soluble ectodomain proteins promoted neurite outgrowth (Burden-Gulley and Brady-Kalnay, 1999; Drosopoulos et al., 1999). The PTPμ ectodomain acted as a substrate for neurite outgrowth, and was present in a complex with N-cadherin in retinal neurons.
Additional studies using chick retinal ganglion cells, provided evidence that PTP\(_\mu\) was required for N-cadherin-mediated neurite outgrowth (Burden-Gulley and Brady-Kalnay, 1999) and axon guidance (Burden-Gulley et al., 2002). It was suggested that PTP\(_\mu\) dephosphorylates a region within the cadherin-catenin complex, which may lead to increased adhesion and growth cone motility. Studies demonstrated that the catalytically inactive form of PTP\(_\mu\) blocked neurite outgrowth mediated by N-cadherin, suggesting that PTP\(_\mu\) phosphatase activity of was required for this process (Burden-Gulley and Brady-Kalnay, 1999). However, these cell culture studies did not demonstrate whether ligand-binding directly activates or inactivates PTP\(_\mu\) catalytic function.

The PTP\(_\mu\) intracellular segment was shown to bind RACK1, a scaffolding protein identified as Receptor for Activated C Kinase, and localize contacts mature adherens junctions (Mourton et al., 2001). A yeast-two hybrid screen was used to isolate RACK-1 as a PTP\(_\mu\)-interacting protein (Mourton et al., 2001). Later studies indicated that the protein kinase C delta (PKC-\(\delta\)) pathway is important in PTP\(_\mu\)-dependent neurite outgrowth (Rosdahl et al., 2002), and that rho GTPases are required for PTP\(_\mu\) signaling in chick retinal ganglion cells (Rosdahl et al., 2003). Antibody perturbation and protein localization results suggested that Cdc42 (a small G-protein) may regulate PTP\(_\mu\)-dependent growth cone rearrangement (Rosdahl et al., 2003). A recent retroviral expression study,
using the LNCaP prostate carcinoma cell line, suggested that PTP\(\mu\) cytoplasmic domain regulates cell adhesion mediated by E-cadherin. Overall, these studies suggest that PTP-\(\mu\) binds RACK1, which then recruits PKC-\(\delta\), resulting in neurite outgrowth.

1.3 Summary of dissertation

This dissertation focuses on gene expression in the mouse cerebellum. Chapter 2 describes the mRNA expression patterns of the R2B subtype, including RPTP\(\rho\), in the normal C57BL/6 mouse cerebellum. In chapter 3, RPTP\(\rho\) mRNA expression in cerebellar mouse mutants is discussed. Chapter 4 describes the production and characterization of recombinant fusion proteins and anti-peptides for the generation of RPTP\(\rho\) polyclonal antibodies. Chapter 5 discusses gene expression profiling using serial analysis of gene expression (SAGE) in the aging mouse cerebellum.
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CHAPTER 2

R2B RPTP mRNA EXPRESSION PATTERNS IN THE MURINE OLFACTORY BULB, CORTEX, HIPPOCAMPUS, AND CEREBELLUM

Protein tyrosine phosphorylation plays a key role in many cellular processes, especially in the central nervous system (CNS). Within the developing brain, tyrosine phosphorylation regulates growth cone elongation, neuronal survival and differentiation, and cell adhesion; whereas, in the adult brain it regulates neurotransmitter receptor binding and synaptic plasticity (Naegele and Lombroso; 1994; Doherty and Walsh, 1994). The cellular localization of receptor protein tyrosine phosphatases provides essential information for a variety of physiological processes. Little is known about the expression pattern of the R2B RPTP molecules in the CNS. Although earlier studies identified brain regions expressing the R2B RPTP mRNAs, they were not comprehensive and technical issues made it difficult to assign R2B expression to specific cell types. In this chapter, each of the four R2B RPTP family members was localized to specific cell types in the olfactory bulb, cerebral cortex, hippocampus and cerebellum of the adult mouse, using in situ hybridization with digoxigenin-labeled riboprobes.
2.1 RESULTS:

2.1.1 Cellular expression of R2B phosphatases in the olfactory bulb, cortex and hippocampus.

The cellular localization of the four R2B transcripts in specific forebrain regions (olfactory bulb, cortex and hippocampus) is shown in Figure 2.1. At postnatal day (P)180, each of the four R2B transcripts was expressed at moderate to high levels in the mitral, external granule and glomerular layers of the olfactory bulb, and at lower levels in the external plexiform layer. All four R2B transcripts were distributed throughout the cerebral cortex, with the highest levels observed in layers II, IV, and V (RPTP$_\rho$), IV and V (PTP$_\mu$), II to V (PTP$_\kappa$), and II through VI (PCP-2). Within the hippocampus and dentate gyrus, large cells (Golgi II neurons) scattered throughout the hippocampal CA1, CA2, and CA3 regions, oriens and pyramidal layers, the hilus and subiculum, expressed RPTP$_\rho$ and PTP$_\mu$ at very high levels. Similarly, the PTP$_\kappa$ and PCP-2 transcripts were also present in Golgi II neurons; however, expression was restricted to cells in the hilus (PTP$_\kappa$, PCP-2) and subiculum (PCP-2). Much higher expression levels were present in hippocampal pyramidal cells and dentate granule cells.
Figure 2.1 R2B RPTP mRNA expression in C57BL/6 mouse olfactory bulb, cerebral cortex, and hippocampus. In situ hybridization using digoxigenin-labeled riboprobes was used to detect mRNA expression in sagittal sections of a P180 male C57BL/6 mouse brain. **Olfactory bulb:** ac, anterior commissure; g, granular layer; m, mitral cell layer; gl, glomerular layer; epl, external plexiform layer. **Cerebral Cortex:** layers I-VI; **Hippocampus:** h, hilar region; or, oriens layer; py, pyramidal layer; r, radiatum layer; d, dentate gyrus; GII, Golgi II cells. Scale bars= 50 microns.
2.1.2 Localization of R2B mRNAs in C57BL/6 mouse cerebellar neurons.

The mouse cerebellum is an excellent model system in which to study the molecular constituents of synaptic organization essential for the control of movement and balance. The adult cerebellar cortex is a highly organized, laminar structure with a limited number of neuronal types. It is organized into ten folia (cerebellar lobules 1-10), with the primary fissure demarcating a morphological boundary between folia 5 and 6. This morphological boundary separates the cerebellar cortex into distinct anterior (folia 1-5) and posterior (folia 6-10) lobes (Altman and Bayer, 1985). Each folium consists of three layers (molecular, Purkinje cell, and granule cell layer), each containing five major neuronal types (Purkinje, granule, stellate, basket, and Golgi cells) and Bergmann glia (Altman and Bayer, 1985; Kandel et al., 1991) (Figure 2.2). In the cerebellum, between 80-85% of all neurons are granule cells (Herrup and Keumerle, 1997). The large Purkinje cell bodies, as well as the smaller cell bodies of the Bergmann glia, form a monolayer between the molecular and granule cell layers. At the molecular level, the cerebellum is highly compartmentalized (Hawkes and Gravel, 1991; Hawkes and Mascher, 1994; Herrup and Kuemerle, 1997). Various molecular markers indicate parasagittal and rostrocaudal organization not affected in the morphology.

The cerebellum receives two major inputs: the first from mossy fibers, arising from multiple sources, and the second from climbing fibers originating in the inferior olivary complex (Hawkes and Mascher, 1994). Mossy fibers provide excitatory input to Golgi and granule cells in the granule cell layer, whereas the
climbing fibers provide input to Purkinje cell dendrites in the molecular layer. Purkinje cells also receive excitatory (glutamatergic) input from granule cell parallel fibers, while stellate and basket cells, located in the molecular layer, provide inhibitory (GABAergic) input. Purkinje cells provide the sole output from the cerebellum, extending their axons ventrally from the cerebellar cortex into the deep cerebellar nuclei (DCN) (Hawkes and Mascher, 1995; Matoba et al., 2000).
Figure 2.2: Organization of the cerebellar cortex. Schematic showing the three cerebellar layers (molecular, Purkinje cell, and granule cell layer), each containing five major neuronal types (Purkinje, granule, stellate, basket, and Golgi cells).
Each of the four R2B transcripts was differentially expressed in the cerebellum. RPTP$_{\rho}$ mRNA was almost entirely restricted to the granule cell layer of lobules 1-6 of the cerebellar cortex and deep cerebellar neurons (see McAndrew et al., 1998a, b). In addition, the more sensitive digoxigenin technique allowed the detection of very sparse labeling in basket and stellate cells in the molecular layer (Figure 2.3 A, B). PTP$_{\mu}$ was expressed at high levels in the Purkinje cells, and at much lower levels in Golgi, stellate and basket cells (Figure 2.3C, D). The PTP$_{\kappa}$ transcript was present at low levels in basket, stellate, Golgi and granule cells throughout the cerebellar molecular and granule cell layers (Figure 2.3E, F). PCP-2 was expressed at moderate levels in granule and Purkinje cells, and at lower levels in basket and stellate cells, and in deep cerebellar neurons (Figure 2.3G, H). The sense signal for each of the four genes (not shown) was very low and distributed uniformly across sections, indicating that non-specific expression was negligible. Table 2.1 summarizes the in situ results obtained for the R2B RPTP mRNA expression in the adult C57BL/6 mouse cerebellum.
Figure 2.3: R2B RPTP mRNA expression in the C57BL/6 mouse cerebellum. In situ hybridization using digoxigenin-labeled riboprobes was used to localize R2B mRNAs in sagittal sections of a P180 male C57BL/6 mouse cerebellum. A, B. RPTP\(\rho\) mRNA is present in granule cells in anterior folia; arrowhead in A shows anterior-posterior boundary at the dorsal surface of lobule 6. C, D. PTP\(\mu\) mRNA is present in Purkinje cells throughout the rostrocaudal extent. E, F. PTP\(\kappa\) mRNA is present in basket and stellate cells throughout the molecular layer (m), and at low levels in granule cells. G, H. PCP-2 mRNA is localized in granule cells and Purkinje cells. P, Purkinje cell; g, granule cell; G, Golgi cell; s, stellate cell; b, basket cell; m, molecular layer; dcn, deep cerebellar nuclei. Scale bars: A, C, E, G= 500 microns; B, D, F, H= 100 microns.
Table 2.1 R2B mRNA expression in the adult C57BL/6 mouse cerebellum.
Signal intensity= + (least) to ++++ (most)

2.2 DISCUSSION:

Northern blot and in situ hybridization studies have shown that the RPTPρ, PTPκ, PTPμ and PCP-2 contain both exclusive and overlapping mRNA expression patterns in many tissues throughout embryonic and postnatal development. In Northern blots using human and adult mouse mRNA, two PTPκ transcripts (5.3kb and 7.0kb) were detected in liver, kidney, intestine, lung, placenta, brain, skeletal muscle, and heart (Jiang et al., 1993; Fuchs et al., 1998), and an alternatively spliced variant (4.2kb) was found in the brain (Fuchs et al., 1998). A single 5.7kb PTPμ transcript was detected in placenta, brain, skeletal muscle, and heart (Fuchs et al., 1998). Another study, using a similar panel of tissues, detected the PTPμ transcript predominantly in lung, and lower levels in heart and brain (Gebbink et al., 1991). A single PCP-2 transcript (5.5kb) was detected in skeletal muscle (Wang et al., 1996), brain and placenta (Fuchs et al., 1998; Wang et al., 1996). RPTPρ (12kb) was present only in the brain (McAndrew et al., 1998a). These data are summarized in Table 2.2.

<table>
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<th>R2B transcript</th>
<th>granule cells (g)</th>
<th>Purkinje cells (P)</th>
<th>Golgi cells (G)</th>
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<td>(Anterior lobes 1-6)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTPμ</td>
<td>-</td>
<td>++++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PTPκ</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>PCP-2</td>
<td>++++</td>
<td>++++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

47
The expression patterns of PTPκ, PTPμ, and PCP-2 were also examined by in situ hybridization in perinatal (Fuchs et al., 1998), and adult mouse brain (Fuchs et al., 1998; Shen et al., 1999): PTPκ mRNA was present in the olfactory system, brain, lung, kidney, and heart at E12.5-E18.5 (Fuchs et al., 1998); PTPμ was detected in developing blood vessels, lung, and brain; and PCP-2 was detected in the visual system, brain, and lung. RPTPρ was present in the embryonic (E14-18) brain and spinal cord (McAndrew et al., 1998a, b).

Although the above studies identified general brain regions expressing the R2B RPTP mRNAs, the resolution was not adequate to show expression in individual cells. The present studies using digoxigenin-labeled probes, extend the information in the earlier work, and give a far more detailed account of the expression patterns of R2B molecules in the adult mouse brain.
<table>
<thead>
<tr>
<th>RPTP</th>
<th>Species</th>
<th>Age</th>
<th>Transcript size(kb)</th>
<th>Tissue Distribution</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTPκ</td>
<td>mouse</td>
<td>adult</td>
<td>5.3 &amp; 7.0</td>
<td>Different levels in kidney, liver, lung, heart, brain. 5.3kb high levels in liver &amp; kidney</td>
<td>Jiang et al. (1993) Mol &amp; Cell. Bio 13: 2942-2951</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>adult</td>
<td>5.3 &amp; 7.0 4.2(ASV)</td>
<td>High levels in brain, liver, kidney, heart, intestine, placenta, skeletal muscle, low intensity in lung, 4.2kb in brain.</td>
<td>Fuchs et al. (1998) Mech. Of Dev. 70:91-109</td>
</tr>
<tr>
<td>PTPμ</td>
<td>mouse</td>
<td>adult</td>
<td>5.7</td>
<td>Predominantly in lung. Lower levels in heart and brain</td>
<td>Gebbink et al. (1991) FEBS 290:123-130</td>
</tr>
<tr>
<td>PCP-2</td>
<td>human</td>
<td>adult</td>
<td>5.5</td>
<td>High levels in brain, skeletal muscle, pancreas</td>
<td>Wang et al. (1996) Oncogene 12:2555-2562</td>
</tr>
<tr>
<td></td>
<td>mouse</td>
<td>adult</td>
<td>5.5</td>
<td>Strong signal in brain &amp; placenta (PCP-2 expression seems to be restricted to adult mouse tissues)</td>
<td>Fuchs et al. (1998) Mech. Of Dev. 70:91-109</td>
</tr>
</tbody>
</table>

Table 2.2: Northern blot analysis of the R2B RPTPs. ASV= alternatively spliced variant
2.3 MATERIALS AND METHODS:

*Tissue Preparation:* Adult C57BL/6 mice were sacrificed under isofluorane anesthetic; brains were removed rapidly, frozen in powdered dry ice, and sectioned (20µm) in the sagittal plane. Sections were mounted onto gelatin-coated slides and stored at −70°C until ready for use.

*R2B RPTP Riboprobe Design:* RT-PCR and PCR primers were designed based on the mouse sequences provided in GenBank for RPTP$_\rho$ (NM_021464), PTP$_\mu$ (NM_008984), PTP$_\kappa$ (NM_008983), and PCP-2 (NM_011214) and ordered from IDT. RT-PCR primers were designed near the 3’ end of the second phosphatase domain (before the STOP codon), and PCR primers were designed to amplify the region corresponding to the first and second phosphatase domains of each R2B RPTP molecule. Table 2.3 shows the primers used in RT-PCR and PCR experiments.

PCR products were digested with the corresponding restriction enzymes, gel purified using the QIAquick gel extraction kit (QIAGEN Cat#28704), and inserted into the pBLUEscript II KS vector (Stratagene, Cat# 212207). The RPTP$_\rho$ riboprobe template was designed to represent a 1.72kb coding region (541 amino acids), corresponding to amino acid #909-1448 (NT#2909-4528), including the first and second phosphatase domains and the stop codon.
The FC#20 human RPTPr subclone (McAndrew et al. 1998a) was used as the PCR template, using the following sequence specific PCR primers, with flanking BamHI and XbaI restriction sites: FC20 Forward Primer (F20PT) (30mer: hRPTPrho NT#2908-2929; 5'-CGGGATCCCTACGGGTTCAAGGAGGAATA C-3'); FC20 Reverse Primer (RPT) (33mer: hRPTPrho NT#4528-4504; 5'-GCAAGCTTCTGTTCC GGGTCTCCACCATGTTG-3'). The FC#20 subclone was obtained from a Stratagene Lambda Zap II human frontal cortex library (McAndrew et al, 1998a, b), and inserted in the pBLUEScript (pBSII KS) plasmid. The PCR reaction contained 0.25μM each of the forward and reverse primers (IDT, Inc.), 1x PLATINUM Taq DNA Polymerase Buffer (GIBCO BRL, Cat#Y02028; minus Magnesium), 0.2mM each dNTP (GIBCO BRL, 10mM dNTP mix Cat#18427-013), 1.5mM MgCl₂ (GIBCO BRL, Cat#Y02016), 2.5U PLATINUM Taq DNA Polymerase (GIBCO BRL, Cat#10966-034), and 50-150ng plasmid DNA from the FC#20 subclone. The 50μl reaction was denatured at

<table>
<thead>
<tr>
<th>R2B RPTP</th>
<th>RT-PCR primer</th>
<th>Forward PCR Primer</th>
<th>Reverse PCR Primer</th>
<th>PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTPρ</td>
<td>FC20 clone</td>
<td>5'-CGGGATCCGCTACGG GTTCAAGGAGGAATA-3'</td>
<td>5'-GCAAGCTTCTGTCC AGGGTCTCCACCATGTTG-3'</td>
<td>94°C for 30sec, 65°C for 30sec, 68°C for 2min; 35 cycles</td>
</tr>
<tr>
<td>PTPδ</td>
<td>5'-GAAGACATCCAC AGTTCTCTG GTT-3'</td>
<td>5'-GCAAGCTTCTATGG TCTCAAGGAGG-3'</td>
<td>5'-CGTCTAGATCTCACA GACAATGCTC-3'</td>
<td>94°C for 20sec, 62°C for 30sec, 72°C for 2min; 35 cycles</td>
</tr>
<tr>
<td>PTPκ</td>
<td>5'-AACATCCACCAC ATTTTGCGCT-3'</td>
<td>5'-CGGGATCCCTATGG TTTCAAAAGAGG-3'</td>
<td>5'-CGTCTAGACCTATGG CACAGAACAT-3'</td>
<td>94°C for 20sec, 63°C for 30sec, 72°C for 2min; 35 cycles</td>
</tr>
<tr>
<td>PCP-2</td>
<td>5'-GTCTTCCACACG TTGTTAAATGTCCA C-3'</td>
<td>5'-CGGGATCCCAAAGAG AAAGACAAAGCTCAA-3'</td>
<td>5'-CGGGATCCCTATGACGA CACCATGTGAAA-3'</td>
<td>94°C for 20sec, 60°C for 30sec, 72°C for 1min; 35 cycles</td>
</tr>
</tbody>
</table>

Table 2.3 RT-PCR and PCR primers and PCR conditions.
94°C for 30s, annealed at 65°C for 30s, with an extension at 68°C for 2min for 35 cycles. The PCR product was electrophoresed on a 1% DNA agarose gel and gel purified using the QIAquick extraction kit (QIAGEN Cat#28704). The PCR products were digested with BamHI and XbaI restriction enzymes and inserted into the pBLUEscript II KS vector (Stratagene, Cat# 212207). The DNA template was digested with BamHI to generate the antisense riboprobe and with XbaI to generate the sense riboprobe. The same PCR reaction and similar conditions were used to generate riboprobes for the other R2B RPTPs.

Riboprobe Synthesis: Antisense and sense riboprobes for the four mouse R2B RPTPs were labeled with digoxigenin using the DIG RNA Labeling Kit (Roche #1175025) with some modifications. Each 20µl reaction contained 1µg purified and linearized template DNA, 2µl 10x NTP labeling mix (Roche, Cat#12777073; 10mM ATP, 10mM CTP, 10mM GTP, 6.5mM UTP, 3.5mM DIG-11-UTP; pH 7.5), 2µl 10x transcription buffer (400mM Tris-HCl; pH 8.0, 20°C, 60mM MgCl2, 100mM DTT, 20mM spermidine), 0.5µl of 40U/µl RNase OUT (Invitrogen Life Technologies, Cat#10777-019), 2µl of 20U/µl T7 RNA Polymerase (Roche, Cat#881767) or T3 RNA polymerase (Roche, Cat#1031163), and was incubated at 37°C for 2 hours. The T7 RNA Polymerase was used to generate the antisense riboprobes, whereas the T3 RNA Polymerase was used to generate the sense riboprobes. The DNA template was digested with 1U/µl RNase-free DNase I (Epicentre, Cat#D9902K) for 15 minutes at 37°C. The reaction was stopped by the addition of 3.6µl of 0.2M EDTA (pH 8.0). The transcripts were
purified by standard RNA precipitation. This reaction consisted of the addition of diethyl-pyrocarbonate (DEPC)-treated H20 to the sample to give a 50µl final volume, then 5µl of 5M ammonium acetate was added and vortexed, followed by the addition of 3 volumes of 100% ethanol. The reaction was precipitated at -20°C for 30 minutes and microcentrifuged at >13,000rpm for 30 minutes at 4°C. The supernatant was removed and the pellet was washed with 3 volumes 70% ethanol, microcentrifuged at >13,000rpm for 15 minutes at 4°C, and resuspended in 50µl DEPC-treated H20. The precipitation step was repeated once and the final pellet was resuspended in 50µl DEPC-treated H20.

**Hybridization:** In situ hybridization was done as described in McAndrew et al. (1998a, b) with some modifications. When sections were ready to use, slides were brought to room temperature. All pre-hybridization solutions were prepared with diethyl-pyrocarbonate (DEPC)-treated water and the following procedure was performed at room temperature (25°C). The sections were fixed in 4% paraformaldehyde (made in 1x PBS, pH 7.4) for 10 minutes, washed twice in 1x PBS for 5 minutes each, rinsed in 0.1M triethanolamine (pH 8.0) for 1min, acetylated in 0.25% (v/v) acetic anhydride in 0.1M triethanolamine for 10min, and washed twice in 2x SSC for 2 minutes each. The sections were not dehydrated in ethanol series; instead proceeded directly to the hybridization steps.

The hybridization buffer was prepared by mixing 500µl Solution A (deionized and filtered formamide; Roche, Cat#1814320), 380µl Solution B (4g dextran sulfate; Fisher Scientific Cat#BP1585-100; 8ml 20x SSC, 800µl 50x Denhardt’s
solution, 800µl yeast tRNA; Roche Cat#109495, and 4ml 1M DTT), 50µl Solution C (10mg/ml salmon testes single stranded DNA, Sigma). Solution C was heated for 10 minutes at 95°C prior to addition to the hybridization buffer. The digoxigenin-labeled riboprobe was added to the hybridization buffer at a final concentration 100-400ng. Approximately 100µl of the hybridization buffer plus riboprobe was added to each slide and hybridized overnight at 50°C.

*High Stringency washes:* Following the overnight hybridization incubation, post-hybridization washes were done with SSC solutions prepared with DEPC-treated water and containing β-mercaptoethanol (v/v 70µl/ml). Sections were briefly rinsed once in 2x SSC, rinsed in 2x SSC for 10 minutes at room temperature, and rinsed in 2x SSC for 10 minutes at 37°C. Sections were then incubated in RNase A solution (50µl of 20mg/ml RNase A in 10mM Tris-HCl, 1mM EDTA, 500mM NaCl, pH 8.0) for 30 minutes at 37°C. Sections were incubated in the following SSC dilutions for 10 minutes each at 65°C: 2x SSC, 1x SSC, 0.5x SSC, 0.25x SSC, and 0.125x SSC. Sections were then incubated in 0.125x SSC for 60 minutes at 65°C, followed by a rinse in 0.125x SSC for 10 minutes at room temperature. The sections were not dehydrated in ethanol series; instead proceeded directly to the immunological detection steps.
**Immunological washes and detection:** Following the hybridization washes, the sections were processed with anti-digoxigenin-AP Fab Fragment antibody (Roche, Cat#1093274) as described in Bian et al. (1996). All wash solutions for the immunological detection steps were prepared in DEPC-treated water. Sections were incubated in TBST (1.4M NaCl, 27mM KCl, 0.25M Tris-HCl, pH 7.5, 1% Tween-20) with 5% normal sheep serum for 1 hour at room temperature. Following the block step, sections were incubated overnight at 4°C with anti-digoxigenin-AP Fab Fragment antibody (1:5000 dilution in TBST and 1% normal sheep serum). Sections were washed five times with TBST for 10 minutes each and washed three times in NTMT (100mM NaCl, 100mM Tris-HCl, pH 9.5, 50mM MgCl₂, 0.1% Tween-20) for 10 minutes each at room temperature. Sections were incubated overnight in a humid chamber at room temperature with substrate (SIGMA Fast BCIP/NBT Alkaline Phosphatase tablets, Cat#B-5655). The reaction was stopped by washing the sections briefly in NTMT, followed by sequential dehydration in ethanol series of 70%, 80%, 90%, and 100% ethanol for 1 minute each, and dried at room temperature. The slides were then mounted with Depex medium.
LIST OF REFERENCES


CHAPTER 3

RPTP$_{\rho}$ mRNA EXPRESSION IN CEREBELLAR MOUSE MUTANTS

The mouse cerebellum is a highly organized structure containing a limited number of neuronal types, and well-known afferent and efferent synaptic connections. Cerebellar abnormalities are associated with loss of fine motor control, such as limb and eye movement, balance, and muscle coordination (Ghez, 1991). Spontaneously occurring mutations in the murine cerebellum result in disruption of normal synaptic connectivity and behavior (Rakic, 1975; Wassef et al., 1987).

In chapter 2 and in previous studies (McAndrew et al., 1998a, b), the expression pattern of RPTP$_{\rho}$ was shown to delineate a boundary dividing the anterior and posterior granule cell layer of the murine cerebellar cortex. Expression of RPTP$_{\rho}$ was entirely restricted to the granule cell layer of anterior lobules 1-6, while absent in posterior lobules 7-10 (Figure 3.1C). This distinct anterior-posterior expression pattern appears at approximately P10 in cells that have migrated to their final destination in the anterior granule cell layer.
Several mutants exist in which cerebellar morphology is altered in the anterior-posterior plane, including rostral cerebellar malformation \((rcm/rcm, Lurcher (Lc/+)\), and weaver \((wv/wv)\). In situ hybridization, using \(^{35}\)S and digoxigenin-labeled cRNA probes, was used to determine if RPTP\(\rho\) expression in mutant cerebella is affected by the mutation or by lack of cell contact with target Purkinje cells.

### 3.1 RESULTS

#### 3.1.1 Rostral cerebellar malformation \((rcm/rcm)\):

Rostral cerebellar malformation \((rcm/rcm)\) is a recessive mutation on mouse chromosome 3 (Przyborski et al, 1998). The mutation occurs in the Unc5h3 gene, the mouse homolog of the \(C.elegans\) unc-5 gene. Unc5h3 is the receptor for mouse netrin-1, a molecule involved in cell migration and axon guidance. This mutant demonstrates a swaying gait at ~P12 (Lane et al., 1992). In the \(rcm/rcm\) cerebellum, both anterior and posterior lobes are disorganized, and the number of clearly defined folia is reduced (Ackerman et al., 1997; Eisenman and Brothers, 1998; Lane et al., 1992; Leonardo et al., 1997). In the hemispheres, granule cells migrate beyond their normal boundary at the anterior surface of lobule 1 extending along the posterior surface of the inferior colliculi. Figure 3.1 shows the RPTP\(\rho\) expression is maintained in ectopic granule cells in the inferior colliculus, indicating that expression is independent of normal afferent and efferent granule cell contacts. In addition, there is a missing folium in the vermis.
The RPTP$_\rho$ boundary at the dorsal surface of lobule 6 is maintained in rcm/rcm (Figure 3.1F), the missing folium caudal to this boundary is likely to be posterior lobule 8 (Crus II).

Figure 3.1: RPTP$_\rho$ mRNA expression in normal C57BL/6 and rostral cerebellar malformation (rcm/rcm) mutant. In situ hybridization using $^{35}$S-labeled riboprobes was used to identify mRNA expression in sagittal sections of adult normal and mutant mouse cerebella. (A) Cresyl fast violet showing anatomical structures in the normal C57BL/6 cerebellum. (B) The granule cell marker, $\alpha$6 subunit of the GABA$_A$ receptor identifies granule cells throughout the normal C57BL/6 cerebellum (C) RPTP$_\rho$ mRNA is localized to the anterior granule cell layer (lobules 1-6) in the normal C57BL/6 cerebellum; arrowhead shows anterior-posterior boundary. Sagittal sections in which $\alpha$6 subunit identifies granule cells in the vermis (D) and hemisphere (E) of the adult rcm/rcm cerebellum. (F) RPTP$_\rho$ delineates the normal expression boundary at the dorsal surface of lobule 6. Ectopic granule cells in the inferior colliculus also express RPTP$_\rho$. v, vermis; h, hemisphere; ic, inferior colliculus, pf, primary fissure, dn, deep cerebellar nuclei.
3.1.2 Lurcher (Lc/Lc):

Lurcher is a semi-dominant mutation, which is lethal in the homozygous (Lc/Lc) mutant (Phillips 1960), and has been identified as a single base pair transition in the transmembrane domain of the mouse δ2 glutamate receptor gene (Zuo et al., 1997). Although the Lc/Lc mouse dies at, or before, birth the heterozygous (Lc/+) mutant also shows cerebellar abnormalities and has moderate ataxia. In the Lc/+, Purkinje cell axons show abnormalities as early as P3 (Wetts and Herrup, 1982), and many cells die between P8 and P26 (Caddy and Biscoe, 1979; Wassef et al., 1987). There is almost complete loss of Purkinje cells by four months. In addition, there is a reduction in the number of granule cells, which is thought to be secondary to the loss of Purkinje cells.

Rostrocaudal abnormalities have been detected in the Lc/+ cerebellum (Eisenmann, 2000), and RPTPρ expression was examined in Lc/+ to determine if the molecular expression boundary in lobule 6 coincides with any of the reported morphological boundaries, or is modified by the Lurcher mutation. In the P30 wildtype cerebellum, RPTPρ and PTPμ transcripts were expressed at normal adult levels in the anterior granule cell layer and Purkinje cell layer, respectively (Figure 3.2). In the Lc/+ cerebellum, the anterior-posterior boundary was retained in hemispheric regions (Figure 3.2C), but was less obvious in the vermis (Figure 3.2D). Absence of the PTPμ transcript in the Lc/+ cerebellum (Figure 3.2F) reflected the complete degeneration of Purkinje cells.
Figure 3.2 RPTP$\rho$ and PTP$\mu$ mRNA expression in the Lurcher (Lc/+ ) cerebellum. In situ hybridization using digoxigenin-labeled riboprobes was used to identify mRNA expression in sagittal sections of P30 Lurcher. A, B) Wildtype (Wt) littermate cerebellum. RPTP$\rho$ mRNA is localized in the anterior granule cell layer (lobules 1-6) in the control animal. Arrowhead shows anterior-posterior boundary (A). B) Higher magnification of inset showing strong labeling of anterior granule cells. C, D) Lc/+ cerebellum. The anterior-posterior boundary of RPTP$\rho$ mRNA was retained in hemispheric regions (C), but was less obvious in the vermis (D). E) Wildtype littermate cerebellum. PTP$\mu$ mRNA is localized throughout the entire Purkinje cell layer. F) Lc/+ cerebellum. PTP$\mu$ mRNA is absent (arrows) in Purkinje cells. A, C: scale bar = 500 microns. B, D: scale bar = 100 microns. g, granule cell layer; P, Purkinje cell. h, hemisphere; v, vermis.
3.1.3 *Weaver (wv/wv)*:

Weaver (wv/wv) is a recessive mutation on mouse chromosome 16. The gene defect was identified as a single base pair substitution in GIRK2, a G protein-coupled, inward-rectifying potassium channel (Patil et al., 1995). Characteristic histological defects in the cerebellum and disruption in motor behavior become evident in weaver mice by week two (Rakic and Sidman, 1973a, b). Affected animals exhibit abnormal gait and posture, and poor limb coordination, and tremor. The cerebellum is reduced in size, but retains some degree of foliation (Eisenman, 2000). The weaver mutation directly affects the migration of granule cells, the majority of which fail to form axons and die close to their site of origin in the external germinal layer during the first two postnatal weeks (Rakic and Sidman, 1973a, b). The few granule cells that reach their correct position in the internal germinal layer appear to develop normally. Although the cerebellar vermis becomes very agranular in the adult wv/wv, a larger number of granule cells and some degree of laminar structure are retained in the hemispheres (Herrup and Trenkner, 1987; Eisenman et al., 1998). Purkinje cells are somewhat reduced in number and are dispersed between the sparse granule cell layer and the narrow molecular layer (Rakic and Sidman, 1973a, b).

Although loss of granule cells in the adult wv/wv cerebellum results in a reduction in size, some degree of foliation is retained (Figure 3.3A). RPTP\(\rho\) expression was examined in littermate control (?/+) and homozygous (wv/wv) cerebella. RPTP\(\rho\) was expressed at normal levels in anterior granule cells in the
littermate cerebellum. At P22, although the $\alpha 6$ subunit of GABA$_A$ receptor labeled all residual granule cells throughout the anterior-posterior extent (Figure 3.3B), indicating that these cells were viable, the RPTP$_\rho$ transcript was completely absent throughout the wv/wv cerebellum (Figure 3.3C). Expression was also examined in younger animals (P11, and P15) to determine if early normal expression levels became reduced as cells degenerated. Although younger control mice expressed the transcript at age-appropriate levels (Figure 3.3D), expression was never observed in wv/wv (Figure 3.3E, F).
Figure 3.3 RPTP$_{\rho}$ mRNA expression in the weaver (wv/wv) cerebellum. In situ hybridization using $^{35}$S-labeled (B, C) and digoxigenin-labeled (D-F) riboprobes was used to identify RPTP$_{\rho}$ mRNA expression in sagittal sections of P22, P11, and P15 weaver. A) Cresyl fast violet showing anatomical structures in weaver (wv/wv). B) The granule cell marker, $\alpha$6 subunit of the GABA$_A$ receptor was used as a positive control showing expression in all residual granule cells throughout the anterior-posterior extent of the weaver (wv/wv) cerebellum. D) Littermate control (?/+) P15 cerebellum. Arrowhead shows anterior-posterior boundary. RPTP$_{\rho}$ mRNA is localized in the anterior granule cell layer (lobules 1-6). RPTP$_{\rho}$ expression is absent (arrows) in wv/wv cerebellum at postnatal day (P)22 (C), P15 (E), and P11 (F). D-E: scale bar = 500 microns.
3.2 DISCUSSION:

When classical histological procedures are used, the basic trilaminar structure of the cerebellar cortex along both the anterior-posterior (lobules 1-10) and mediolateral axis (vermis through hemispheres) appears morphologically homogeneous. We have shown (McAndrew et al., 1998b and Chapter 2) that the RPTP\(\rho\) transcript is entirely restricted to the postmigratory granule cell layer of anterior cerebellar lobules 1 through 6, and is completely absent in posterior lobules 7 to 10. The signal in anterior granule cells becomes detectable only at postnatal day 10, when parallel fiber synapse formation with target Purkinje cells, and afferent contact by spinocerebellar mossy fibers, are at a peak. During postnatal weeks two and three, the autoradiographic gradient between anterior and posterior folia forms a clearly discernible boundary at the dorsal surface of lobule 6, which extends throughout the vermis and hemispheres, effectively dividing the cerebellar cortex into two compartments. The onset of RPTP\(\rho\) expression in anterior folia at the peak of afferent and efferent synaptic contact suggests that RPTP\(\rho\) may be initiated by pre- or postsynaptic contact.

Although granule cells are generally considered to be a homogenous population, the existence of a granule cell layer RPTP\(\rho\) expression boundary raises the possibility of two embryologically derived sub-populations consisting of anterior (RPTP\(\rho\)-expressing), and posterior (RPTP\(\rho\) non-expressing) granule cells. All cerebellar neurons are generated in different regions at the surface of the fourth ventricle. Classically, it was thought that the cerebellum was entirely derived from the rhombencephalon (the most caudal subdivision of the neural
tube), whereas the mesencephalon (the rostral subdivision) was believed not to be involved in cerebellar morphogenesis. Studies using specific molecular markers (Nair et al., 1993) and chick-quail chimeras (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Hallonet and Le Douarin, 1993; and Martinez et al., 1999) have shown that Purkinje cells located in anterior and posterior cerebellar compartments are derived from different embryonic origins. Purkinje cells in the anterior compartment are derived from the caudal mesencephalon; whereas cells in the posterior compartment originate from the rostral metencephalon. The isthmus organizer provides signals for the embryonic development of these two regions (Martinez, 2001). Many genes are involved in patterning these regions, including Otx2 and Gbx2 (Simone, 2000), Wnt 1, En1, and Fgf8 (Martinez, 2001), and members of the Pax and Hox families (Williams and Holland, 1998).

Unlike Purkinje cells, granule cell precursors are derived solely from the rostral metencephalon (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990). Granule cells arise from neuroblasts at E13 (Wingate, 2001); proliferation requires, at least in part, the Math1 gene (Ben-Arie et al., 1997) and interaction with Purkinje cells. As Purkinje, Golgi, stellate and basket cells migrate outward toward the surface of the cerebellar cortex, granule cells migrate rostrally across the external cerebellar surface forming the external granular layer (egl) which reaches a maximal width between P7-10. The first granule cell precursors to leave the germinal layer tend to migrate towards the most anterior cerebellar lobules, whereas those last to leave reach the posterior lobules (Goldowitz,
In a second wave of migration, postmitotic granule cells migrate through the molecular and Purkinje cell layers in association with Bergmann glial fibers (Hatten, 1990; Komuro and Rakic, 1998). Differentiating granule cells extend axons perpendicularly across the molecular layer trailing parallel fibers, which form synapses with Purkinje cells. Between P8 and P12, parallel fiber terminals become more numerous and mature (Herndon et al., 1981). By P15, the number of granule cells undergoing division is reduced and almost ceases (Miale and Sidman, 1961), and by P20, virtually all granule cells complete their migration through the forming Purkinje cell layer to their final destination in the internal granular layer.

Mice in which genetic abnormalities result in rostrocaudal granule cell degeneration may reflect the earlier embryonic cerebellar compartmentation. In adult meander tail homozygotes, anterior granule cells fail to migrate to their adult positions within cortical lamina, leaving the anterior cerebellum agranular and underdeveloped; although residual granule cells at the dorsal surface of lobule 6 were capable of synthesizing other granule cell markers, RPTP_ρ expression was completely absent in these mice (McAndrew et al., 1998b). This finding raises the possibility that there are two overlapping anterior and posterior cell populations within the dorsal granule cell layer of lobule 6, one that is genetically programmed to express RPTP_ρ and another devoid of RPTP_ρ expression. In Lc/+ and rcm/rcm, the expression boundary is retained, as the anterior granule cell population was able to migrate to the internal
germinal layer (igl). However, in mea/mea and wv/wv, the RPTP\(\rho\) positive anterior granule cell precursors preferentially degenerate much earlier in development, long before their final migration into the igl.

It is unclear what initiates RPTP\(\rho\) expression in postmigratory granule cells in the normal cerebellum. At least two possibilities exist, and both are speculative: The first is that all granule cells are capable of RPTP\(\rho\) expression, but only those that migrate to the anterior cerebellum come into contact with the environmental factors that trigger expression of the RPTP\(\rho\) phenotype. However, in rcm/rcm, RPTP\(\rho\) is expressed normally in ectopic granule cells that have migrated into the inferior colliculi, and similarly, Lc/+ hemispheric granule cells express RPTP\(\rho\) in the complete absence of afferent Purkinje cells (as evidenced by lack of PTP\(\mu\) expression). Thus, normal synaptic contact does not appear to be an absolute requirement for RPTP\(\rho\) expression and argues against the first possibility. The greater likelihood is that a subpopulation of granule cell precursors already committed to expressing RPTP\(\rho\) migrates into the anterior region of the cerebellum where expression occurs at a pre-programmed developmental timepoint, irrespective of the cellular environment.

3.3 MATERIALS AND METHODS

*RPTP\(\rho\) Riboprobe Design:* Described in chapter 2.

*Riboprobe Synthesis and \(^{35}\)S-labeling:* Antisense (BamHI-linearized) and sense (XbaI-linearized) riboprobes were labeled with Uridine 5'-\(\alpha\)-[\(^{35}\)S] triphosphate (Amersham Biosciences #SJ-40383; 800Ci/mmol, 40mCi/ml) using the following
transcription reaction mix. Each 10µl reaction contained ~10nM linearized template DNA, 1x transcription buffer (400mM Tris-HCl; pH 8.0, 20°C, 60mM MgCl2, 100mM DTT, 20mM spermidine), 12.5mM DTT, 2.0U/µl RNaseOUT Ribonuclease Inhibitor (Invitrogen Life Technologies, Cat#10777-019), 400µM each NTP minus UTP (Invitrogen Life Technologies; 10mM ATP, Cat#18330-019; 10mM CTP, Cat#18331-017; 10mM GTP, Cat#18332-015), 12.5µM 5'-α-[S35]UTP, and 2.5U/µl T7 RNA Polymerase (Roche, Cat#881767) or T3 RNA polymerase (Roche, Cat#1031163), and was incubated at 37°C for 1 hour. The T7 RNA Polymerase was used to generate the antisense riboprobe, whereas the T3 RNA Polymerase was used to generate the sense riboprobe. Following transcription, the reaction was digested with the addition of a 15µl digestion mix containing 1x transcription buffer, 20mM DTT, 0.33U/µl RNase-free DNase I (Epicentre, Cat#D9902K), and 1.33U/µl RNaseOUT Ribonuclease Inhibitor for 30 minutes at 37°C. Transcripts were purified according to Henderson (1996), using standard phenol extraction to remove the enzymes and alcohol precipitation to separate the radiolabeled riboprobe from unincorporated nucleotides.

**Hybridization:** Hybridization conditions were the same as described in chapter 2. The radiolabeled riboprobe was added to the hybridization buffer at a final concentration of 2.0 x 10⁶ CPM/100µl. Approximately 100µl of the hybridization buffer plus riboprobe was added to each slide and hybridized overnight at 50°C.
High stringency washes: Described in chapter 2.

Preparation of autoradiograms: Coverslips (Fisher #0 or #1) were dipped in NTB2 emulsion (VWR Scientific), dried, and stored in a light-proof box with dessicant. The dipped coverslips and clean slides were apposed and stored in a light-proof box at either room temperature dehumidified chamber or at 4°C for 6-12 days. Coverslips were developed in Kodak Dektol, fixed in Kodak Fixer, washed in water, and sequentially dehydrated in an ethanol series (as described above). The coverslips containing the autoradiogram were soaked in xylenes and mounted with Depex mounting medium. Slides containing the sections were dipped in NTB2 emulsion, dried, and stored in a light-proof box at 4°C for 6-12 days with dessicant. Images were developed as above, washed in water, and sequentially dehydrated in ethanol. The slides were then soaked overnight in 100% butanol to remove lipids from the tissue. The sections were re-hydrated in ethanol, counterstained in cresyl fast violet stain (w/v, 0.5g cresyl fast violet/250ml diH2O) to show the anatomical structures within the cerebella of each mouse, rinsed in water, dehydrated in ethanol, and destained in glacial acetic acid (v/v, 1ml/250ml 95% ethanol). Slides were soaked in xylenes and mounted with Depex medium.
LIST OF REFERENCES


The R2B RPTPs consist of variable extracellular domains, a single transmembrane domain, and an intracellular region composed of two phosphatase domains. This subtype has only been identified in vertebrate species, and is developmentally regulated in the central nervous system (Stoker and Dutta, 1998; McAndrew et al., 1998a, b; Fuchs et al., 1998). The domains found in the extracellular segment of the R2B subtype are commonly found in cell adhesion molecules, suggesting that these phosphatase molecules also possess adhesive properties (Brady-Kalnay and Tonks, 1995). The R2B subtype, consisting of PTP\(_\mu\), PTP\(_\kappa\), PCP-2, and RPTP\(_\rho\), shares a high degree of amino acid sequence similarity. The localization of the R2B proteins has not been determined in mouse brain, due to limited availability of specific antibodies towards these molecules.
The focus of the work described in the present chapter was to generate RPTP$_{\rho}$-specific polyclonal antibodies that would recognize the native protein in mouse cerebellum, and more specifically to localize the protein within individual cell types. The RPTP$_{\rho}$ cDNA sequence was used to design recombinant protein constructs and peptides for the generation of polyclonal antibodies. In addition, antibodies available for two other R2B RPTPs (PTP$_{\mu}$ and PTP$_{\kappa}$) were used to localize these proteins to specific neuronal types in the mouse cerebellum.
Figure 4.1: Human and mouse RPTP\(\rho\) protein domain organization. The RPTP\(\rho\) extracellular segment contains a MAM, an Ig-like domain, and four FN III repeats, and the intracellular segment contains a juxtamembrane 'wedge' domain and two phosphatase domains. The cDNA sequence predicts a full-length protein of 164kDa (excluding glycosylated residues). The presence of a putative furin cleavage site (FCS) at AA#633-636 predicts an N-terminal product of at least 71kDa, and a C-terminal product at 91-93kDa. Recombinant fusion proteins were generated to specific protein domains: N-terminal (MAM-Ig): 35kDa, N-terminal (FC70), juxtamembrane (CB87): 30-32kDa, C-terminal (FC20): 62kDa. Peptide sequences (designated by asterisks followed by numbers) corresponding to specific regions of the RPTP\(\rho\) protein were chosen for the production of anti-peptide polyclonal antibodies: N-terminal peptides: #1 (Research Genetics), #2 (QCB) raised against adjacent regions of the MAM domain; Internal peptides: #3 (QCB), #4 (Zymed) were raised against the transmembrane domain and alternatively spliced exon 16; C-terminal peptides: #5 (QCB) and #6 (Sigma Genosys) were raised against adjacent regions in the noncatalytic, second phosphatase domain.
4.1 RPTP\(\rho\) Protein Domain Organization:

Figure 4.1 shows a schematic of the human and mouse RPTP\(\rho\) protein domain organization, as well as regions chosen for recombinant protein and peptide design for the production of polyclonal antibodies. The domain organization for the RPTP\(\rho\) protein includes an extracellular segment consisting of a MAM, an Ig-like domain, and four FN III repeats, whereas the intracellular segment consists of a juxtamembrane ‘wedge’ domain and two phosphatase domains.

4.1.1 Recombinant Fusion Protein Design: Based on amino acid alignments with the other R2B family members (PTP\(\kappa\), PTP\(\mu\), and PCP-2), recombinant fusion proteins and peptides were designed to specific N-terminal and C-terminal regions of the RPTP\(\rho\) protein, as well as a unique internal region. The RPTP\(\rho\) sequence showed extensive amino acid homology to PTP\(\kappa\), PTP\(\mu\), and PCP-2; therefore, extracellular and intracellular regions of RPTP\(\rho\) were expressed separately in \textit{E. coli} bacterial expression hosts as 6xHis or GST fusion proteins. The extracellular region consisted of two fusion proteins: (1) an N-terminal protein (designated as MAM-Ig) comprising the MAM and Ig-like domains (generated by Julie Besco) and (2) an intracellular protein (designated as FC70) encompassing part of the Ig-like domain and the four FN III repeats. The intracellular region consisted of three fusion proteins: (1) two juxtamembrane proteins (designated as CB87) comprising the transmembrane domain and part of the first phosphatase domain and (2) a C-terminal protein (designated as}
FC20) comprising the first and second phosphatase domains, the stop codon, and part of the 3’-untranslated region (Figure 4.1). The C-terminal FC20 (62kDa) protein was used in further studies and will be described in detail.

4.1.2 Anti-peptide Design: In addition to the recombinant fusion proteins, peptides were designed against the same regions within the RPTP ρ protein. Peptides were designed so that their sequences contained either low or high homology to the other R2B family members. The first set of peptides was generated against regions of the human RPTP ρ protein (GenBank protein ID# NP_008981) with limited homology to human PTP κ (GenBank protein ID# NP_002835), human PTP μ (GenBank protein ID# NP_002836), and human PCP-2 (GenBank protein ID# NP_005695) proteins. These peptide sequences were directed against the MAM domain and the transmembrane domain. Tables 4.1 and 4.2 show limited amino acid homology, designated as percent identity, for human RPTP ρ peptide sequences compared to corresponding regions in human PTP κ, PTP μ, and PCP-2.
Table 4.1: Human RPTP\(\rho\) amino acid identity to human PTP\(\kappa\), PTP\(\mu\), and PCP-2.
The percentage identity between human RPTP\(\rho\) peptide sequences and corresponding regions of human PTP\(\kappa\), PTP\(\mu\), and PCP-2. \(h=\text{human}\)

<table>
<thead>
<tr>
<th>Peptide Sequences</th>
<th>hPTP(\kappa)</th>
<th>hPTP(\mu)</th>
<th>hPCP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal Research Genetics</td>
<td>27%</td>
<td>53%</td>
<td>47%</td>
</tr>
<tr>
<td>N-terminal QCB</td>
<td>29%</td>
<td>47%</td>
<td>17.6%</td>
</tr>
<tr>
<td>Internal QCB</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Internal Zymed</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>C-terminal QCB</td>
<td>52%</td>
<td>62%</td>
<td>43%</td>
</tr>
<tr>
<td>C-terminal Sigma Genosys</td>
<td>77%</td>
<td>77%</td>
<td>77%</td>
</tr>
</tbody>
</table>

A. N-terminal Research Genetics: human RPTP\(\rho\) amino acid #42-56

<table>
<thead>
<tr>
<th>Peptide Sequences</th>
<th>GYSVALGTNFTWEQ</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hRPTP(\rho)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRPTP(\rho)</td>
<td>GYSVALGTNFTWEQ</td>
<td>100%</td>
</tr>
<tr>
<td>hRPTP(\kappa)</td>
<td>DYOQDLY-DDFWEVH</td>
<td>27%</td>
</tr>
<tr>
<td>hRPTP(\mu)</td>
<td>GYSQSEG-DDFNWEQ</td>
<td>53%</td>
</tr>
<tr>
<td>hPCP-2</td>
<td>EYSQAY-DFNWEQ</td>
<td>47%</td>
</tr>
</tbody>
</table>

B. N-terminal QCB peptide: human RPTP\(\rho\) amino acid #57-73

<table>
<thead>
<tr>
<th>Peptide Sequences</th>
<th>INTTEKPMLDQAVPTGS</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hRPTP(\rho)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRPTP(\rho)</td>
<td>INTWEKPMLPAVPTGS</td>
<td>88%</td>
</tr>
<tr>
<td>hRPTP(\kappa)</td>
<td>VSAQEPHYLPPEMPQGS</td>
<td>29%</td>
</tr>
<tr>
<td>hRPTP(\mu)</td>
<td>VNTLTKPTSDPWMPSGS</td>
<td>47%</td>
</tr>
<tr>
<td>hPCP-2</td>
<td>VRHPGTRAPADLPHGS</td>
<td>17.6%</td>
</tr>
</tbody>
</table>

Table 4.2: Limited amino acid sequence homology to human PTP\(\kappa\), PTP\(\mu\), and PCP-2.  
(A) N-terminal Research Genetics and (B) N-terminal QCB peptide sequences showed regions of human and mouse RPTP\(\rho\) with least homology to the other R2B RPTP family members. Amino acids bolded in red are homologous to the human RPTP\(\rho\) sequence; homology is designated as % identity. The internal QCB peptide (hRPTP\(\rho\) amino acid #740-754; CLLTTGASTQNSNTV) and internal Zymed peptide (hRPTP\(\rho\) amino acid #790-803; RRNAYSYSYLSQR) showed 0% homology to the other R2B RPTP family members, or to any other sequences in the EST/nucleotide database or TIGR. \(h=\text{human};\ m=\text{mouse}\).
The second set of peptides was generated against regions of the human RPTP\(\rho\) protein with high homology to human PTP\(\kappa\), PTP\(\mu\), and PCP-2. Peptide sequences were directed against adjacent regions in the second phosphatase domain. Tables 4.1 and 4.3 show high amino acid homology, designated as percent identity, for human RPTP\(\rho\) peptide sequences compared to human PTP\(\kappa\), PTP\(\mu\), and PCP-2. Table 4.4 gives an overall summary of the amino acid designations for all peptide sequences.

A. **C-terminal QCB peptide:** hRPTP\(\rho\) amino acid #1380-1400

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hRPTP(\rho)</td>
<td>RRLEKWQEQYDGREGRTVVHC</td>
<td>100%</td>
</tr>
<tr>
<td>mRPTP(\rho)</td>
<td>RRLEKWQEQYDGREGRTVVHC</td>
<td>100%</td>
</tr>
<tr>
<td>hPTP(\kappa)</td>
<td>LQVEKWQEECKEENGRTIVHC</td>
<td>52%</td>
</tr>
<tr>
<td>hPTP(\mu)</td>
<td>ROVDEKWQEEYNNGEPTVVHC</td>
<td>62%</td>
</tr>
<tr>
<td>hPCP-2</td>
<td>AEVDKWQAESGDGRTIVHC</td>
<td>43%</td>
</tr>
</tbody>
</table>

**C-terminal Sigma Genosys:** hRPTP\(\rho\) amino acid #4331-1445

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>hRPTP(\rho)</td>
<td>KTLRNNSKSNVMET</td>
<td>77%</td>
</tr>
<tr>
<td>mRPTP(\rho)</td>
<td>KTLRNNSKSNVMET</td>
<td>100%</td>
</tr>
<tr>
<td>hPTP(\kappa)</td>
<td>KTLRNNSKPNMVEA</td>
<td>77%</td>
</tr>
<tr>
<td>hPTP(\mu)</td>
<td>KTLRNNSKPNMVDL</td>
<td>77%</td>
</tr>
<tr>
<td>hPCP-2</td>
<td>QTLRNYKPNMVET</td>
<td>77%</td>
</tr>
</tbody>
</table>

Table 4.3: High amino acid sequence homology to human PTP\(\kappa\), PTP\(\mu\), and PCP-2. C-terminal QCB and C-terminal Sigma Genosys peptide sequences showed regions of human RPTP\(\rho\) with highest homology to the other R2B RPTP family members. Amino acids bolded in red are homologous to the human RPTP\(\rho\) sequence; homology is designated as % identity. *h*=human; *m*=mouse.
<table>
<thead>
<tr>
<th>Peptide Region/ (shortened name)</th>
<th>Amino acid # (peptide length)</th>
<th>RPTP&lt;sub&gt;ρ&lt;/sub&gt; domain</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-terminal Research Genetics (RESGEN42/56)</td>
<td>42-56 (15 amino acids)</td>
<td>MAM domain</td>
<td>GYSVALGNTNGFTWEQ</td>
</tr>
<tr>
<td>N-terminal QCB (NQCB57/73)</td>
<td>57-73 (17 amino acids)</td>
<td>MAM domain</td>
<td>INTTEKPMLDQAVPTGS (C)-amide</td>
</tr>
<tr>
<td>Internal QCB (IQCB740/754)</td>
<td>740-754 (15 amino acids)</td>
<td>Transmembrane domain &amp; alternatively spliced exon 16</td>
<td>Acetyl-CLLTGASTQNSNTVamide</td>
</tr>
<tr>
<td>Internal Zymed (Zym790/803)</td>
<td>790-803 (13 amino acids)</td>
<td>Transmembrane domain &amp; alternatively spliced exon 16</td>
<td>(C)RRNAYSYSYSQRLTRVHC-amide</td>
</tr>
<tr>
<td>C-terminal QCB (CQCB 1380/1400)</td>
<td>1380-1400 (21 amino acids)</td>
<td>Phosphatase II domain</td>
<td>Acetyl-RRLEKWQEQYDGREGRTVVHCamide</td>
</tr>
<tr>
<td>C-terminal Sigma Genosys (SIGGEN 1433/1445)</td>
<td>1433-1445 (13 amino acids)</td>
<td>Phosphatase II domain</td>
<td>(C)KTLRNKSNMVET</td>
</tr>
</tbody>
</table>

**Table 4.4: RPTP<sub>ρ</sub> peptide sequence identity.** Synthetic peptides were generated against specific human RPTP<sub>ρ</sub> regions. Each peptide was given a shortened name, in parentheses, incorporating the name of the company and amino acid location. The amino acid number, peptide length in amino acids, corresponding RPTP<sub>ρ</sub> domain, and peptide sequence are given for each peptide. The cysteine residue used in cysteine-thiol coupling of the peptide to the carrier protein was designated as (C). The carboxy terminus was designated as –COOH. Some peptide sequences were acetylated and/or amidated; designated as *Acetyl-* and *-amide*, respectively.
4.2 RESULTS:

4.2.1 Production of Extracellular and Intracellular Recombinant RPTPρ

**Fusion Proteins:** *Bacterial Expression Vector:* The pPROEX-HT (Figure 4.2) bacterial expression vector was used to generate the RPTPρ C-terminal recombinant fusion protein (designated as FC20) in an *E. coli* host. This vector was used for protein expression, since purification of a recombinant protein could be achieved with a single chromatography step. The pPROEX-HT vector, which is under the control of the *Trc* promoter, was used for high-level expression of RPTPρ proteins in *E. coli*. The *Trc* promoter and lacIq repressor induce gene expression with the addition of the lactose analog IPTG, isopropyl β-D-thiogalactoside; whereas the multiple cloning site (MCS) is preceded by six histidines. A 6x-His affinity purification strategy facilitated in detection of the C-terminal FC20 protein. Once the fusion protein was expressed, DNA sequencing using the (-) 50 reverse M13 primer (5'-TTGTGAGCGGATAACAATTTC-3'), confirmed the location of the histidine sequence at the amino terminus of FC20. A rapid one-step affinity purification of the target protein fused to the 6x-His sequence was conducted using a nickel nitrilo-tri-acetic acid (Ni-NTA) resin.
Figure 4.2: pPROEX-HT Bacterial Expression Vector. The C-terminal FC20 RPTPρ protein (62kDa) was generated using the pPROEX-HT vector system (~4775bp; GIBCO BRL Cat# 10711-018). This vector is used for is under the control of the Trc promoter (pTrc). A prokaryotic ribosome binding site (RBS) is located upstream of the 6x-His sequence. Additional features of this vector include: the lacIq repressor, a pBR322 origin (ori), ampicillan resistance (Apq), the bacteriophage f1 intergenic region, and a Tobacco Etch Virus (TEV) protease cleavage site. The multiple cloning site (MCS) shows the BamHI and Xbal sites used for insertion of the C-terminal PTPρ fragment.
**Intracellular RPTP<sub>ρ</sub> Segment: Generation of the C-terminal FC20 Recombinant Fusion Protein:** The intracellular region of the R2B RPTPs contains two highly conserved phosphatase domains; the first domain contains the 11 hallmark amino acids defining the catalytic core. An intracellular recombinant RPTP<sub>ρ</sub> fusion protein, designated as FC20, was generated using the pPROEX-HT bacterial expression system.

**4.2.2 Protein Expression of the C-terminal FC20 fusion protein:** Uninduced and induced total cell lysates were run on 12% SDS-PAGE and stained with Coomassie blue. A strong band at ~62kDa was present in the induced samples, which corresponded to the predicted molecular weight value for the C-terminal recombinant protein (Figure 4.3, lane 3). This band was not present in the uninduced sample (Figure 4.3, lane 2). A β-galactosidase control was run concurrently with the FC20 sample; a strong protein band was detected in the induced sample at the expected 116kDa molecular weight (not shown). The intensity of the induced β-galactosidase protein band was comparable to the induced FC20 protein band.
Figure 4.3: Bacterial protein expression and anti-his antibody reactivity to the C-terminal FC20 recombinant fusion protein: 12% SDS-PAGE. Lanes 1-3 are Coomassie stained. Lane 1: Benchmark Prestained Protein Ladder (GIBCO BRL). Lane 2-5: Uninduced (U) and Induced (I) with 0.6mM IPTG. *E. coli* strain DH5αF’ cultures were transformed with pPROEXHT-c/FC20. Lanes 2-3: Total bacterial lysates showed protein expression of the C-terminal FC20 recombinant protein (62kDa); Lane 5: C-terminal FC20 protein detected with the Tetra-His antibody in the induced sample (QIAGEN).

4.2.3 Anti-His antibody reactivity to the C-terminal (FC20) recombinant fusion protein: The pPROEX-HTc vector contained a 6x-His affinity tag and once expressed, this sequence was located at the amino terminus of the RPTPρ fusion protein. There were several benefits for using the 6x-His tag as part of the fusion protein. This tag was smaller (molecular mass=1kDa) than other commonly used tags; therefore, it should not interfere with the structure or function of the RPTPρ recombinant protein. In addition, tag removal by protease cleavage was not necessary. The tag is poorly immunogenic (Hurd and Hornby, 1996); therefore, the recombinant protein (including the tag) was used as an
antigen to generate antibodies against the RPTP$_{\rho}$ protein. Lastly, the 6x-His tag has strong affinity to Ni-NTA products, which facilitated in detection and purification of the recombinant protein.

QIAexpress anti-His antibodies were used to detect the C-terminal recombinant protein fused to a 6x-His affinity tag. These anti-His antibodies were mouse monoclonal IgG1 antibodies, which had high affinity and specificity to the 6x-His tag. The antibodies used in this study included the RGS-His, Penta-His, and Tetra-His antibodies that recognized the RGS(H)$_4$, (H)$_5$, and (H)$_4$ epitopes, respectively. Western blotting was used to test anti-His antibody reactivity to the recombinant protein. The Tetra-His antibody recognized the expected C-terminal protein band at ~62kDa in the induced sample (Figure 4.3, lane 5). No protein bands were detected in non-transformed DH5$\alpha$F$'$ competent cells by the Tetra-His antibody (not shown). Similar results were seen with the Penta-His antibody (data not shown).

4.2.4 Solubilization of the C-terminal FC20 RPTP$_{\rho}$ recombinant protein:

Initial lysozyme and sonication conditions, used to determine protein solubility characteristics of RPTP$_{\rho}$, indicated that the recombinant protein was insoluble (Figure 4.4A), whereas the $\beta$-galactosidase control protein was mostly soluble (data not shown). Under these solubilization conditions, the soluble protein was evident in the supernatant fraction, whereas the insoluble protein was evident in the pellet fraction. These initial solubilization conditions suggested that the RPTP$_{\rho}$ recombinant protein may be located within inclusion bodies. Therefore,
more stringent solubilization conditions were needed to produce a soluble form of the RPTP\(_\rho\) protein. A soluble form of the protein was needed to obtain a pure protein to be useful in the production of polyclonal antibodies.

Figure 4.4: A. Solubilization conditions used on the C-terminal FC20 RPTP\(_\rho\) fusion protein.
12% Coomassie stained SDS-PAGE gel. Total bacterial cell lysates of DH5\(\alpha\) F' \(E. coli\) cultures were transformed with pPROEXHT-c/FC20. Protein expression: Lane 1: Uninduced, Lane 2: Induced; Lanes 3-10: Supernatant (S) and pellet (P) fractions following solubilization conditions: Lysozyme (100\(\mu\)g/ml) and sonication, 4M Urea, 10% Triton X-100, and 6M Guanidine hydrochloride (GdnHCl).

B. Nickel Affinity purification of the C-terminal FC20 fusion protein:
12% Coomassie stained SDS-PAGE gel. Total bacterial cell lysates of DH5\(\alpha\) F' \(E. coli\) cultures transformed with pPROEXHT-c/FC20. Protein expression: Lane 1: Uninduced (U), Lane 2: Induced (I), Fractions during purification: Lane 3: Supernatant (Sup) fraction solubilized in 6M guanidine hydrochloride, Lane 4: pellet (P) fraction; Lane 5: wash (W) step prior to elution; Lane 6: Elution (E) of purified target (FC20 RPTP\(_\rho\)) protein (62kDa) with 4M Urea; Lane 7: Strip fraction.
Based on high expression levels and specific anti-His antibody reactivity, the C-terminal recombinant protein was used in further solubilization studies. These conditions consisted of strong detergents used in the solubilization process, which included 4M and 8M urea, 10% Triton X-100, 10% SDS, and 6M guanidine hydrochloride. Results from the solubilization studies indicated that the recombinant protein was insoluble in 4M urea (Figure 4.4A) and in 8M urea (data not shown). The recombinant protein was 50% soluble in 10% SDS (data not shown) and 10% Triton X-100 (Figure 4.4A); whereas it was completely soluble in 6M guanidine hydrochloride (GdnHCl) (Figure 4.4A). Using the GdnHCl solubilization conditions, the soluble protein was evident in the supernatant fraction, whereas the insoluble protein was evident in the pellet fraction.

Once a soluble RPTPρ protein was obtained, a metal chelation chromatography approach, using nickel affinity purification, was used to achieve a purified form of the protein. The 6x-His tag sequence, located at the N-terminal end of the FC20 recombinant protein, bound to Ni$^{2+}$ cations immobilized on the His bind resin. Once unbound proteins were washed away, the target RPTPρ FC20 protein (62kDa) was recovered by elution with high concentration of imidazole. A relatively purified form of the RPTPρ FC20 recombinant protein was achieved, with a minor percentage of target remaining in the stripped resin (Figure 4.4B).
4.2.5 Production of polyclonal antibodies against the C-terminal FC20 recombinant protein: The purified and denatured C-terminal recombinant protein was injected into rabbits for the production of polyclonal antibodies to RPTP\(\rho\). ELISA titers for this polyclonal antibody were very low. This polyclonal antibody was not reactive towards the purified C-terminal recombinant protein in Western blot analysis (Figure 4.5). The antiserum was also tested on the entire bacterial culture, which did not produce any specific bands at 62kDa (Figure 4.5). In addition, reactivity was tested on mouse brain tissue homogenates, but no specific protein bands were detected (data not shown).
Figure 4.5. Lack of antibody reactivity against the C-terminal FC20 RPTP$_\rho$ fusion protein. Reactivity of the C-terminal polyclonal antibody, generated against the C-terminal fusion protein, was tested against the purified C-terminal recombinant protein (Lanes 2-4), as well as the entire bacterial culture (Lanes 5-7). The expected protein product was 62kDa. Lane 1: Rabbit IgG.

4.2.6 Anti-peptide antibody reactivity to the recombinant fusion proteins:

Antibody reactivity towards the recombinant fusion proteins, was tested by Western blotting with anti-peptide antibodies. N-terminal anti-peptide antibodies generated by QCB (NQCB57/73) and Research Genetics (RESGEN42/56) were raised against adjacent amino acid sequences in the MAM domain. Both anti-peptide antibodies detected the expected 35kDa protein band in induced cultures of the MAM-Ig recombinant protein (shown by Julie Besco). Results are shown for NQCB57/73 (Figure 4.6).
Internal anti-peptide antibodies generated by QCB (IQCB740/754) and Zymed (Zym790/803) were raised against the transmembrane domain and alternatively spliced exon 16, respectively. None of the internal anti-peptide antibodies recognized the internal CB87 short or long fragments (data not shown). This may be due to low antigenic affinity of the anti-peptide antibodies towards the recombinant protein, or to the low levels of protein expression.

All affinity purified anti-peptide antibodies were used to check antibody reactivity towards the C-terminal FC20 recombinant protein; as expected, only the C-terminal anti-peptide antibodies detected the 62kDa FC20 recombinant protein. C-terminal anti-peptide antibodies generated by QCB (CQCB1380/400) and Sigma Genosys (SIGGEN1433/1445) were raised against adjacent amino acid sequences in the second phosphatase domain. Both anti-peptide antibodies detected the expected 62kDa protein band in induced cultures of the FC20 recombinant protein (Figure 4.6, lane 9 and 11). A β-galactosidase control was probed with both C-terminal anti-peptide antibodies to assess any non-specific signal in large protein bands. None of the C-terminal anti-peptide antibodies detected the large protein band at 116kDa in the β-galactosidase lane (data not shown). An anti-peptide antibody generated against the internal RPTP region (Zym790/803) did not detect the ~62kDa induced band, tending to confirm the specificity of the C-terminal antibodies for the FC20 region (Figure 4.6, lane 7).
Figure 4.6. N-terminal and C-terminal recombinant 6X His fusion protein expression and antibody reactivity: 12% SDS-PAGE; Lane 1: Benchmark Prestained Protein Ladder (GIBCO BRL); Uninduced (U) and Induced (I) with 0.6mM IPTG. E. coli strain DH5αF' cultures transformed with pPROEXHT-c/FC20 or MAM-Ig. Lanes 3 shows N-terminal anti-peptide reactivity with the 35kDa N-terminal fusion protein. Lanes 5 and 7 show lack of internal anti-peptide antibody reactivity with 62kDa FC20 protein.; Lanes 9 and 11 detected with CQCB1380/1400 and SIGGEN1433/1445 affinity purified anti-peptide antiserum reacted with the 62kDa C-terminal FC20 RPTPρ fusion protein.

4.2.7 Anti-peptide antibody reactivity towards mouse brain tissue.

MacDNASIS and EMBOSS, two computer prediction programs, were used to predict the molecular weight of the entire RPTPρ protein, as well as potential cleavage products and alternatively spliced products (Table 4.5). The RPTPρ cDNA sequence predicted a full-length protein of 164kDa (excluding glycosylation), which is similar to the other R2B RPTP members. The presence of a putative cleavage site at AA#633-636 within the fourth FN III repeat predicted an N-terminal product of at least 71kDa and a C-terminal product at 91-93kDa, depending on alternative splicing (Besco, 2003; Besco et al., 2003).
TABLE 4.5: Molecular Weight Predictions using MacDNASIS & EMBOSS. Predicted molecular weight values for full-length and potential cleavage products for the RPTP\(\rho\) protein.

<table>
<thead>
<tr>
<th>Region of RPTP(\rho) protein</th>
<th>Predicted MW(kDa)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entire RPTP(\rho) protein</td>
<td>164kDa</td>
<td>Includes alternatively spliced region</td>
</tr>
<tr>
<td>N-terminal end</td>
<td>71kDa</td>
<td>Includes proteolytically cleaved site:KSRR</td>
</tr>
<tr>
<td>C-terminal end</td>
<td>93.5kDa</td>
<td>Includes alternatively spliced region</td>
</tr>
<tr>
<td>C-terminal end</td>
<td>91.6kDa</td>
<td>No alternatively spliced region</td>
</tr>
</tbody>
</table>

Besides the potential furin-like cleavage site in the R2B RPTPs, additional proteolytic processing events may exist, such as calpain-mediated cleavage within the juxtamembrane region. Calpain-mediated proteolytic cleavage was shown by Western analysis for PTP\(\alpha\) and PTP\(\epsilon\) (members of the R4 subtype containing short, highly glycosylated extracellular domains) in various cell lines and mouse tissues, resulting in smaller protein forms at 66kDa and 65kDa, respectively (Gil-Henn et al., 2000; Gil-Henn et al., 2001). Sequence alignments of mouse and human PTP\(\epsilon\) showed the potential region of cleavage, which consisted of a methionine residue preceded by a valine residue, followed by two leucine residues. Sequence alignment of PTP\(\epsilon\) and PTP\(\alpha\) with RPTP\(\rho\) showed that this leucine residue is conserved (Figure 4.7). Conservation of this leucine may indicate a potential conserved calpain cleavage site in the R2B and R4 RPTP subtypes, since calpain does not recognize a specific amino acid sequence. Molecular weight analysis of potential cleavage near this leucine
residue predicted an intracellular RPTPρ protein product of approximately 70kDa.

In addition, an alternative translation initiation site exists near this cleavage site, predicting a C-terminal RPTPρ protein product of 75kDa.

**Figure 4.7: R2B and R4 alignment of potential region for calpain-cleavage.** An alternative translation initiation site (boxed in red) is shown for RPTPρ which generates a fragment of ~75kDa. The potential region for calpain-cleavage for the R2B and R4 RPTPs is boxed in blue. If cleavage takes place for RPTPρ, a fragment of ~70kDa is generated. The red arrow shows the region for the wedge domain found in these RPTPs.
All anti-peptide polyclonal antibodies were analyzed by Western blot and immunocytochemistry on normal C57BL/6 mouse brain tissue. The Research Genetics N-terminal affinity purified antibody only recognized a protein band at 66kDa, which corresponds to the putative N-terminal cleavage product (Figure 4.8A). An expected full-length product (~164-200kDa) was not detected with this antibody. Immunocytochemical analysis using this antibody in normal adult C57BL/6 brain tissue did not show expression in the cerebellum, although a very intense expression pattern was observed in the cortex (predominantly layer 1) and the inferior colliculus (Figure 4.8A). In Western analysis, the other N-terminal affinity purified antibody, generated by QCB, detected a potential full-length protein product (~184kDa) and N-terminal cleavage product (~101kDa) (Figure 4.8B). Both perfused and paraffin-embedded sections were used in immunocytochemical studies; however this antibody did not show specific RPTPρ expression (data not shown).

The QCB internal antibody detected a strong band at 164kDa, which was not present in the preimmune bleed (Figure 4.8C); this may represent the full-length protein. Although this antibody generated a strong response in Western blot analysis, it did not show specific RPTPρ expression (data not shown). This antibody was not affinity-purified successfully, possibly due to the region chosen for design. The Sigma Genosys C-terminal affinity-purified antibody detected a potential full-length protein product (~196kDa) and N-terminal cleavage product (~104kDa) (Figure 4.8D); however this antibody did not show specific RPTPρ expression in the mouse cerebellum (Figure 4.8D)
Figure 4.8 Western blot and immunohistochemical analysis of anti-peptide polyclonal antibodies on mouse brain. A C57BL/6 (P44) mouse whole brain crude protein prep was run on an 8% SDS-gel and blotted with N-terminal (A, B), internal (C) and C-terminal anti-peptide antibodies (D). Affinity purified antibodies for the N-terminal (A) and C-terminal (D) were also analyzed by immunohistochemistry on P60 C57BL/6 mouse cerebella. Both antipeptides were not specific for RPTP in tissue. ct, cortex., ic, inferior colliculus, P, Purkinje cell layer.
4.2.8 QCB C-terminal Anti-peptide Antibody Reactivity in mouse brain:

Initial Western blotting studies using the QCB C-terminal anti-peptide antibody, detected two strong protein bands at 70kDa and 75kDa, which were about 20kDa shorter than the predicted C-terminal product; whereas the full-length protein was not detected by this antibody. Additional Western analysis was conducted using a crude synaptosomal membrane preparation from adult female C57BL/6 brain (P44) and a crude P2 (pellet #2) membrane preparation from a postnatal day 7 (P7) C57BL/6 mouse brain. Later bleeds (Bleeds 6-8) and the affinity purified antibody detected two strong protein bands at 70kDa and 75kDa in the P7 preparation (Figure 4.9A). These same two bands were detected in P44 brain with bleed 6 and the affinity purified bleed, which were not detected with the preimmune bleed (Figure 4.9B). Similar to previous results, a strong protein band was detected in the preimmune at 159kDa in the P44 brain and P7 brain preparations. This band was most likely an abundant protein in the brain, not related to RPTP$\rho$, which elicited a strong response in the rabbit by the preimmune serum and was purified out in later bleeds.

Western blot analysis of the QCB C-terminal anti-peptide antibody on adult (P336) mouse C57BL/6 brain resulted in two strong protein bands at 70kDa and 75kDa detected with the affinity purified antibody, which was not detected by preimmune serum or bleed 3 (Figure 4.9 C, D). The preimmune bleed detected a protein band at ~159kDa, which was absent in later bleeds. Western analysis was performed, using adult C57BL/6 male (P336) brain and liver tissue blotted with the affinity purified C-terminal antibody, to test anti-peptide antibody
reactivity in brain tissue. The two major protein bands seen previously were detected in the brain at 70kDa and 75kDa, which were absent in liver (Figure 4.9 D). This blot showed that the QCB C-terminal anti-peptide antibody generated against RPTP$_\rho$, a CNS-specific molecule, detected two protein products only in the brain.

In addition to mouse tissue, two human brain preparations were also used to test antibody reactivity. Crude tissue homogenates of a male human frontal cortex and female human brain (Clontech) were electrophoresed on 6% SDS-polyacrylamide gels, and transferred to nitrocellulose membrane. Within both human brain preparations, the same two strong protein bands seen in mouse brain preparations were detected at 70kDa and 75kDa (Figure 4.10 A, B). Once again, an unrelated protein band at 159kDa was detected in the Clontech human brain preparation with the preimmune serum, these results were similar to those obtained in mouse brain preparations. A faint band was detected in both human preparations at 179kDa, which may correspond to the predicted full-length RPTP$_\rho$ protein, excluding glycosylation. This antibody was also analyzed in immunocytochemical studies on normal C57BL/6 mouse brain. No specific RPTP$_\rho$ expression was evident in the cerebellum (Figure 4.10 C).
Figure 4.9 Western blot and immunohistochemical analysis of the QCB C-terminal anti-peptide polyclonal antibodies on mouse brain. C57BL/6 mouse brain protein preparations were run on 8% SDS-gels and blotted with the QCB C-terminal anti-peptide antibodies (A-D).
Figure 4.10 Western blot analysis of the QCB C-terminal anti-peptide polyclonal antibodies on human brain and immunocytochemical analysis on mouse brain. Human brain protein preparations were run on 8% SDS-gels and blotted with the QCB C-terminal antipeptide antibodies (A-B). The C-terminal affinity purified antibody was also analyzed by immunohistochemistry (C) on P60 C57BL/6 mouse cerebella.
4.2.9 PTPµ antibodies cross-react with the C-terminal RPTPρ recombinant fusion proteins. PTPµ antibodies, provided by Susann Brady-Kalnay (Case Western Reserve University), were used in testing cross-reactivity to the C-terminal RPTPρ recombinant protein. SK15 and SK18 are monoclonal antibodies of the IgG1 subtype, which recognize the PTPµ phosphatase domain. Cross-reactivity of PTPµ antibodies with C-terminal RPTPρ (FC20) recombinant protein was tested by Western blot analysis. Low level cross-reactivity was observed for both SK15 and SK18 PTPµ antibodies (Figure 4.11A). The localization of SK15 and SK18 was examined in the normal C57BL/6 mouse brain cerebellum (Figure 4.11B). Both antibodies were present in Purkinje cell bodies and throughout the entire molecular layer, and in good correspondence with the localization of PTPµ RNA in Purkinje cell somas.

4.2.10 The PTPκ antibody cross-react with the C-terminal RPTPρ recombinant fusion proteins. As with the PTPµ antibodies, the PTPκ polyclonal antibody (Santa Cruz, #sc-1113) was designed against the first phosphatase domain and cross-reacted with the C-terminal RPTPρ recombinant protein (62kDa) (Figure 4.12A). Antibody staining was present in stellate and basket cells throughout the molecular layer, and in the granule cell and Purkinje cell layers of the C57BL/6 mouse cerebellum. Protein localization was in good correspondence with that of the PTPκ RNA in stellate and basket cells and granule cells.
**Figure 4.11** PTPµ antibodies cross react with the C-terminal RPTPρ fusion protein. A) Western Blot Analysis: Uninduced (U) and Induced (I) bacterial cultures expressing the C-terminal RPTPρ recombinant fusion protein were electrophoresed on a 12% SDS-gel, transferred and blotted with C-terminal PTPµ monoclonal antibodies (SK15 and SK18). Both antibodies cross react with the RPTPρ fusion protein (62kDa). B) Immunocytochemistry on P60 C57BL/6 mouse cerebellum. A, B) PTPµ SK18 antibody was present throughout the entire molecular layer (m), and at higher magnification (B), the protein was present in the Purkinje cell layer (P). C,D) PTPµ SK15 antibody was in the molecular layer of the anterior lobules, and at higher magnification (D) the protein was also present in the Purkinje cell layer. E) Secondary antibody control (anti-mouse) F) The α1 subunit of the GABA<sub>A</sub> receptor is a positive control staining the Purkinje cell layer. C) In situ hybridization. PTPµ mRNA was present throughout the Purkinje cell layer, and also in the stellate and basket cells and Golgi cells.
Figure 4.12  The PTPκ antibody cross reacts with the C-terminal RPTPρ fusion protein. A) Western Blot Analysis: Uniduced (U) and Induced (I) bacterial cultures expressing the C-terminal RPTPρ recombinant fusion protein were electrophoresed on a 12% SDS-gel, transferred and blotted with the RPTPkappa polyclonal antibody. The antibody cross reacted with the RPTPρ fusion protein (62kDa). B) Immunocytochemistry on P60 C57BL/6 mouse cerebellum. A, B) PTPκ antibody was present throughout the entire molecular layer in stellate (s) and basket (b) cells, and in the granule cell layer (g). At higher magnification (B), the protein was present in the Purkinje cell layer (P). C) Secondary antibody control. F) The β3 subunit of the GABAβ receptor is a positive control staining the granule cell layer. C) In situ hybridization. PTPκ mRNA was present throughout the molecular layer in the stellate (s) and basket (b) cells; and in the granule cells (g) and Golgi cells (G).
4.3 DISCUSSION:

In summary, this chapter describes the design, production, and characterization of RPTPρ fusion proteins and anti-peptides for the generation of polyclonal antibodies. Anti-peptide antibody reactivity towards the recombinant fusion proteins was tested by Western blot analysis. Although several anti-peptide antibodies identified predicted bands in mouse and human brain preps using Western blot analysis, none of these antibodies gave the predicted RPTPρ distribution in immunocytochemical studies. In addition, antibodies available for the other R2B RPTPs (PTPµ and PTPκ) were used to localize the protein within specific neuronal types in the mouse cerebellum. It was expected that, by and large, the mRNA would be expressed in cell bodies of specific cell types, whereas the protein encoded by the mRNA would be expressed in the dendritic or axonal processes of that cell.

Examination of RPTPρ expression in various mouse and human membrane preparations consistently revealed two protein bands at ~70kDa and 75kDa. There are two possible explanations for the presence of these two strong protein bands. One explanation is that post-translational proteolytic processing of RPTPρ may result in specific in vivo cleavage of the full-length protein into shorter products. Besides the potential proteolytic cleavage at the furin-like cleavage site in the R2B RPTPs, additional proteolytic processing events may exist, such as calpain-mediated processing within the juxtamembrane region. Calpain is a member of a highly conserved family of calcium-dependent cysteine proteases. In vivo, calpain is tightly regulated by its specific inhibitor, calpastatin.
This protease is implicated in apoptosis and the proteasome-ubiquitin pathway of protein degradation (Solary et al., 1998). Calpain-mediated proteolytic cleavage was shown by Western analysis for PTP$_\alpha$ and PTP$_\varepsilon$ (members of the R4 subtype containing short, highly glycosylated extracellular domains) in various cell lines and mouse tissues, resulting in smaller protein forms at 66kDa and 65kDa, respectively (Gil-Henn et al., 2000; Gil-Henn et al., 2001). Calpain was described to prefer leucine or valine residues as the second residue on the N-terminal site of cleavage (Wang and Yuen, 1994). For example, sequence alignments of mouse and human PTP$_\varepsilon$ showed the potential region of cleavage, which consisted of a methionine residue preceeded by a valine residue, followed by two leucine residues. Sequence alignment of PTP$_\varepsilon$ and PTP$_\alpha$ with the R2B RPTPs showed that this leucine residue is conserved in all PTPs examined. Conservation of this leucine may indicate a potential conserved calpain cleavage site in these RPTP subtypes, since calpain does not recognize a specific amino acid sequence.

Molecular weight analysis of potential cleavage near this leucine residue predicted an intracellular RPTP$_\rho$ protein product of approximately 70kDa, which may explain the presence of one of the protein products detected by Western analysis. Calpain-mediated proteolytic cleavage may account for the shorter fragment detected with the QCB C-terminal antibody at around 70kDa. Proteolytic processing has been observed in a few cases to regulate subcellular localization of PTPs (Frangioni et al., 1993; Aicher et al., 1997; Nguyen et al., 1999). Therefore, proteolytic processing may alter RPTP$_\rho$ subcellular
localization, resulting in its activation. Subcellular localization of RPTPρ may additionally influence potential substrate specificity, as well as other proteins this enzyme may interact with.

A second possibility, for presence of the 75kDa protein product, is explained by an internal initiation codon within RPTPρ for translation. Translation initiation normally starts at ATG codons, but on occasion it may initiate at CTG codons. Internal initiation of translation at leucine (internal CTG codon) of the full-length RPTPρ protein may generate a smaller protein product at 75kDa. In order to determine whether initiation of translation at alternate codons could result in smaller RPTPρ protein products, a series of constructs may be generated in which specific codons are inactivated either separately or in various combinations. Protein lysates may be blotted to determine whether there is an increase in accumulation of specific protein products. Increase in the expression of smaller protein products, for instance at 75kDa, will indicate that translation initiation may take place at alternate codons.

Western studies showed that two members of the R2B subtype (PTPμ, and PTPκ) cross-reacted with RPTPρ, which may be indicative of their high homology, especially within the C-terminal phosphatase domains. PTPμ expression in the mouse cerebellum appeared to cross-react or overlap with RPTPρ expression. Two explanations may exist for the PTPμ antibody cross-reactivity evident in immunocytochemical studies. One possibility may be that more Purkinje cells are present in the anterior region of the cerebellum; therefore accounting for more PTPμ staining in this region compared to the posterior
region. Purkinje cell counts were analyzed in both regions, and ~20% more Purkinje cells were present in the anterior lobules. Another explanation is that RPTPρ labeling of granule cell synaptic terminals on the parallel fibers may cross react with PTPμ on the Purkinje cell dendritic tree. Parallel fibers are known to interact with the Purkinje cell dendritic tree in the molecular layer.

4.4 MATERIALS & METHODS

Generation of RPTPρ constructs:

C-terminal FC20 construct: The FC20 clone (3.2kb) was obtained from a Stratagene Lambda Zap II human frontal cortex library (McAndrew et al, 1998a, b), and inserted in the pBLUEscript (pBSII KS) plasmid. The FC20 clone spanned amino acid # 909-3’UTR (NT#2909-~5900), including the first and second phosphatase domains, the stop codon, and part of the 3’ untranslated region. The FC20 human RPTPρ clone (provided by Patty McAndrew) was used as the PCR template, using the following sequence specific PCR primers, with flanking BamHI and XbaI sites:FC20 Forward Primer (F20PT) (30mer: hRPTPρ NT#2908-2929; 5’-GGGATCCCTACGGGTTCAAGGAGGAATA C-3’); FC20 Reverse Primer (RPT) (33mer: hRPTPρ NT#4528-4504; 5’-GCAAGCTTCTGTTCC AGGGTCTCCACCATGTG-3’). The PCR reaction consisted of 35 cycles at 94°C for 30s, 65°C for 30s, and 68°C for 2min; the products were electrophoresed on a 1% agarose gel.
Bacterial expression of mouse and human RPTPβ recombinant fusion proteins:
The FC20 PCR product (1.72kb) and the pPROEX-HTc (4.779kb) prokaryotic expression system (GIBCO BRL, Cat#10711-018) were digested using BamHI and XbaI restriction endonucleases, gel purified, and ligated overnight at 4°C. The FC20 recombinant fusion protein lacked the 3' untranslated region and was designed to represent a 1.72kb coding region (541 amino acids), corresponding to amino acid#909-1448 (NT#2909-4528), including the first and second phosphatase domains and the stop codon. DH5αF' competent cells (GIBCO BRL, Cat#18265-017), were transformed with pPROEX-HTC/FC20 and grown at 37°C in LB with 50-100µg/ml ampicillin at 225rpm. Expression of the C-terminal FC20 recombinant fusion protein was induced by the addition of 0.4-0.6mM IPTG. Cells were grown to optimal expression (24hrs), and harvested by centrifugation. Total bacterial cell lysates of uninduced and induced protein samples (30µg) were electrophoresed on 12% SDS-polyacrylamide gels and stained with Coomassie blue.

Molecular Weight Determination: MacDNASIS and EMBOSS programs were used to predict the molecular weight of the C-terminal FC20 recombinant protein. Both programs gave identical values of 62kDa, representing the 6x-His sequence tag as well as additional vector sequences upstream from the insert; therefore the FC20 insert was 61kDa.
Lysis of Bacterial Cells and Solubilization of RPTP$_{\rho}$ recombinant fusion proteins:

**Enzymatic cell lysis of E. coli:** Lysozyme (Roche, Cat#107255) and sonication were used to disrupt the bacterial cell wall in order to determine the solubility properties of each recombinant protein. Hen egg lysozyme was used to cleave the glucosidic linkages in the bacterial cell wall polysaccharide (Brewer, S.J., and Sassenfeld, 1990). In addition, high salt concentrations of deoxycholate were used to dissociate nucleic acids from proteins; this allows digestion of nucleases with DNase I.

Following lysis, OD$_{600}$ and OD$_{450}$ values were compared. OD values decreased by more than 50% of pre-lysis conditions for cells that were lysed. Supernatant and pellet samples for each lysis condition were also run on SDS-PAGE to confirm the results of the OD readings. Prior to purification of the expressed target protein, preliminary analysis of expression levels, cellular localization, and solubility of the target proteins were performed.

**Solubilization of N- and C-terminal RPTP$_{\rho}$ recombinant fusion proteins prior to affinity purification:** The RPTP$_{\rho}$ fusion proteins formed insoluble aggregates, inclusion bodies, in *E.coli*. It was possible to solubilize the proteins from purified inclusion bodies using 6M guanidine hydrochloride under denaturing conditions. Liquid cultures (3ml LB with 100$\mu$g/ml ampicillin), of either N- or C-terminal RPTP$_{\rho}$, were grown at 37°C overnight at 225rpm. A 50ml liquid culture (LB + 100$\mu$g/ml ampicillin) was inoculated with 500$\mu$l of the overnight culture and grown at 37°C at 225rpm for 3-5hrs. Expression of all recombinant fusion proteins was
induced by the addition of 0.4-0.6mM IPTG. Total bacterial cell lysates (1ml samples) of uninduced and induced protein samples were centrifuged at max speed in a microcentrifuge. Cells were grown to optimal expression (4-24hrs), and harvested by centrifugation at 10,000xg for 10min (JA-20 rotor, Beckman). Pellets were frozen overnight at -20°C, and then resuspended in 10ml sterile water and incubated with 200µg/ml hen egg lysozyme (Roche, Cat#107255) for 1-3hrs at 25°C. Diluted samples (1:10) were measured at OD\textsubscript{600} and OD\textsubscript{450} before and after lysozyme incubation, and following sonication. Pellets were sonicated 10x for 1min at 10W (60 Sonic Dismembrator; Fisher Scientific). Once OD values reached half the original values, continued with centrifugation at 10,000xg for 30min.

Supernatants were removed and saved for analysis and purification. Pellets were resuspended in 10ml wash buffer (100mM Tris, pH 7.4, 5mM EDTA, pH 8.0, 5mM DTT, 2M Urea, 2% Triton X-100) and centrifuged at 10,000xg for 10min. The washing step was repeated (2-3x) until the pellet was one color and supernatant was clear. The final homogenous pellet was resuspended in 5ml 1x binding buffer (8x= 40mM imidazole, 4M NaCl, 160mM Tris, pH 7.9, 6M guanidine hydrochloride). The suspension was briefly sonicated to disperse the pellet, and then proceeded with Ni\textsuperscript{2+} affinity purification.

\textit{Ni2+ Affinity Column Purification Under Denaturing Conditions:} RPTP\textsubscript{ρ} recombinant fusion proteins were purified using a nickel affinity column under denaturing conditions according to the manufacturer’s protocol (Metal Chelation
Chromatography; Novagen, Inc.). His Bind Resin (Novagen, Cat#69670-3), estimated at 8mg/ml bed volume, was used for rapid one-step purification of proteins containing a His tag sequence by metal chelation chromatography.

**Anti-peptide antibody protocol:** A general flowchart of the anti-peptide antibody protocol is given in Figure 4.13. The first step in the production of an anti-peptide antibody was the synthesis of a peptide consisting of 13-21 amino acids, followed by HPLC purification of the peptide sequence. Once the peptide sequence was verified by mass spectroscopy, the peptide was conjugated to a carrier protein, such as KLH or ovalbumin, through cysteine thiol coupling. A pre-immune bleed was taken prior to injection of the peptide into New Zealand white rabbits. The primary injection consisted of the peptide emulsified in Freund’s complete adjuvant, followed by a boost with the peptide emulsified in Freund’s incomplete adjuvant. The first bleeds were obtained, and tested by ELISA to identify titer levels. ELISA analysis of the sera was performed using the peptides conjugated to BSA, a non-relevant protein carrier. The rabbits were boosted with the peptide emulsified in Freund’s incomplete adjuvant until high titer levels were achieved. All bleeds were tested by Western blot analysis on mouse and human brain tissue, as well as liver tissue. Based on titers and Western results, some bleeds were affinity purified, and further used in both Westerns and immunocytochemistry.
Enzyme-Linked Immunosorbent Assay (ELISA): Anti-peptide titers were determined with ELISA. Microwell plates were coated with 1µg/100ul/well of solid phase immobilized antigen (peptide Cys-KLH), which was used to capture antibody (pre- and post-immune serum). Serially diluted antibody (200µl of 10Fold) was added to each well and incubated for 30min at 37°C. The supernatant was decanted, and then wells were washed twice with wash buffer (PBS containing 0.05% Tween-20). Secondary antibody, (100µl of 1:2000 HRP-conjugated goat anti-rabbit IgG; Zymed #62-6120) was added to each well and incubated for 30min at 37°C. Diluent for antibody or enzyme conjugate was 1% BSA in PBS containing normal goat IgG (0.1mg/ml) + KLH spike (0.1µg/ml). Following secondary antibody incubation, the wells were washed twice with wash buffer. Substrate solution, 100µl ABTS (Zymed #00-2011), was added to each well and incubated at room temperature for 10 and 20min. Microtiter plates were read at A405.
Anti-peptide Polyclonal Antibody Production in Rabbits

Peptide synthesis/ HPLC purification/ Mass Spec verification of sequence

↓

Conjugation of peptide to carrier like KLH, ovalbumin, etc

↓

Pre-immune bleed, primary injection of peptide emulsified in Freund's complete adjuvant

↓

Boost with peptide emulsified in Freund's incomplete adjuvant

Repeat until achieve good titer

Bleed rabbit

↓

ELISA, Western analysis, Affinity purification

Figure 4.13: A general flowchart of the anti-peptide polyclonal antibody protocol.
Membrane preparation:

Crude (P2 pellet) membrane preparation: C57BL/6 mouse and human brain and liver tissue were removed, and homogenized in 20 volumes (w/v) of ice-cold lysis buffer (5mM Tris-Cl, 5mM Tris base, 5mM MgCl₂; pH 7.4; plus 1 Protease Inhibitor Cocktail tablet from Roche Cat# 1 836 153 per 50ml buffer) using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged in Beckman Model J2-21 centrifuge with the JA-20 rotor for 15min at 48,000xg at 4°C. The supernatant was discarded and the pellet was resuspended in 20 volumes (w/v) of ice-cold lysis buffer, plus Protease inhibitor cocktail tablet, using a tissue homogenizer (Sonic Dismembrator; Fisher Scientific) at a power setting of 3 Watts for 30sec. The homogenate was centrifuged in a Beckman Model J2-21 centrifuge with the JA-20 rotor for 15min at 48,000xg at 4°C. The supernatant was discarded and the pellet was resuspended in 1-2ml of deionized water. Protein concentrations of the membrane preparations were determined using the BCA protein assay kit (SIGMA, Cat# BCA-1).

Crude synaptosomal membrane preparation: C57BL/6 mouse and human brain and liver tissue were removed, and homogenized in 15 volumes of ice-cold 0.32M sucrose using a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged in Beckman Model J2-21 centrifuge with the JA-20 rotor for 10min at 1000xg at 4°C. The pellet was discarded, and the supernatant was centrifuged for 20min at 20,000xg at 4°C. The pellet was
resuspended in 20ml ice-cold distilled water using a tissue homogenizer (Sonic Dismembrator; Fisher Scientific) at a power setting of 3 Watts for 30sec. The suspension was centrifuged for 20min at 8000xg at 4°C. The tube was gently agitated to suspend the buffy coat surrounding the pellet into the supernatant, without disturbing the pellet. The pellet was discarded, and the suspension was centrifuged for 20min at 48,000xg at 4°C. The supernatant was discarded, and the pellet (crude synaptic membrane pellet) was resuspended in 1-2ml of deionized water. Protein concentrations of the crude synaptosomal membrane preparations were determined using the BCA protein assay kit (SIGMA, Cat# BCA-1).

**Human Protein Medleys:** Human brain (16 year old Caucasian female, Cat# 7800-1) and liver (37 year old Caucasian female, Cat# 7800-1) protein medleys were purchased from Clontech. The protein medleys were provided as an electrophoresis-ready solution containing SDS-solubilized proteins prepared from normal human tissues. Each preparation contained 10mg/ml protein concentration isolated from whole tissue homogenates, provided in Laemmli sample buffer.

**Western blotting:** The following protocol is a modification of the ECL Western blotting system (Amersham ECL kit; Cat#RPN2209). Stacking gels and resolving gels (6%, 8%, 10%, and 12%), as well as all SDS-polyacrylamide gel electrophoresis solutions were prepared according to "Antibodies: A Laboratory
RAD, Cat#165-2944), a Gelman power pack, and 1x Laemmili running buffer (1
liter 10x stock= 30.3g Tris base, 144.2g glycine, 10.0g SDS, pH 8.3) were used
for SDS-polyacrylamide gel electrophoresis. Tissue protein samples (50-100µg)
were prepared in 5x SDS loading buffer (0.25M Tris-Cl, pH 6.8, 10% SDS, 0.5%
bromophenol blue, 0.5M DTT, 50% glycerol) and separated by SDS-PAGE at
20mA (constant current) through the stacking gel and 40mA through the
resolving gel. Once the loading dye ran off, the gels were prepared for wet
electrophoretic transfer according to “Antibodies: A Laboratory Manual”, Harlow
and Lane, 1988. The gel, NitroBind (0.45) nitrocellulose membrane (Osmonics
Inc., Cat#EP4HY00010), and Whatman 3MM filter paper were soaked in chilled
transfer buffer (for proteins 20,000-400,000 MW: 50mM Tris base, 380mM
glycine, 0.1% (w/v) SDS, 20% methanol) for 15-30min. The transfer sandwich
was assembled as described in Harlow and Lane (1988), and submerged in the
BIO RAD transfer tank containing chilled transfer buffer. All transfers were run at
4°C at constant voltage (100Volts; 0.5Amps) for 1hr using the LKB 2005
Transphor Power Supply.

Gels were stained in Coomassie Brilliant Blue Staining solution (0.25%
Coomassie Brilliiant Blue R-250, 0.5% methanol, 0.1% glacial acetic acid).
Nitrocellulose membranes were rinsed in 1x PBS, labeled and cut into strips (for
processing in different conditions), and blocked in 1% gelatin (Laboratory grade
100 Bloom, Fisher Cat#G7-500) for 1hr. Strips were briefly rinsed and washed
(1x15min, 2x5min) in PBS-Tween-20 (0.1%), incubated in primary antibody
diluted (1:500) in PBS-Tween-20 (0.1%) for 1hr, briefly rinsed and washed (1x15min, 2x5min) in PBS-Tween-20 (0.1%), and incubated in 1:50,000 dilution of anti-rabbit IgG-HRP secondary antibody (Santa Cruz, sc-2004) for 45min. Following the secondary antibody incubation, strips were briefly rinsed and washed (1x15min, 4x5min) in PBS-Tween-20 (0.1%) and detected for 1min with equal amounts of ECL Western blotting detection reagents (Amersham Pharmacia, Cat#RPN2209). Excess detection solution was drained off, and then the strips were exposed to Kodak Biomax Light film (Amersham Pharmacia, Cat#V8689358) for 10sec to 5min.

**Immunocytochemistry:** Perfused and unperfused, paraffin-embedded mouse brain tissue was sectioned at 40um (coronal, horizontal, and sagittal planes) using the IEC-CTF Microtome-cryostat at –13°C, and sections were transferred to 24-well plates containing 1xPBS. The following immunocytochemistry protocol is a modification of Vector Labs Kit protocol. Sections were incubated in 3%H2O2/10%methanol made up in 1xPBS for 5min, and then washed 3x 5-10min in 1xPBS until bubbles were gone. Sections were blocked in 5% blocking serum (Normal goat serum), washed 1x 5-10min in 1xPBS, and incubated 24-28hrs at room temperature in primary antibody/blocking serum. Following the primary antibody incubation, sections were washed 3x 10min in 1xPBS, incubated with secondary antibody/blocking serum (biotinylated anti-rabbit IgG, Vector Labs), washed 3x 10min in 1xPBS, and reacted for 60min in the ABC Mix (Avidin-Biotin Complex mixture, Vector Labs). Following the reaction, sections
were washed 3x 10min in 1xPBS, incubated in DAB solution (SIGMAFAST DAB tablets, Sigma Cat#D-4293) for 10min, and rinsed 4x in cold 1xPBS. Sections were mounted onto gelatin-coated slides and air-dried overnight.
LIST OF REFERENCES


Current Protocols in Molecular Biology, 1997


Aging in the central nervous system (CNS) is controlled by expression of specific genes; however, the complexity of this system has made it difficult to identify these genes. A lack of biochemical markers slows the progress in deciphering molecular components within the aging process in the brain. The cerebellum controls movement and balance, which is disrupted with aging. The aging mouse cerebellum has a reduced number of Purkinje, basket and stellate cells, whereas granule cell numbers remain relatively intact (Sturrock 1989).

Several gene expression-profiling techniques have been described, which may be divided into ‘open’ and ‘closed’ systems. DNA microarray technology is a ‘closed’ system, which detects a limited and pre-selected number of previously known genes. To date, microarray assays have identified only a subset of the genes differentially expressed in the aged mouse cerebellum. ‘Open’ system approaches such as SAGE (Serial Analysis of Gene Expression), which does not rely on prior knowledge of genes (Velculescu et al., 1995), has the potential to identify the full set of expressed cerebellar genes. This sequencing-based
approach is more appropriate for detection and quantification of known genes and novel genes for which no sequence information exists. SAGE provides quantitative, as well as qualitative data, for every potentially transcribed sequence from a sample. This method is based on two principles: The isolation of short nucleotide sequence tags (14-15bp SAGE tags) from a defined position within the transcript, followed by ligation of all tags into long DNA molecules (concatemers) which are then cloned into a plasmid vector and isolated. This approach relies on sequencing of the concatemers, which results in identification and quantitation of individual tags corresponding to specific genes, including novel genes. SAGE takes advantage of the mRNA 3’ region (Yamamoto et al., 2001); the SAGE tag is located next to the 3’-most Nla III restriction site. Each tag contains a sufficient number of nucleotides that corresponds to an individual transcript, and therefore, represents a unique marker for any expressed gene. Sequencing of clones isolates individual tags, which are matched with tags derived from the Unigene database; thereby the gene corresponding to each tag is identified. The frequency of each tag is proportional to the amount of corresponding mRNA present in a sample; this results in a quantitative measure of transcript abundance in the cell or tissue of interest (Saha et al., 2002).

The objective of this chapter is to identify and quantify all expressed cerebellar genes in the adult and aged C57BL/6 mouse cerebellum by conducting a global analysis of gene expression using SAGE. This approach may identify candidate genes involved in the aging process.
5.1 Principles of the SAGE method:

Serial Analysis of Gene Expression (SAGE) is a sequence-based approach developed for the identification and quantitation of all genes from a sample, such as a cell line or specific tissue, without prior knowledge of the genes (Velculescu et al., 1995). A schematic of the method is outlined in Figure 5.1. Briefly, polyA+ RNA is bound to oligo-(dT) magnetic beads. Double-stranded cDNA is synthesized on the beads using reverse transcriptase and *E. coli* DNA polymerase. The cDNA is cleaved with a 4bp-recognizing enzyme (an anchoring enzyme), such as *Nla* III. This restriction enzyme is one of the most frequently used enzymes and is known to cleave DNA approximately every 256bp at a specific restriction site (CATG). The cDNA is then divided into two fractions and ligated with two adapters (designated as A and B). Adapters A and B are ~40bp in length. These two different adapters contain cohesive 4bp overhangs that are complementary to the *Nla* III digested cDNA fractions. The adapters contain a type IIS recognition site near the 3'-*Nla* III sequence; this site is specific for the tagging enzyme, such as *BsmF I*. The reaction mixtures are cleaved with *BsmF I*, which binds to the corresponding recognition sequence in the adapter. The cDNA is cleaved 10-14bp downstream from the recognition site, which releases a 50bp tag containing a 4bp overhang at the 5'-end. Klenow DNA polymerase is used to fill in the 5'-overhangs created by the *BsmF I* digestion, and then the two fractions of tags are ligated to form ~100bp ditags. The 100bp ditags are amplified and then cleaved with *Nla* III to release 26bp ditags, which contain sequences derived from the transcript cDNA. Polyacrylamide gel
electrophoresis is used to purify the 26bp ditags, and then these ditags are ligated to form concatemers. Following gel purification, the concatemers are cloned into the pZERO-1 vector to produce plasmid clones, which are sequenced to identify tags. Each isolated clone contains a series of concatenated ditags separated by the 4bp Nla III recognition sequence (CATG). Upon sequencing the tag concatemers, ~50 tag sequences are identified in each sequencing reaction. Each 14bp tag sequence is compared to the mouse Unigene reliable tag-to-gene mapping database and matched to well-characterized mRNAs/cDNA sequences or expressed sequence tags (ESTs). At the end, the SAGE technique generates a list of tags, with their corresponding count values, thereby producing an overall representation of gene expression in a given sample.
Figure 5.1 Principles of the SAGE method. From Besco, 2003.
5.2 RESULTS:

SAGE cerebellar libraries were constructed (See materials and methods section) from C57BL/6 male mice at postnatal days (P) 92, 150, 810, and 840. Two libraries at P810 were independently generated (designated as P810#1 and P810#2), whereas the P840 library consisted of two male animals pooled prior to library construction. A P23 cerebellar library constructed by Besco (2003) from a pooled male and female mouse was used for an additional comparison. Tag frequency in each SAGE library reflects the relative abundance of the corresponding cerebellar mRNA, a feature that allows comparison between independently generated libraries. All libraries were estimated to contain over 100,000 tags, indicating that they are sufficiently large to obtain a good representation of expressed genes. The actual number of tags sequenced (total tags) was much smaller: 19,435 for the P23 library (Besco, 2003), 16,430 for the P92 library, 18,103 for the P150 library, 18,581 for the P810#1 library, 8,528 for the P810#2 library, and 7,630 for the P840 library. This cutoff was arbitrary, based mainly on sequencing cost. Prior to comparison of the SAGE libraries, several steps were taken for SAGE data analysis (Figure 5.2).
Figure 5.2: Flow chart of SAGE Analysis.
5.2.1 Normalization of data, consolidating duplicate tags, and Closest Neighbor Analysis: The SAGE2000 software (Johns Hopkins University) was used to extract tag sequences from the raw sequence data. These tags were then compared to the mouse Unigene database (NCBI) and matched to a Unigene ID number corresponding to a specific gene. Those genes not represented in the Unigene database may be representative of novel genes. Because the tag count in each mouse cerebellar library was different, data were normalized using two approaches: (1) Calculation of percent values (absolute abundance= \( \frac{\text{count}}{\text{total tags per library}} \times 100 \)), or (2) Conversion of counts to tags per million (TPM= \( \frac{1,000,000 \times \text{count}}{\text{total tags per library}} \)). TPM is a standard notation in the SAGE literature for normalizing data. Once data was normalized, duplicate tags were consolidated. Duplicate tags, shared the same tag count, but identified more than one gene. For example, the tag corresponding to the sequence TCAGGCTGCC identified the gene acetyl-Coenzyme A acetyltransferase 1 (Unigene ID#219649) and the gene ferritin heavy chain (Unigene ID# 1776), which was consistent for all libraries generated. These duplicate tags, identifying more than one gene, were grouped as one entry. Most duplicate tags corresponded to genes belonging to the same gene family, or to genes with similar domain structures.

Although the majority of tags in all libraries were present in multiple copies, a significant number of tags were present only once. In addition, multiple tag sequences would identify the same gene, although frequently the tags were present at low frequency. For example, the two tag sequences AGAGGCAAG
(tag count=12) and CTATGCATTG (tag count=1) identified the same gene, synapsin I (Unigene ID# 19661). In most studies, these single tags are discounted as it was assumed that many could arise through sequencing errors due to base substitution, deletion, or addition. However, this process tends to eliminate tags corresponding to genes expressed at very low levels. In order to compromise between the two extremes of either retaining or discounting all single tags, an algorithm was developed in the lab by Dr. Rejniak. This algorithm, Closest Neighbor Analysis, substitutes, deletes, or adds bases to determine whether any tag in the SAGE library is related to another within the same library. Tags with a count of 1, identified as a closest neighbor, showing such errors were removed from the corresponding library. Finally, all duplicate tags and genes were consolidated to obtain the number of unique tag sequences.

5.2.2 Distribution of tags into four categories: Unique tags were further categorized into four groups: (1) Novel genes, (2) ESTs, (3) RIKEN, KIA, and hypothetical genes, and (4) known genes (Figure 5.3). The known genes category contained the largest percentage of tags (32%-41%) in all SAGE cerebellar libraries, followed by ESTs (25%-28%), RIKEN, KIA, and hypothetical genes (21%-25%), and novel genes (9%-20%). The overall distribution of tags within these groups was the same for all mouse cerebellar libraries. The high percentage of novel genes is surprising as they represent relatively abundant transcripts, which might be assumed would have been detected previously. It is
also notable that the percentage of novel genes was almost twice as high in one of the aged cerebellar libraries (P810#1) compared to the adolescent and adult (P23, P92, and P150), suggesting that, in this animal, novel genes had become active during the aging process.

5.2.3 Functional Classification using MmSAGEClass:

Tags in the "known genes" category were further subdivided into functional categories based on the Gene Ontology classification using a web-based program developed in this laboratory (MmSAGEClass) (http://mbi.osu.edu/~rejniak/MmSAGEClass.html). MmSAGEClass assigns each SAGE tag within a given library to an ontology class, and is designed specifically for the classification of mouse genes. This program uses information provided from three databases, which includes the National Center for Biotechnology Information (NCBI) SAGEmap (http://www.ncbi.nlm.nih.gov/SAGE), the Cancer Genome Anatomy Project (CGAP) Genes (http://cgap.nci.nih.gov/Genes), and the Gene Ontology (GO) Consortium (http://www.geneontology.org), to classify data provided as tags and their counts into three general ontologies. More specifically, data required for the MmSAGEClass database is obtained from the file SAGEmap_tag_ug-rel (data deposited on October 11, 2002), which contains only reliable tag-to-gene assignments, from the NCBI SAGEmap.
Figure 5.3: Distribution of tags in C57BL/6 mouse cerebellar libraries into four categories. Unique tags were categorized into four groups: (1) Novel genes (2) ESTs (3) RIKEN, KIA, and hypothetical genes (4) Known genes in mouse cerebellar libraries at postnatal days P23, P92, P150, P810#1, P810#2, and P840. Each category was calculated as a percentage of the total tags obtained for a particular age. * from Besco, 2003.
In addition, functional data is obtained from data accessible at the CGAP GO Browser containing data versions: function.ontology-version 2.463, process.ontology-version 2.531, and component.ontology-version 2.236. 

*MmsAGEClass* was used to classify mouse genes identified in all the C57BL/6 mouse cerebellar SAGE libraries generated. Each gene may be assigned to more than one functional class; therefore, the total number of genes listed in the classes may be greater than the total number of tags used as input data. Overall, the tag frequency was proportional to the abundance of specific mRNA species within a given tissue, in this case the cerebellum. The percentage of genes within each functional class was virtually identical in all libraries (Figure 5.4). Most genes were categorized into the cellular component (CC)-cell (20%-21%) and biological process (BP)-physiological processes (20%), followed by molecular function (MF)-enzyme activity (13%-16%), MF-binding activity (9%-12%), MF-transporter activity (10%-12%), and MF-obsolete (6%-10%). Further comparison of percent change in the number of expressed genes within each functional category for the young (average of P23, P92, and P150) and aged (average of P810#1, P810#2, and P840) C57BL/6 mouse cerebellum was analyzed. As seen previously, the percentage of total tags within each functional category was virtually identical between the young and aged cerebellum (Figure 5.5).
Figure 5.4: Functional classification of known genes using MmSAGEClass. SAGE libraries generated from C57BL/6 mouse cerebella at postnatal days (P) 23, 92, 150, P810#1, P810#2, and P840 were analyzed. Tags with counts equal to one were analyzed and removed by closest neighbor analysis. Genes are classified into subcategories within the main groups, which include **CC-cellular component**; **BP-biological process**; **MF-molecular function**. *P23 from Besco, 2003*
Figure 5.5. Functional classification of known genes using MmSAGEClass. Young cerebellum represents the average of postnatal days (P) 23, 92, and 150. Aged cerebellum represents the average of postnatal days (P) 810#1, P810#2, and P840. Tags with counts equal to one were analyzed and removed by closest neighbor analysis. Genes are classified into subcategories within the main groups, which include: CC, cellular component; BP, biological process; MF, molecular function. Error bars represent standard deviation.
5.2.4 High abundance tags and low abundance tags: Data showed that distinct differences existed in the kinds and amounts of genes present in the adolescent, adult, and aged mouse cerebellar libraries. Within all mouse cerebellar libraries, the three hundred most abundantly expressed genes, with tag counts greater than three, were compared (Table 5.1, page 1 of 20). All values were normalized and represented in tags per million (TPM). Genes identified included known genes, ESTs, RIKEN, KIA, hypothetical genes, and novel genes. Many genes increased or decreased throughout aging, as well as fluctuated between ages. The most striking change included a decrease in gene expression with aging for both prolactin and growth hormone.

There are many approaches to analyze differences in gene expression between individual libraries. One such approach included comparison between the adult (P92) and aged (P810#1) libraries. Results showed differentially expressed transcripts with greater than 5-fold increase or decrease (Table 5.2). Genes demonstrating a large upregulation in the P810 cerebellum included testis specific protein (Dcd8), tumor rejection antigen (gp96), macrophage activation 2, oflactomedin 1, and several ESTs, and novel genes. Genes exhibiting a large downregulation included prolactin, growth hormone, Smt2h2, ribosomal protein S3a, and a novel gene. The p-chance for prolactin and growth hormone was calculated at <2.3E-05, which shows that there is a significant change in expression of these genes in the aged cerebellum.
The majority of tags were identified at low abundance (between counts of 1-3). An example of genes expressed at low abundance, throughout all cerebellar libraries, were the receptor protein tyrosine phosphatases, including all members of the R2B subtype (Table 5.3). Although tag counts were low and not normalized, several RPTPs appeared to decrease (RPTPα, CD45, RPTPε, and PTPBR7/PTP-SL), whereas others increased (RPTPβ/ζ) in expression during aging.
## Table 5.1: Most abundantly expressed genes in C57BL/6 mouse cerebellar libraries

SAGE2000 software was used to extract tag sequences for mouse cerebellar libraries from the raw sequence data. These tags were then compared to the mouse Unigene database and matched to a Unigene ID number corresponding to a specific gene. Genes not represented in the Unigene database may be representative of novel genes. *P23 from Besco, 2003. Table shows page 1 of 20.

<table>
<thead>
<tr>
<th>Tag Sequence</th>
<th>Unigene ID</th>
<th>Description/Gene Product</th>
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<th>P92</th>
<th>P150</th>
<th>P810#1</th>
<th>P810#2</th>
<th>P840</th>
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<td>ribosomal protein L32/Mus</td>
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<td>14583</td>
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<td>16417</td>
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<td>interleukin-1 receptor-associated</td>
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<td>11321</td>
<td>6573</td>
<td>5328</td>
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<td>9567</td>
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<td>4747</td>
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<td>1991</td>
<td>3283</td>
<td>3277</td>
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<td>calmodulin 2/ESTs</td>
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<td>2762</td>
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**Animals:** 1M+1F 1 Male 1 Male 1 Male 1 Male 2 Males

**Total tags:** 19,436 16,430 18,103 18,581 8,528 7,630
### Table 5.2: Differentially expressed transcripts in an adult (P92) and aged (P810) mouse cerebellum.

Transcripts listed show greater than 5-fold increase or decrease. P-chance, significance level calculated by SAGE2000 software, with normalization feature checked.
<table>
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<th>Tag Sequence</th>
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Table 5.3: Low abundance tags: Receptor Protein Tyrosine Phosphatases identified by SAGE. Values are given as tag counts. The R2B subtype is highlighted in yellow.
DISCUSSION:

The human genome is estimated to encode between 35,000-75,000 genes (Venter et al., 2001), whereas the mouse genome is predicted to contain between 30,000-40,000 genes (Waterston et al., 2002). In both cases, only a small fraction of these genes have been completely characterized. The finished draft for both human and mouse genomic sequences (NIH/NCBI and Celera Discovery Systems) will assist in determining qualitative and quantitative profiles of global gene expression ('transcriptome') within any tissue or individual cell type, thereby facilitating in the identification of known and novel genes.

Several gene expression-profiling techniques have been described, which may be divided into 'open' and 'closed' systems. SAGE is an 'open' system capable of identifying and quantifying both known and unknown genes. Closed systems include approaches such as differential display and subtractive hybridization. Although these methods have been useful in identifying differences among transcript populations, expression analysis was limited to a set number of genes and was generally not quantitative (Velculescu et al., 1995, 2000). Microarrays using cDNAs or oligonucleotides also compare expression profiles, although quantifiable, but are limited to previously isolated genes.

In a recent study, DNA microarrays containing ~6500 genes were used to examine gene expression profiles in the adult (5-month) and aged (30-month) mouse cerebellum (Lee et al., 2000; Weindruch et al., 2002). The results suggested that aging was accompanied by an increase in oxidative stress and
inflammatory response, and a decrease in neurotrophic support, all aspects of human neurodegenerative diseases. None of the most highly expressed genes identified in the present SAGE analysis were present in the microarray study. Among the low frequency genes, approximately 86 genes were present in both studies. Within the latter group, there was little correspondence between the two data sets. Similarly, a recent study using rat hippocampal mRNA (Evans et al., 2002) showed that although a good correlation existed between Affymetrix Gene Chip and SAGE methods when detecting moderate to high transcript levels, low levels of expression were not detected reliably by either technique.

In the present SAGE study, gene expression profiles were determined in adolescent, adult, and aged mouse cerebella. Four gene categories were defined: The largest percentage of tags within all SAGE cerebellar libraries were known genes, followed by ESTs, RIKEN, KIA, and hypothetical genes, and novel genes. The overall distribution of tags within these groups was the same for all mouse cerebellar libraries. The high percentage of novel genes (9%-20%) was surprising as many represented relatively abundant transcripts, which might be assumed would have been detected previously. The known genes category was further subdivided into functional classes using a web-based program called MmSAGEClass. Many genes increased or decreased during aging.

Comparison of a single male adult (P92) and an aged (P810) mouse cerebellum showed that although the percentage of genes in each functional category did not change in the aged cerebellum, distinct differences were evident for specific
genes. The most striking of these changes was a strong downregulation in growth hormone (GH) and prolactin. This finding was repeated in all three aged cerebella.

Growth hormone (GH) and prolactin belong to the somatotropin/prolactin family, and are secreted by the pituitary gland into the bloodstream. Secretion of these hormones promotes growth and development in a variety of tissues, including the brain (Olsen et al., 2002). Growth hormone stimulates cells to secrete insulin-like growth factor (IGF-1), which is a small peptide structurally related to proinsulin that promotes proliferation of cells (Sonntag et al., 2001). Hormones generated in the hypothalamus control the secretion of GH and prolactin. Growth hormone releasing factor (GRF) stimulates GH release (Guillemin et al, 1982), whereas inhibition is regulated by somatostatin (Brazeau et al., 1973). In humans, the release of GH is known to occur in pulse-like bursts from the pituitary gland, and a distinct male and female secretory pattern has been identified (Jansson et al., 1985).

A characteristic observed in aging mammals is a decrease in endocrine function, which is evident by changes in hormone release as well as how a cell responds to a specific hormone action. Many studies in both animals and humans have documented that the release of growth hormone decreases with age (Ho et al., 1987; Meites, 1988; Nyberg, 1997). The result of age-related decrease in GH is reflected in the activity of IGF-1, which also decreases with age (Florini et al., 1985; Rudman, 1985). There is evidence that hormones regulated by GH, such as IGF-1, are important in brain function (Thornton et al.,
1999; Sonntag et al., 2001). For instance, insulin-growth factors have been reported to stimulate neurite formation, increase survival of neurons and glia, increase synaptogenesis, and have an important role in neuronal repair (McMorris and Dubois-Dalcq, 1988; Carson et al., 1993; Thornton et al., 1999).

The signal transduction pathways (Figure 5.5) affected during human GH/prolactin activation involve several tyrosine kinases including Janus kinase 2 (JAK2), the signal transducer and activator of transcription 5 (STAT5), and mitogen-activated protein kinase (MAPK) (Darnell et al., 1994; Horseman and Yu-Lee, 1994). Activation of the GH receptor stimulates JAK2 to form a complex with the receptor, leading to phosphorylation of both proteins, followed by phosphorylation of several intracellular proteins. A decrease in signal transduction has been described for insulin and IGF-1 receptors in aged animals (Sonntag et al., 2001). Reduced activity of the IGF-1 pathway greatly increases lifespan in C. elegans (Murphy et al., 2003).

In summary, information obtained by SAGE reflects the general state of the cell or tissue at a particular moment in time. In spite of the variability between animals, there is considerable stability in the functional classes across all ages. SAGE is an excellent screening method for the detection of known and unknown genes, and may be used as a 'first step' in the analysis of developmental processes and disease mechanisms.
Figure 5.5: Prolactin/Growth Hormone/Insulin-like Growth Factor-1 (IGF-1) Signaling Pathway. GH-Growth Hormone; GHR- Growth Hormone Receptor; PRL-Prolactin; PRLR- Prolactin Receptor; P=phosphorylated. S5-STAT5.
5.4 MATERIALS AND METHODS:

Animals and Tissue Extraction:

Aged male C57BL/6 mice (27-28month) were obtained from the National Institute of Aging (NIA). Animals were maintained and housed at the NIA behind pathogen barriers, maintained at 70°F, and fed NIH 31 feed ad libitum. Mice were regularly monitored for genetic purity and health status. Adolescent and adult C57BL/6 males at postnatal days (P) 23, P92 and P150 were bred in-house from a stock obtained from Jackson Laboratories (Bar Harbor).

Animals were sacrificed by CO₂ inhalation, followed by cervical dislocation. The brain was removed rapidly within 1 minute after sacrifice, and the cerebellum was dissected immediately from individual animals. RNA was extracted using Trizol Reagent (Invitrogen Life Technologies, Cat#15596-026), prepared according to the method of Velculescu et al., (1995) and Zhang et al., (1997). Two milliliters of pre-cooled Trizol Reagent was added to each tissue sample and homogenized. The time from sacrifice to homogenization did not exceed 30 seconds, thus ensuring that the mRNA remained maximally intact. Samples were incubated for 5 minutes at room temperature to allow the tissue to dissolve. Chloroform (200 µl) was added and the sample was mixed vigorously. The samples were incubated for 2 minutes at room temperature and centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase was transferred to a clean, RNase-free tube and the RNA precipitated by the addition of 1 ml isopropanol. The samples were incubated at room temperature for 10 minutes
and centrifuged at 12,000 x g for 10 minutes at 4°C. The resulting pellet was washed with 75% ethanol and resuspended in RNase-free water. A 1% denaturing agarose gel was used to verify the quality of total RNA (Figure 5.6A).

SAGE: SAGE was performed as outlined in the detailed protocol provided in the Invitrogen manual (http://www.invitrogen.com). A brief description of the main steps is provided below.

cDNA synthesis: Total RNA was mixed with oligo-(dT) magnetic beads and polyA+ RNA was isolated. Double stranded cDNA was synthesized on the beads using reverse transcriptase and digested with the restriction enzyme Nla III (the anchoring enzyme). This enzyme cleaves DNA immediately after a CATG site. cDNA synthesis and Nla III digestion was verified on a 3.5% agarose gel (Figure 5.6B).
Figure 5.6: Verification of RNA quality, cDNA synthesis, and efficiency of \textit{Nla} III digestion of total RNA from a P840 C57BL/6 male mouse cerebellum. A) A 1\% denaturing agarose gel was used to verify 10\mu g of total RNA from a P840 C57BL/6 male mouse cerebellum. Lane 1 shows the expected 18S (1.9Kb) and 28S (4.5Kb) rRNA bands. B) PCR products (10\mu l) were loaded into each lane of a 3.5\% agarose gel; Lane 1 contains a 100bp DNA marker (Invitrogen Cat#15628-019). Following cDNA synthesis, a 540bp band was PCR amplified using the GAPDH primers (Lane 2); and a 350bp band was amplified using the EF primers (Lane 3). After successful \textit{Nla} III digestion, the GAPDH primer binding sites were lost (Lane 4), and the EF primer binding sites were retained (Lane 5). A no template negative control was also used, which resulted in no bands (Lane 6).

\textit{Ligation of adapters:} The digested double stranded cDNA was divided into two fractions; each fraction was ligated with one of two adapter sequences (designated as A and B). Adapters A and B are \textasciitilde 40bp in length. The adapters contained cohesive 4bp overhangs (GTAC) that are complementary to the \textit{Nla} III digested cDNA (CATG). Each fraction was cleaved with restriction enzyme \textit{Bsm} F I, the tagging enzyme, which cleaves the cDNA 10-14 bp downstream from the recognition site. This releases an \textasciitilde 50 bp tag with a 4 bp overhang located at the 5’ end. The \textasciitilde 50 bp tag consists of a \textasciitilde 40 bp adapter sequence and 10-14 bp of unique sequence from a single transcript.
**Construction of the ditags:** Klenow DNA polymerase was used to fill the 5’ overhang created by *BsmF* I digestion, and then the two fractions of single tags were ligated to form ~100 bp ditags. These ~100 bp ditags were amplified with specific primers to produce sufficient 100 bp ditags for the subsequent generation of concatemers. A qualitative PCR ditag optimization step was used (Figure 5.7A), prior to gel purification of the 100bp ditags (Figure 5.7B). No PCR bias was expected, since the ditags were of short, uniform (~100 bp) size. The ~100 bp ditags were cleaved with the *Nla* III anchoring enzyme to release a 26 bp ditag. A 5µl sample of the digest was used for verification of complete digestion (Figure 5.8A). These ditags were comprised entirely of sequences derived from transcript cDNAs. Each ditag was separated by the *Nla* III CATG recognition sequence. The 26 bp ditags were purified away from the adapters by polyacrylamide gel electrophoresis (Figure 5.8B), and ligated to form concatemers (Figure 5.9A). The fraction containing 20-50 tags/concatemer was gel purified, and then the concatemers were cloned into the digested pZErO-1 cloning vector (Figure 5.9B), and transformed into *E. coli* TOP 10 competent cells. Plasmid DNA was digested with *Xba* I and *Hind* III to release inserts (Figure 5.10A), and each clone was sequenced (Figure 5.10B).
Figure 5.7: PCR Optimization of ditag amplification. A) PCR products (10µl) were loaded into each lane of a 3.5% agarose gel; Lane 1: 100bp DNA Marker (Invitrogen Cat#15628-019); Lane 2: 1/20 dilution of P840 cerebellum template; Lane 3: 1/40 dilution of P840 cerebellum template; Lane 4: 1/80 dilution of P840 cerebellum template; Lane 5: 1/20 dilution of the I-SAGE control template; Lane 6: No template control; Lane 7: No ligase control. A strong 100bp ditag band is evident, as well as a faint 80bp band (contains only adapter sequences without any transcript sequence). B) Gel purification of the 100bp Ditag: Scale-up PCR reactions were set up using 398 samples. All PCR samples were pooled and run on a 12% polyacrylamide gel. A strong 100bp band (representing the 100bp Ditag) was evident and cut out from lanes 3-10 for further purification. Lane 1 contains a 10bp DNA marker (Invitrogen Cat#10821015) and Lane 2 contains a 100bp DNA marker (Invitrogen Cat# 15628-019).

Figure 5.8: Verification of NlaIII digest: A) An overnight digestion of the P840 cerebellum template with NlaIII was set up. A 5µl sample of each reaction (Lanes 2-5) was analyzed on a 3.5% agarose gel to check the efficiency of NlaIII digestion. The digestion resulted in >80% cleavage of the 100bp ditags yielding 26bp ditags. Lane 1 contains a 100bp DNA marker (Invitrogen Cat# 15628-019). Lane 6 contains a 10bp DNA marker (Invitrogen Cat#10821015). B) Gel purification of the 26bp Ditag: The purified 100bp ditag was digested with NlaIII to release the ditags from the adapters. The digested samples were electrophoresed on a 12% polyacrylamide gel. The adapters migrated at ~40bp, and the ditags migrated at ~26bp (Lanes 2-10). No partial digestion products (60-100bp) were observed. Lane 1 contains a 10bp DNA marker (Invitrogen Cat#10821015).
Figure 5.9: Ligation of the 26bp Ditag to yield Concatemers:  A) Lane 1 and 3 contain a 100bp DNA marker (Invitrogen Cat#15628-019). The smear in lane 2 represents the concatemers resulting from a 3hr ligation of the purified 26bp Ditag.  B) Linearization of pZERO-1 cloning vector: Lane 1 and 5 contain a 1Kb Plus DNA marker. Lane 2: 5µl of SphI digested pZERO-1 vector; Lane 3: No DNA; Lane 4: 2µg undigested pZERO-1 vector. Linearized pZERO-1 vector is 2808bp.
Figure 5.10: Isolation of SAGE clones. A) The ditag concatemers were ligated into the pZERO-1 vector and transformed into *E. coli* TOP10 competent cells by electroporation. The transformed cells were plated and grown on LB plates containing 50µg/ml zeocin. Colonies were picked from these plates and miniprepped. The plasmid DNA was digested with *Xba*I and *Hind*III at 37°C for 4hrs. Lanes 2-11 contain inserts ranging from 800bp-1kbp. Lanes 1 and 12 contain a 1Kb Plus DNA Marker. B) Sequencing results from a single clone from a P840 cerebellar library. Electrophoretic trace showing *Nla* III recognition sequence (CATG) (underlined). The intervening sequences correspond to the ditags. 358 clones from the P840 mouse cerebellar library were sequenced, generating 7,630 tags.

*Library Characterization and Sequence Analysis:*
Each SAGE library consisted of ~2x10^6 SAGE tags (or transcripts), with cloned insert sizes of ~500 bp (40 SAGE tags per clone). Randomly selected clones were sequenced. Prior to full-scale sequencing, a fraction of the clones (containing insert sizes ranging from 300bp to 1Kb) were checked for the presence of known genes expressed in the cerebellum. A unique 10-14bp sequence tag identified each transcript. The frequency of each tag within a given population of clones was quantified. Selected clones were sequenced with the 5'-GAC GTC GAC CTG AGG TAA TTA TAA CC-3' sequencing primer.

Sequence trace files generated by the ABI PRISM 3700 DNA Analyzer were analyzed with the Phred base-calling software (Applied Biosystems, Inc.). The SAGE2000 analysis software (Velculescu et al., 1995) was used to extract and analyze the primary sequence data from the electrophoretic trace files. The software extracts tag sequences from the sequence files, counts each tag, and provides a report containing the frequency of each tag and its identity. Only tags encountered two or more times were counted to reduce the chance of including tags produced solely by sequencing errors. Tag sequences were compared to a the National Center for Biotechnology Information (NCBI) mouse SAGE tag-to-gene mapping reference database provided at ftp://ncbi.nlm.nih.gov/pub/sage/map/Mm/Nla3, which matches possible 14-mer tags with both characterized mouse gene entries, as well as expressed sequence tags (ESTs). Potential matches were further analyzed, using sequences present in the NCBI Unigene mouse database, to determine whether there a match existed at the 15th base. In addition, the 15th base was used to determine
whether the matched sequence could be confirmed as being the most 3’ end of a known mouse transcript or EST. Matched ESTs were further analyzed by an advanced BLAST search for homology with known genes.

Functional classification of SAGE tags using MmSAGEClass: A web-based program (MmSAGEClass), http://mbi.osu.edu/~rejniak/MmSAGEClass.html, was developed in the lab by Dr. Rejniak for classification of mouse genes. Briefly, each submitted tag was compared to the Unigene database and was uniquely matched to a Unigene ID number corresponding to the SAGEmap database. Tags yielding more than one Unigene ID number or not matching any Unigene ID number were discarded. The remaining tags were assigned one or more GO numbers provided within the CGAP database and arranged into specific gene ontology classes based on biological function. The functional classification of genes within MmSAGEClass was based on three general ontology categories, including biological processes (BP), cellular component (CC), and molecular function (MF), which are defined by the Gene Ontology (GO) Consortium (Ashburner et al., 2000). This information was accessible through the Gene Ontology Browser (http://cgap.nci.nih.gov/Genes). According to the GO classification the following definitions exist for each broad classification, biological processes refer to “a biological objective to which the gene or gene product contributes”, molecular function is defined as “the biochemical activity (including specific binding to ligands or structures) or a gene product”, and cellular component refers to “the place in the cell where a gene product is active”
(Ashburner et al., 2000). Within these three broad categories exists a hierarchical structure of classification of specific terms, reflecting the biological roles of genes. For example, further classification within the broad category of biological processes includes cell communication, death, and behavior, which are also further subclassified. This system of hierarchical classification is evident in the other two broad categories. Further subclassification exists, although MmSAGEClass restricted analysis to the three broad categories and the first subclassification. The overall goal of the GO Consortium was to provide a defined vocabulary to describe the roles of genes and gene products within any organism.
LIST OF REFERENCES


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