PROTEIN-TYROSINE PHOSPHATASES: REGULATION OF CELL GROWTH AND DIFFERENTIATION

by

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To My Parents

Mr. Daosheng Pan and Ms. Wumei Lin

To My Uncle

Mr. Mufa Pan

To My Wife

Li Lin

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Table of Contents

Chapter 1. Introduction and Literature Review.....1

Structure of Protein-Tyrosine Phosphatases.....3

Function of Protein-Tyrosine Phosphatases.....9

Regulation of Protein-Tyrosine Phosphatases.....19

Manuscripts

I. Chapter 2: G Protein Activation of A Hormone-Stimulated Phosphatase in Human Tumor Cells.....58

II. Chapter 3: Somatostatin Inhibits DNA Synthesis in MIA PaCa-2 Cells Through A cAMP-Independent Pathway.....75

III. Chapter 4: Cloning and Expression of Two Structurally Distinct Putative Receptor Protein-Tyrosine Phosphatases Generated by RNA Processing from A Single Gene.....98

IV. Chapter 5: Expression of Putative Receptor Protein-Tyrosine Phosphatase PTP-P1 is Increased During NGF-Induced Neuronal Differentiation of PC12 Cells.....130

Chapter 6: Summary and Future Directions..... 143

List of Figures

Chapter I: Introduction and Literature Review

Fig. 1. Structural Features of Intracellular Protein-Tyrosine Phosphatases.....5
Fig. 2. Comparison Between Putative Receptor Protein-Tyrosine Phosphatases.....7
Fig. 3. Comparison Between Receptor-Tyrosine Kinase Signaling And Receptor-Tyrosine Phosphatase Signaling.....26

Chapter II

Fig. 1. Dose-dependence of stimulation of phosphatase activity by somatostatin.....71
Fig. 2. Effect of nonhydrolizable GTP analogues on activation of phosphatase activity in MIA PaCa-2 membranes by somatostatin.....72
Fig. 3. Effect of pertussis toxin on phosphatase activity.....73
Fig. 4. Dephosphorylation of EGFR prepared from A-431 cells by phosphatases from MIA PaCa-2 membranes.....74

Chapter III

Fig. 1. Somatostatin inhibits both basal and EGF-stimulated DNA synthesis.....95
Fig. 2. Both Somatostatin and forskolin inhibit DNA synthesis.....96
Fig. 3. Pertussis toxin inhibits DNA synthesis in MIA PaCa-2 cells....97

Chapter IV

Fig. 1. Restriction map of PTP-PS and PTP-P1.....121
Fig. 2. Nucleotide and amino acid sequences of PTP-PS and PTP-P1.....122
Fig. 3. Homology between PTP-P1, HPTPδ, and LAR.....124
Fig. 4. Messenger RNA expression in PC12 Cells, detected by northern blot hybridization.....125
Fig. 5. PTP-P1 and PTP-PS are generated by RNA processing.....126
Fig. 6. Messenger RNA expression of PTP-P1 and PTP-PS in tissues and cells, detected by northern blot hybridization.....127
Fig. 7. Phosphatase activity of PTP-P1 and PTP-PS.....129

Chapter V

Fig. 1. PTP-P1 mRNA levels are increased by NGF and NGF plus cycloheximide but not by EGF.....141
Fig. 2. Summary of Fig 1.....142

ABSTRACT

Protein-tyrosine phosphorylation and dephosphorylation have been recognized as major process for regulating cell growth and differentiation. Much have been learned about protein-tyrosine kinases over the past 15 years, while relatively less has been known about protein-tyrosine phosphatases (PTPases). Protein-tyrosine phosphatases have been implicated in a number of important cellular processes including signal transduction, cell cycle regulation, and T cell activation. I have studied hormonal regulation of protein-tyrosine phosphatase in human tumor cells. I have demonstrated that somatostatin can stimulate a membrane-associated protein-tyrosine phosphatase activity in a human pancreatic carcinoma cell line, MIA PaCa-2 cells. Vanadate completely blocked both stimulated and basal phosphatase activity. The somatostatin stimulation of phosphatase activity was dose-dependent and the half-maximum stimulation occured with 2 nM somatostatin.

I have also shown that the somatostatin stimulation of phosphatase activity is mediated through a pertussis toxin-sensitive G protein coupling pathway. Both guanosine triphosphate (GTP) and its analog 5'-guanylyllimidodiphosphate (GppNHp) increased phosphatase activity of MIA PaCa-2 cell membranes. The addition of somatostatin further increased this activity. However, somatostatin stimulation of phosphatase activity was completely blocked by the nonhydrolizable guanine nucleotide analogue guanosine 5'-O-(2-thiodiphosphate) (GDP-ßS). Somatostatin also stimulated phosphatase activity in whole cells. This stimulation was blocked by the pretreatment of cells with pertussis-toxin. In addition, somatostatin inhibited both basal and EGF-stimulated DNA synthesis in MIA PaCa-2 cells, and this inhibition can be blocked by a protein-tyrosine phosphatase inhibitor vanadate. The stimulation of protein-tyrosine phosphatase activity by somatostatin may be responsible for its anti-proliferative action in these cells and may represent a general mechanism for regulating cell growth.

Protein-tyrosine phosphatases can be divided into intracellular and transmembrane (or putative receptor) forms. The transmembrane protein-tyrosine phosphatases are potential signal transducing receptors although their ligands have not been identified. I have isolated two novel putative receptor protein-tyrosine phosphatases, PTP-P1 and PTP-PS, from PC12 cells. Both PTP-P1 and PTP-PS share an identical extracellular domain and a transmembrane domain. PTP-P1 contains two PTPase catalytic domains in its cytoplasmic region, while PTP-PS contains only the first PTPase domain followed by a unique 26 amino acid sequence. In addition, I have demonstrated that PTP-P1 and PTP-PS are generated by RNA processing from a single gene. The use of a poly-adenylation site in the intron between the domain 1 and domain 2 of PTP-P1 generates PTP-PS, whereas alternative splicing of the intron between the domain 1 and domain 2 of PTP-P1 generates PTP-P1. This is the first example of RNA processing generating two structurally distinct protein-tyrosine phosphatases within catalytic domains.

Both PTP-P1 and PTP-PS contain PTPase activity towards a number of synthetic substrates when expressed in *E.coli*. PTP-P1 is encoded by three discret mRNA transcripts which are approximately 8, 6, and 4 kilobases, whereas PTP-PS is encoded by a single mRNA transcript which is approximately 4.8 kilobases. Both PTP-P1 and PTP-PS are primarily expressed in some neuronal and endocine tissues and cells, with PTP-P1 most abundantly expressed in W2 cells and in the brain cortex. The mRNA levels of PTP-P1 were induced by NGF during NGF-induced PC12 cell differentiation, and by cycloheximide, a protein synthesis inhibitor, suggesting that the NGF induction of PTP-P1 mRNA levels might be mediated through a mechanism which increased messenger stability. These results suggest that PTP-P1 may be involved in neuronal differentiation and PTP-PS may be involved in more general neuronal functions.

Chapter I

INTRODUCTION AND LITERATURE REVIEW

Discovered in the 1950s by Edwin Krebs and Edmond Fischer as a means of regulating glycogen phosphorylase activity, protein phosphorylation and dephosphorylation have been recognized as major processes for regulating cell growth and differentiation. A variety of cellular proteins are known to be regulated by this process, particularly the proteins involved in signal transduction. Signals received by cells have their effects amplified and disseminated by a network of protein phosphorylation-dephosphorylation events, the great complexity of which we are begining to reveal and appreciate. Over the past decade, tremendous progress has been made towards understanding this complexity, although much remains to be learned.

Protein kinases and protein phosphatases are categorized by their ability to phosphorylate or dephosphorylate either serine and threonine residues or tyrosine residues. Accordingly, protein kinases are divided into two classes: protein serine/threonine kinases and protein-tyrosine kinases (PTKs). Similarly, protein phophatases are classified into protein serine/threonine phosphatases (PP) and protein-tyrosine phosphatases (PTPases). Both protein kinases and protein phosphatases are subject to physiological control to promote normal cell function. About one third of cellular proteins are phosphorylated in the resting state, and about 99% of these phosphoproteins are phosphorylated on serine/threonine (1). Protein serine/threonine kinases include cAMP-dependent protein kinases (PKAs), cGMP-dependent protein

kinases, protein kinase C (PKC) family, calcium/calmodulin-dependent protein kinases (CAM kinases), and casein kinases (CKs) (2-7). They mainly respond to extracellular signals such as hormones, growth factors, and neurotransmitters. Protein serine/threonine phosphatases (PPs) also exist as multiple isoforms, including PP-1, PP-2A, PP-2B, and PP-2C (8,9). Fewer PP genes have been identified compared to protein serine/threonine kinases and tyrosine kinases and phosphatases. They appear to have broad substrate specificity and execute multiple functional roles within the cell. Some of them, such as PP-2A, also possess limited PTPase activity.

Although phospho-tyrosine proteins occupy only a small percentage of total cellular phospho-proteins, tyrosine phosphorylation plays extremely important roles. Protein-tyrosine kinases have been major players over the past decade in the studies of oncogenesis and signal transduction by growth factors (10,11). Tyrosine phosphorylation was discovered by studying avian sarcoma virus which contains a viral oncogene *v-src*, the first tyrosine kinase purified (12). It was later demonstrated that *v-src* is a mutated gene that the virus aquired from the mammalian genome. At the present time, at least ten cellular members of the *src* kinase family have been isolated by molecular cloning. In 1982, the EGF receptor was purified and cloned (13,14). It was the first receptor-tyrosine kinase (RTK) discovered and triggered the subsequent idenfication of other receptor-tyrosine kinases (PDGF receptor, insulin receptor, and FGF receptor, etc.) (15).

Unlike well-studied PTKs, the understanding towards biological roles of PTPases has just begun. PTPase activity was first observed in A-431 cells, an epidermal carcinoma cell line, and in cells transformed with temperature-

sensitive mutants of Rous sarcoma virus containing pp60^{src} (16-18).The hunt for the PTPase took nearly 10 years as many researchers tried to isolate it from various tissues and cell lines, but only partial purification of multiple isoforms of PTPases was obtained (19-26). The breakthrough came about five years ago, when Tonks et al successfully purified the first PTPase PTP1B to homogeneity from the human placenta (27,28). The sequence of this PTPase was surprising, as it was structurally unrelated to the serine/threonine phophatases, but homologous to each of two tandem repeated domains in the cytoplasmic portion of the leukocyte common antigen CD45 (29). CD45, a transmembrane protein mainly expressed in T lymphocytes, was later shown to possess intrinsic PTPase activity (30-32). Soon after, a number of other transmembrane and non-transmembrane PTPases have been isolated from a variety of species (including viruses and bacteria), demonstrating that the PTPases represent a diverse family of enzymes with both intracellular and integral membrane forms and carry out many important cellular functions (33). The long confusion that the PTPases might play a merely housekeeping role to clean up after tyrosine kinases has come to the end.

Structure of Protein-Tyrosine Phosphatases

There are two classifications for PTPases based on structural characeristics (33,34). According to Fischer et al (33), the PTPase family can be divided into two subfamilies: transmembrane and intracellular forms. The transmembrane forms can be further divided into four types depending on different organization of the extracellular domain. Krueger et al (34) proposed that the family could be divided into three classes. Class I are

cytoplasmic PTPases. Class II contains receptor-linked PTPases with a single PTPase domain, and class III contains receptor-linked PTPases with two tandem repeated PTPase domains. All intracellular PTPases (class I) contain a single PTPase catalytic domain, which is a segment of approxiamately 250 to 280 residues conserved in all PTPases, a cysteine residue in the domain is essential for the PTPase activity. This essential cysteine is located within a highly conserved core domain sequences of 11 residues (I/V)HCXAGXXR(S/T)G, which is a hallmark of the PTPase domain. Mutation of any of these residues can significantly impair PTPase activity (35). PTPases catalyze phosphate transfer via formation of cysteine-phosphate intermediate, a mechanism also used by serine/threonine phosphatases (36). Some intracellular PTPases including PTP1C (also called SH-PTP1 or HCP) (37-40), PTP1D (also called SH-PTP2 or SYP) (41-43), *Drosophila* PTPase *corkscrew* (*csw*) (44), and PTP2C (45) contain two SH2 (src-homology 2 domain) domains in their N-terminus. The SH2 and SH3 domains have been demonstrated to mediate important protein-protein interactions in signal transduction (46,47). Some intracellular PTPases like PTP-H1 (48) and HPTP-MEG (49), contain an amino-terminal domain which is related to cytoskeletal proteins, suggesting that certain PTPases are involved in regulation of cytoskeleton organization. Nevertheless, a transmembrane PTPase, CD45, was shown to be regulated by direct interaction with the cytoskeleton (50), although CD45 contains no sequence related to the cytoskeletal proteins. A PTPase isolated from hematopoietic cells, termed PEP, contains a large carboxyl-terminal domain of approximately 500 amino acids that is rich in proline, glutamic acid, serine, and threonine residues (PEST sequences) (40). The PEST sequences are found in proteins that are rapidly degraded. A PTPase identified in *vaccinia*,

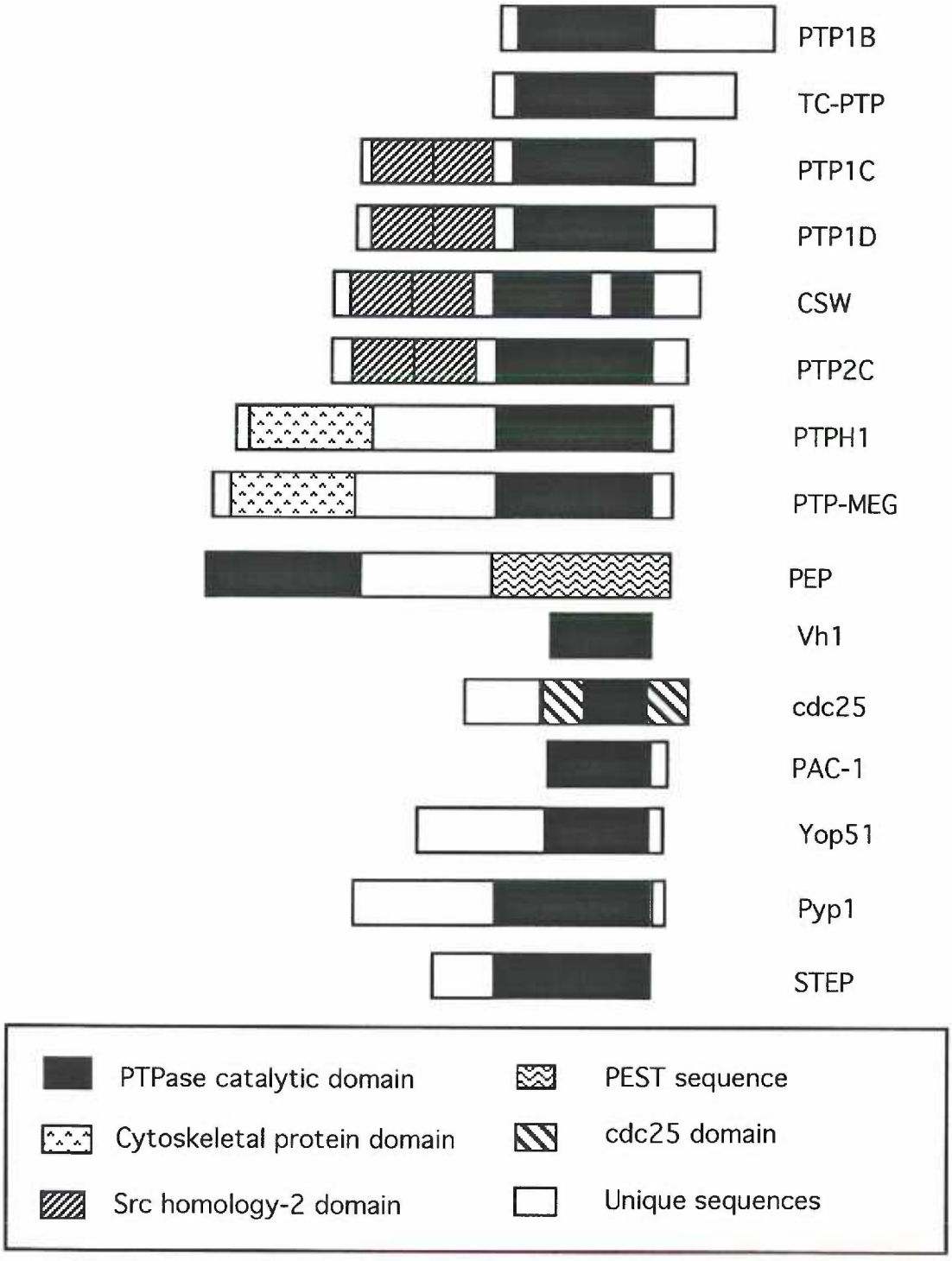


Figure 1. Structural Features of Intracellular PTPases

Vh1, was found to have no structural similarity to other PTPases except cdc25 (51), a protein controlling cell entry into mitosis. The Vh1 homologues from human, mouse, and yeast have also been isolated (52-56). These genes constitute a unique subfamily of PTPases that continues to grow. Their gene products are usually small molecules with about 180 amino acids and a key cysteine residue in the core domain, and their function remains to be determined though they might play some important roles in both the cytoplasm and nucleus. For example, they may regulate signal attenuation by dephosphorylating certain signal transducing molecules like MAP (mitogen-activated protein) kinases or MEKs (MAP kinase kinases). They might also play certain roles in controlling cell cycling. Interestingly, Vh1 is a dual-specific phosphatase which catalyzes the removal of phosphate from both serine/threonine and tyrosine (51). The Vh1-related protein phopsphatases have also been identified in the genomes of several orthopoxviruses and a baculovirus (57). Not only do viruses possess PTPase genes, a PTPase was also identified from bacteria *Yersinia* (58). This PTPase, Yop51, is essential for the pathogenesis of the black plague(59).

The overall structure of transmembrane (or putative receptor) PTPases is similar to that of receptor-tyrosine kinases such as EGF receptor, PDGF receptor, and FGF receptor (see Figure 2). They all contain an extracellular domain and a cytoplasmic region. Most of receptor-linked PTPases identified to date are found to contain varying numbers of immunoglobulin-like domain (Ig-like domain) and fibronectin type three repeats (FN-III) in their extracellular domain. The extracellular domains of HPTPß and the *Drosophila* PTPase PTP99A and DPTP10D are composed only of FN-III domains (34,60,61). Ig domains are about 100 amino acids long and contain

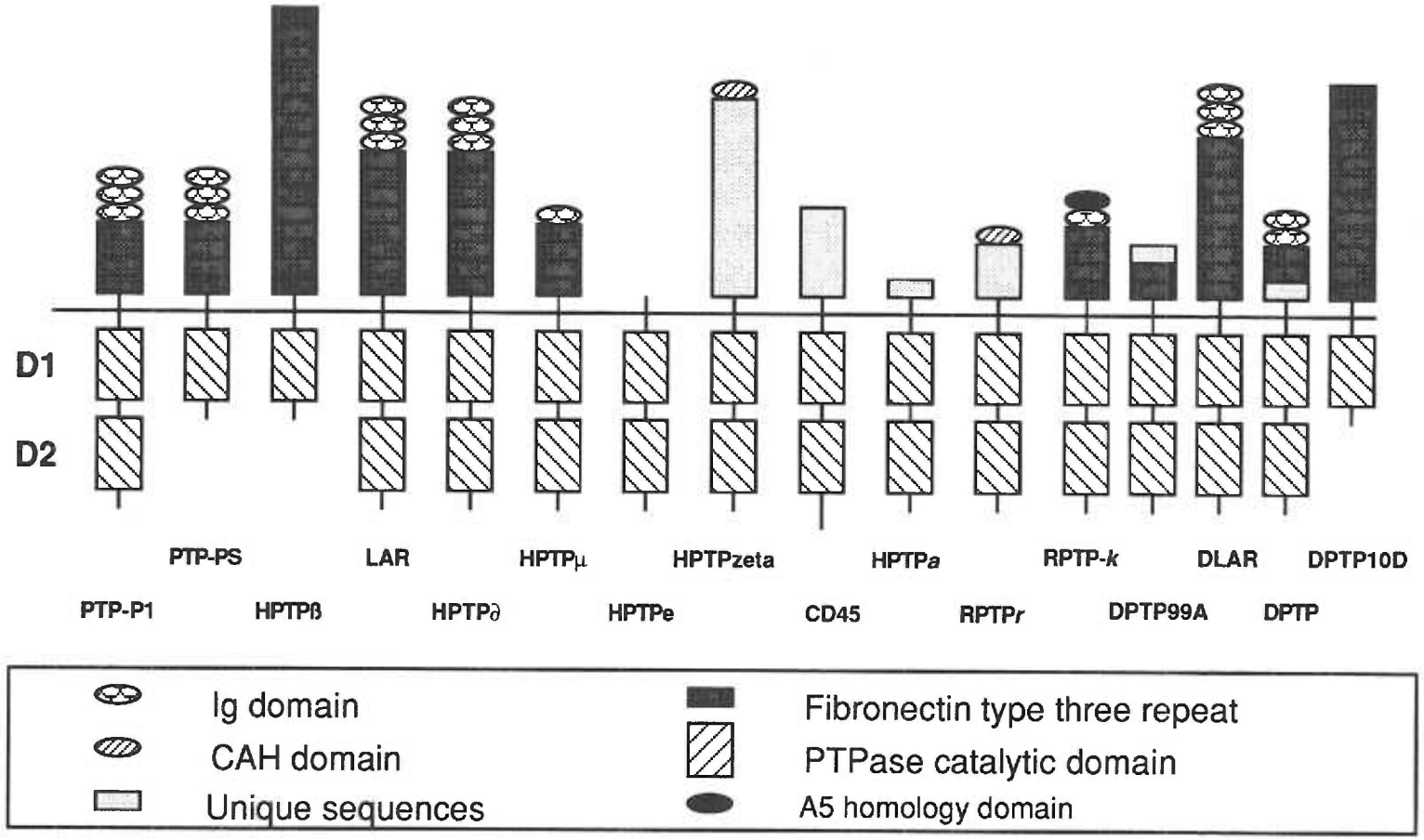


Figure 2. Comparison between Receptor-linked Protein-Tyrosine Phosphatases. The fibronectin type three repeat, immunoglobulin (Ig)-like domain, carbonic anhydrase homology domain, PTPase domain, and unique sequences unrelated to any known proteins are schematically represented. D1 and D2, PTPase domain 1 and 2.

intrachain disulfide bonds, and are located near the amino-terminus of the molecule. Ig-like domains are thought to function as cell surface recognition structures, and are found in many adhesion molecules and growth factor receptors including the neural-cell adhesion molecule (N-CAM) and the PDGF receptor (62,63). FN-III repeats are about 90 amino acids and contain a characteristic sequence motif presented in fibronectin. FN-III are found in several proteins mediating protein-protein interaction such as cytotactin and N-CAM (64). FN-III are also found in the receptors for growth hormone and prolactin. It has been suggested that FN-III repeats mediate the interaction of the receptors with ligands or with other components of the receptor complex (64). Other than four FN-III domains and one Ig-like domain, the extracellular region of RPTP-κ contains at N-terminus a domain related to A5 surface protein (65). The extracellular region of PTPζ, RPTPγ, and RPTPß contain at the N-terminus a domain homologous to carbonic anhydrases (CAH) (66,67). Barnea et al have constructed a model for the CAH-like domain of RPTPγ based on the crystal structure of CAH, and proposed that the CAH-like domain of RPTPγ may have a function other than catalysis of hydration of metabolic CO_2 (67).

Most putative receptor PTPases contain two tandem repeated PTPase catalytic domains in their cytoplasmic region except HPTPß (34), PTP-PS (68), and the *Drosophila* PTPase DPTP10D (60,61) (see Figure 1). The N-terminal domain (domain 1, D1) and the C-terminal domain (domain 2, D2) usually share about 35-45% sequence identity and an almost identical core domain. Interestingly, for some PTPases such as HPTPγ, the critical cysteine residue in the core domain is absent in its D2. Instead, it is replaced by an asparagine residue (34). The D1 of all putative receptor PTPases contain

PTPase activity, but D2 of most of them contains no PTPase activity when expressed in *E. coli*. This difference has led to a hypothesis that D1 and D2 may play different roles *in vivo*. However, the D2 of HPTPα was shown to contain relatively lower PTPase activity and distinct substrate specificity compared to its D1 (69). The D2 of CD45 contains no PTPase activity when expressed in bacteria, mutation of the critical cysteine in its D1 abolishes all PTPase activity, but mutation of the cysteine in D2 does not affect PTPase activity (35). However, it is demonstrated that the D2 of CD45 contains viable PTPase activity when subjected to limited proteolysis by endoproteinase Lys-C or trypsin. A construct of CD45 cytoplasmic region in that 109 residues of the D1 catalytic domain were deleted, was expressed in mammalian cells and was found to contain PTPase activity (70). These data suggest that the D2 of CD45 may be a functioning PTPase *in vivo* and requires external regulation to express its activity.

I recently reported the isolation of PTP-PS, the second mammalian putative receptor PTPase with a single PTPase domain, I will detail the identification of PTP-PS in chapter IV. PTP-PS and PTP-P1 are generated by RNA processing from a single gene. PTP-P1 contains two PTPase domains. This is the first example of RNA processing generating two structurally distinct PTPases of two different classes (according to the classification of Krueger et al (34). The significance of this type of RNA processing is still not clear. It is hypothesized that the D2 may play regulatory function *in vivo*. It is also possible that PTP-PS and PTP-P1 may have different enzyme kinetics and substrate specificity and may interact with different set of intracellular proteins to regulate signal transduction or signal termination.

Function of Protein-Tyrosine Phosphatases

Compared to the structural knowledge of cloned PTPase cDNAs, the understanding of their function is limited. However, studies using both biochemical and genetic approaches have generated considerable information over the past few years.

As counterparts to protein-tyrosine kinases, certain PTPases might act as tumor suppressor genes. For example, the putative receptor PTPase HPTPγ maps to a region of the human chromosome 3p21 that is frequently deleted in renal cell carcinoma and lung carcinoma (71). Polymorphism analysis of this gene has provided further evidence that HPTPγ might be a potential anti-oncogene (72). The deletion of genes like this could be responsible for the uncontrolled activity of tyrosine kinases. The inactivation of certain activated tyrosine kinases which deliver positive signals for cell growth may rely on PTPases. Griswold-Prenner et al (73) recently reports biochemical evidence showing that MAP kinase can decrease EGF receptor phosphorylation through activation of a membrane-associated PTPase activity. In an *in vitro* experiment with A431 cell membrane extracts, tyrosine phosphorylation of EGF receptor decreases rapidly after autophosphorylation (74). This suggests that a PTPase in the membrane extracts is able to remove phosphate from EGF receptors following autophosphorylation. Whether or not this PTPase is same as MAP kinase-activated PTPase is not clear. It is possible that action of this PTPase may serve as a feedback mechanism to modulate signal transduction by receptor-tyrosine kinases. Recently, Faure et al (75) reported that an endosome-

associated PTPase might regulate the phosphorylation state of insulin receptor and EGF receptor. There are three major tyrosine phosphorylation sites in the insulin receptor. The sequential phosphorylation of these sites activates the insulin receptor tyrosine kinase activity. The sequential dephosphorylation of these three sites therefore inactivates the insulin receptor in a similar magnitude (76). Biochemical studies also found that a PTPase was involved in the suppression of PDGF receptor autophosphorylation in p21 *ras* -transformed cells (77).

Receptor-tyrosine kinases activate a number of signal transducing proteins accompanied by a cascade of phosphorylation events (see Figure 3). One of these, MAP kinase, is activated by a dual-specific kinase MEK (MAP kinase kinase). Activated MAP kinase is phosphorylated both on tyrosine and serine/threonine residues. MAP kinase activity reaches a maximum about 5 minutes after EGF stimulation and then decreases rapidly to basal level (78). However, MAP kinase protein level does not vary significantly (79), suggesting that MAP kinase activity must be deactivated by protein modification, such as dephosphorylation. It was recently shown that a dual-specific phosphatase HVH1 (human homolog of *vaccinia* phosphatase Vh1), can specifically dephosphorylate MAP kinase on tyrosine and threonine *in vitro* (80). These results implicate that the HVH1 phosphatase may dephosphorylate MAP kinase *in vivo* and serve as a negative regulator to terminate signal transduction mediated by growth factor receptors. It is very intriguing that a mouse homolog of Vh1 phosphatase, 3CH134, whose RNA level can be induced by many mitogens including serum, growth factors, and phorbol esters (55). In addition, the human Vh1-related phosphatase PAC-1, is induced upon T cell activation and mitogen stimulation(54). The

expression of these Vh1-related phosphatases is coincident with the inactivation of MAP kinase, suggesting that the Vh1-related phosphatases might serve as "the off signals" to counteract "the on signals" impinged by receptor-tyrosine kinases, forming a network of signal transduction and attenuation within MAP kinase signaling pathway.

Some hormones like somatostatin, dopamine, and TGF-ß have anti-proliferative effects. The molecular mechanisms of these effects are not completely understood. I have provided evidence that the hormone somatostatin stimulates a membrane PTPase activity in human pancreactic carcinoma cells, and this activity can dephosphorylate EGF receptor *in vitro*. These findings suggest that somatostatin might inhibit the growth of human tumor cells by directly restoring the balance of tyrosine phosphorylation on critical substrates (81). Similar results have also been obtained by studying effect of dopamine on a pituitary cell line with stably transfected dopamine receptor D2 (82). It was also reported that angiotensin II, TGF-ß1, and interleukin-6 (IL-6) stimulated PTPase activity (83-85). TGF-ß1 is known as a growth inhibitory factor for keratinocytes (86); IL-6 is a cytokine that induces growth arrest of M1 myeloblastic cells (87). Increased levels of PTPase activity have also been documented in differentiated HL-60 cells and in peripheral blood monocytes and granulocytes (88). PTPases may selectively dephosphorylate tyrosyl residues in response to changes in the cellular environment and provide cell with necessary regulatory apparatus for rapid increases or decreases in tyrosine phosphorylation level. Alteration in PTPase activity could be involved in neoplastic processes with certain PTPases acting as potential anti-oncogenes. Cell culture studies have demonstrated that the inhibition of endogenous PTPase activity in non-

transformed cells by vanadate, a specific PTPase inhibitor, can readily induce cellular transformation (89). Increased cell density in fibroblasts is accompanied by increased membrane PTPase activity (90), suggesting that PTPases are involved in cell-cell contact inhibition. Microinjection of PTP1B into *Xenopus* oocytes retarded the insulin-induced maturation by dephosphorylating critical substrates that are used by the insulin receptor tyrosine kinase (91). Transient expression of PTP1B and the T-cell protein tyrosine phosphatase can reduce transcriptional activity of AP-1 and CREB binding sites (92). These findings indicate that certain PTPases counteract the activities of tyrosine kinases to promote normal cell growth and that disruption of function of these PTPases can lead to malignant cell growth and transformation. In fission yeast, two PTPases pyp1 and pyp2 were found to negatively regulate mitosis. Overexpression of pyp1 or pyp2 delays the onset of mitosis by a wee1-dependent mechanism (93). Wee1 is a dual-specific protein kinase which phosphorylates Tyr-15 and Thr-14 of $p34^{cdc2}$, a mitotic inducer, and subsequently inactivates it.

However, PTPases do not simply oppose action of tyrosine kinases. In many aspects, they can work in concert with tyrosine kinases to mediate positive cellular response. For example, it has been demonstrated that PTPases participate in both the interferon α (IFNα) and γ (IFNγ) signal transduction pathways (94,95). It is not known if the PTPase in the IFNα pathway is the same as the one in the IFNγ pathway. However, these unidentified PTPases may be upstream of and serve to activate a tyrosine kinase during the initial phase of the signaling cascade, but are not necessary to maintain the further signal transduction after the tyrosine kinase is activated, since two PTPase inhibitors vanadate or zinc chloride, inhibited the signaling only when

incubated with membranes before the addition of IFNα or IFNγ (94,95). IFNα interacts with its cell surface receptor and rapidly activates the formation of the interferon-stimulated gene factor (ISGF-3), which subsequently translocates to the nucleus and stimulates the transcription of a variety of early response genes. IFNγ activates a protein complex termed the IFNγ–activating factor (GAF) in the cytoplasm through a pathway similar to that of IFNα. The GAF then translocates to the nucleus and activates expression of the guanylate-binding protein. The ISGF-3 is a complex of four different proteins and contains a tyrosine kinase (96). This kinase was recently identified by genetic means (97).

CD45, one of the best studied PTPases, has been implicated in regulating immune response and lymphocyte development. The most direct evidence for the involvement of CD45 in regulating T cell receptor (TCR) signal transduction has come from analysis of TCR signaling in CD45-deficient T cells. T cell clones deficient of CD45 fail to proliferate or produce interleukin-2 (IL-2) in response to antigen or monoclonal antibodies (98) and fail to lyse target cells (99). The deletion of CD45 from the cells appears to uncouple the TCR from the intracellular signal transduction machinery. Engagement of the TCR, on two different CD45-deficient cell lines, was also shown to be unable to induce tyrosine kinase activity, phosphatidylinositol hydrolysis, and mobilization of intracellular calcium (100,101). These experimental data indicate that CD45 actively participates in normal T lymphocyte signal transduction and is required for the initiation of signals for cell division. CD45 might act through dephophorylation of a tyrosine residue in C-terminus of *lck* and *fyn* kinases. The removal of phosphate from this tyrosine residue is required for the activation of

members of *src* kinase family (102-105). Both *lck* and *fyn* have been shown to be critical components of TCR and IL-2 signaling complexes (106-111). More recently, transgenic mice with disruption of CD45 exon-6 have been generated (112). The homozygotes did not express CD45. B cell development of the mice was not affected, but thymocyte maturation was blocked at the transitional stage from immature $CD4^+$ and $CD8^+$ to mature $CD4^-$ or $CD8^-$ cells, and only a few T cells could be detected in peripheral lymphoid organs. The cytotoxic T cell response to lymphocytic choriomeningitis virus was also absent. These data further demonstrate that CD45, a putative receptor protein-tyrosine phosphatase, is differentially required for the development and function of B and T lymphocytes.

The SH2 domain is also used by PTPases as a functional motif to interplay with other signal transducing proteins. Three mammalian SH2-containing PTPases have been isolated. Studies on these genes have revealed some interesting insights. The first one isolated, PTP1C, was shown to be able to associate with EGF receptor (37). It was later identified from and shown to be mainly expressed in hematopoietic cells (38-40). Biochemical studies have also identified that PTP1C can associate with c-*Kit* after CSF (colony-stimulating factor) stimulation (113). C-*Kit* is a receptor-tyrosine kinase for CSF which is required for differentiation of blood stem cells and the nervous system in the mouse. These results indicate that PTP1C is important in hematopoeitic cell function. Nevertheless, this PTPase was recently identified to be responsible for a murine genetic disease (114). Mutations at the murine motheaten locus are within this gene and exhibit severe hematopoeitic defects. This represents the first example that a PTPase gene is implicated in a genetic disorder. Another SH2-containing PTPase, PTP1D

(or SYP or SH-PTP2) (41,42,115), was found to be regulated by physical interaction with PDGF receptor and EGF receptor. Unlike PTP1C, PTP1D does not dephosphorylate receptor-tyrosine kinases despite their physical association (41,42), suggesting that PTP1C may regulate RTKs, whereas PTP1D may be regulated by RTKs. PTP1D was phosphorylated on tyrosine in cells overexpresssing the ß PDGF receptor and this tyrosine phosphorylation correlated with an enhancement of its PTPase activity. The SH2 domains of PTP1C and PTP1D are essential for their interaction with growth factor receptors. Using genetic approaches, this class of PTPases has also been studied in *Drosophila*. The putative *Drosophila* homologue of PTP1D, *corkscrew*, was found to be a downstream signal transducer of the *torso* receptor tyrosine kinase (45). Its loss-of-function mutation suppresses the *torso* mutation. These findings suggest that certain PTPases are active players in signal transduction mediated by growth factor receptors.

Some PTPases may contain potential oncogenic activity when deregulated. As reported by Zheng et al (116), over-expression of a putative receptor PTPase, HPTPα, can lead to malignant transformation of fibroblasts. Over-expressed HPTPα stimulated *src* kinase activity by resulting in increased dephosphorylation of its C-terminal tyrosine residue. Whether *c-src* is a physiological substrate of HPTPα remains unclear, although HPTPα can dephosphorylate Tyr-527 of *c-src in vitro*.

Cell cycle control is a key step for regulation of cell growth. Over the past five years, tremendous progress has been achieved in this field. Molecular characterization of many gene products (cyclins, kinases, E2F, Retinoblastoma protein, and phosphatases) involved in cell cycle regulation

has made this field one of the most fruitful (117-122). It has been clear that phosphatases are involved in cell cycle control, particularly the PTPase cdc25 (123-125). Even before it was identified to be a PTPase, cdc25 had been well studied. The cdc25 controls mitosis of cell by dephosphorylating Tyr-15 of $p34^{cdc2}$ and subsequently activates it. Activation of $p34^{cdc2}$ is essential for cell to enter mitosis. The $p34^{cdc2}$ is negatively regulated by wee1, which is a dual-specific protein kinase acting in opposite to cdc25 (126). The cdc25 protein is relatively stable, but its PTPase activity and association with $p34^{cdc2}$/cyclin B oscillate throughout the cell cycle (127). Its activity is increased by association with cyclin B. The cell cycle-dependent activation of cdc2 could be due to the cell cycle-dependent association between cdc25 and $p34^{cdc2}$/cyclin B complexes (127). Three cdc25 have been isolated from human fibroblasts, termed cdc25 A, B, and C, and all of them can be activated by cyclin B (128). These PTPases might play critical roles in different checkpoints during the cell cycle. Recently, Fang and Newport (129) presented evidence that two different cdc2 proteins ($p34^{cdc2}$ and $p32^{cdc2}$) might control different checkpoints of the cell cycle, as shown that $p34^{cdc2}$ targeted on G2-M transition whereas $p32^{cdc2}$ on G1-S phase. These findings suggest that different cdc25 may control different checkpoints of the cell cycle by accompanying different cdc2 proteins.

Protein phosphorylation and dephosphorylation have been implicated in regulation of development. The *Drosophila* PTPase *corkscrew* is in the class of terminal genes which is required for the formation of the head and tail of the embryo (45). The mouse homolog of *corksrew* PTP1D is expressed throughout the whole embryo development (41). Tyrosine phosphorylation on proteins was also noted in *Dictyostelium*, implicating that protein tyrosine

phosphorylation and dephosphorylation might be important for regulating cellular functions in this organism (130). A PTPase, PTP1, was isolated by genetic means. PTP1 is an intracellular form and shares about 40% sequence homology with most of other known PTPases. It is expressed at a very low level in vegetative cells, but induced by 4hr, and maximally expressed at the tight aggregate stage. The *Dictyostelium* mutants with PTP1 gene disruption show accelerated development, whereas strains overexpressing PTP1 to a high level fail to aggregate. Strains overexpressing moderate levels exhibit severe morphological defects following aggregation (131). These results indicate that PTP1 plays important regulatory roles during *Dictyostelium* development.

Protein tyrosine phosphorylation and dephosphorylation have also been implicated in neural function and differentiation. Over the past decade, efforts have been focused mostly on studies of neuronal protein-tyrosine kinases and their substrates. It is only recently that the attention has been directed towards the protein phosphatases, but evidence is still mostly circumstantial.

Neural transmitters modulate many important functions of the nervous system. The nicotinic acetylcholine receptor (nAchR) is an neurotransmitter-gated ion channel which mediates signal transduction at the postsynaptic membrane of nicotinic cholinergic synapses. The nAchR is phosphorylated to a high stoichimetry on tyrosine residues both *in vitro* and *in vivo*. Moreover, tyrosine phosphorylation has been shown to regulate the functional properties of the receptor. A PTPase which dephosphorylates tyrosine-phosphorylated nAchR was purified from Torpedo electroplax, a

tissue highly enriched in the nAchR. The purified PTPase has an apparent molecular mass of 43 kd on SDS gel and is specific for tyrosine-phosphorylated nAchR (132). However, its molecular identity remains to be determined by molecular cloning.

PC12 cell line is an excellent system for studying neuronal differentiation. It displays an endocrine cell phenotype in its resting state and can be converted into an neuronal phenotype upon NGF induction. PTP-P1, a putative receptor PTPase cloned from PC12 cells, is encoded by three different transcripts approximately 8, 6, and 4kb (68). The 6kb transcript is abundantly and specifically expressed in some neuronal cells and tissues (68). Upon NGF stimulated differentiation of PC12 cells, mRNA level of PTP-P1 can be increased, suggesting that this gene may be involved in neuronal differentiation. Further evidence can be drawn from the results obtained by Walton et al (133), who found that PTP-NE3 (identical to PTP-P1) was expressed in olfactory epithelium in a developmental manner with high level in the immature neurons and a lower level in mature neurons. Recently, Wu and Bradshow demonstrated that sodium orthovanadate, an inhibitor of PTPases, inhibited PC12 cell differentiation induced by NGF and FGF, suggesting that the level of protein-tyrosine phosphorylation and dephosphorylation must be kept in a balanced level during neuronal cell differentiation (134). This result is consistent with the observation on PTP-P1. Moreover, three PTPases with molecular mass of 500, 300, and 60 kd were found to be activated and PTPase activity was increased 2-3 fold during NGF-induced neuronal differentiation of PC12 cells (135), suggesting that PTPases may be involved in neuronal differentiation process.

Several others, mostly putative receptor PTPases, have been shown to be specifically or abundantly expressed in the nervous system. The STEP, "striatum-enriched phosphatase", was found to highly enriched in the striatum (136). RPTPß, was found to be expressed only in the brain, with the high level of expression in the ventricular and subventricular zones of the embryonic mouse brain, suggesting the importance of this PTPase in the development of central nervous system (137). PTPζ, was isolated from a fetal brain cDNA library and is highly expressed in a glioblastoma cell line (66).

Several PTPases have also been isolated from the *Drosophila* (see Figure 1). Three putative receptor PTPases, DLAR, DPTP10D, and DPTP99A are expressed in different patterns of cells in the ventral nerve cord, and all three proteins are restricted to axons (60,61). DLAR and DPTP99A are apparently expressed on most or all axons, while DPTP10D is mainly localized to the anterior commisure and its junction with the longitudinal tracts (60,61). All three PTPases possess mutiple fibronectin type three repeats, similar to that of N-CAM, L1, and fasciclin (63,64). These extracellular elements may mediate some of their effects in the nervous system to direct cell-cell interaction and cell migration.

Regulation of Protein-Tyrosine Phosphatases

Our knowledge about regulation of PTPases is still in the very preliminary stage. Similar to PTKs, PTPases are regulated in both transcriptional and post-transcriptional steps. Some low molecular weight intracellular PTPases are found to be immediate early genes. The serum-inducible gene 3CH134

(in murine 3T3 cells) and its human homolog CL100 which encode proteins related to Vh1, are rapidly and transiently expressed during Go/G1 transition and are induced by mitogens including serum, PDGF, FGF, and TPA (55,56). Their transcription is activated within minutes after serum stimulation, reaches a peak level by about 20 min. The PAC-1, a nuclear PTPase, which shares significant homology with Vh1, is also mitogen inducible (54). The yeast Vh1 related gene, YVh1 is induced by nitrogen starvation (52). PTP-P1 is induced post-transcriptionally by NGF and cycloheximide by increasing stability of messenger RNA (68).

Post-translational modifications and second messengers also modulate PTPase activity. The PTPase activity of a 55 kd catalytic subunit in the particulate of various cell lines can be stimulated by isoproterol, forskolin, and cAMP analog (138). Studies on permeabilized mast cells suggest that the enabling reaction for exocytosis might involve protein dephosphorylation. A recombinant rat brain PTPase was shown to stimulate Ca^{2+}-dependent amylase secretion from pancreatic acinar cells. The endogerous PTPase activity was shown to be associated with the postgranule supernant, zymogen granules, and in particulate zymogen granule membranes (139). The involvement of a PTPase in this process may be regulated by second messengers such as calcium. CD45, which regulates tyrosine kinase activity of members of *src* family, is phosphorylated transiently on tyrosine upon activation of Jurkat T cells (140). CD45 PTPase activity is negatively regulated by calcium in T cells possibly through activation of a Ca^{2+}-calmodulin dependent phosphatase as shown that T cells treated with ionomycin have decreased levels of serine/threonine phosphorylation on CD45 (141). The physiological significance of these

modifications on CD45 is not known. The SH2-containing PTPase PTP1C is poorly active when assayed with various protein substrates *in vitro*. However, its activity is stimulated to >1000 fold by anionic phospholipids when myelin basic protein or MAP kinase were used as substrates (142). Phospholipids are known to distribute asymmetrically on cell plasma membrane. Such distribution would allow PTP1C to be regulated, assuming that PTP1C can associate with them and be translocated to the plasma membrane. PTPases are also regulated by tyrosine phosphorylation. CD45 is transiently phosphorylated on tyrosine upon activation of T cells by antigen. The significance of this action is not clear. PTP1C is also rapidly phosphorylated on tyrosine in macrophages by *c-Kit* when stimulated by CSF (143). It is not known whether this phosphorylation affects PTP1C activity. PTP1D is tyrosine phosphorylated by PDGF receptor both *in vivo* and *in vitro*, and its activity is subsequently increased (43). In an *in vitro* assay, it was found that the SH2 domains of PTP1D but not those of PTP1C were phosphorylated by insulin receptor tyrosine kinase and bound to the phosphorylated C-terminus of insulin receptors (144). It is possible that both PTP1C and PTP1D are new members of signal transducing proteins used by different receptor-tyrosine kinases.

Subcellular localization of PTPases provides another step for regulation. The intracellular PTPase PTP1B, the first PTPase identified, was found to be localized in the endoplasmic reticulum (ER), tightly associated with microsomal membranes through its C-terminal 35 amino acids, with its PTPase domain oriented towards the cytoplasm (145). These findings provide new insights for regulation of cellular processes by tyrosine phosphorylation. For example, PTP1B may serve to prevent auto-activation

of growth factor receptors which are synthesized in and transported via the ER. Growth factor receptors are activated by homodimerization. It is possible that adventitious clustering of growth factor receptors could occur in the ER, leading to ligand-independent activation. PTP1B may erase this gratuitous event by dephosphorylating auto-phosphorylated growth factor receptors as it does *in vitro* (146). Interestingly, a receptor-tyrosine kinase *ltk*, was found to be in the ER upon over-expression and is suggested to be activated by forming disulphide-linked multimers in response to changes in redox potential within the cells (147). Ltk therefore might be a physiological substrate for PTP1B. Other signal transducing proteins like G proteins and cAMP-dependent protein kinase have also been found in the ER (148,149). Some PTPases are localized to the nucleus. PAC-1, an Vh1-related and mitogen inducible PTPase, was mainly localized in the nucleus (54). Nevertheless, a *Drosophila* PTPase, dPTP61F, undergoes alternative splicing at its 3' end of the message, altering the C-termini of the encoded proteins. Each protein with a unique C-terminus is targeted to different location: the cytoplasm or the nucleus (150).

All of the protein serine/threonine phosphatases have regulatory subunits (8,9). However, it is unclear if this is the case for the PTPases. Purification of PTP1B and other PTPases does not appear to copurify with additional proteins that might represent a potential regulatory subunit. Some protein serine/threonine phosphatases are also regulated by low molecular weight heat-stable inhibitors (DARPP-32, Inhibitor-1). Ingebritsen (151) identified two heat-stable inhibitors for two of the PTPase activities from brain. It is not clear if these inhibitors are similar to DARPP-32 or Inhibitor-1.

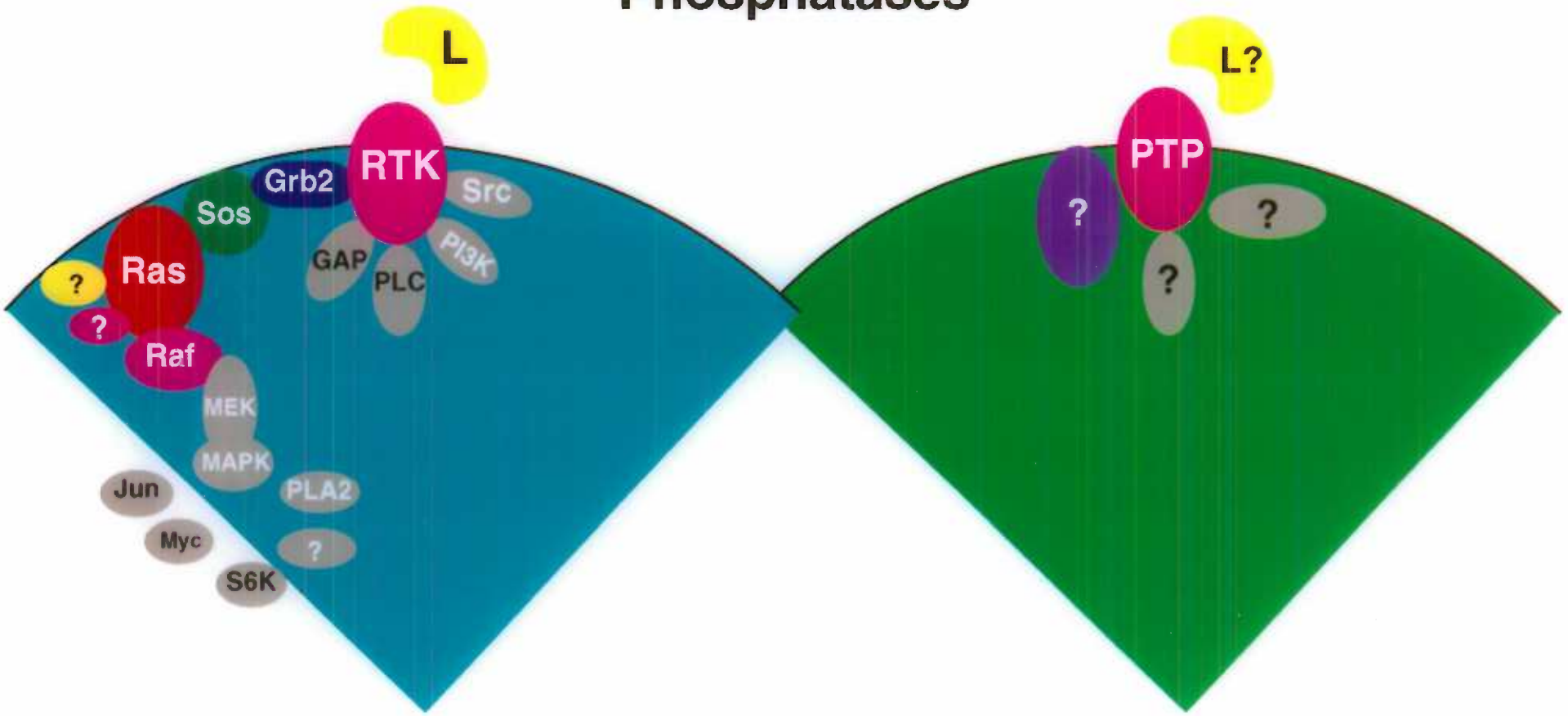
PTPases are also regulated by their sequences. Limited proteolysis resulted in increased PTPase activity of the T-cell PTPase which contains a negative regulatory segment in its C-terminus. Deletion of this segment leads the T-cell PTPase to be constitutively active (152). Limited proteolysis also stimulates PTP1C activity 8-10 folds (153). The negative regulatory segment is also present in receptor-linked PTPases. The juxtamembrane segment of HPTPß potentially functions as a negative regulatory sequence. Deletion of this sequence increased by 5-fold HPTPß activity (154).

Extracellular domains of some putative receptor PTPases are also physiologically regulated. LAR is expressed on the cell surface as a complex of two non-covalently associated subunits derived from a protein by proteolytic cleavage and shedding of the CAM-like extracellular region. The LAR E-subunit (145 kd) contains the cell adhesion molecule-like receptor region, while the LAR P-subunit (85 kd) contains a short segment of the extracellular region, the transmembrane domain, and the cytoplasmic PTPase domain. The cleavage occurs intracellularly and at a paired basic amino acid site by a subtilisin-like endoprotease (155,156). PTP-P1 and PTP-PS might have a similar shedding event in their extracellular domain since both proteins contain a cleavage site similar to that of LAR. A similar site is also contained in another putative receptor protein tyrosine phosphatase, RPTP-κ, as a consensus sequence for cleavage by the processing endopeptidase furin (66). However, the functional significance of this type of shedding remains to be determined. One hypothesis could be that the protein shedding might regulate signal transduction initiated by potential PTPase ligand.

It is widely accepted that potential ligands for putative receptor PTPases do exist although they have not been identified yet. Considerable efforts are now being made in several laboratories to tackle this problem. Putative receptor PTPases may serve as signal transducing receptors as do their counterpart molecules, receptor-tyrosine kinases. It was shown by cross-linking study that CD45 can form homodimers *in vivo* (157), suggesting that putative receptor PTPases may share similar mechanistic action of receptor-tyrosine kinases. More clues were provided by the recent work from Weiss and coworkers (158), who engineered and studied a chimeric construct where the extracellular domain of CD45 was replaced by that of EGF receptor. Expression of this construct in a T lymphocyte cell line deficient of CD45 restored the lost T cell receptor (TCR) signaling. But the TCR signaling as well as the PTPase activity of the construct were inhibited when the cells were treated with ligand EGF, suggesting that putative receptor PTPases may be regulated by their ligands in a manner different from receptor-tyrosine kinases: ligand inactivation rather than ligand activation.

Upon binding to ligands, receptor-tyrosine kinases undergo homodimerization, autophosphorylation, and subsequently activation of their intrinsic tyrosine kinase activity (159). Autophosphorylated cytoplasmic domains of these receptors directly or indirectly interact with and activate several downstream signal transducing molecules to propagate signals. These molecules include PI3 kinase (159-164), phospholipase C (PLC) (159,165-169), GRB2 (or *Sem*5 in C. elegans, *drk* in D*rosophila)* (159,170-178), GAP (GTPase Activating Protein) (159,179-182), Ras (159),*src* kinase (159,183), raf-1 kinase (184), and others. Activated PI-3 kinase phosphorylates the inositol ring of phosphatidyl-inositol in the 3' position

Comparison Between Signal Transduction By Receptor Tyrosine Kinases And By Putative Receptor Tyrosine Phosphatases



Receptor-Tyrosine Kinase Signaling

Putative Receptor PTPase Signaling

(159). PLC catalyzes generation of diacylglycerol (DAG) which activates protein kinase C (PKC) and generation of IP3 (inositol 1,4,5-triphosphate) to mobilize intracellular calcium (159). Grb2 is protein which contains no tyrosine kinase domain but only SH2 and SH3 (src-homology 3 domain) domains and serves as an adaptor protein linking receptor-tyrosine kinases to Ras signaling pathway in mammalian cells (The *Drosophila* and *C. elegance* homologues of Grb2 play the similar functional role) (170-178). In fibroblasts, EGF stimulates the formation of a ternary complex containing Sos Grb2-EGF receptor tyrosine kinase (171,172). Sos (mammalian homologue of son of *sevenless*, a *Drosophila* guanine nucleotide exchange factor) is brought by Grb2 from the cytoplasm into the vicinity of its target, plasma membrane-associated Ras (170-178). This process permits Ras to be activated. The logical questions therefore appear: what molecular mechanism underlies inactivation or activation of putative receptor PTPases by ligand? Is it auto-dephosphorylation following homodimerization? What could be the possible molecular identity of the ligand? What intracellular proteins interact with putative receptor PTPases and transduce signals? It is predictable that the answers will come in the next few years. Finally, it might be worth noting that mullerian inhibitory substance (MIS), a secreted protein related to the TGFß family, inhibits EGF receptor autophosphorylation both *in vitro* and *in vivo* (185). Could MIS be a potential ligand for putative receptor PTPase which might act specifically on the EGF receptor? This would explain the effect of MIS on the EGF receptor. We can only await another breakthrough for the PTPase studies to come.

REFERENCES

1. Hunter, T. & Sefton, B.M. (1980) The transforming gene product of Rous sarcomavirus phosphorylates tyrosine. *Proc. Natl. Aca. Sci. USA.* 77, 1311-1315

2. Hanks, S.K., Quinn, A.N. & Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science.* 241, 42-52

3. Soderling, T.R. (1990) Protein kinases: regulation by autoinhibitory domains. *J. Biol. Chem.* 265, 1823-1826

4. Taylor, S.S., Buechler, J.A. & Yonemoto, W. (1990) cAMP-dependent protein kinase: framwork for a diverse family of regulatory enzymes. *Annu. Rev. Biochem.* 59, 971-1005

5. Kikkawa, U., Kishimoto, A. & Nishizuka, Y. (1989) The protein kinase C family: heterogeneity and its implications. *Annu. Rev. Biochem.* 58, 31-44

6. Edelman, A.M. (1987) Protein serine/threonine kinases. *Annu. Rev. Biochem.* 56, 567-613

7. Hunter, T. (1987) A thousand and one protein kinases. *Cell.* 50, 823-829

8. Cohen, P. (1989) The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* 58, 453-508

9. Shenolika, S.S. & Nairn, A.C. (1990) Protein phosphatases: recent progress. *Adv. Cyclic. Nucleotide Protein Phosphorylation Res.* 23, 1-121

10. Aaronson, S.A. (1991) Growth factors and cancer. *Science.* 254, 1146-1152

11. Bishop, J.M. (1991) Molecular themes in Oncogenesis. *Cell.* 64, 235-248

12. Collett, M.S., Purchio, A.F. & Erikson, R.L. (1980) Avian sarcoma virus transforming protein, pp60^{src}, shows protein kinase activity specific for tyrosine. *Nature.* 285, 167-169

13. Cohen, S., Carpenter, G. & King, L. Jr. (1980) Epidermal growth factor-receptor-protein kinase interaction. *J. Bio. Chem.* 255, 4834-4842

14. Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A. et al. (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature.* 309, 418-425

15. Carpenter, G. (1987) Receptors for epidermal growth factor and other polypeptide mitogens. *Annu. Rev. Biochem.* 56, 881-914

16. Carpenter, G., King, L., Jr. & Cohen, S. (1979) Rapid enhancement of protein phosphorylation in A-431 cell membrane preparations by epidermal growth factor. *J. Biol. Chem.* 254, 4884-4891

17. Ushiro, H. & Cohen, S. (1980) Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. *J. Biol. Chem.* 255, 8363-8365

18. Sefton, B.M., Hunter, T., Beemon, K. & Eckhart, W. (1980) Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. *Cell,* 20, 807-816

19. Brautigan, D.L., Bornstein, P. & Gallis, B. (1981) Phosphotyrosyl-protein phosphatase, specific inhibition by Zn^{2+}. *J. Biol. Chem.* 256, 6519-6522

20. Swarup, G., Speeg, K.V., Jr., Cohen, S. & Garbers, D. (1982) Phosphotyrosyl-protein phosphatase of TCRC-2 cells. *J. Biol. Chem.* 257, 7298-7301

21. Chernoff, J. & Li, H. (1983) Multiple forms of phosphotyrosyl-protein phosphatase from cardiac mucles: Partial purification and characterization of an EDTA-stimulated phosphotyrosyl-protein phosphatase. *Arch. Biochem. Biophys.* 226, 517-530

22. Rotenberger, S.A. & Brautigan, D.L. (1987) Membrane protein phosphotyrosine phosphatase in rabbit kidney. *Biochem. J.* 243, 747-754

23. Roome, J., O'Hare, T., Pilch, P.F. & Brautigan, D.L. (1988) Protein phosphotyrosine phosphatase purified from the pariculate fraction of human

placenta dephosphorylates insulin and growth-factor receptors. *Biochem. J.* 256, 493-500

24. Foulkes, J.G., Erikson, E. & Erikson, R.L. (1983) Separation of multiple phosphotyrosyl- and phosphoseryl- phosphatases from chicken brain. *J. Biol. Chem.* 258, 431-438

25. Shriner, C.L. & Brautigan, D.L. (1984) Cytosolic protein phosphotyrosine phosphatases from rabbit kidney. *J. Biol. Chem.* 259, 11383-11390

26. Ingebritsen, T.S. (1988) Phosphotyrosyl-protein phosphatases, separation of multiple forms from brain and purification of the major form to near homogeneity. *J. Biol. Chem.* 264, 7747-7753

27. Tonks, N. K., Diltz, C.R. & Fischer, E.H. (1988) Purification of the major protein tyrosine phosphatase of human placenta. *J. Biol. Chem.* 263, 6722-6730

28. Tonks, N.K., Diltz, C.D. & Fischer, E.H. (1988) Characterization of the major tyrosine phosphatase of human placenta. *J. Biol. Chem.* 263, 6731-6737

29. Charbonneau, H., Tonks, N.K., Kumar, S., Diltz, C.D., Harrylock, M., Cool, D.G., Krebs, E.G., Fischer, E.H. & Walsh, K.A. (1989) Human placenta protein tyrosine phosphatase amino acid sequences and relationship

to a family of receptor-like proteins. *Proc. Natl. Acd. Sci. USA* 86, 5252-5256

30. Charbonneau, H., Tonks, N.K., Walsh, K.A. & Fischer, E.H. (1988) The leukocyte common antigen (CD45): A putative receptor-linked protein tyrosine phosphatase. *Proc. Natl. Aca. Sci. USA* 85, 7182-7186

31. Tonks, N.K., Diltz, C.D. & Fischer, E.H. (1990) CD45, an integral membrane protein tyrosine phosphatase. *J. Biol. Chem.* 265, 19674-10680

32. Tonks, N. K., Charbonneau, H., Diltz, C.D., Fischer, E.H. & Walsh, K.A. (1988) Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase. *Biochem. 27, 8695-8690*

33. Fischer, E.H., Charbonneau, H. & Tonks, N.K. (1991) Protein tyrosine phosphatases: A diverse family of intracellular and transmembrane enzymes. *Science*, 253, 401-406

34. Krueger, N.X., Strueli, M. & Saito, H. (1990) Structural diversity and evolution of human receptor-like protein tyrosine phosphatases. *EMBO J.* 9, 3241-3252

35. Strueli, M., Krueger, N.X., Thai, T., Tang, M.G. & Saito, H. (1990) Distinct functional roles of the two intracellular phosphatase like domains of the receptor-linked protein-tyrosine phosphatases LCA and LAR. *EMBO J.* 9, 2399-2407

36. Guan, K. & Dixon, J.E. (1991) Evidence for protein tyrosine phosphatase catalysis proceeding via a cysteine-phosphate intermediate. *J. Biol. Chem.* 266, 17026-17030

37. Shen, S., Bastien, L., Posner, B.I. & Chretien, P. (1991) A protein-tyrosine phosphatase with similarity to the SH2 domain of the protein-tyrosine kinases. *Nature*, 352, 736-739

38. Yi, T., Cleveland, J.L. & Ihle, J.N. (1992) Protein tyrosine phosphatase containing SH2 domains: characterization, preferential expression in hematopoietic cells, and localization to human chromosome 12p12-p13. *Mol. Cell. Biol.* 12, 836-846

39. Plutzky, J., Neel, B.G. & Rosenberger, R.D. (1992) Isolation of a src homology 2-containing tyrosine phosphatase. *Proc. Natl. Aca. Sci. USA* 89, 1123

40. Mattews, R.J., Bowne, D.B., Flores, E. & Thomas, M.L. (1992) Characterization of hematopoeitic intracellular protein tyrosine phosphatases: description of phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine- rich sequences. *Mol. Cell. Biol.* 12, 2396-2405

41. Feng, G., Hui, C. & Pawson, T. (1993) SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. *Science*, 259, 1607-1610

42. Vogel, W., Lammers, R., Huang, J. & Ullrich, A. (1993) Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation. *Science*, 259, 1610-1614

43. Freeman, R.M., Jr., Plutzky, J. & Neel, B.G. (1993) Identification of a human src homology 2-containing protein-tyrosine phosphatase: A putative homolog of drosophila corkscrew. *Proc. Natl. Aca. Sci. USA* 89, 11239-11243

44. Perkins, L.A., Larsen, I. & Perrimon, N. (1992) Corkscrew encodes a putative protein tyrosine phosphatase that functions to transduce signal from the receptor tyrosine kinase torso. *Cell*, 70, 225-236

45. Ahmad, S., Banville, D., Zhao, Z., Fischer, E.H. & Shen, S. (1993) A widely expressed human protein tyrosine phosphatase containing src homology 2 domains. *Proc. Natl. Aca. Sci. USA* 99, 2197-2201

46. Koch, C.A., Moran, M.F., Anderson, D., Ellis, C. & Pawson, T. (1991) SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science*, 252, 668-674

47. Pawson, T. & Gish, G.D. (1992) SH2 and SH3 domains: from strucure to function. *Cell*, 71, 359-362

48. Yang, Q. & Tonks, N.K. (1991) Isolation of a cDNA clone encoding a human protein tyrosine phosphatase with homology to the cytoskeletal

associated proteins band 4.1, ezrin and talin. *Proc. Natl. Aca. Sci. USA* 88, 5949-5952

49. Gu, M., York, J.D., Warshawsky, I. & Majerus, P.W. (1991) Identification, cloning, and expression of a cytosolic megakaryocyte protein tyrosine phosphatase with sequence homology to cytoskeletal protein 4.1. *Proc. Natl. Aca. Sci. USA* 88, 5867-5871

50. Lockshwar, V.B. & Bourguignon, L.Y.W. (1992) Tyrosine phosphatase activity of lymphoma CD45 (GP180) is regulated by a direct interaction with the cytoskeleton. *J. Biol. Chem.* 267, 21551-21557

51. Guan, K., Broyles, S.S. & Dixon, J.E. (1991) A Tyr/Ser protein phosphatase encoded by vaccinia virus. *Nature*, 350, 359-362

52. Guan, K., Hakes, D.J., Wang, Y., Park, H., Cooper, T.G. & Dixon, J.E. (1992) A yeast protein phosphatase related to the vaccinia virus Vh1 phosphatase is induced by nitrogen starvation. *Proc. Natl. Aca. Sci. USA* 89, 12175-12179

53. Ishibashi, T., Bottaro, D.P., Chan, A., Miki, T. & Aaronson, S.A. (1992) Expression cloning of a human dual-specific phosphatase. *Proc. Natl. Aca. Sci. USA*, 89, 12170-12174

54. Rohan, P.J., Davis, P., Moskaluk, C.A., Kearns, M., Krutzch, H., Siebenlist, B. & Kelly, K. (1993) PAC-1, a mitogen-induced nuclear protein tyrosine phosphatase. *Science*, 259, 1763-1766

55. Charles, C.H., Abler, A.S. & Lau, L.F. (1992) DNA sequence of a growth factor-inducible immediately early gene and characterization of its encoded protein. *Oncogene*, 7, 187-190

56. Keyse, S.M. & Emslie, E.A. (1992) Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature*, 359, 644-647

57. Hakes, D., Martell, K.J., Zhao, W., Massung, R.F., Esposito, J. & Dixon, J.E. (1993) A protein phosphatase related to the vaccinia virus Vh1 is encoded in the genomes of several orthopoxviruses and a baculovirus. *Proc. Natl. Aca. Sci. USA* 90, 4017-4021

58. Guan, K. & Dixon, J.E. (1991) Protein tyrosine phosphatase activity of an essential virulence determinant in *Yersinia*. *Science*, 249, 553-556

59. Bliska, J.B., Guan, K., Dixon, J.E. & Falkow, S. (1991) Tyrosine phosphatae hydrolysis of host proteins by an essential *Yersinia* virulence determinant. *Proc. Natl. Aca. Sci. USA* 88, 1187-1191

60. Yang, X., Seow, K.T., Bahri, S.M., Oon, S.H. & Chia, W. (1991) Two *Drosophila* receptor-like protein tyrosine phosphatase genes are expressed in a subset of developing axons and pioneer neurons in the embryonic cells. *Cell* , 67, 661-673

61. Tian, S., Tsoulfas, P. & Zinn, K. (1991) Three receptor-like protein tyrosine phosphatases are selectively expressed on central nervous system axons in the *Drosophila* embryo. *Cell*, 67, 675-685

62. Springer, T.A. (1990) Adhesion receptors of the immune system. *Nature*, 346, 425-434

63. Cunningham, B.A., Hampty, J.J., Murray, B.A. & Edelman, G. (1987) Neural cell adhesion molecules: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. *Sience*. 236, 799-806

64. Patthy, L. (1990) Homology of a domain of the growth hormone/prolactin receptor family with type III modules of fibronectin. *Cell*, 61, 13-14

65. Jiang, Y.P., Wang, H., D'eustachio, P., Musacchio, J.M., Schlessinger, J. & Sap, J. (1993) Cloning and characterization of RPTP-κ, a new member of the receptor protein tyrosine phosphatase family with a proteolytically cleaved cellular adhesion molecule-like extracellular region. *Mol. Cell. Biol.* 13, 2942-2951

66. Krueger, N.X. & Saito, H. (1992) A human transmembrane PTP, RPTPζ, is expressed in brain and has an N-terminal receptor domain homologous to carbonic anhydrase. *Proc. Natl. Aca. Sci. USA* 87, 7417-7421

67. Barnea, A.M., Canoll, P.D., D'enstachio, P., Morse, B., Lowy, J.B., Laforgia, S., Huebner, K., Musacchio, J. M., Sap. J. & Schlessinger, J.

(1993) Identification of a carbonic anhydrase-like domain in the extracellular region of RPTPγ defines a new subfamily of receptor tyrosine phosphatases. *Mol. Cell. Biol.* 13, 1497-1506

68. Pan, M., Rim, C., Lu, K., Florio, T. & Stork P. J.S. (1993) Expression and cloning of two structurally distinct receptor-linked protein-tyrosine phosphatases generated by RNA processing from a single gene. *J. Biol. Chem.*. 268, 19284-19291

69. Wang, Y. & Pallen, C.J. (1991) The receptor-like protein-tyrosine phosphatase HPTPα has two active catalytic domains with distinct substrate specificity. *EMBO J.* 10, 3231-3237

70. Tan, X., Stover, D.R., & Walsh, K.A. (1993) Demonstration of protein tyrosine phosphatase activity in the second of two homologous domains of CD45. *J. Biol. Chem.* 268, 6835-6838

71. Laforgia, S., Morse, B., Levy, J., Barnea, G., Cannizzaro, L.A., Li, F., Nowell, P.C., Noghosian-Seel, L., Glick, J., Weston, A., Harris, C.C., Drabkin, H., Patterson, D., Croce, C.M., Schlessinger, J. & Huebner, K. (1991) Receptor protein-tyrosine phosphatase γ is a cannidate tumor suppressor gene at human chromosome region 3p21. *Proc. Natl. Aca. Sci. USA*. 88, 5036-5040

72. Tsukamoto, T. Takahashi, T., Ueda, R, Hibi, K., Saito, H. & Takahashi, T. (1992) Molecular analysis of the protein tyrosine phosphatase γ gene in human lung cancer cell lines. *Cancer Res.* 52, 3506-3509

73. Griswold-Prenner, I., Carlin, C. & Rosner, M.R. (1993) Mitogen-activated protein kinase regulates the epidermal growth factor receptor through activation of a tyrosine phosphatase. *J. Biol. Chem.* 268, 13050-13054

74. Swarup, G., Cohen, S. & Garbers, D.L. (1982) Inhibition of membrane phosphotyrosyl-protein phosphatase activity by vanadate. *Biochem. Biophys. Res. Commun.* 107, 1104-1109

75. Faure, R., Baquiran, G., Bergeron, J.J.M. & Posner, B.I. (1992) The dephosphorylation of insulin and epidermal growth fator receptors. *J. Biol. Chem.* 267, 11215-11221

76. Goldstein, B.J. (1992) Protein-tyrosine phosphatases and the regulation of insulin action. *J. Cell. Biochem.* 48, 33-42

77. Thomaska, L. & Resnick, R.J. (1993) Involvement of a phosphotyrosine protein phosphatase in the suppression of platelet-derived growth factor receptor autophosphorylation in ras-transformed cells. *Biochem. J.* 293, 215-221

78. Ahn, N.G., Weiel, J.E., Chan, C.P. & Krebs, E.G. (1990) Identification of multiple EGF-stimulated protein serine/threonine kinases from Swiss 3T3 cells. *J. Biol. Chem.* 265, 11487-11494

79. Boulton, T.G. & Cobb, M.H. (1991) Identification of multiple extracellular signal-regulated kinases(ERKs) with antipeptide antibodies. *Cell Regul.* 2, 357-371

80. Zheng, C. & Guan, K. (1993) Dephosphorylation and inactivation of the mitogen-activated protein kinase by a mitogen-induced tyr/thr protein phosphatase. *J. Biol. Chem.* 268, 16116-16119

81. Pan, M., Florio, T. & Stork, P.J.S. (1992) G protein activation of a hormone-stimulated phosphatase in human tumor cells. *Science*, 256, 1215-1217

82. Florio, T. Pan, M., Newman, B., Hershberger, R.E., Civelli, O. & Stork, P.J.S. (1992) Dopaminergic inhibition of DNA synthesis in pituitary tumor cells is associated with phosphotyrosine phosphatase activity. *J. Biol. Chem.* 267, 24169-24172

83. Bottar, S.P., King, I.N., Reichlin, S. & Dahlstroem, I. (1992) The angiotensin AT2 receptor stimulates protein tyrosine phosphatase activity and mediates inhibition of particulate guanylate cyclase. *Biochim. Biophys. Acta.* 183, 206-211

84. Gruppuso, P.A., Mikumo, R., Brautigan, D.L. & Brau, L. (1991) Growth arrest induced by transforming growth factor ß-1 is accompanied by protein phosphatase activation in human keratinocytes. *J. Biol. Chem.* 266, 3444-3448

85. Zafriri, D., Argaman, M., Canaani, E. & Kimchi, A. (1993) Induction of PTPase activity by interleukin-6 in M1 myeloblastic cells and analysis of possible counteraction by the BCR-ABL oncogene. *Proc. Natl. Aca. Sci. USA*. 90, 477-481

86. Moses, H., Yang, E.Y. & Pietenpil, J.A. (1990) TGF-ß stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell,* 63, 245-247

87. Frank, D.A. & Sartorelli, A.C. (1988) Alterations in tyrosine phosphorylation during the granulocytic maturation of HL-60 leukemia cells. *Cancer. Res.* 48, 52-58

88. Frank, D.A. & Satorelli, A.C. (1986) Regulation of protein phosphotyrosine content by changes in tyrosine kinase and protein phosphotyrosine phosphatase activities during induced granulocytic and monocytic differentiation of HL-60 leukemia cells. *Biochim. Biophys. Res. Commun.* 140, 440-447

89. Klarland, J.K. (1985) Tranfomation of cells by an inhibitor of phosphatase activity acting on phosphotyrosine in proteins. *Cell,* 41, 707-717

90. Pallen, C.J. & Tong, P.H. (1991) Elevation of membrane tyrosine phosphatase activity in density-dependent growth arrested fibroblasts. *Proc. Natl. Aca. Sci. USA*. 88, 6996-7000

91. Tonks, N.K., Cicirelli, M.F., Diltz, C.D., Krebs, E.G. & Fischer, E.H. (1990) Effect of microinjection of a low-Mr human placenta protein tyrosine phosphatase on induction of meiotic cell division in *Xenopus* Oocytes. *Mol. Cell. Biol.* 10, 484-463

92. Champion-Arnaud, P., Gesnel, M.-C., Foulkes, N. Ronsin, C., Sassone-Corsi, P. & Breathnach, R. (1991) Activation of transcription via AP-1 or CREB regulatory sites is blocked by protein tyrosine phosphatases. *Oncogene*, 6, 1203-1209

93. Millar, J.B.A., Russel, P. Dixon, J.E. &Guan, K. (1992) Negative regulation of mitosis by two functionally overlapping PTPases in fission yeast. *EMBO J.* 11, 4943-4952

94. David, M., Romero, G., Zhang, Z., Dixon, J.E. & Larner, A.C. (1992) In vitro activation of transcription factor ISGF3 by interferon-alpha involves a membrane-associated tyrosine phosphatase and tyrosine kinase. *J. Biol. Chem.* 268, 6593-6599

95. Igarahi, K., David, M., Finbloom, D.S., Larner, A.C. (1993) In vitro activation of the transcription factor γ interferon activation factor by γ interferon: evidence for a tyrosine phosphatase/kinase signaling cascade. *Mol. Cell. Biol.* 13, 1634-1640

96. Fu, X. (1992) A transcription factor with SH2 and SH3 domains is directly activated by an interferon induced cytoplasmic protein tyrosine kinase. *Cell,* 70, 323-335

97. Veelazquez, L., Fellous, M., Stark, G.R. & Pellegrini, S. (1992) A protein tyrosine kinase in the interferon α/β signaling pathway. *Cell*, 70, 313-322

98. Pingel, J.T. & Thomas, M.L. (1989) Evidence that the leukocyte common antigen is required for antigen-induced T lymphocyte proliferation. *Cell*, 58, 1055-1065

99. Weaver, C.T., Pingel, J.T., Nelson, J.O. & Thomas, M.L. (1991) $CD8^+$ T cell clones deficient in the expression of the CD45 protein tyrosine phosphatase have impaired response to T cell receptor stimuli. *Mol. Cell. Biol.* 11, 4415-4422

100. Koreztky, G., Picus, J. Schultz, T. & Weiss, A. (1991) Tyrosine phosphatase CD45 is required for both T cell antigen receptor and CD2 mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc. Natl. Aca. Sci. USA.* 88, 2037-2041

101. Koretzky, G., Picus, J., Thomas, M.L. & Weiss, A. (1990) Tyrosine phosphatse CD45 is essential for coupling T cell antigen receptor to the phosphatidylinositol pathway. *Nature*, 346, 66-68

102. Sieh, M., Bolen, J. & Weiss, A. (1993) CD45 specifically modulates binding of lck to a phosphopeptide encompassing the negative regulatory tyrosine of lck. *EMBO J.* 12, 315-321

103. Hurley, T.R., Hyman, R. & Sefton, B.M. (1993) Differential effects of expression of the CD45 tyrosine protein phosphatase on the tyrosine phosphorylation of the lck, fyn, and src tyrosine protein kinases. *Mol. Cell. Biol.* 13, 1651-1656

104. Mcfarland, E.D.C., Hurley, T.R., Pingel, J.T., Sefton, B.M. Shaw, A. & Thomas, M.L. (1993) Correlation between src family member regulation by the protein-tyrosine phosphatase CD45 and transmembrane signaling through the T cell receptor. *Proc. Natl. Aca. Sci. USA*. 90, 1402-1406

105. Mustelin, T., Coggeshall, K.M. & Altman, A. (1989) Rapid activation of the T cell tyrosine protein kinase pp56^{lck} by the CD45 phosphotyrosine phosphatase. *Proc. Natl. Aca. Sci. USA*. 86, 6302-6306

106. Hatakeyama, M., Kono, T., Kobayashi, N., Levin, S., Perlmutter, R.M. & Taniguchi, T. (1991) Interaction of the IL-2 receptor with the src-family kinase p56^{lck}: identification of novel intermolecular association. *Science*, 252, 1523-1528

107. Straus, D.B. & Weiss, A. (1992) Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell*, 70, 585-593

108. Weiss, A. (1993) T cell antigen receptor signal transduction: a tale of tails and cytoplasmic protein-tyrosine kinases. *Cell*, 73, 209-212

109. Cooke, M.P., Abraham. K.M., Forbush, K.A. & Perlmutter, R.M. (1991) Regulation of T cell receptor signaling by a src family protein-tyrosine kinase ($p59^{fyn}$). *Cell*, 65, 281-291

110. Appleby, M.W., Gross, J.A., Cooke, M.P., Levin, S.D., Qian, X. & Perlmutter, R.M. (1992) Defective T cell receptor signaling in mice lacking the thymic isoformof $p59^{fyn}$. *Cell,* 70, 751-763

111. Stein, P.L., Lee, H., Rich, S. & Soriano, P. (1992) $pp59^{fyn}$ mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell*, 70, 741-750

112. Kishihara, K., Penninger, J., Wallace, V.A., Kundig, T.M., Kawai, K., Wakeham, A., Timms, E., Pfeffer, K., Ohashi, P.S., Thomas, M.L., Furioger, C., Paige, C. & Mak, T.K. (1993) Normal B lymphocyte development but impaired T cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice. *Cell.* 74, 143-156

113. Yi, T. & Ihles, J.N. (1993) Association of hematopoietic cell phosphatase with c-Kit after stimulation with c-Kit ligand. *Mol. Cell. Biol.* 13, 3350-3358

114. Schultz, L.D., Schweitzel, P.A., Rajan, T.V., Yi, T., Ihle, J.N., Mattews, R.J., Thomas, L. & Beier, D.R. (1993) Mutations at the munine motheaten locus are within the hematopoeitic cell protein-tyrosine phosphatase (hcph) gene. *Cell*, 73, 1445-1454

115. Lechleider, R.J., Freeman, R.M., Jr., & Neel, B.G. (1993) Tyrosyl phosphorylation and growth factor receptor association of the human corkscrew homologue, SH-PTP2. *J. Biol. Chem.* 268, 13434-13438

116. Zheng, X., Wang, Y. & Pallen, C.J. (1992) Cell transformation and activation of pp60$^{c\text{-}src}$ by overexpression of a protein tyrosine phosphatase. *Nature*, 359, 336-339

117. Lewin, B. (1990) Driving the cell cycle: M phase kinase, its partners, and substrates. *Cell*, 61, 743-752

118. Millar, J.B.A. & Russel, P. (1992) The cdc25 M-phase inducer: an unconventional protein phosphatase. *Cell*, 68, 407-410

119. Cooper, J.A. & Whyte, P. (1989) RB and the cell cycle: entrance or exit. *Cell*, 58, 1009-1011

120. Sherr, C. J. (1993) Mammalian G1 cyclins. *Cell*, 73, 1059-1065

121. Chellappan, S.P., Hiebert, S., Mudryj, M., Horomitz, J.M. & Nevins, J. (1991) The E2F transcription factor is a cellular target for the RB protein. *Cell*, 65, 1053-1061

122. Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J., Hanks, S.K., Roussel, M.F. & Sherr, C.J. (1992) Identification and properties of an atypical catalytic subunit (p34$^{psk\text{-}j3}$/cdk4) for mammalian D type G1 cyclins. *Cell*, 71, 323-334

123. Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J. F. & Kirschner, M.W. (1991) cdc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. *Cell*, 67, 197-211

124. Dunphy, W.G. & Kumagai, A. (1991) The cdc25 protein contains an intrinsic phosphatase activity. *Cell*, 67, 189-196

125. Kumagai, A & Dunphy, W.G. (1992) Regulation of the cdc25 protein during the cell cycle in Xenopus extracts. *Cell*, 70, 139-151

126. Coleman, T.R., Tang, Z. & Dunphy, W.G. (1993) Negative regulation of the wee1 protein kinase by direct action of the Nim1/Cdr1 mitotic inducer. *Cell*, 72, 919-929

127. Jessus, C. & Beach, D. (1992) Oscillation of MPF is accompanied by periodic association between cdc25 and cdc2-cyclin B. *Cell*, 68, 323-332

128. Galaktinov, K. & Beach, D. (1991) Specific activation of cdc25 tyrosine phosphatase by B-type cyclins: evidence for multiple roles of mitotic cyclins. *Cell*, 67, 1184-1194

129. Fang, F. & Newport, J.W. (1991) Evidence that the G1-S and G2-M transtions are controlled by different cdc2 proteins in higher eukaryotes. *Cell*, 66, 731-742

130. Tan, J.L. & Spudich, J.A. (1990) Developmentally regulated protein-tyrosine kinase genes in *Dictyostelium discoideum. Mol. Cell. Biol.* 10, 3578-3583

131. Howard, P.K., Sefton, B.M. & Firtel, R.A. (1992) Analysis of a spatially regulated phosphotyrosine phosphatase identifies tyrosine phosphorylation as a key regulatory pathway in Dictyostelium. *Cell*, 71, 637-647

132. Mei, L. & Huganir, R.L. (1991) Purification and characterization of a protein tyrosine phosphatase which dephosphorylates the nicotinic acetylcholin receptor. *J. Biol. Chem.* 266, 16063-16072

133. Walton, K., Martell, K.J., Kwak, S.P., Dixon, J.E. & Largent, B.L. (1993) A novel receptor-type protein tyrosine phosphatase is expressed during neurogenesis in the olfactory neuroepithelium. *Neuron,* 11, 387-400

134. Wu, Y.Y. & Bradshaw, R.A. (1993) Effect of nerve growth factor and fibroblast growth factor on PC12 cells: inhibition by orthovanadate. *J. Cell. Biol.* 121, 409-422

135. Aparicio, L.F., Ocrant, I., Boylan, J.M. & Gruppuso, P.A. (1992) Protein phosphatase activation during nerve growth factor-induced neuronal differentiation of PC12 cells. *Cell Grow. Differ.* 3, 363-367

136. Lombroso, P.J., Murdoch, G. & Lerner, M. (1991) Molecular characterization of a protein-tyrosine-phosphatase enriched in striatum. *Proc. Natl. Aca. Sci. USA*. 88, 7242-7246

137. Levy, J.B., Canoll, P.D., Silvennoinen, O., Barnea, G., Morse, B., Honegger, A.M., Huang, J.-T., Cannizzaro, L.A., Park, S.-H., Druck, T., Huebner, T., Sap, J., Ehrlich, M., Musacchio, J.M. & Schlessinger, J. (1993) The cloning of a receptor-type protein tyrosine phosphatase expressed in the central nervous system. *J. Biol. Chem.* 268, 10573-10581

138. Jena, B.P., Padfield, P.J., Ingebritsen, T.S. & Jamieson, J.D. (1991) Protein tyrosine phosphatase stimulates Ca^{2+}-dependent amylase secretion from pancreatic acini. *J. Biol. Chem.* 266, 17744-17746

139. Brautigan, D.L. & Pinault, F.M. (1991) Activation of membrane protein-tyrosine phosphatase involving cAMP- and Ca^{2+}/phospholipid-dependent protein kinase. *Proc. Natl. Aca. Sci. USA*. 88, 6696-6700

140. Stover, D.R., Charbonneau, H., Tonks, N.K. & Walsh, K.A. (1991) Protein-tyrosine-phosphatase CD45 is phosphorylated transiently on tyrosine upon activation of Jurkat T cells. *Proc. Natl. Aca. Sci. USA*. 88, 7704-7707

141. Ostergard, H.L. & Trowbridge, I.S. (1991) Negative regulation of CD45 protein phosphatase activity by ionomycin in T cells. *Science*, 253, 1423-1425

142. Zhao, Z., Shen, S. & Fischer, E.H. (1993) Stimulation by phospholipids of a protein-tyrosine-phosphatase containing two src homology domains. *Proc. Natl. Aca. Sci.* 90, 4251-4255

143. Yeung, Y., Berg, K.L., Pixley, F.J., Angeletti, R.H. & Stanley, E.R. (1992) Protein tyrosine phosphatase-1C is rapidly phosphorylated in tyrosine in macrophages in response to colony stimulating factor. *J. Biol. Chem.* 267, 23447-23450

144. Maegawa, H., Ugi, S., Ishibashi, O., Tachikawa, R., Takahara, N., Tanaka, Y., Takagi, Y., Kikawa, R., Shigeta, Y. & Kashiwagi, A. (1993) Src homology 2 domains of protein tyrosine phosphatase are phosphorylated by insulin receptor kinase and bind to the C-terminus of insulin receptors in vitro. *Biochem. Biophys. Res. Comm.* 194, 208-214

145. Frngloni, J.V., Beahm, P.H., Shifrin, V., Jost, C.A. & Neel, B.G. (1992) The nontransmembrane tyrosine phosphatase PTP1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence. *Cell*, 68, 545-560

146. Tappia, P.S., Sharma, R.P. & Sale, G.J. (1991) Dephosphorylation of autophosphorylated insulin and epidermal growth factor receptor by two major subtypes of protein-tyrosine-phosphatase from human placenta. *Biochem. J.* 278, 69-74

147. Bauskin, A.R., Alkalay, I. & Ben-Neriah, Y. (1991) Redox regulation of a protein tyrosine kinase in the endoplasmic reticulum. *Cell*, 66, 685-696

148. Audigier, Y., Nigam, S.K. & Blobel, G. (1988) Identification of a G protein in rough endoplasmic reticulum of canine pancreas. *J. Biol. Chem.* 263, 16352-16357

149. Nigam, S.K. & Blobel, G. (1989) Cyclic AMP-dependent protein kinase in canine pancreatic rough endoplasmic reticulum. *J. Biol. Chem.* 264, 16927-16932.

150. McLaughlin, S. & Dixon, J.E. (1993) Alternative splicing gives rise to a nuclear protein tyrosine phosphatase in Drosophila. *J. Biol. Chem.* 268, 6839-6842

151. Ingebretisen, T.S. (1989) Phosphotyrosyl-protein phosphatases: identification and characerization of two heat-stable protein inhibitors. *J. Biol. Chem.* 264, 7754-7759

152. Tonks, N.K., Yang, Q. & Guida, P., Jr. (1991) Structure, regulation and function of protein tyrosine phosphatases. *Cold Spring Harbor Symposia on Quantitative Biol.* LVI, 265-273

153. Zhao, Z., Bouchard, P., Diltz, C.D., Shen, S. & Fisher, E.H. (1993) Purification and characterization of a protein tyrosine phosphatase containing SH2 domain. *J. Biol. Chem.* 268, 2816-2820

154.Wang, Y. & Pallen, C.J. (1992) Expression and characterization of wild type, truncated, and mutant forms of the intracellular region of the receptor-like protein tyrosine phosphatase HPTPß. *J. Biol. Chem.* 267, 16696-16702

155. Strueli, M., Krueger, N.X., Ariniello, P.D., Tang, M., Munro, J.M., Blattler, W.A., Adler, D.A., Disteche, C.M. & Saito, H. (1992) Expression of the receptor-linked protein tyrosine phosphatase LAR: proteolytic cleavage and shedding of the CAM-like extracellular region. *EMBO J.* 11, 897-907

156. Yu, Q., Lenardo, T. & Weinberg, R.A. (1992) The N-terminal and C-terminal domains of a receptor tyrosine phosphatase are associated by non-covalent linkage. *Oncogene*, 7, 1051-1-57

157. Tokeda, A., Wu, J.J. & Maizel, A.L. (1992) Evidence for monomeric and dimeric forms of CD45 associated with a 30kDa phosphorylated protein. *J. Biol. Chem.* 267, 16651-16659

158. Desai, D.M, Sap, J., Schlessinger, J. & Weiss, A. (1993) Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell*, 73, 541-554

159. Schlessinger, J. & Ullrich, A. (1992) Growth factor signaling by receptor tyrosine kinases. *Neuron*, 9, 383-391

160. Hu, P. Margolis, B. Skolinik, E.Y. Lammers, R., Ullrich, A. & Schlessinger, J. (1992) Interaction of phosphatidylinositol 3-kinase-

associated p85 with epidermal growth factor and platelet-derived growth factor receptors. *Mol. Cell. Biol.* 12, 981-990

161. Otsu, M., Hiles, I., Gout, I., Fry, M.J., Ruiz-Larrea, F. Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A.D., Morgan, P.J., Courtneidge, S.A., Parker, P.J. & Waterfield, M.D. (1991) Characterization of two 85 kd proteins that associate with receptor tyrosine kinases, middle-T/pp60$^{c\text{-}src}$ complexes, and PI3-kinase. *Cell*, 65, 91-104

162. Skolnik, E.Y., Margolis, B., Mohammadi, M., Lowenstein, E., Fischer, R., Drepps, A. Ullrich, A. & Schlessinger, J. (1991) Cloning of PI3 kinase-associated p85 utilizing a novel method for expression/cloning of target proteins for receptor tyrosine kinases. *Cell*, 65, 83-90

163. Backer, J.M., Meyers, M.G., Jr., Shoelson, S.E., Chin, D.J., Sun, X., Miralpex, M., Hu, P., Margolis, B., Skolnik, E.Y., Schlessinger, J. & White, M.F. (1992) Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *EMBO. J.* 11, 3469-3479

164. Hiles, I.D, Otsu, M., Volinia, S., Fry, M.J. Gout, I., Dhand, R., Panayoto, G., Ruiz-Larrea, F., Thompson, A., Totty, N.F., Hsuan, J.J., Courtneidge, S.A., Parker, P.J. & Waterfield, M.D. (1992) Phosphatidylinositol 3-kinase: structure and expression of the 110 kd catalytic subunit. *Cell*, 70, 419-426

165. Katan, M., Kriz, R.W., Totty, N., Philp, R., Meldrum, E., Aldape, R.A., Knopf, J. & Parker, P.J. (1989) Determination of the primary structure of

PLC-154 demonstrates diversity of phosphoinositide-specific phospholipase C activities. *Cell*, 54, 171-177

166. Suh, P., Ryu, S.H., Moon, K.H., Suh, H.W. & Rhee, S.G. (1989) Cloning and sequence of multiple forms of phospholipase C. *Cell*, 54, 161-169

167. Wahl, M.J., Daniel, T.O. & Carpenter, G. (1988) Antiphosphotyrosine recovery of phospholipase C activity after EGF treatment of A431 cells. *Science*, 241, 968-970

168. Majerus, P.W., Ross, T.S., Cunningham, T.W., Caldwell, K.K., Jefferson, A.B. & Bansal, V.S. (1990) Recent insights in phosphatidylinositol signaling. *Cell*, 63, 459-465

169. Rhee, S.G. (1991) Inositol phospholiid-specific phospholipase C: interaction of the γ1 isoform with tyrosine kinase. *TIBS*. 16, 297-301

170. Feig, L.A. (1993) The many roads that lead to Ras. *Science*, 260, 767-768

171. Duday, L. & Downward, J. (1993) Epidermal growth factor regulates p21 ras through the formation of a complex of receptor, Grb2 adaptor protein, and Sos nucleotide exchange factor. *Cell*, 73, 611-620

172. Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P.G., Schlessinger, J. &

Pawson, T. (1993) Association of the Shc and Grb2/sem5 SH2 containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature*, 360, 689-692

173. Olivier, J.P., Roabe, J., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafer, E. & Parson, T. (1993) A Drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchanger, Sos. *Cell*, 73, 179-191

174. Rozakis-Adcock, M. Fermley, R., Wade, J., Parson, T. & Bowtell, D. (1993) The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos. *Nature*, 363, 83-85

175. Li, N., Batzer, A., Daly, R., Yajnik, Skolnik, E., Chardin, P., Bar-Sagi, D., Margolisd, B. & Schlessinger, J. (1993) Guanine-nucleotide-releasing factor hSos, binds to Grb2 and links receptor tyrosine kinases to Ras signaling. *Nature*, 363, 85-88

176. Gale, N.W., Kaplan, J., Lowenstein, E.J., Schlessinger, J. & Bar-Sagi, D. (1993) Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. *Nature*, 363, 88-92

177. Egan, S.E., Giddings, B.W., Brooks, M.W., Baday, L., Sizeland, A.M. & Weinberger, R.A. (1993) Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature*, 363, 45-51

178. Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E.Y., Bar-Sagi, D. & Schlessinger, J. (1992) The SH2 and SH3 domains-containing proteins Grb2 links receptor tyrosine kinases to ras signaling. *Cell*, 70, 431-442

179. Ellis, C., Moran, M., McCormick, F. & Pawson, T. (1990) Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. *Nature*, 343, 377-381

180. Rubinfeld, B., Munemitsu, S., Clark, R., Conroy, L., Watt, K, Crosier, W.J., McCormick, F. & Polakis, P. (1991) Molecular cloning of a GTPase activating protein specific for the Krev-1 protein $p21^{rap1}$. *Cell*, 65,1033-1042

181. Kaziauskas, A., Ellis, C., Pawson, T. & Cooper, T.A. (1990) Binding of GAP to activated PDGF receptors. *Science* 247, 1578-1581

182. Kaplan, D.R., Morrison, D.K., Wong, G., McCormick, F. & Williams, L.T. (1990) PDGF ß-receptor stimulates tyrosine phosphorylation of GAP and association of GAP with a signaling complex. *Cell*, 67, 125-133

183. Gould, K.L. & Hunter, T. (1988) Platelet-derived growth factor induces multisite phosphorylation of $pp60^{c\text{-}src}$ and increases its protein-tyrosine kinase activity. *Mol. Cell. Biol.* 8, 3345-3356

184. Li, P., Wood, K., Mamon, H., Haser, W. & Roberts, T. (1991) Raf-1: a kinase currently without a cause but not lacking in effects. *Cell*, 64, 470-482

185. Couhlin, J.P., Donahoe, P.K., Budzik, G.P. & Maclaughlin, D.T. (1987) Mullerian inhibitory substance blocks autophosphorylation of the EGF receptor by inhibiting tyrosine kinase. *Mol. Cell. Endocrinol.* 49, 75-86

Chapter II

G Protein Activation of A Hormone-Stimulated Phosphatase in Human Tumor Cells

Ming-Gui Pan, Tullio Florio, and Philip J.S. Stork

Abstract: We demonstrate that the growth-inhibiting peptide hormone somatostatin stimulates phospho-tyrosine phosphatase activity in the human pancreatic cell line MIA PaCa-2. This hormonal activation was mediated by a pertussis toxin sensitive guanosine triphosphate (GTP)-binding protein (G protein) present in the membranes of these cells. Activation of this G protein by somatostatin stimulated the dephosphorylation of exogenous epidermal growth factor receptor prepared from A431 cells in vitro. This pathway may mediate the antineoplastic action of somatostatin in these cells and in human tumors, and could represent a general mechanism of G protein coupling that is utilized by normal cells in the hormonal control of cell growth.

Pertussis-toxin sensitive G proteins mediate a wide range of functions within the cell, including the control of cell growth *(1, 2)*. Constitutively active mutants of these proteins have been implicated in the pathogenesis of some human neoplasms *(3)*. However, the effectors which G protein pathways activate to mediate growth regulation have not been fully identified; these effectors may include phosphotyrosine phosphatases (PTPs). Inhibition of endogenous PTP activity in non-transformed cells in culture can induce cellular transformation *(4)*, and the injection of exogenous PTP into *Xenopus* oocytes can reverse the mitogenic effect of insulin-stimulated tyrosine kinase activity *(5)*. These findings suggest that PTP activity may counteract the actions of tyrosine kinases to promote normal cell growth, and that interfering with this activity may lead to malignant transformation.

In vitro, the enzymatic activity of PTPs far exceeds that of tyrosine kinases *(6, 7)*. Therefore, PTPs must be regulated in vivo to maintain a balance between phosphorylation and dephosphorylation. Cellular responses to increased tyrosine kinase activity should generate rapid stimulation of specific PTPs followed by a return to resting levels. G proteins provide hormone receptors with the ability to generate rapid stimulation and termination of effector pathways.

To explore the possibility that G proteins regulate PTP activity we examined the effects of somatostatin on the undifferentiated human pancreatic cancer cell line MIA PaCa-2 *(8)*. This cell line expresses somatostatin receptors, and displays a hormonally regulated PTP activity *(9)*. To characterize the regulation of this PTP activity, we evaluated the action of somatostatin on cell membranes and on whole cells. We measured phosphatase activity with

the synthetic substrate para-nitrophenol phosphate (pNpp) *(10)*; this substrate releases a spectrophotometrically detectable cleavage product following incubation with phosphatases.

The MIA PaCa-2 cell may contain membrane-associated and cytoplasmic PTPs as well as serine-threonine phosphatase activity. To ensure that the assay was specific for membrane PTP activity, we incubated MIA PaCa-2 membranes with the substrate pNpp and the phosphatase inhibitors microcystin-LR (M-LR) and $ZnCl_2$. M-LR is a specific inhibitor of the major serine-threonine protein phosphatases 1 and 2A *(11)*. $ZnCl_2$ at micromolar concentrations inhibits cytosolic PTPs *(12)* more effectively than membrane PTPs. These inhibitors only slightly reduced the basal phosphatase activity (less than 10%), suggesting that a low level of serine-threonine phosphatase activity was present in our membrane preparations *(13)*. The phosphatase activity that remained after inhibition by M-LR was unable to dephosphorylate specific substrates of serine-threonine phosphatase *(14)*. Furthermore, this remaining phosphatase activity was completely blocked by vanadate (50uM), a specific inhibitor of PTP *(15)* (Fig. 1). Therefore only PTPs were active under the conditions of our assay.

Under conditions of substrate excess, the phosphatase activity from unstimulated membranes was proportional to the length of time and the quantity of membrane proteins assayed *(13)*. Inclusion of somatostatin-14 (1 uM) doubled this activity at all times during a one hour assay. Vanadate completely blocked both stimulated and basal phosphatase activity (Fig. 1). Somatostatin was active over a wide range of concentrations; half-maximal stimulation of phosphatase activity occured with 2 nM somatostatin. This is

similar to the somatostatin concentration that produces a half-maximal effect on growth in this cell line *(16)*.

We evaluated phosphatase activity in membranes incubated in the presence of guanosine triphosphate (GTP) (200 uM), and 5'-guanylylimidodiphosphate (GppNHp, 200 uM), with and without somatostatin-14 (1 uM). GppNHp and GTP *(12)* increased phosphatase activity of MIA PaCa-2 membranes (Fig. 2); the addition of somatostatin further increased this activity (Fig. 2). However when we incubated cell membranes with the nonhydrolizable guanine nucleotide analogue guanosine 5'-0-(2-thiodiphosphate) (GDP-ßS, 200 uM) somatostatin stimulation of phosphatase activity was completely blocked (Fig. 2). This demonstrates that somatostatin's effect on phosphatase activity in membranes is mediated by a GTP binding protein.

We also examined somatostatin stimulation of phosphatase activity in whole cells; the result was similar in magnitude to the effect seen in membranes (Fig. 3) *(17)*. This suggests that both whole cells and membrane preparations contain the accessory proteins necessary for this stimulation. Both effects were completely blocked by the pretreatment of cells with pertussis toxin (PTX) (Fig. 3). PTX pretreatment alone did not inhibit the basal phosphatase activity of these membranes, demonstrating that only the hormone-sensitive activity is PTX-sensitive.

To investigate whether somatostatin stimulated phosphatase activity could dephosphorylate a physiologically relevant substrate, we prepared an enriched source of epidermal growth factor receptors (EGFRs) from

membrane vesicles of the cell line A-431 *(18)*. EGFR is selectively phosphorylated in vitro on tyrosine residues and is a substrate for membrane-associated tyrosine phosphatases *(18)*. We incubated membrane vesicles from A431 cells with EGF to activate the intrinsic tyrosine kinase activity of the EGFR. The major phosphoprotein was the 170 kD autophosphorylated EGFR *(13)*. Incubation of ^{32}P-labeled membrane vesicles with MIA PaCa-2 membranes in the presence of somatostatin alone or with GppNHp reduced the intensity of labeled ^{32}P within the 170 kD band; co-incubation with somatostatin and GppNHp further reduced the intensity of this band. No effect was seen if the vesicles were incubated with MIA PaCa-2 membranes in the absence of somatostatin or GppNHp (Fig. 4). Somatostatin did not stimulate dephosphorylation in the presence of GDP-ßS *(13)*. This demonstrates that dephosphorylation of exogenous EGFR is stimulated by somatostatin in MIA PaCa-2 membranes via a G protein pathway.

Activation of membrane phosphatases by PTX-sensitive G proteins may be a general mechanism for the hormonal control of cell growth. Activation of tyrosine kinase activity following stimulation of G protein-coupled receptors may provide additional hormonal control of cell growth *(19, 20)*. Recent reports suggesting that G proteins are also involved in receptor tyrosine kinase signaling pathways *(21,22)* indicate that the G proteins and the tyrosine kinase/phosphatase signaling pathway may be interconnected at multiple levels.

FIGURES

Fig. 1. Dose-dependence of stimulation of phosphatase activity by somatostatin. Phosphatase activity was measured as described *(10)* in the presence of various concentrations of somatostatin-14 at 30°C for 10 min. The phosphatase activity is expressed as percent increase over control. Each point represents the mean of seperate experimental determinations ± SE (n=2). Insert: maximal stimulation of phosphatase activity in MIA PaCa-2 membrane proteins by somatostatin and its inhibition by vanadate. Lane 1, membranes alone; lane 2, membranes plus vanadate (50 uM); lane 3, membranes with somatostatin (1 uM); lane 4, membranes with somatostatin plus vanadate. Phosphatase activity is presented as percent of control. Each point represents a mean ± S.E (n=4).

Fig. 2. Effect of nonhydrolizable GTP analogues on activation of phosphatase activity in MIA PaCa-2 membranes by somatostatin. MIA PaCa-2 membrane proteins (20 ug) were incubated alone (C), with somatostatin-14 (1uM) (S), with GppNHp (0.2 mM) (G), with both (S + G) or with somatostatin and GDP-ßS (GDP-ßS + S), in the presence of 2.5 mM $MgCl_2$. Phosphatase acitvity was measured as described *(10)* and is shown as percent of control. Each point represents the mean of duplicate determinations ± SE (n=3). Statistically significant differences ($p<0.05$) were determined between groups represented by lanes 1 through 4 with the Scheffe F test.

Fig. 3. Effect of pertussis toxin on phosphatase activity. MIA PaCa-2 cells (5×10^5 per 10 cm plate) were maintained in fetal calf serum (10%) for 5

days to reach 80 to 90% confluency. Cells were treated with pertussis toxin (120 ng/ml) (Sigma) for 18 hours and then washed and maintained in DMEM. **A.** Membrane proteins (20 ug) from untreated cells were incubated with buffer (C), or somatostatin-14 (1 uM) (S); membrane proteins from PTX-treated cells were incubated with buffer (P) or somatostatin (P+S) and assayed for PTP activity at 30°C for 10 min. **B.** PTX-treated and untreated cells were incubated with or without somatostatin (1uM) in serum-free DMEM at 37°C for 60 min. (E) Membrane proteins (20 ug) from untreated cells (C), cells treated with somatostatin (S), PTX (P), or PTX plus somatostatin (P+S), were incubated in buffer with pNpp at 30° C for 10 min. Phosphatase activity is presented as percent of control. Points represent the mean of duplicate determination $\pm$ S.E (n=5).

Fig. 4. Dephosphorylation of EGFR prepared from A-431 cells by phosphatases from MIA PaCa-2 membranes. A-431 membrane vescicles enriched for the EGFR were prepared as described *(16)*. Membrane vescicles proteins (2 ug) were incubated in 200 ul of kinase buffer [30 mM Hepes (pH 8.0) 15 mM $MnCl_2$, nonidet P-40 (0.75%), and 15 mM ATP] in the presence of EGF (100 nM) and 60 uCi γ^{32}P-ATP (5000 Ci/mmole) for 10 min at 37°C. Portions (40 ul) were immediately added to four separate reactions containing MIA PaCa-2 membranes (20 ug) alone (lane 1) or membranes in the presence of 200 uM GppNHp (lane 2), 1 uM somatostatin (lane 3), or 200 uM GppNHp and 1 uM somatostatin (lane 4). Reactions were incubated at 30°C, for 30 min in a final volume of 60 ul, which included 50 mM Hepes (pH 7.2) 1 mM EDTA, 5 mM DTT, 20 nM M-LR, 10 uM $ZnCl_2$, 2.5 mM $MgCl_2$, and 0.8 mM ATP, and the reaction was terminated with the addition of 5X Laemmli buffer (12 ul). The samples

were separated by SDS-polyacrylamide gel electrophoresis (7% gel) and detected by autoradiography. Relative densities of bands were: Lane 1, 1.00; lane 2, 0.15; lane 3, 0.16; and lane 4, 0.08.

References and notes

1. Z. Cui, *et al.*, *J. Biol. Chem.* **266**, 20276 (1991).

2. S. Hermouet, J. Merendino J.J., J.S. Gutkind, A.M. Spiegel, *Proc. Natl. Acad. Sci., U.S.A.* **88**, 10455 (1991).

3. J. Lyons, *et al.*, *Science* **249**, 655 (1990).

4. J.K. Karlund, *Cell* **41**, 707 (1985).

5. M.F. Cicirelli, *et al.*, *Proc. Natl. Acad. Sci., U.S.A.* **87**, 5514 (1990).

6. E.H. Fischer, H. Charbonneau, N.K. Tonks, *Science* **253**, 401 (1991).

7. T.S. Ingebritsen, *J. Biol. Chem.* **264**, 7754 (1989).

8. A.A. Yunis, G.K. Arimura, D.J. Russin, *Int. J. Cancer* **19**, 128 (1977).

9. M.T. Hierowski, C. Liebow, K. du Sapin, A.V. Schally, *FEBS* **179**, 252 (1985).

10. Characterization of the phosphatase activity in MIA PaCa-2 cells. Membranes were prepared from unstimulated cells grown to 80 to 90% confluence, washed twice in phosphate buffered saline (PBS) and mechanically scraped in membrane buffer [0.32 M sucrose, 10 mM tris (pH 7.5), 5mM EGTA, and 1 mM EDTA]. Nuclei were removed after

centrifugation at 2,000 x g for 10 min at 4°C and the membrane fraction was sedimented following centrifugation at 15,000 x g 45 min at 4°C. The membranes were resuspended in membrane preparation buffer [20 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM PMSF, Bacitracin, 20 ug/ml] and assayed for protein content. Membranes containing 20 ug protein were incubated with somatostatin-14 (1 uM) for 5 min at 30°C in 80 ul containing 20 ul of 5X reaction buffer [250 mM Hepes (pH 7.2), 50 mM DTT, 25 mM EDTA] in the presence of M-LR (20 nM) and $ZnCl_2$ (10 uM) and the reaction was initiated by the addition of pNpp substrate (50 mM, final concentration) for 30 min at 30°C. The reactions were stopped by the addition of 0.9 ml of 0.2 N NaOH and the absorbance of the samples was measured at 410 nm.

11. C. MacKintosh, K.A. Beattie, S. Klumpp, P. Cohen, G.A. Codd, *FEBS* **264**, 187 (1990).

12. N.K. Tonks, C.D. Diltz, E.H. Fischer, *Meth. Enzymol.* **201**, 427 (1991).

13. M.G. Pan, and P.J.S. Stork, unpublished observations.

14. A 19 amino acid peptide corresponding to the phosphorylation site of the RII subunit of PKA (residues 81-99) (Blumenthal, D.K., *et al.* J. Biol. Chem. 26:8140-8145, 1986) radiolabeled in the presence of γ-^{32}P-ATP by the catelytic subunit, was kindly provided by Tom Soderling. 200,000 cpm and 20 ug of membranes plus inhibitors were incubated in phosphatase buffer for 60 min at 30° C. in the presence and absence of somatostatin-14

(1 uM). The reaction mixture was spottted on p81 Whatmann filters and free phosphate was removed by washing in 0.5% phosphoric acid. Filters were assayed in a scintillation counter to measure retained cpm. Neither unstimulated membranes nor somatostatin-stimulated membranes contained serine-threonine phosphatases that were active on this substrate in this assay, P.J.S. Stork, unpublished observations.

15. G. Swarup, S. Cohen, D.L. Garbers, *Biochem. Biophys. Res. Comm.* **107**, 1104 (1982).

16. C. Liebow, M.T. Lee, A.R. Kamer, A.V. Schally, *Proc. Natl. Acad. Sci., U.S.A.* **88**, 2244 (1991).

17. M.G. Pan, T. Florio, P.J.S. Stork, Membranes prepared from whole cells incubated with somatostatin for 60 min provided maximal stimulation of phosphatase activity, unpublished observation.

18. S. Cohen, H. Ushiro, C. Stoscheck, M. Chinkers, *J. Biol. Chem.* **257**, 1523 (1982).

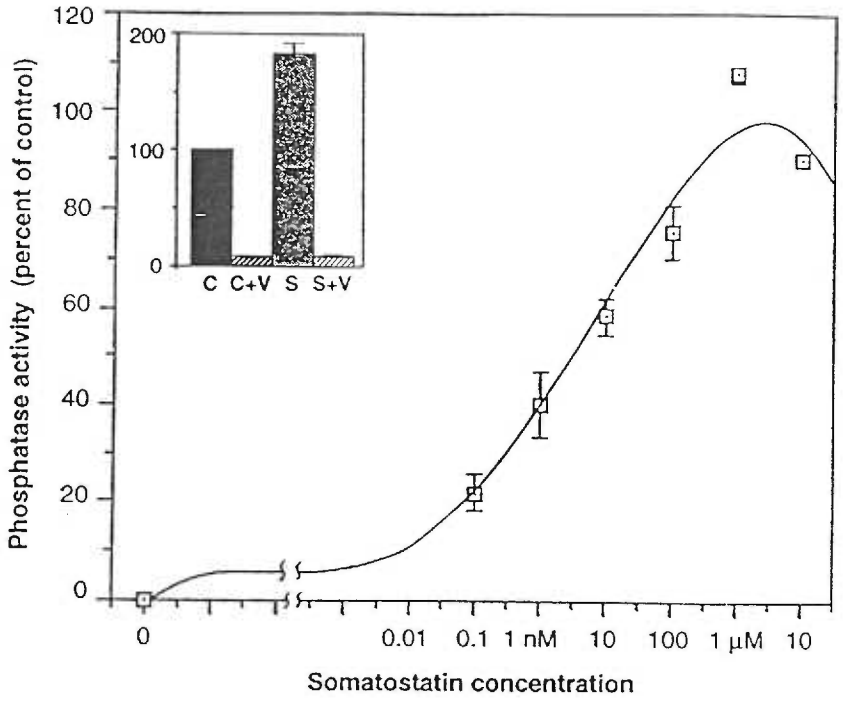
19. D. Cirillo, G. Gaudino, L. Naldini, P. Comoglio, *Mol. Cell. Biol.* **6**, 4641 (1988).

20. I. Zachary, J. Gil, W. Lehmann, J. Sinnet-Smith, E. Rozengurt, *Proc. Natl. Acad. Sci., U.S.A.* **88**, 4577 (1991).

21. I. Teitelbaum, *J. Biol. Chem.* **265**, 4218 (1990).

22. M.F. Jarvis, and G. Gessner, in *Society for Neuroscience* New Orleans, LA, Nov. 10-15, 1991), pp. 45.

23. We thank R. Goodman, T. Segerson, M. Forte, and T. Soderling for reading the manuscript, G. Clinton for providing A-431 cells, A. Shaw for providing the pNpp protocol, C. Rim and L. Lin, for technical assistance, and J. Tasnady for help in preparing the manuscript.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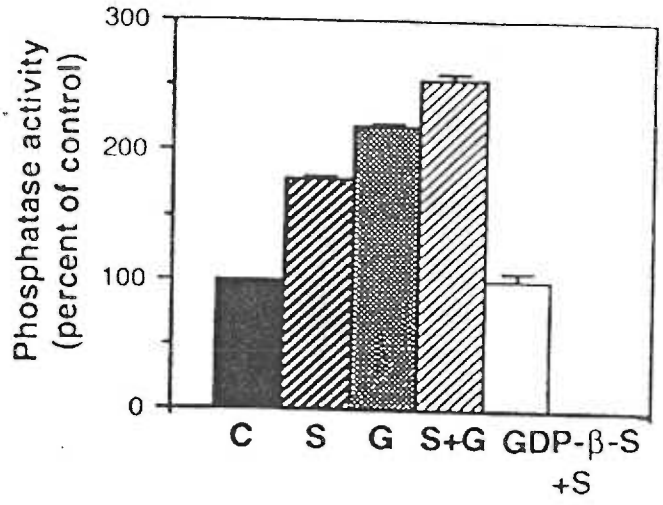
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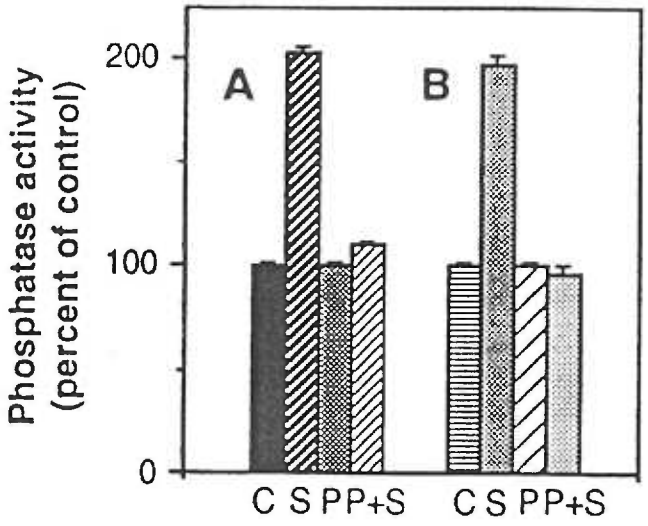
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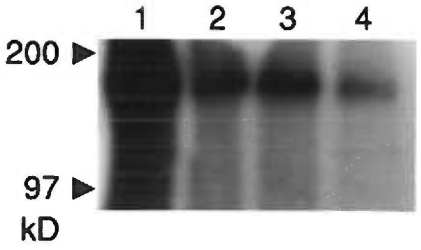
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Chapter III

Somatostatin Inhibits DNA Synthesis in MIA PaCa-2 Cells Through A cAMP-Independent Pathway

MING-GUI PAN & PHILIP J.S. STORK

Abstract: Somatostatin has been shown to inhibit cAMP synthesis and exert anti-proliferative action on many cell types. We have previously demonstrated that somatostatin can stimulate a membrane-associated PTPase activity through a G protein-coupling pathway. In this manuscript, we provide evidence that this pathway may elicit inhibitory signals for growth. We show that somatostatin can inhibit DNA synthesis in MIA PaCa-2 cells, and this inhibition can be blocked by a PTPase inhibitor vanadate. We also show that forskolin, a potent stimulator of cAMP production, does not block somatostatin's anti-mitogenic effect. Instead, forskolin also inhibits DNA synthesis, indicating that cAMP is not involved in somatostatin's anti-mitogenic effect.

Somatostatin was first identified as a growth hormone release-inhibiting factor in the hypothalamus (1). It exerts effects on a variety of cell types throughout the body. Somatostatin has been shown to inhibit hormone secretion in endocrine cells and spontaneous neuronal firing via several different signal transduction pathways (2). Somatostatin inhibits adenylyl cyclase activity or production of cAMP and reduces calcium currents and increases potassium channel conductance (3-9). More recently, somatostatin has been shown to stimulate a protein tyrosine phosphatase activity in human tumor cells and a serine/threonine protein phosphatase activity in pituitary cells (10-12).

The best studied action of somatostatin is its inhibition of secretion of hormone (growth hormone, prolactin) and growth factors (EGF and IGF). This action has been clinically applied in the palliative treatment of patients with hormone-producing tumors. Despite encouraging clinical results, little is known about somatostatin's actions to directly regulate cell growth. Many of somatostatin actions to inhibit cellular proliferation may be mediated through a direct antagonistic effect on the growth promoting factors such as epidermal growth factor (EGF) (13-15). EGF is a polypeptide with mitogenic activity in controlling normal differentiation of mesodermal and ectodermal cells. The receptor for EGF is encoded by a mammalian proto-oncogene c-erbB and displays tyrosine kinase activity when activated by ligand (16,17). This activation is mediated by "autophosphorylation" of the recepor on specific tyrosine residues. Activated EGF receptors with increased kinase activity are present in many human tumors including bladder, lung, colon, and pancreas, and transduce oncogenic signals to tumor

cells (11,18-20). In addition to inhibiting the release of EGF, somatostatin directly inhibits EGF stimulation of cell growth. Somatostatin inhibits DNA synthesis and cell replication in human cells induced by EGF and thyrotropin and insulin-like growth factor-1 (13,21). In addition, somatostatin stimulates a protein tyrosine phosphatase activity in human pancreatic carcinoma cells in culture and this stimulation may reverse the autophosphorylation of the EGF receptor in the membrane of these cells (10). These experimental data extend the understanding and clinical significance of this neuroendocrine hormone.

The recent molecular characterization of five cDNAs for somatostatin receptor provides the tools for studying molecular mechanism underlying somatostatin's actions (22-25). All five cDNAs exhibit the sequence characteristics of G protein coupled receptor superfamily. The cloning of these receptor cDNAs provides reagents to extend our pharmacological knowledge and suggests that its many biological effects may be mediated by distinct receptor subtypes.

We are interested in the molecular mechanism used by growth inhibitory hormones to inhibit cell growth. We have previously shown that somatostatin can stimulate a protein tyrosine phosphatase activity via a G protein coupling pathway (10). In this paper, we show that somatostatin can inhibit DNA synthesis in MIA PaCa-2 cells, and this inhibition can be blocked by vanadate, an inhibitor of protein tyrosine phosphatases. We also show that cAMP is not involved in somatostatin's inhibition of DNA synthesis in these cells.

Materials and Methods

Materials

Somatostatin was purchased from Bachem (Torrance, CA). Pertussis toxin from List Phamaceuticals (Campell, CA). Vanadate, EGF, and forskolin were purchased from Sigma. (3H)thymidine was purchased from Amersham. Tissue culture media and sera were purchased from Grand Island Biological Co. (Grand Island, NY). MIA PaCa-2 cells were obtained from ATCC (Americal Tissue Culture Corporation).

Methods

Cell culture. MIA PaCa-2 cells were grown in monolayer culture on tissure culture dishes (Falcon, Oxnard, CA) at 37°C in a humidified atmosphere with 5% carbon dioxide. Cells were grown in DMEM medium supplemented with 10% fetal calf serum. Media were changed 12-24 hr prior to experimentation.

Thymidine incorporation assay. Cells were seeded at the density of 5 x 10^5 in six-well plates with 3ml of DMEM medium supplemented with 10% fetal calf serum. After 48 hr cells were washed with serum free DMEM twice and kept in serum free DMEM medium for additional 72 hr. Test substances (somatostatin, EGF, pertussis toxin, vanadate, and forskolin) were added in 3 ml of fresh serum medium, and the cells were incubated for 16 hr. Then the cells were pulsed with (3H)thymidine (1uCi/ml) for 4 hr, washed with PBS, detached with trypsin at 37°C for 10 min, extracted in 10% trichloroacetic

acid and filtered through a fiber glass filter (Whatman GF/A). The filters were washed sequentially under vaccum with 10% and 5% trichloroacetic acid and 95% ethanol, and the trichloroacetic acid-insoluble fraction was counted in a scitillation counter.

Statistical analysis. Experiments were performed in duplicate and repeated at least twice. Analysis of variance was performed, and a p value < 0.05 was considered statistically significant.

Results

Somatostatin inhibited basal and EGF-stimulated DNA synthesis

We have previously shown that somatostatin can stimulate a membrane PTPase activity in MIA PaCa-2 cells (10). This activity can dephosphorylate EGF receptor in vitro. Somatostatin has been shown to inhibit DNA synthesis in a thyroid cell line, FRTH-5 (21). As illustrated in Fig. 1, we show that somatostatin can also inhibit DNA synthesis in MIA PaCa-2 cells. MIA PaCa-2 is derived from human pancreatic adenocarcinoma cells and displays high basal levels of DNA synthesis. EGF (10nM) can stimulate DNA synthesis by 2-3 folds of basal activity. Both basal and EGF-stimulated DNA synthesis can be inhibited by somatostatin (1uM) by 40-50%. Somatostatin's inhibition of DNA synthesis was blocked by vanadate, an inhibitor of PTPases (lane 3). Vanadate alone can stimulate DNA synthesis (Lane 4).

Somatostatin inhibition of DNA synthesis was not blocked by forskolin

It is thought that somatostatin signaling pathway involves both inhibition of adenylyl cyclase to reduce cellular level of cAMP and inhibition of calcium influx. cAMP has both stimulatory and inhibitory effects on cell growth depending on the cell type (26,27). To determine whether cAMP is involved in somatostatin's inhibition of cell growth in MIA PaCa-2 cells, we measured DNA synthesis in MIA PaCa-2 cells treated with forskolin or forskolin plus somatostatin. Forskolin is known to stimulate synthesis of cAMP by activating an adenylyl cyclase. Figure 2 (A and B) shows that

forskolin can independently inhibit DNA synthesis in MIA PaCa-2 cells. 60% basal DNA synthesis activity was inhibited by 1uM forskolin, and 40-50% by 100nM. Interestingly, when the cells were treated with both forskolin and somatostatin, the effect was additive. These results indicate that cAMP is an inhibitory factor for MIA PaCa-2 cell growth and that additional pathways account for somatostatin's inhibition of DNA synthesis.

Perussis toxin inhibits DNA synthesis in MIA PaCa-2 cells

Pertussis toxin (PTX) is a specific and potent inhibitor of a subset of G proteins that include G_o and G_i. PTX was shown previously to inhibit cell growth and DNA synthesis in some cell types (28). Many of somatostatin's actions have been shown to be mediated through PTX-sensitive pathway. To determine if somatostatin inhibition of DNA synthesis in MIA PaCa-2 cells is also mediated through a PTX-sensitive pathway, we studied effect of PTX pretreatment in thymidine incorporation assay. As illustrated in Fig. 3, like somatostatin, PTX itself can inhibit DNA synthesis in MIA PaCa-2 cells. However, when the cells were treated with both somatostatin and PTX, this inhibition was slightly decreased.

Discussion

Understanding the mechanism for regulating cell growth has provided many important insights for cancer biology. A number of hormones, growth factors, and neuropeptides have been shown to affect cellular proliferation. Growth factors including EGF, PDGF, FGF, and insulin are mitogenic for a variety of cell types. They exert effect through binding to their receptors, which are protein-tyrosine kinases encoded by proto-oncogenes. Growth factor receptors initiate a cascade of tyrosine and serine/threonine phosphorylation reactions in the cells to deliver mitogenic signals into the nucleus. A number of proteins including many onco-proteins have been identified to be active components of growth factor receptor signaling complexes. Deregulated expression of these proto-oncogenes interferes with normal cellular processes and the ability of cells to arrest their growth.

Oncogenes have been the subject of many cancer studies during the past decade. However, discovery of tumor suppressor genes has generated a new interests on the signals that control cell growth. Tumor suppressor genes are wild-type alleles of genes that play regulatory roles in cell proliferation and differentiation. Their loss or inactivation is oncogenic to the cells. One such example may be protein-tyrosine phosphatases which counteract the action of tyrosine kinases. Certain protein-tyrosine phosphatases have been found to be potential tumor suppressor genes. For example, the receptor-like PTPase HPTPγ maps to a region of the human chromosome 3p21 that is frequently deleted in renal cell carcinoma and lung carcinoma (29) and might be mutated in many lung carcinomas (30).

Protein-tyrosine phosphatases have been recognized to play many important cellular functions. One of these is the negative regulation of cell growth. The inhibition of endogerous PTPase activity by vanadate can readily transform fibroblasts (31). Increased cell density in fibroblasts is accompanied by increased membrane PTPase activity (32), suggesting that some PTPases are involved in cell-cell contact inhibition. We have previously shown that somatostatin can stimulate a membrane PTPase activity in human pancreatic carcinoma cells, and that this activity can dephosphorylate EGF receptor in vitro (10). These findings suggest that somatostatin might inhibit the growth of human tumor cells by directly restoring the balance of tyrosine phosphorylation on critical substrates. Similar results have also been obtained by studying effect of dopamine on a pituitary cell line that stably expresses a transfected D2 dopamine receptor cDNA (33). It was also reported that angiotensin II, TGF-ß1, and interleukin-6 stimulated PTPase activity (34-36). TGF-ß1 is known as a growth inhibitory factor for keratinocytes. Interleukin-6 is a cytokine that induces growth arrest of M1 myeloblastic cells. The PTPase activity stimulated by IL-6 could counteract the action of oncogene product BCR-ABL.

Recently, Griswold-Prenner and coworkers found that MAP kinase, a downstream signal transducing protein of growth factor receptor signaling pathway, was able to decrease EGF receptor phosphorylation by stimulating a membrane-associated PTPase activity (37). This finding may underly one of mechanisms for signal attenuation, the diminution of positive signals delivered to the nucleus. Inactivation of growth factor-stimulated receptor-tyrosine kinases may requires certain PTPases. It is possible that

somatostatin stimulates a similar phosphatase to antagonize the mitogenic activity of certain growth factors. In this report, we have shown that somatostatin can inhibit DNA synthesis in human pancreatic carcinoma cells, and this inhibition was reversed by vanadate, an inhibitor of PTPases. These results suggest that somatostatin's inhibition of DNA synthesis may be mediated through stimulation of a PTPase activity. However, since vanadate alone can stimulate DNA synthesis in these cells, it is also possible that somatostatin's effect was not specifically blocked by vanadate and that elimination of somatostatin's effect on DNA synthesis by vanadate might not be related to its inhibition of specific PTPase activity induced by somatostatin.

We have shown that forskolin, a stimulator of cAMP synthesis, inhibited DNA synthesis in MIA PaCa-2 cells. In addition, we have previously shown that isoproterol, an agonist of adrenergic receptor and a potent stimulator of cAMP production, inhibited DNA synthesis(33). Interestingly, the effect of forskolin and somatostatin on DNA synthesis were additive, indicating that cAMP is an anti-proliferative signal for these cells, and that cAMP is not involved in somatostatin's effect on DNA synthesis. Somatostatin inhibits cAMP production in pituitary cells. It is not known if somatostatin has this effect in MIA PaCa-2 cells. It is possible that inhibition of cAMP production might mediate somatostatin's effect in some cell types. Cyclic-AMP exerts both negative and positive effects on cell growth depending on the cell type studied. In thyroid cells, cAMP is stimulated by TSH and is indispensible for thyroid cell proliferation (38). However, cAMP functions as a negative regulator of cell proliferation in variety of other cell types. The molecular mechanism underlying cAMP's effect is not clear. Recently, our laboratory

has obtained results showing that cAMP can inhibit DNA synthesis by blocking protein phosphatase PP-1 activity in pituitary cells (39).

We have also shown that both somatostatin and pertussis toxin can independently inhibit DNA synthesis in MIA PaCa-2 cells. However, this inhibition was partly blocked when cells were treated with both reagents. It seems that somatostatin's effect on DNA synthesis was partly blocked by pertussis toxin. However, since pertussis toxin alone can also inhibit DNA synthesis in MIA PaCa-2 cells, it is impossible to determine whether somatostatin's effect on DNA synthesis is sensitive to pertussis toxin. Pertussis toxin may completely block somatostatin's pathway while exerting its own effect on DNA synthesis. However, if somatostatin inhibits DNA synthesis through a pertussis toxin-insensitive pathway, then the effect of somatostatin and pertussis toxin should be additive or not changed.

In summary, we have provided evidence that somatostatin can inhibit DNA synthesis in MIA PaCa-2 cells possibly through stimulation of a PTPase activity. Forskolin, a potent stimulator of cAMP synthesis can also exerts inhibitory effects on DNA synthesis in these cells. The effect of forskolin and somatostatin were additive, suggesting that cAMP is not involved in somatostatin's effect on DNA synthesis.

References

1. Patel YC, Relchlin S 1978 Somatostatin in hypothalamus, extra-hypothalamic brain, and peripheral tissue of the rat. Endocrinol 102:523-530

2. Rens-Domiano S, Law SF, Yamada S, Seino GI, Reisine T 1992 Phamacological properties of two cloned somatostatin receptors. Mol Phamacol 42:28-34

3. Koch BD, Dorflinger LJ 1985 Pertussis toxin blocks both cyclic AMP-mediated and cAMP-independent actions of somatostatin: Evidence for coupling of Ni to decreases in intracellular free calcium. J Biol Chem 260:13138-13145

4. Ikeda SR, Schofield GG 1989 Somatostatin blocks a calcium current in rat sympathetic ganglion neurons through a pertussis toxin-sensitive mechanism. J Physiol 407:221-240

5. Reisine T 1990 Cellular mechanisms of somatostatin inhibition of calcium influx in the anterior pituitary cell line AtT20. J Pharm and Exper Therpeutics 254:646-651

6. Wang HL, Reisine T, Dichter M 1990 Somatostatin-14 and somatostatin28 inhibit calcium currents in rat neocortical neorons. Neuroscience 38:335-342

7. Wang HL, Bogen C, Reisine T, Dichter M 1989 Somatostatin-14 and somatostatin-28 induce opposite effects on potassium currents in rat neocortical neurons. Proc Natl Aca Sci USA 86:9616-9620

8. Inoue M, Nakajima S, Nakajima Y 1988 Somatostatin induces an inward rectification in rat locus coeruleus neurons through a pertussis toxin-sensitive mechanism. J Physiol 407:177-198

9. Jacqin T Champagnat J, Madamba S, Denavit-Saubie M, Siggins GR 1988 Somatostatin depresses excitability in neurons of the solitary tract complex through hyperpolarization and augmentation of IM, a non-activating voltage dependent outward current blocked by muscarinic agonists. Proc Natl Aca Sci USA 85:948-952

10. Pan MG, Tlorio T, Stork PJS 1992 G protein activation of a hormone-stimulated phosphatase in human tumor cells. Science 256:37-39

11. Hierowski MT, Liebow C, du Sapin K, Schally AV 1985 Stimulation by somatostatin of dephosphorylation of membrane proteins in pancreatic cancer MIA PaCa-2 cells. FEBS 179:252-256

12. White RW, Schonbrunn A, Armstrong DL 1991 Somatostatin stimulates Ca2+-activated K+ channels through protein dephosphorylation. Nature 351:570-573

13. Mascado RN, Sherline P 1982 Somatostatin inhibits rapid centrosomal separation and cell proliferation induced by epidermal growth factor. Endocrinol 111:1394-1396

14. Schally AV, Redding TW 1987 Somatostatin analog as adjuncts to agonists of luteinizing hormone-releasing hormone in the treatment of experimental prostate cancer. Proc Natl Aca Sci USA 84:7275-7279

15. Liebow C, Hierowski M, du Sapin K 1986 Hormonal control of pancreatic cancer growth. Pancreas 1:44-86

16. Aaronson SA 1991 Growth factors and cancer. Science 254:1146-1152

17. Heldin CH, Westermark B 1984 Growth factors: mechanism of action and ralation to oncogenes. Cell 37:9-20

18. Capon DJ, Chen EY, Levinson AD, et al 1983 Complete nucleotide sequences of the Y24 Human bladder carcinoma oncogenes and its normal homologue. Nature, 302:33-37

19. Sherwin SA, Minna JD, Gazdar AF, Todaro GJ 1981 Expression of epidermal and nerve growth factor receptors and soft agar growth factor production by human lung cancer cells. Cancer Res 41:3538-3542

20. Qin YA, Schally AV, Willems G 1991 Somatostain analogue RC-160 inhibits the growth of transplanted colon cancer in rats. Int J Cancer 47:765-770

21. Tsuzaki S, Moses AC 1990 Somatostatin inhibits deoxyribonuclei acid synthesis induced by both thyrotropin and insulin-like growth factor-1 in FRTL5 cells. Endocrinol 126:3131-3137

22. Ymada Y, Post SR, Wang K, Tager HS, Bell GI, Seino S 1992 Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract, and kidney. Proc Natl Aca Sci USA 89:251-255

23. Yasuda K, Rens-Domiano S, Breder CD, Law SF, Saper CB, Reisine T, Bell GI 1992 Cloning of a novel somatostatin receptor, SSTR3, coupled to adenylyl cyclase. J Biol Chem 267:20422-20428

24. O'Caarroll A, Lolait SJ, Konig M, Mahan LC 1992 Molecular cloning and expression of a pituitary somatostatin receptor with preferential affinity for somatostatin-28. Mol Pharmacol 42:939-946

25. Bruno JF, Xu Y, Song J, Berelowitz M 1992 Molecular cloning and functional expression of a brain-specific somatostatin receptor. Proc Natl Aca Sci USA 89:11151-11155

26. Durmont JE, Jauniaux J, Roger PP 1989 The cyclic AMP-mediated stimulation of cell proliferation. TIBS 9:67-72

27. Blomhoff HK, Blomhoff R, Stokke T, Davies CD, Brevik K, Smeland EB, Funderud S, Godal T 1988 cAMP-mediated growth inhibition of a B-

lymphoid precursor cell line Reh is associated with an early transient delay in G2/M, followed by an accumulation of cells in G1. J Cell Physiol 137:583-587

28. Crouch MF, Belford DA, Milburn PJ, Hendry, IA 1990 Pertussis toxin inhibits EGF-, phorbol ester-, and insulin-stimulated DNA synthesis in BALB/c3T3 cell: evidence for post-receptor activation of Gi. Biochem. Biophys Res Comm 3167:1369-1376

29. Laforgia S, Morse B, Levy J, Barnea G, Cannizzaro LA, Li F, Nowell PC, Boghosian-Seel L, Glick J, Weston A, Harris CC, Drabkin H, Patterson D, Croce CM, Schlessinger J, Huebner K 1991 Receptor protein tyrosine phosphatase γ is a candidate tumor suppressor gene at human chromosome region 3p21. Proc Natl Aca Sci USA 88:5036-5040

30. Tsukamoto T, Takahashi T, Udea R ,Hibi K, Saito H, Takahashi T 1992 Molecular analysis of the protein tyrosine phosphatase γ gene in human lung cancer sell lines. Cnacer Res 52:3506-3509

31. Klarland J.K. 1985 Tranformation of sells by an inhibitor of phosphatase activity acting on phosphotyrosine in proteins. Cell, 41, 707-717

32. Pallen CJ , Tong PH 1991 Elevation of membrane tyrosine phosphatase activity in density-depdent growth arrested fibroblasts. Proc Natl Aca Sci USA 88, 6996-7000

33. Florio T, Pan M, Newman B, Hershberg RE, Civelli O, Stork PJS 1992 Dopaminergic inhibition of DNA synthesis in pituitary tumor sells is assodiated with phosphotyrosine phosphatase activity. J Biol Chem 267, 24169-24172

34. Bottar SP, King IN, Reichlin S, Dahlstroem I, 1992 The angiotensin AT2 receptor stimulates protein tyrosine phosphatase activity and mediates inhibition of particulate guanylate cyclase. Biochim Biophys Acta 183:206-211

35. Rijksen G, Voller MC, Van Zoelen EJ 1993 Orthovanadate both mimics and antogonize the transforming growth factor beta action on normal rat kidney cells. J Cell Physiol 154:393-401

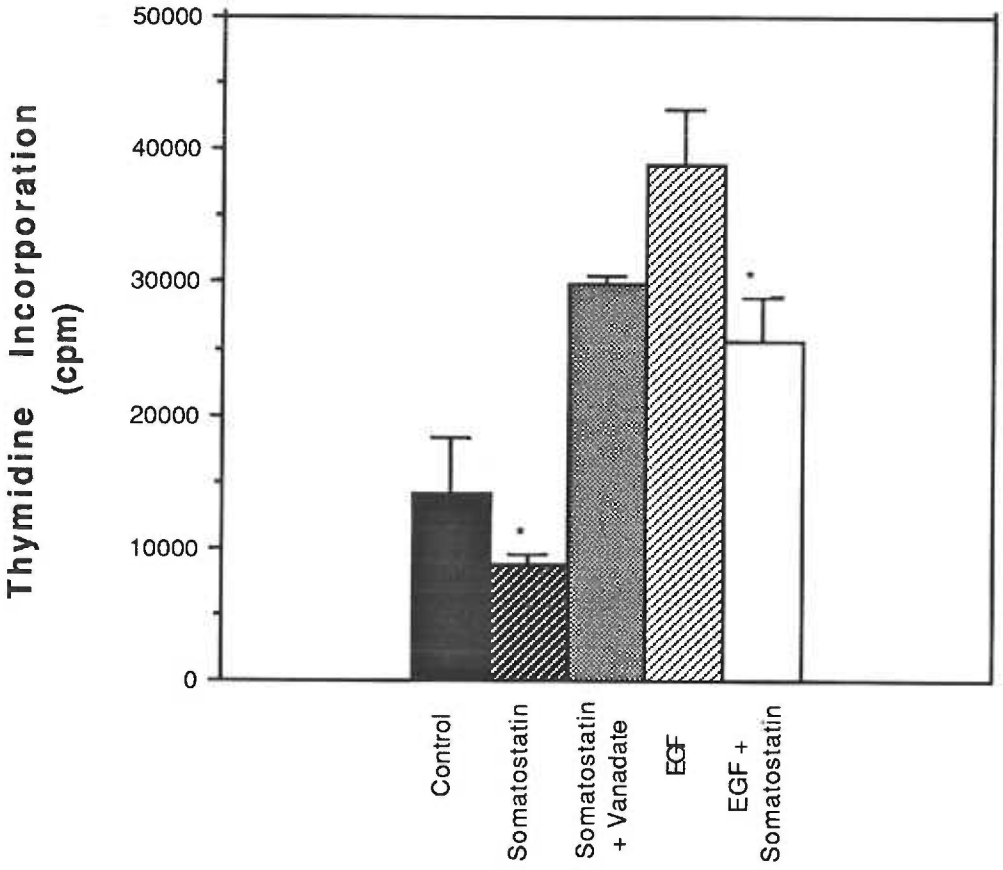
36. Zafriri D, Argaman M, Canaani E, Kimchi A 1993 Induction of PTPase activity by interleukin-6 in M1 myeloblastic cells and analysis of possible counteraction by the BCR-ABL oncogene. Proc Natl Aca Sci USA 90:477-481

37. Griswold-Prenner I, Carlin C, Rosner MR 1993 Mitogen-activated protein kinase regulates the epidermal growth factor receptor through activation of a tyrosine phosphatase. J. Biol. Chem. 268:13050-13054

38. Wynford-Thomas et al. 1987 Proliferative response to cyclic AMP elevation of thyroid epithelium in suspension culture. Mol Cell Endocrinol. 51,163-168

39. Florio T, Perrino B, Stork PJS 1993 Cyclosporin A and cAMP inhibit serine/threonine protein phosphatase and cellular proliferation in pituitary cells. (submitted)

Fig. 1


Thymidine Incorporation (cpm)
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30000
20000
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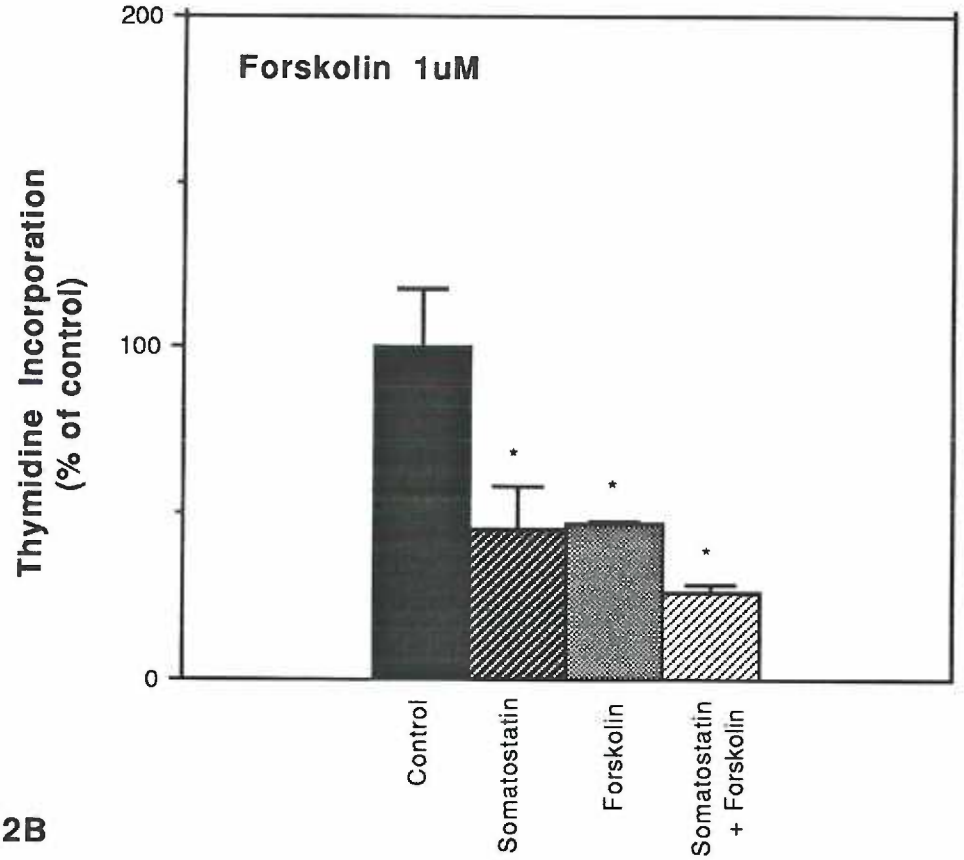
Somatostatin
Somatostatin + Vanadate
EGF
EGF + Somatostatin

Fig. 2A


Forskolin 1uM
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(% of control)
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100
0
*
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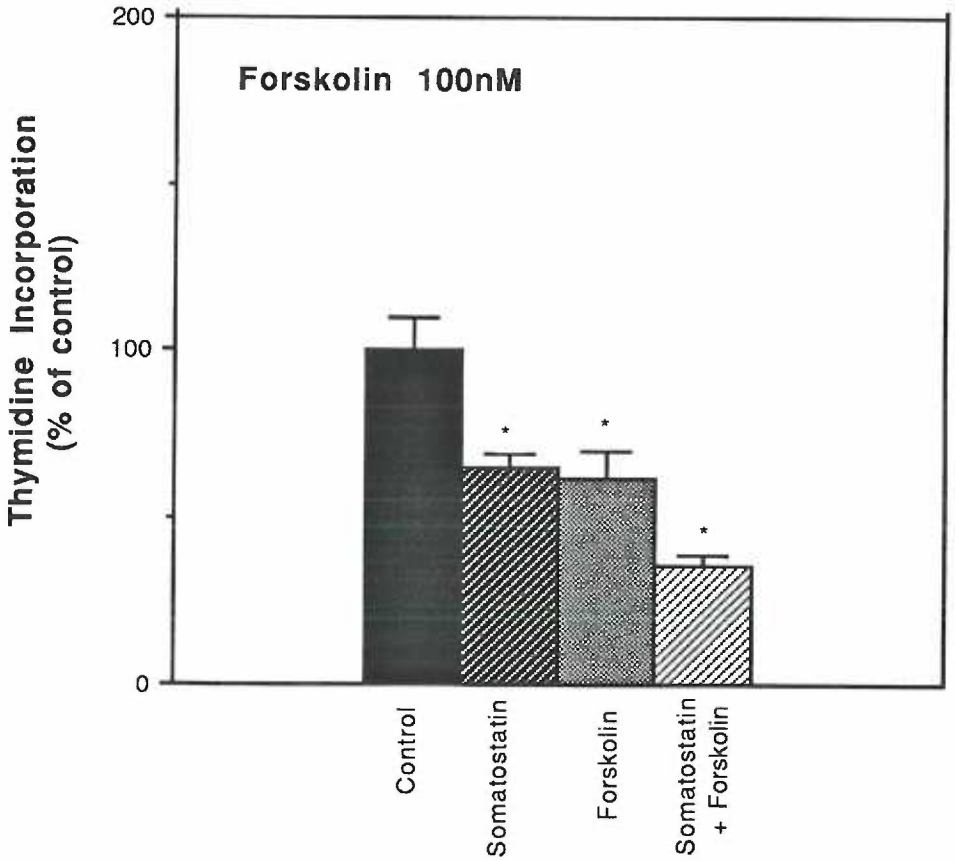
Control
Somatostatin
Forskolin
Somatostatin
+ Forskolin

Fig. 2B


Forskolin 100nM
Thymidine Incorporation
(% of control)
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100
0
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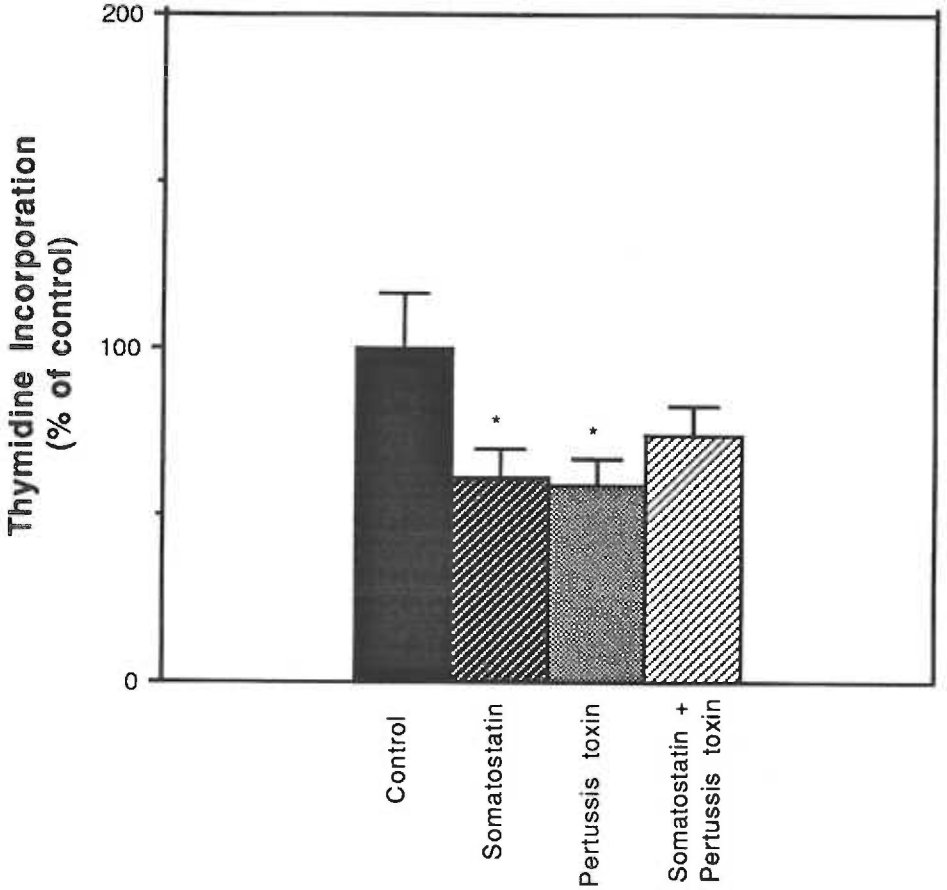
Control
Somatostatin
Forskolin
Somatostatin
+ Forskolin

Fig. 3


Thymidine Incorporation
(% of control)
200
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Control
Somatostatin
Pertussis toxin
Somatostatin +
Pertussis toxin

Legends to Figures

Figure 1. Somatostatin inhibits both basal and EGF-stimulated DNA synthesis. 1, control; 2, somatostatin (1uM); 3, Somatostatin plus vanadate (50uM); 4, vanadate 5, EGF (10nM); 6, EGF plus somatostatin; * indicate $p<0.05$, lane 2 (somatostatin) compared to lane 1 (control) and lane 6 (EGF plus somatostatin) compared to lane 5 (EGF).

Figure 2. Both somatostatin and forskolin inhibit DNA synthesis. (A) 1, control; 2, somatostatin (1uM); 3, forskolin (1uM); Somatostatin plus forskolin. (B) 1, control; 2, somatostatin (1uM); 3, forskolin (100nM); 4, somatostatin plus forskolin. * indicate $P<0.05$, in each lane compared to control.

Figure 3. Pertussis toxin inhibits DNA synthesis in MIA PaCa-2 cells. 1, control; 2, somatostatin (1uM); 3, pertussis toxin (100ng/ml); 4, somatostatin plus pertussis toxin.

Chapter IV

Cloning and Expression of Two Structurally Distinct Putative Receptor Protein-Tyrosine Phosphatases Generated by RNA Processing from A Single Gene

MING-GUI PAN, CAROLINE RIM, KUN PING LU, TULLIO FLORIO, & PHILIP J.S. STORK.

Running Title: RNA Processing of Novel Protein-Tyrosine Phosphatases

Summary We describe here the first example of RNA processing generating two functional putative receptor protein-tyrosine phosphatases (protein-tyrosine-phosphate phosphohydrolase, EC3.13.48) that are structurally distinct within their catalytic domains. Two cDNAs, PTP-P1 and PTP-PS, were isolated from rat pheochromocytoma cells, which encode two putative receptor protein-tyrosine-phosphatases and are produced by alternative splicing and differential use of poly-adenylation sites. Both cDNAs share an identical extracellular domain and a single transmembrane domain, but differ within their cytoplasmic regions: PTP-P1 contains two tandem repeated PTPase catalytic domains, whereas PTP-PS contains only the amino-terminal domain. Bacterial expression of PTPase domains of both cDNAs demonstrates that PTP-P1 and PTP-PS contain tyrosine-phosphatase activity. PTP-P1 is encoded by three transcripts of approximately 8, 6, and 4 kilobases, whereas PTP-PS is encoded by a single 4.8 kb transcript. PTP-P1 (6kb) and PTP-PS are mainly expressed within the brain and in neuronal and endocrine cells. These data suggest that PTP-P1 and PTP-PS may be involved in neuronal function.

Protein tyrosine phosphorylation is an important mechanism to control cellular proliferation and differentiation. This mechanism requires both protein-tyrosine kinases (PTKases; ATP:protein-tyrosine *O*-phosphotransferase, EC 2.7.1.112) and protein-tyrosine-phosphatases[1] (PTPases; protein-tyrosine-phosphate phosphohydrolase, EC 3.13.48)) to maintain a balance between tyrosine phosphorylation and dephosphorylation. The protein tyrosine kinase family, which has been extensively investigated and well characterized, can be divided into two classes, intracellular and transmembrane. Most of transmembrane PTKases are receptor tyrosine kinases (RTKs), in which growth factors bind to the external domain, activating the cytoplasmic kinase domain and initiating a cascade of tyrosine and serine/threonine phosphorylation events (reviewed in refs 1-5).

In contrast, the PTPase family is much less understood. Only since the purification and cloning of human placental major protein-tyrosine-phosphatase (PTP-1B) (6-8), have people begun to understand PTPases at the molecular level. Increasing evidence demonstrates the importance of PTPases in various aspects of cell growth and differentiation. For example, cdc25, a protein that controls the cell's entry into mitosis, is a PTPase which activates cdc2 by dephosphorylating Tyr-15 of cdc2 (9,10). CD45, one of the best characterized PTPases, is required for proliferation of T cell lymphocytes and induction of interleukin-2 (11-13). Two classifications of PTPase family have been proposed. According to Fischer *et al* (14), the family can be divided into two subfamilies: transmembrane and intracellular PTPases. The transmembrane PTPases can be further divided into four

different types depending on different organization of extracellular domain. According to Krueger *et al* (15), the PTPase family can be divided into three classes. Class I contains cytoplasmic PTPases, which are small molecules, that usually contain only a single PTPase catalytic domain. Class II contains receptor-linked PTPases with a single PTPase catalytic domain, and class III contains receptor-linked PTPases with two tandem repeated PTPase domains.

The recent discovery that the tyrosine kinase *trk* is the receptor for nerve growth factor highlights the importance of protein-tyrosine phosphorylation in neuronal differentiation (16-18). NGF, a soluble factor required for survival of sensory and sympathetic neurons, can transform PC12 cells from an endocrine cell phenotype to a sympathetic neuronal phenotype (19). This differentiation of PC12 cells induced by NGF has been extensively investigated, but the mechanism remains poorly understood. NGF stimulates tyrosine kinase activity by binding to the extracellular domain of its receptor and consequentially activating a cascade of phosphorylation events (20). This process appears to involve p^{21}ras, raf-1 kinase, and MAP kinases (21,22).

Although the importance of PTKases, like *trk*, in neuronal differentiation is well characterized, the involvement of PTPases in neuronal function and differentiation is much less clear. In *Drosophila*, three receptor-linked PTPases, DLAR, DPTP10D, and DPTP99A are selectively expressed in the central nervous system (23,24). DLAR and DPTP99A are expressed on most axons, while DPTP10D is primarily localized to the anterior commissure and its junctions with the longitudinal tracts. These findings suggest that

receptor-linked protein-tyrosine-phosphatases may be involved in axonal outgrowth and guidance during embryonic development.

To begin to address the importance of PTPases in neuronal differentiation, we have characterized two cDNAs from PC12 cells that encode putative receptor PTPases, PTP-P1 and PTP-PS, which are the results of alternative splicing and differential use of poly-adenylation sites. PTP-P1 contains two PTPase catalytic domains, whereas PTP-PS contains only the first catalytic domain. PTP-P1 is encoded by three transcripts (8, 6, and 4 kb) and PTP-PS is encoded by a single 4.8 kb transcript. PTP-P1(6kb) and PTP-PS are mainly expressed in neuronal tissues and cells and in some endocrine cells. These data suggest that PTP-P1 and PTP-PS may be involved in neuronal function.**

EXPERIMENTAL PROCEDURES

Synthesis of A PC12 cDNA Library--A size-selected cDNA library (inserts larger than 2kb) was prepared from PC12 cell mRNA by using the cDNA cloning kit from Life Technologies, Inc. (SuperScript Plasmid System for cDNA synthesis and plasmid cloning). The plasmid was replaced by a modified eukaryotic expression vector pSRαRSN. This plasmid is derived from pcDL-SRα (25), in which two original SalI sites were destroyed, and two new restriction sites (SalI and NotI) were created between the EcoRI site to allow directional cloning of modified cDNA inserts. The library was introduced into DH10B cells (BRL) by electroporation. The complexity of the library is 2.5×10^5 individual clones.

PCR Amplification-- 200ng of the plasmid PC12 cDNA library described above was used as a template in the polymerase chain reaction primed with two degenerate oligonucleotides for 35 temperature cycles. Each cycle consisted of 60 sec at 94°C, 30 sec at 45°C, and 60 sec at 72°C in a PCR thermocycler (Perkin Elmer). The PCR buffer was 50mM KCl, 1.5mM $MgCl_2$, 0.1mM dNTPs, 15mM Tris.HCl, pH 8.4. The sequences of the two oligonucleotides are 5'-AAG(A)-TGC(T)-TGC(T)-GA(C)T(C)-CAA(G)-TAC(T)-TGG-CC-3' and 5'-CC-C(G)AC(T)-C(GT)CC-A(G)GC-GC(G)T(A)-A(G)CA-G(A)TG-3', respectively, which correspond to amino acids KCH(P)QYWP and HCSGI(V)GR within the PTPase catalytic domain. The PCR fragments were subcloned into M13mp18 and M13mp19 and sequenced.

Screening of the PC12 cDNA Library with PC3 Probe and Isolation of Full Length Clones--One of the PCR products (PC3) was used to screen the plasmid PC12 cDNA library (described above). The PC12 library was plated onto LB/Ampicillin plates and transferred to nitrocellulose membranes in duplicate. The nitrocellulose membranes were washed with 2xSSC/1%SDS at 65°C for 2 hours prior to hybridization. Hybridization was carried out at 43°C under 50% formamide and the final wash was 0.2xSSC/0.1%SDS at 65° C. To obtain full length clones, an Acc1 fragment (Fig.1) was used as a probe to screen a rat cortex lamda cDNA library. The screening yielded 370bp of additional 5' coding sequences. The remaining 5' end sequences were then isolated by using 5' RACE protocol (BRL). Briefly, a oligonucleotide corresponding to the 5' end of the sequences was used as primer to synthesize cDNA from PC12 RNA using reverse transcriptase (RT). The cDNA was tailed with sequences complementary to the anchor primer, PCR was performed using an internal primer upstream to the RT primer and the anchor primer. PCR products were then digested with SalI and SmaI and subcloned into pBluescript SK +/-.

DNA Sequencing--All PCR products and fragments of PTP-P1 and PTP-PS were subcloned into M13mp18 and M13mp19 (New England BioLabs) and pBluescript SK+/- (Stratagene). Both strands of each fragment were sequenced by the dideoxynucleotide chain termination method with modified T7 DNA polymerases (USB) using restriction fragments and primer walks.

Northern Blot Analysis--Total RNA was extracted from various tissues and cell lines as described in Fig. 6 legend by using guanidine/cesium chloride method to isolate total RNA(26). Equal amounts (10 to15 ug) of total RNA were electrophoresed in a formaldehyde/formamide/0.8% agarose gel, blotted, and hybridized to [^{32}P]-labelled probes. The hybridization conditions for random-primed DNA probes were the same as those used in the screening of the library. For RNA probes, the hybridization temperature was 63°C.

Bacterial Expression of PTPases--The PTPase domains of PTP-P1 (amino acids 875-1494) and PTP-PS (amino acids 875-1260) were amplified by the PCR using oligonucleotides specific to PTP-P1 and PTP-PS that incorporated NdeI and BamHI restriction sites to facilitate cloning into pET-15b (Novagen). The plasmids (pET-P1 and pET-PS) were over-expressed in *E.coli* strain BL21/LysE (provided by Dr. R.H. Goodman, Vollum Institute, OHSU). The overnight culture of bacteria containing pET-P1 and pET-PS were diluted 1:100 in 100ml LB broth containing 0.4% glucose, cultured under selection with 50ug/ml carbenicillin and induced with 1mM IPTG (Isopropylthio-ß-D-galactoside) when the absorbance of the cultures reached an OD_{600} of 0.6-0.8. The bacterial cultures were pelleted and resuspended in 10ml IMAC-5 (20mM Tris-HCl pH 7.9, 0.5M NaCl, 10% glycerol, 1mM PMSF, and 5mM imidazol) and sonicated. Protein concentration was determined by the Bradford method (27).

Phosphatase Assay--When *p*-nitrophenyl phosphate (*p*-Npp) was used as a substrate, the phosphatase assay was carried out in 100ul reaction at 30°C in buffer containing 50mM HEPES (pH 7.0), 10mM DTT, 5mM EDTA, and

10mM NaF. Reactions were stopped by addition of 0.2N NaOH, and the absorbance was measured at 410nm (28). When cdc2(6-20) peptide (29,30) was used as a substrate, dephosphorylation was measured as previously described (31). The cdc2(6-20) peptide was phosphorylated on tyr-15 by *Lyn* kinase (UBI) as reported (32). The cdc2(6-20) peptide was kindly provided by Dr. J. H. Wang, Department of Biochemistry, Faculty of Medicine, University of Calgary, Calgary, Alberta, T2N 4N1, Canada. Raytide (Oncogene Science) was phosphorylated by *lck* kinase purified by immunoprecipitation from CV-1 cells transfected with a plasmid pBluescript-*lck* (containing a full-length *lck* cDNA) following infection with a vaccinia virus expressing T7 RNA polymerase (provided by Dr. A. Shaw, University of Washington, St. Louis, Missourri).

RESULTS

Cloning of PTP-P1 and PTP-PS--To obtain clones for new members of the protein-tyrosine-phosphatase family, two degenerate oligonucleotides were synthesized, which correspond to the conserved amino acids KCH(P)QYWP and HCSGI(V)GR within the PTPase catalytic domain. These degenerate oligonucleotides were used in a polymerase chain reaction (PCR), using the PC12 cDNA library described above as a template. The major PCR product was approximately 320 base pair in length, which was subcloned into M13 and sequenced. After sequencing twelve clones, two were found to encode potentially novel members of the PTPase family. One of them, PC3, was used as a probe to screen the PC12 cDNA library. The initial screening yielded about 300 positive clones, 20 of them were analyzed by southern blot, and further screening of these clones produced two partial clones, designated PTP-P1 and PTP-PS. To obtain 5'-end sequences encoding full length of both PTP-P1 and PTP-PS, we used a Acc1 fragment of PTP-PS which is located in the 5'-end of initial partial clone (Fig. 1) as a probe to screen a rat cortex lamda gt11 cDNA library (Clontech), the screening yielded 8 overlap positive clones, one of them, with a largest insert 1.6 kilobase, contains additional 370 bp of 5' end coding sequences for both PTP-P1 and PTP-PS. The remaining 5'-end sequences of both PTP-P1 and PTP-PS were then isolated using a 5'-RACE protocol.

Both PTP-P1 and PTP-PS PTP-P1 share amino acid residues 1 to 1235 and nucleotide sequences 1 to 4305 (Fig. 2A) encoding a potential signal peptide

(the first 23 residues), an extracellular domain of 824 amino acids, and a single transmembrane domain. The extracellular domain contains two IgG-like domains and four fibronectin type three repeats (32). In addition, PTP-P1 contains two PTPase catalytic domains within its cytoplasmic region, and a 900 bp A-T rich 3'-untranslated region (Fig. 2C). In contrast, PTP-PS contains a single N-terminal PTPase catalytic domain and a unique carboxy-terminal sequence of twenty-six amino acids (residues 1236-1261, Fig. 2B) and a very short 3'-untranslated region (Fig. 2B). PTP-P1 and PTP-PS are highly homologous to LAR (32) and HPTP-δ (14) in their catalytic domains (Fig. 3). LAR and HPTP-δ are class III protein tyrosine phosphatases. However, because PTP-PS contains only a single catalytic domain, it belongs to class II.

Northern blots were performed to analyze the expression of PTP-P1 and PTP-PS. A DNA probe (Probe 1) prepared from a fragment of PTP-P1 {generated by SacI and KpnI digestion (1.07 kb) and covering the first catalytic domain} (Fig. 1) that is shared by both clones, detected four discrete transcripts, which were approximately 8, 6, 4.8, and 4 kb (Fig. 4A). Another DNA probe (Probe 2, Fig. 1) prepared from a fragment (containing the carboxy-terminal portion of the second catalytic domain and the 3'-untranslated region of PTP-P1) specific to PTP-P1, detected three discrete transcripts, which were 8, 6, and 4 kb in length (Fig. 4B). An RNA probe (Probe 3, Fig. 1) derived from sequences unique to the 3' end of PTP-PS, detected a single 4.8 kb transcript (Fig. 4C).

PTP-P1 and PTP-PS are Produced by RNA Processing--To confirm the hypothesis that PTP-P1 and PTP-PS are produced by alternative splicing, we

performed PCR using PC12 genomic DNA as a template. Three oligonucleotides flanking the putative splicing sites were used in the PCR (Fig. 5A). Oligonucleotide 1 corresponds to nucleotides 3535-3561 of PTP-P1 (within the first catalytic domain); oligonucleotide 2 corresponds to nucleotides 3843-3857 of PTP-PS (within the 3' UT); and oligonucleotide 3 corresponds to nucleotides 3883-3899 of PTP-P1(within the second catalytic domain). The PCR products, shown in figure 5B, were subcloned into M13 and sequenced. The results demonstrate that PTP-P1 and PTP-PS are alternative spliced transcripts of a single gene. Excision of the intron containing the sequence unique to PTP-PS and the 63 bp intronic sequence produces PTP-P1, whereas PTP-PS is produced by differential use of the poly-adenylation site that is included within this intron. The consensus intron donor and acceptor sites, poly-adenylation site, and splicing pattern are shown in Fig. 5C. The unique 26 amino acids at the 3' end of PTP-PS shares no significant homology with known proteins.

Gene Expression of PTP-P1 and PTP-PS in Tissues and Cells--A number of tissues and cell lines were examined for gene expression of PTP-P1 and PTP-PS. The PTP-P1 Sac I/Kpn I (1.07 kb) fragment was used as a probe. PTP-P1 (8 kb) transcript is expressed in most cell lines and tissues examined; PTP-P1 (6kb) transcript is expressed mainly in some neuronal cell lines and highly expressed in the rat medullary thyroid carcinoma cell line W2 and within the brain, being most abundant in the cortex (Fig. 6A). The expression of PTP-P1 (4kb) is lower than that of the other two PTP-P1 transcripts but is detectable in cortex and pituitary cells, and in cells of neural crest origin, including W2 and PC12 cells. Using PTP-PS specific sequences as a probe, PTP-PS expression was detected in some neuronal and

endocrine cells such as PC12 cells, W2 cells, brain cortex, and GH4 cells. (Fig. 6B). PTP-P1 (8kb), (6kb), and (4kb) may also be generated by alternative splicing of a single gene. These results indicate that alternative splicing can produce neuron-specific protein-tyrosine-phosphatases. However, the understanding of alternative splicing events of PTP-P1 must await the availability of full-length sequences encoding each variant.

Phosphatase Activity of PTP-P1 and PTP-PS--To confirm PTP-P1 and PTP-PS contain PTPase activity, we expressed the catalytic domains of PTP-P1 and PTP-PS in *E.coli*. The catalytic domain of PTP-P1 expressed a 68 kDa protein, and the catalytic domain of PTP-PS expressed a 40 kDa protein (data not shown). The bacterial lysates containing these proteins can hydrolyze *p*-Npp in the presence of serine/threonine protein phosphatase inhibitor NaF (10mM) and can dephosphorylate the peptide substrate cdc2(6-20) peptide that has been specifically phosphorylated on tyr-15 by tyrosine kinase *Lyn* and raytide that has been phosphorylated by *lck* kinase (see Experimental Procedures and Fig. 7).

DISCUSSION

We have characterized two cDNAs from PC12 cells; PTP-P1 and PTP-PS, that encode putative receptor protein-tyrosine-phosphatases. The two phosphatases are generated by alternative splicing and differential use of poly-adenylation sites. PTP-P1 and PTP-PS are highly homologous to HPTP-δ (15) and LAR(33) in their catalytic domains, indicating that they are members of the "LAR-like" subfamily of putative receptor protein-tyrosine-phosphatases. Bacterial expression studies demonstrate that PTP-P1 and PTP-PS contain protein-tyrosine-phosphatase activity.

Using a probe specific to PTP-P1 (probe 2, Fig. 1), we detected three transcripts which are approximately about 8, 6, and 4 kb in length. These three transcripts were also detected when using a probe common to both PTP-P1 and PTP-PS (probe 1, Fig. 1). These results suggest that the three PTP-P1 transcripts all share two catalytic domains and/or 3'-untranslated region but differ in 5'-end sequences that encode the extracellular domains of these proteins. Similar heterogeneity within the extracellular domains of N-CAM and the phosphatase CD45 are generated by alternative splicing (35,36). Therefore, we suggest that the heterogeneity of transcripts encoding PTP-P1 may also arise by alternative splicing within the extracellular domain. In contrast, PTP-PS is encoded by a single transcript that is generated by RNA processing within the cytoplasmic domain.

This family of PTPases represents the first example of RNA processing generating PTPases of two structurally distinct classes. The excision of an

intron that separates the two catalytic domains produces PTP-P1. The inclusion of this intron allows the synthesis of PTP-PS. This intron contains the C-terminal 26 amino acids of PTP-PS and a typical poly-adenylation site. Its use allows the synthesis of PTP-PS with a single catalytic domain.

The physiological significance of this type of RNA processing remains to be characterized. Since both processed RNAs encode proteins with PTPase activity, it is possible that these two PTPases may be differentiated either by their substrate specificity or enzyme kinetics. An example of alternative splicing within the phosphatase domain was described for LRP by Matthews et al (37). LRP is a putative receptor protein tyrosine phosphatase encoded by a gene containing a 108 bp intron whose inclusion disrupts the first PTPase catalytic domain.

We have examined the gene expression of PTP-P1 and PTP-PS. PTP-P1 (8kb) transcript is widely expressed, but PTP-P1 (6 kb) is mainly expressed within the brain and in neuronal cells. PTP-PS is also expressed in neuronal and endocrine cells, suggesting that both PTP-P1 (6kb) and PTP-PS may be involved in neuronal functions. PTP-P1 and PTP-PS contain an extracellular domain with four fibronectin type III repeats anf three IgG-like domains. Many receptor-linked protein tyrosine phosphatases contain several NCAM-like fibronectin type III repeats and IgG immunoglobin-like domains within their extracellular domains (14), these sequence elements may be involved in cell-cell interaction and cell migration during neuronal development. In addition, PTP-P1 mRNA levels are increased following NGF-induced PC12 cell differentiation[2]. Moreover, vanadate, a specific inhibitor of PTPases, was recently shown to be able to block NGF-induced PC12 cell

differentiation (38). Therefore, we speculate that PTP-P1 might be involved in neuronal differentiation. Since PTP-P1 differs from PTP-PS by its inclusion of a second PTPase domain, we suggest that this second domain may have regulatory function *in vivo*.

Acknowledgement: We are grateful to Dr. J.H. Wang for providing cdc2(6-20) peptide, Dr. A. Shaw for providing the plasmids pcDL-SRa, pBluescript-*lck*, and vaccinia virus, and Dr. K. Walton for helpful discussion and sharing the sequence information prior to publication.

REFERENCES

1. Ullrich, A. & Schlessinger, J. (1990) *Cell,* 61, 203-212.

2. Yarden, Y. (1988) *Ann. Rev. Biochem.* 57, 443-478.

3. Carpenter, G. (1987) *Ann. Rev. Biochem.* 56, 881-914.

4. Hunter, T. (1987) *Cell,* 50, 823-829.

5. Hanks, S.K., Quinn, A.M. & Hunter, T. (1988) *Science,* 241, 42-52.

6. Tonks, N.K., Diltz, C.R. & Fischer, E.H. (1988) *J. Biol. Chem.* 263, 6722-6730.

7. Tonks, N.K., Diltz, C.D. & Fischer, E.H. (1988) *J. Biol. Chem.* 263, 6731-6737.

8. Charbonneau, H., Tonks, N.K., Kumar, S. & Fischer, E.H. (1989) *Proc. Natl.Acad. Sci. USA* 86, 5252-5256.

9. Dunphy, W.G. & Kumagai, A. (1991) *Cell,* 67, 189-196.

10. Gautier, J., Solomon, M.J. Booher, R.N., Bazan, J.F. & Kirschner, M.W. (1991) *Cell,* 67, 197-211.

11. Pingel, J.T. & Thomas M.L. (1989) *Cell,* 58, 1055-1065.

12. Koretzky, G.A., Picus, J., Thomas M.L. & Weiss, A. (1990) *Nature (London),* 346, 66-68.

13. Koretzky, G.A., Picus, J., Schultz, T. & Weiss, A. (1991) *Proc. Natl. Acad. Sci. USA* 88, 2037-2041.

14. Fischer, E.H.,Charbonneau, and Tonks, N.K. (1991) *Science,* 253,401-406

15. Krueger, N.K., Streuli, M. & Saito, H. (1990) *EMBO J.* 9, 3241-3252.

16. Klein, R. Jing, S., Nabduri, V., O'Rourke, E. & Barbacid (1991) *Cell,* 65,189-197.

17. Kaplan, D.R., Hempstead, B.L., Martin-Zanca, D., Chao, M.V. & Parada, L.F. (1991) *Science*, 252, 554-558.

18. Nabreda, A.R., Marin-Zanca, D., Kaplan, D.R., Parada, L.F. & Santos, E. (1991) *Science*, 252, 558-561.

19. Oppenheim, R.W., *Studies in Developmental Neurology* (Oxford Univ. Press, Oxford, U.K., 1981).

20. Kaplan, D.R., Mantin-Zanca, D. & Parada, L.F. (1991) *Science*, 350, 158-160.

21. Wood, K.W., Sarnecki, C., Roberts, T.M. & Blenis, J. (1992) *Cell*, 68, 1041-1050.

22. Thomas, S.M., Demarco, M., D'Arcangelo, G., Hakgoua, S. & Bruhhe, J.S. (1992) *Cell*, 68, 1031-1040.

23. Tian, S., Tsoulfas, P. & Zinn, K. (1991) *Cell*, 67, 675-685.

24. Yang, X., Seow, K.T., Bahri, S.M., Oon, S.H & Chia, W. (1991) *Cell*, 67, 661-673

25. Takebe, Y., Seiki, M., Fujisawa, J.I., Hoy, P., Yokota, K., Arai, K.L., Yoshida, M. & Arai, N. (1988) *Mol. Cell. Biol.* 8, 466-472.

26. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Second Edition. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press)

27. Bradford, M.M. (1976) *Anal. Biochem.* 72, 248-254.

28. Pan, M.G., Florio, T. & Stork, P.J.S. (1992) *Science*, 256, 1215-1217.

29. Litwin, C.M.E., Cheng, H. & Wang, J.H. (1991) *J. Biol. Chem.* 266, 2557-2566

30. Cheng, H., Litwin, C.M.E., Hwang, D.M. & Wang, J.H. (1991) *J. Biol. Chem.* 266, 17919-17925.

31. Streuli, M., Krueger, N.X., Tsai, A.Y.M. & Saito, H. (1989) *Proc. Natl. Acad. Sci. USA* 86, 8698-8702.

32. Guan, K., Broyles, S.S. & Dixon, J.E. (1991) *Nature (London),* 350, 359-361.

33. Streuli, M., Krueger, N.X., Hall, L.R., Schlossman, S.F. & Saito, H. (1988) *J. Exp. Med.* 168, 1523-1530.

34. Strueli, M., Krueger, N.X., Thai, T., Tang, M. & Saito, H. (1990) *EMBO. J.*, 9, 2399-2407

35. Thomas, M.L. (1989) *Ann. Rev. Immunol.* 7, 339-369.

36. Cunningham, B.A., Hemperly, J.J., Murray, B.A., Prediger, E.A., Brackenbury, R. & Edelman, G.M. (1987) *Science,* 216, 799-806.

37. Matthews, R.J., Cahir, E.D. & Thomas, M.L. (1990) *Proc. Natl. Acad. Sci.USA* 87, 4444-4448.

38. Wu, Y.Y. & Bradshow, R.A. (1993) *J. Cell. Biol.*, 121, 409-422

Footnotes:

[1]The abbreviations used are: PTPase, protein-tyrosine-phosphatase; RTK, receptor-tyrosine kinase; PCR, polymerase chain reaction.

[2] Pan, M.-G., Stork, P.J.S.: manuscript in preparation.

Legends to figures:

Fig. 1. Restriction map of PTP-PS and PTP-P1. Ac, AccI; H, HincII; S, SacI; Sm, SmaI; A, ApaI; K, KpnI. The gray boxes represent the transmembrane domains and the black boxes represent the PTPase catalytic domains. The lines corresponding to the restriction map represent the fragments used as probes for northern blot hybridization.

Fig. 2. Nucleotide and amino acid sequences of PTP-PS and PTP-P1. A, Sequences shared by both PTP-P1 and PTP-PS. Nucleotide sequences 1-3646 and its predicted amino acid sequences shared by PTP-P1 and PTP-PS are shown. The potential signal peptide and transmembrane domain are underlined. B, Sequences unique to PTP-PS. Nucleotide sequences 3646-3661 and its predicted unique 26 amino acids of PTP-PS are shown. The putative poly-adenylation signal is underlined. C, Sequences unique to PTP-P1. Nucleotide sequences 3446-5324 and its predicted amino acid sequences 1235-1494 of PTP-P1 are shown, the putative poly-adenylation signal is underlined.

Fig. 3. Homology between PTP-P1, HPTPδ, and LAR. The amino acid sequences of two tandem repeated PTPase domains are boxed. The single letters are used. The transmembrane domains are highlighted by bold letters.

Fig. 4. Messenger RNA expression in PC12 cells, detected by northern blot hybridization. A, Hybridization with a probe (Probe 1) shared by both PTP-P1 and PTP-PS. B, Hybridization with a probe (Probe 2) specific to PTP-P1. C, Hybridization with an RNA probe (Probe 3) specific to PTP-PS.

The PTP-P1 probes were labeled with [^{32}]P by random-priming. The sequences unique to the PTP-PS 3'-end were subcloned into pBluescript SK +/- and transcribed *in vitro* by T7 RNA polymerase in the presence of α-[^{32}P]-CTP.

Fig. 5. PTP-P1 and PTP-PS are generated by RNA processing. A, Schematic map of the PCR strategy used. D1 stands for PTPase domain 1, D2 stands for PTPase domain 2, the light gray region stands for PTP-PS 3'-end unique sequence. B, The PCR amplification of genomic DNA. The photograph represents ethydium bromide-stained gel demonstrating the position of amplified PC12 genomic DNA using the primers shown. Lane 1, DNA marker; lane 2, product of primers 1 and 2; lane 3, product of primers 1 and 3. C, Intronic and junctional sequences located between the two PTPase domains of PTP-P1. The splice donor site and the splice acceptor site are in dark gray boxes, the sequences unique to the 3' end of PTP-PS is enclosed within a light gray box. Intronic sequences are shown in lower case letters.

Fig. 6. Messenger RNA expression of PTP-P1 and PTP-PS in tissues and cells, detected by northern blot hybridization. A, Hybridization with a DNA probe shared by both PTP-P1 and PTP-PS (probe 1). Hybridization of the same blot to human b-actin DNA probes are shown in the bottom. B, Hybridization with an RNA probe specific to PTP-PS (probe 3). The 28S ribosomal band of the same filter stained with methylene blue is shown in the bottom. Cell line and tissues are noted in the figure. Abbreviations used include: W2, rat medullary thyroid carcinoma; COS, monkey kidney cells;

Molt-B1, human B lymphocytes; Hela, human epithelial carcinoma; MIA PaCa-2, human pancreatic carcinoma; SKN-MC, human neuroblastoma; HT-29, human colonic carcinoma; LX-1, human small lung carcinoma; Rin 5F, rat insulinoma; GH_4C_1, rat pituitary tumor; PC12, rat pheochromocytoma.

Fig. 7. Phosphatase activity of PTP-P1 and PTP-PS. Bacterial extracts expressing the catalytic portions of PTP-P1 and PTP-PS were assayed for phosphatase activity. A, Hydrolysis of the substrate *p*Npp. B, Dephosphorylation of phosphorylated cdc2(6-20) peptide. C, Dephosphorylation of phosphorylated Raytide. The wild type bacteria *(E.coli*, strain:BL21/LysE) were used as control.

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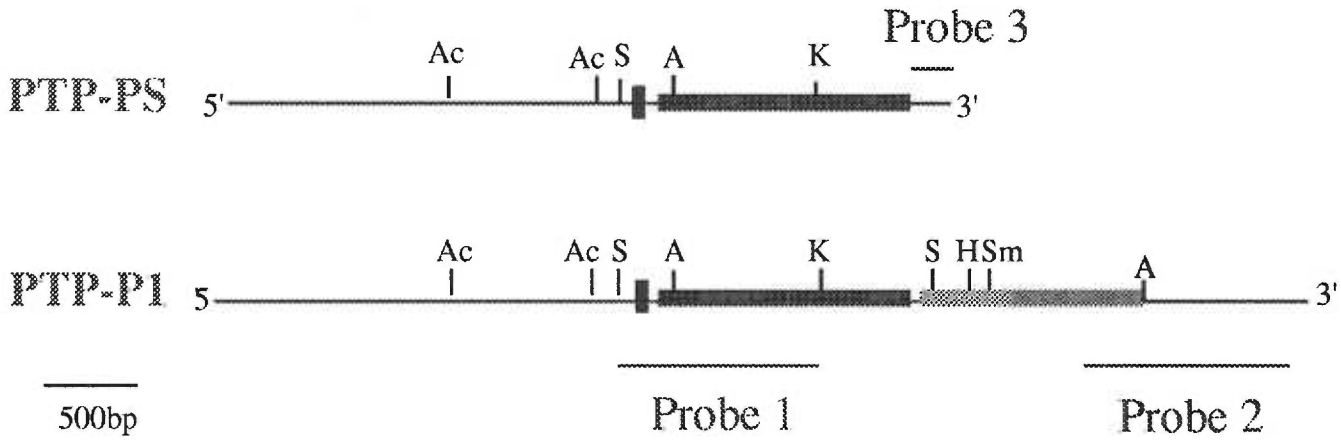
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Asn Tyr Met Val Gln Thr Glu Asp Gln Tyr Ser Phe Ile His Glu Ala Leu Leu Glu Ala Val Gly Cys Gly Asn Thr Glu Val Pro Ala Arg Ser  1211

CTC TAC ACC TAT ATC CAG AAG CTG GCC CAG GTG GAG CCT GGC GAG CAT GTC ACA GGA ATG GAG CTT GAG TTC AAG                              3756
Leu Tyr Thr Tyr Ile Gln Lys Leu Ala Gln Val Glu Pro Gly Glu His Val Thr Gly Met Glu Leu Glu Phe Lys                              1236
```

B.

```
PTP-PS: GTG ACT GCG GGA CCA CAG TGG ACG GGT GGG CTG AAA ACT GAC TCC CAC AGC TGT CAA CTG ACC CCC ACA CAT ACA CAG TAAtaaaacaaat   3853
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gtgctggtgaaaaaaaaaaaaaaaa                                                                                                    3878
```

C.

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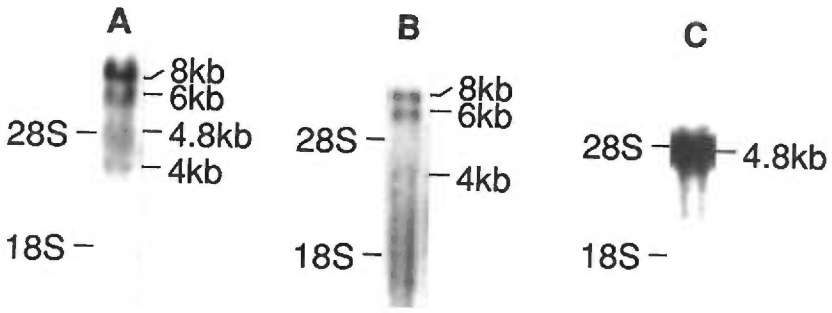
PTP P1(PS)
LAR
HPTPd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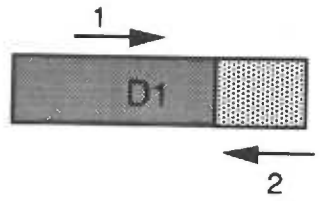

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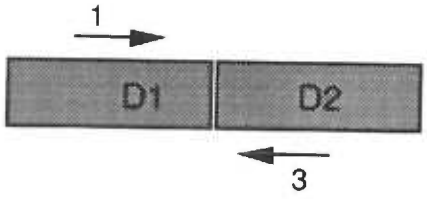
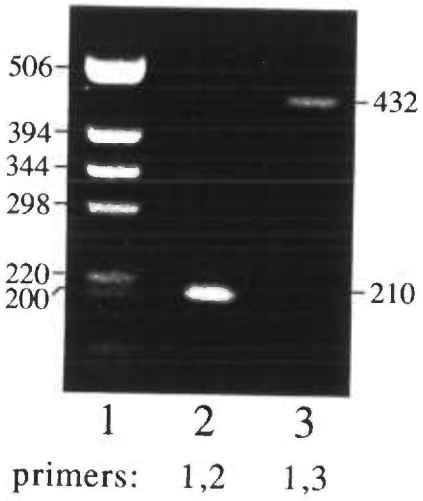


PTP-P1 cDNA

B.

Genomic DNA: PCR



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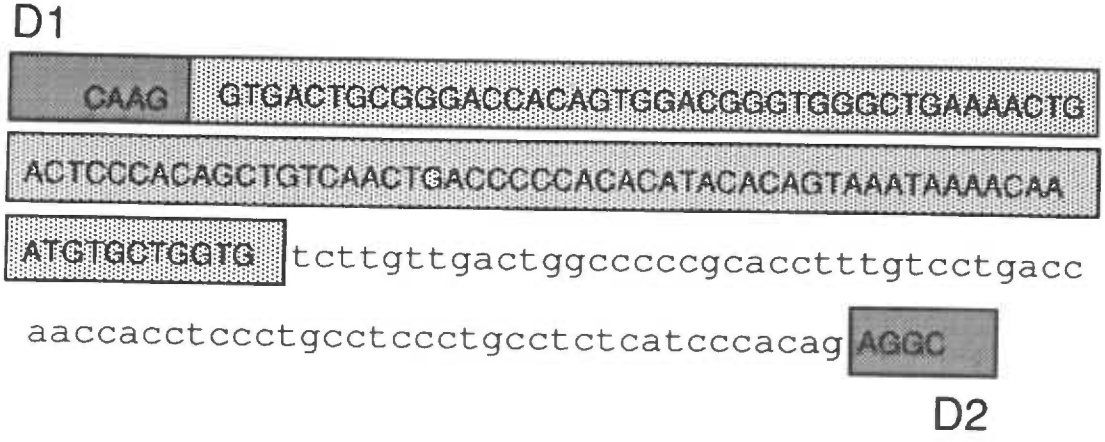

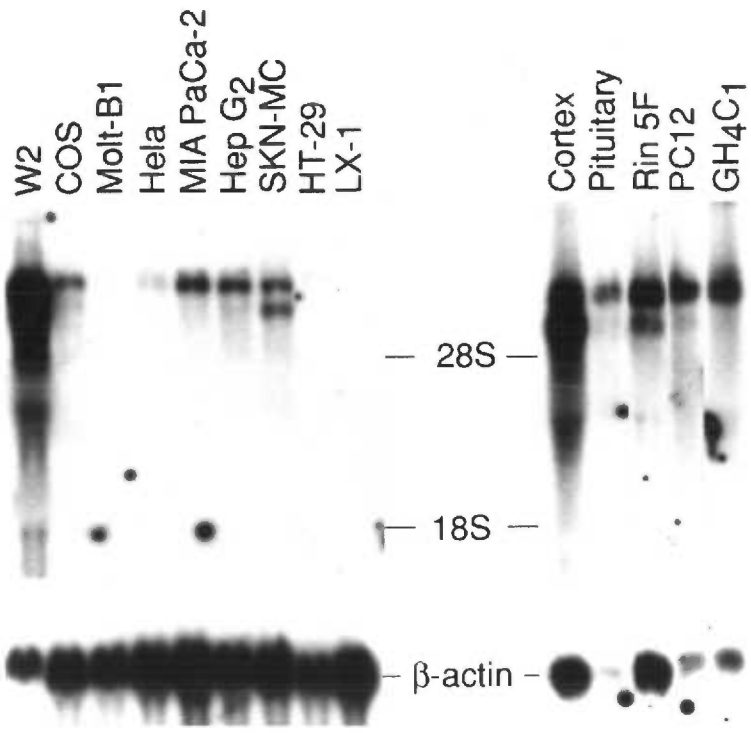
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PC12
GH4C1
28S
18S
β-actin

PC12
Spleen
Liver
Cortex
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Testes
28S
18S
28S

Fig. 7A

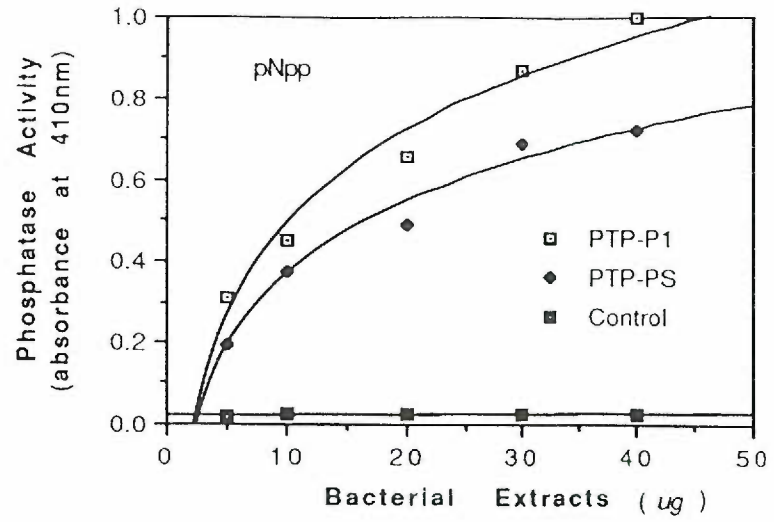


Fig. 7B

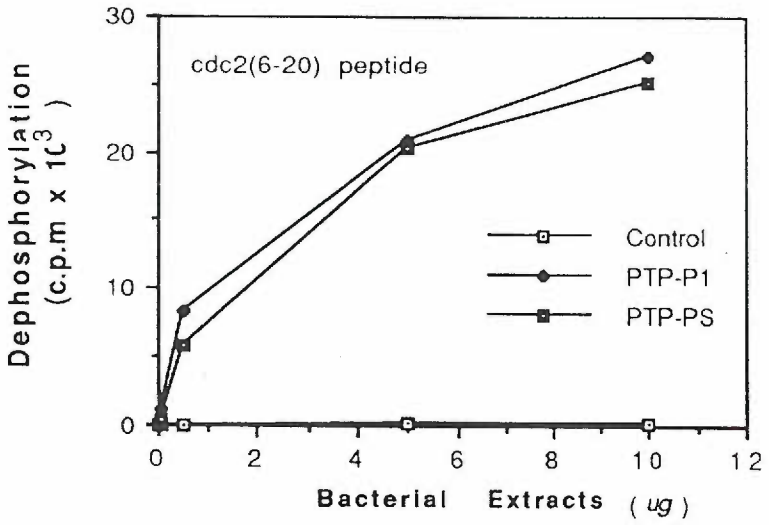
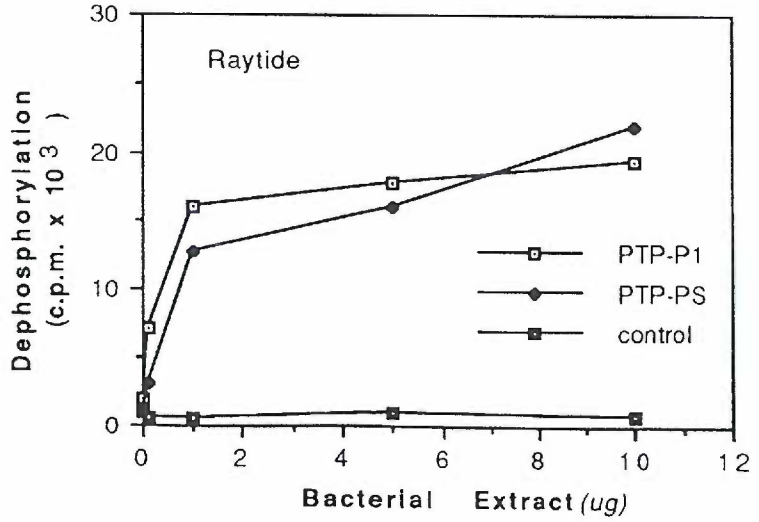


Fig. 7C



Chapter V

Expression of Putative Receptor Protein-Tyrosine Phosphatase PTP-P1 is Increased During NGF-Induced Neuronal Differentiation of PC12 Cells

Ming-Gui Pan & Philip J.S. Stork

Summary The mechanism that underlies neuronal differentiation induced by NGF is not clear. It was suggested that protein-tyrosine phosphatases were involved in NGF-induced neuronal differentiation. We have proviously isolated two putative receptor PTPases, PTP-P1 and PTP-PS. PTP-P1 is mainly distributed in some neuronal tissues and cells. We here show that PTP-P1 expression can be increased during NGF-induced PC12 cell differentiation, and this stimulation may be due to increased RNA stability. These results suggest that PTP-P1 might be involved in neuronal differentiation.

NGF is the most studied of the family of neuronal growth factors that are thought to play a central role in the development, growth, and maintenance of the nervous system. NGF acts through binding to its receptors: the low affinity NGF receptor $p75^{LNGFR}$ and the high affinity NGF receptor $p140^{trk}$ (1-4). The molecular mechanism of p75 in mediating NGF action is not clear, whereas $p140^{trk}$ has been shown to mediate signal transduction initiated by NGF (5-8). Trk is a proto-oncogene and a receptor-tyrosine kinase. The other members of the trk family have been shown to be receptors for other neural trophic factors (9-14).

The PC12 cell line is an execellent model for studying neuronal differentiation. This cell line can be induced by NGF to commit to neuronal differentiation, but in contrast, is stimulated by EGF to proliferate. Both trk and EGF receptor contain tyrosine kinase activity when activated by NGF and EGF. Activated trk and EGF receptor trigger a very similar set of responses including activation of $p21^{ras}$, $p60^{src}$, raf-1, MAP kinases, phospholipase C, and PI-3 kinase. It is still a mystery why NGF and EGF induce completely different phenotypic effect on PC12 cells yet share many signal transduction characteristics (15). One hypothesis is that signal transduced by tyrosine phosphorylation by EGF may be sustained throughout the entire stimulation period, while tyrosine phosphorylation by NGF stimulation might be tightly modulated and maintained in a moderate level. This level might be maintained by the action of a protein-tyrosine phosphatase (PTPase). This hypothesis is supported by a report showing that orthovanadate, a PTPase inhibitor, prolonged tyrosine phosphorylation by nonreceptor-tyrosine kinases that are activated following the stimulation of NGF and EGF receptor-tyrosine kinases and inhibited neurite extension

induced by NGF and FGF (16). In that study, the inhibition of neurite extension of PC12 cells correlated with the inhibition of PTPase activity (16). The exact site of vanadate action leading to the inhibition of neurite extension are not clear. It will be necessary to identify the specific PTPases involved in this action of vanadate. Furthermore, in another study, it was reported that membrane PTPase activity could be stimulated 2-3 fold during NGF-induced PC12 cell neuronal differentiation, and that three PTPases with molecular mass of 500, 300, and 60 kd were activated in response to NGF (17). It was also shown that NGF antagonized EGF's mitogenic activity, and this action might be mediated through induction of a PTPase (17). We have previously isolated two novel putative receptor PTPases from PC12 cells, PTP-P1 and PTP-PS (18). PTP-P1 is primarily expressed in some neuronal tissues and cells and is most abundantly expressed in the brain cortex. To determine the possible involvement of PTP-P1 in neuronal differentiation, we studied the regulation of transcripts encoding PTP-P1 in PC12 cells undergoing neuronal differentiation. We here show that PTP-P1 expression is increased during NGF-induced PC12 cell differentiation. We suggest that PTP-P1 might be a potential candidate involved in differentiating action between NGF and EGF.

Methods and Materials

Materials- Restriction enzymes and modifying enzymes were purchased from Boehringer Mannheim and BRL. The α-^{32}P-dCTP and nitrocellulose membranes were purchased from Schleicher & Schuell. Cycloheximide, EGF, and NGF were purchased from Sigma.

Tissue culture- PC12 cells were maintained in DMEM supplemented with 5% fetal calf serum plus 5% horse serum. After reaching 80% density, the cells were washed and maintained in fresh medium, and NGF (50ng/ml) and EGF (50ng/ml) or cycloheximide (100ng/ml) were added and treated for 1, 3, 12, 48 hr, and 5 days.

Northern blots- Total RNA from PC12 cells was isolated by lysis in guanidium thiocyanate. Twelve micrograms of total RNA from each sample was electrophoresed in a 1% denaturing agarose gel. The RNAs were then transfered to nitracellulose membranes and UV-crosslinked. The blots were bybridized to a ^{32}P-labeled probe that was prepared from the 1.1 kb Sac1/Kpn1 fragment of PTP-P1, which is also shared by PTP-PS. The hybridizations were allowed to proceed for 18 hr at 43^{o}C in 50% formamide, 2XSSC, 1X Denhart buffer. The blots were then washed for 1 hr with 1X SSC/0.1% SDS 65^{o}C, and then for 1 hr with 0.1X SSC/0.1% SDS in 65^{o}C. Autoradiographs were developed following exposure in -80^{o}C for three days using Kodak films. The same blots were then hybridized to a ß-actin probe using similar procedures.

Densitometry- Density of autoradiographed bands were measured using a scanning densitometry (GS 300 Transmittance/Reflectance, Hoefer Scientific Instruments, San Francisco, California). All bands were measured twice. The normalized ratios were calculated against the densities of actin bands.

Results

To investigate if PTP-P1 and PTP-PS are involved in neuronal differentiation, we evaluated the regulation of their expression during NGF-induced cell differentiation at the mRNA level. We found that mRNA levels of both PTP-P1 8 and 6 kb transcripts are increased by NGF but not by EGF. The 6 kb transcript increased 3-fold after 48 hr NGF treatment and remained elevated (2.5 fold) after 5 days (Fig. 1 and Fig. 2A), while 8 kb transcript increased 2.5 fold after 12 hr NGF induction (Fig. 1 and Fig. 2B). The mRNA level of PTP-PS was not changed (Fig. 1). These results show that PTP-P1 expression is selectively regulated by NGF.

This type of regulation by NGF can occur at the transcriptional or post-transcriptional level. Since PTP-P1 but not PTP-PS contains a 3'-untranslated region which is A-U rich, we suspected that this region might be related to NGF regulation. To evaluate the possible regulation of PTP-P1 by RNA stability, we examined whether addition of cycloheximide in tissue culture can stimulate expression of PTP-P1. We found that cycloheximide plus NGF further increased messenger levels of both PTP-P1 8 and 6 kb by 7 to 9 fold (Fig. 1B and Fig. 2C).

Discussion

We have previously shown that PTP-P1 is mainly expressed in some neuronal tissues and cells, suggesting that this PTPase may be involved in neuronal function. In this study, we show that expression of PTP-P1 can be differentially regulated by NGF and EGF. Its mRNA level was increased during NGF-induced neuronal differentiation of PC12 cells but not during response to EGF (Fig. 1 and Fig. 2A and 2B). We also suggest that the increase of PTP-P1 expression might be due to increased mRNAs stability, as that addition of cycloheximide further increased NGF-stimulated expression of PTP-P1 (Fig. 1B and Fig. 2C). However, it is also possible that NGF increases PTP-P1 expression by acting at the transcriptional step. Further studies are needed to elucidate this question.

Several PTPases, mostly putative receptor form, have been localized to the nervous system. RPTPß, was found to be only expressed in the brain, with the highest level of expression in the ventricular and subventricular zones of the embryonic mouse brain, suggesting the importance of this PTPase in the development of central nervous system (19). PTPζ was isolated from a fetal brain cDNA library and is highly expressed in a glioblastoma cell line (20). STEP, "striatum enriched phosphatase", a cytoplasmic PTPase, was found to be highly enriched in the striatum (21).

The differential regulation of PTP-P1 by NGF and EGF suggests that PTP-P1 may be a candidate to balance tyrosine phosphorylation *in vivo* in response to NGF induction of neuronal differentiation. It is possible that other PTPases are involved in this regulation. However, PTP-P1 is the only

PTPase known to be regulated by NGF but not EGF at present time. Increased expression of PTP-P1 induced by NGF may serve as a feedback mechanism to maintain tyrosine phosphorylation in a moderate level within the cells. Several proteins including trk and src are activated by tyrosine phosphorylation following the addition of NGF. These phosphotyrosine proteins might be tightly regulated by PTPases in neuronal cells to maintain an appropriate state for neuronal differentiation. In fibroblasts, it was shown that NGF could also mediate mitogenic signals through trk, suggesting that neural-specific PTPases are not present to modify NGF action in these cells. The arrest of mitogenic activity in neuronal cells is required for the cells to commit to differentiation. The notion that PTP-P1 is involved in neuronal differentiation is also supported by a report showing that PTP-NE3 (identical to PTP-P1) is expressed in olfactory neuroepithelium in a developmental manner with high mRNA expression in immature neurons and lower level in mature neurons, suggesting that this PTPase is involved in regulating the differentiation of olfactory neuroepithelium (22). PTP-P1 contains four fibronectin type three repeats and three immunoglobulin-like domains similar that contained in N-CAM (neural-cell adhesion molecule), L1, and fasciclin. These structural elements may mediate some of PTP-P1's effect in regulation of cell-cell interaction or cell migration. It was recently demonstrated that extracellular domain of RPTPμ stimulated cell-cell adhesion (23,24). RPTPμ is a putative receptor PTPase with a Ig-like domain and four fibronectin type three repeats within its extracellular domain. However, more experiments are needed to determine the physiological relevance of these observations.

References

1. Radeke, M.J., Misko, T.P., Hsu, C., Herzenberg, L.A. & Shooter, E.M. (1987) Nature. 325, 593-597

2. Johnson, D., Lanahan, A., Buck, C.R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M. & Chao, M. (1986) Cell. 47, 545-554

3. Klein, R., Jing, S., Nanduri, V., O'Rourke, E. & Barbacid, M. (1991) Cell, 65, 189-197

4. Kaplan, D.R., Hempstead, B.L., Martin-Zanca, D., Chao, M. & Parada, L.F. (1991) 252, 554-5558

5. Cordon-Cardo, C., Taoley, P., Jing, S., Nanduri, V., O'Rourke, E., Lamballe, F., Kovary, K., Klein, R., Jones, K.R., Reichardt, L.F. & Barbacid, M. (1991) Cell, 66, 173-183

6. Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F. & Chao, M.V. (1991) Nature. 350, 678-683

7. Jing, S., Tapley, P. & Barbacid, M. (1992) Neuron. 9, 1067-1-79

8. Loeb, D.M., Maragos, J., Martin-Zanca, D., Chao, M., Parada, L.F. & Greene, L.A. Cell. 66, 961-966

9. Durkop, H., Lazta, U., Hummel, M., Eitelbach, F., Seed, B. & Stein, H. (1992) Cell. 68, 421-427

10. Squinto, S.P., Stitt, T.N., Aldrich, T.H., Davis, S., Bianco, S.M., Radziejewski, C., Glass, D., Masiakowski, P., Furth, M.E., Valenzuela, D.M., DiStefano, P.S. & Yancopoulos, G.D. (1991) Cell. 65, 885-893

11. Soppet, D., Escandon, E., Maragos, J., Middlemas, D., Reid, S.W., Blair, J., Burton, L.E., Stanton, B.R., Kaplan, D.R., Hunter, T., Nikolics, K. & Parada, L.F. (1991) cell, 65, 895-903

12. Lamballe, F., Klein, R. & Barbacid, M. (1991) Cell. 66, 967-979

13. Klein, R., Conway, D., Parada, L.F.& Barbacid, M. (1990) Cell. 61, 647-656

14. Bothwell, M. (1991) Cell. 65, 915-918

15. Chao, MV. (1992) Cell. 68, 995-997

16. Wu, Y.Y. & Bradshow, R.A. (1993) J. Cell. Biol. 121, 409-422

17. Aparicio, L.F., Ocrant, I., Boylan, J.M. & Gruppuso, P.A. (1992) Cell Grow. Differ. 3, 363-367

18. Pan, M., Rim, C., Lu, K., Florio, T., & Stork, P.J.S. (1993) J. Biol. Chem. 268, 19284-19291

19. Lombroso, P.J., Murdoch, G. & Lerner, M. (1991) Proc. Natl. Aca. Sci. USA. 88, 7242-7246

20. Levy, J.B., Canoll, P.D., Silvennoinen, O., Barnea, G., Morse, B., Honegger, A.M., Huang, J.-T. Cannizzaro, L.A., Park, S.-H., Druck, T., Huebner, T., Sap, J., Ehrlich, M., Musacchio, J.M. & Schlessinger, J. (1993) J. Biol. Chem. 268, 10573-10581

21. Krueger, N.X. & Saito, H. (1992) Proc. Natl. Aca. Sci. USA 87, 7417-7421

22. Walton, K., Martell, K.J., Kwak, S.P., Dixon, J.E & Largent, B.L. (1993) Neuron, 11, 397-400

23.Gebbink, M.E.B.G., Zondag, G.C.M., Wubbolts, R.W., Beijersbergen, R.L., Etten, I. & Moolenaar, W.H. (1993) J. Biol. Chem. 268, 16101-16104

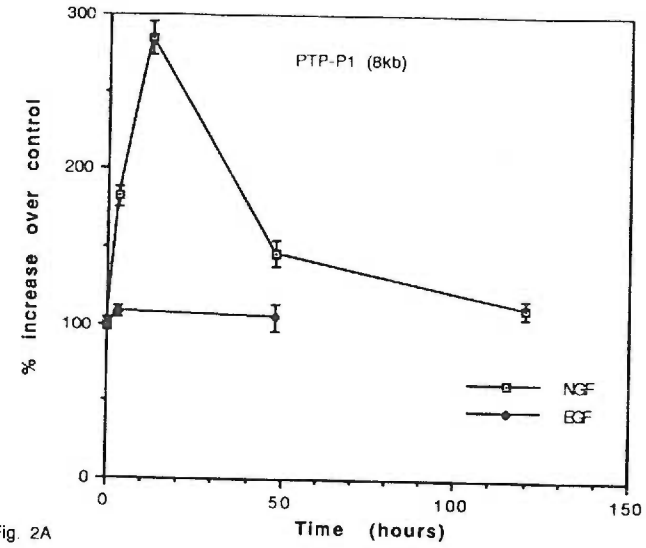
24. Brady-Kalnay, S.M., Flint, A.J. & Tonks, N.K. (1993) J. Cell. Biol. 122, 961-972

Legends to Figures

Fig. 1. PTP-P1 mRNA levels are increased by NGF and NGF plus cycloheximide but not by EGF. A, Northern blot analysis of PTP-P1 expression in PC12 cells treated with NGF and EGF. PC12 cells were treated with NGF and EGF for 3 hr, 2 days, and 5 days. B, Northern analysis of PTP-P1 expression in PC12 cells treated with NGF and NGF plus cycloheximide for 12 hr. Lane 1, untreated; Lane 2, NGF treated for 12 hr; Lane 3, NGF plus cycloheximide for 12 hr.

Fig. 2. Summary of Figure 1. The 6 and 8 kb bands of PTP-P1 and actin bands were measured using a scanning densitometry. The relative densities of PTP-P1 were calculated against actin. A, The 6 kb transcript is increased by NGF but not by EGF treatment. B, The 8 kb transcript is increased by NGF but not by EGF treatment. C, Cycloheximide further increased mRNA levels of PTP-P1 6 and 8 kb transcripts.

Fig. 2 B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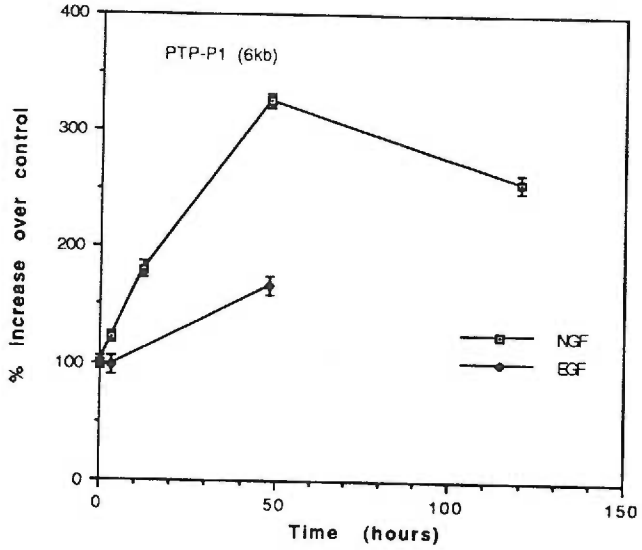
50
100
150
Time (hours)
NGF
EGF

Fig. 2A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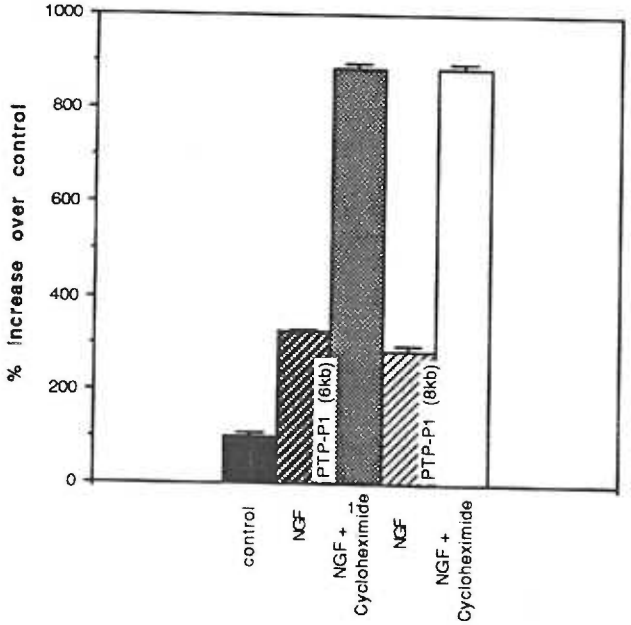
Time (hours)
NGF
EGF


% increase over control
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800
600
400
200
0
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PTP-P1 (8kb)
control
NGF
NGF + Cycloheximide
NGF
NGF + Cycloheximide

Chapter VI

SUMMARY AND FUTURE DIRECTIONS

Cell growth and differentiation is one of the major themes in biology. Over the past forty years, numerous experiments have demonstrated the importance of phosphorylation and dephosphorylation in the regulation of cell growth and differentiation. A number of protein kinases and protein phosphatases have been isolated, and the functional diversity of these enzymes have been demonstrated from the membrane to the nucleus, from cell cycle regulation to embryonic development.

Protein tyrosine kinases have been extensively investigated and are well characterized. In contrast, less is known about protein-tyrosine phosphatases. The first protein tyrosine kinase was identified in 1979 (1), whereas the first protein tyrosine phosphatase was identified in 1988 (2-4). However, increasing evidence has accumulated to demonstrate the biological importance of PTPases.

The molecular emergence of protein tyrosine phosphatases has come with many surprises. First, they are a unique family of enzymes structurally unrelated to protein kinases and protein serine/threonine phosphatases (5). Second, they are not housekeeping genes, instead their gene products may be equally important to protein tyrosine kinases with respect to regulating cellular functions. One of the best examples is protein tyrosine phosphatase cdc25, a key regulator of cell mitosis (6).

In this thesis, using both biochemical and molecular approaches, I have tried to address the fundamental question of whether protein tyrosine phosphatases regulate cell growth and differentiation. I have demonstrated that somatostatin, an anti-proliferative hormone, can stimulate protein tyrosine phosphatase activity through a G protein coupling pathway (7). In addition, I have isolated two novel putative receptor protein tyrosine phosphatases (8), one of which may be involved in neuronal differentiation.

Somatostatin is a growth hormone release-inhibiting factor with ability to inhibit cell growth. Somatostation has been used clinically to treat patients with hormone-producing tumors. It exerts effects in a variety of cell types throughout the body. It inhibits adenylyl cyclase activity, reduces calcium currents, and increases potassium channel conductance (9-15). Moreover, somatostatin can stimulate a protein tyrosine phosphatase activity in human tumor cells (7) and a serine/threonine protein phosphatase activity in pituitary cells (16).

I have demonstrated that somatostatin can stimulate a PTPase activity in MIA PaCa-2 cells, a pancreatic carcinoma cell line. This stimulation may mediate one of somatostatin's actions to regulate cell growth. This stimulated PTPase activity can dephosphorylate EGF receptor *in vitro*, suggesting that this PTPase activity may dephosphorylate certain physiological substrates *in vivo* to block positive signals delivered to tumor cells. Somatostatin can inhibit DNA synthesis in MIA PaCa-2 cells, and this inhibition may be mediated through stimulation of protein tyrosine phosphatase activity. The stimulation of protein tyrosine phosphatase activity by somatostatin may

represent a general mechanism for certain hormones to regulate cell growth. Recently, Florio et al (17) have also demonstrated that dopamine, another antiproliferative hormone, can stimulate protein tyrosine phosphatase activity, and this stimulation is correlated with its inhibiton of DNA synthesis. In addition, TGF-ß1 and interleukin-6, have also been shown to stimulate PTPase activity (18,19). TGF-ß1 and IL-6 inhibits growth of keratinocytes and myoloblastic cells, respectively.

All of somatostatin's actions have been shown to be mediated through G protein-coupled pathways. G proteins are a heterotrimeric complex composed of three subunits α, ß, and γ (20–24). Activation of G proteins by G protein-coupled receptors results in interaction with several effectors such as adenylyl cyclase, phospholipase C, and certain ion channels (20-24). Using GTP and GDP analogues and pertussis toxin which specifically inhibits G proteins G_o and G_i, I have provided evidence that somatostatin stimulation of a PTPase activity is mediated through a pertussis toxin-sensitive G protein coupling pathway. These data suggest that there may be additional G protein effectors in the cells. Interestingly, It was recently reported that PI-3 kinase might be another G protein effector that may be activated by direct interaction with G proteins (25). In addition, in T cells, a 32 kd GTP-binding protein was found to be associated with the CD4-$p56^{lck}$ and CD8-$p56^{lck}$ T cell receptor complexes (26), demonstrating that interaction between T cell receptor complexes and a G protein. There have been a number of reports suggesting that G proteins may also be involved in receptor-tyrosine kinase signaling pathways (27,28), demonstrating that two major signal transduction pathways may be interconnected or cross-talk at multiple levels.

Stimulation of a PTPase activity by G protein could be a result of direct interaction or indirectly mediated through another protein. If specific PTPases are stimulated by direct interaction with activated G proteins, these PTPases may display structural features that facilitate G protein coupling. This can only be confirmed by purification and cloning of these PTPases. A recent report has provided some biochemical evidence that somatostatin can stimulate a membrane PTPase activity in rat pancreatic acinar cells and that this PTPase is a protein with approximately 70 kd molecular weight (29). The purification and molecular cloning of this PTPase will accelerate the understanding on somatostatin's action to regulate cell growth.

Five cDNAs encoding somatostatin receptors have been isolated (30-33). The pharmacological characteristics of each receptor have been shown to be distinct and are still controversial (34). It is unlikely that all somatostatin receptors couple to stimulation of PTPase activity. It would be of interest to identify the specific receptor(s) which are responsible for somatostatin's stimulation of PTPase activity. This task requires stable expression of each receptor in some cell lines with which each receptor can be specifically studied.

To start to characterize the role of PTPases in neuronal differentiation, I have isolated two cDNAs encoding putative receptor protein-tyrosine phosphatases from PC12 cells, PTP-P1 and PTP-PS. To date, about a dozen putative receptor PTPases have been identified. Most of them contain two homologous PTPase domains. I have shown that PTP-P1 is a putative receptor PTPase with two PTPase domains, whereas PTP-PS contains only a

single PTPase domain. I have also shown that PTP-P1 and PTP-PS are generated by RNA processing from a single gene. The use of a polyadenylation site within the intron between the domain 1 and domain 2 of PTP-P1 generates PTP-PS, while alternative splicing of this intron generates PTP-P1. This is the first report that two distinct PTPases of different classes can be generated by RNA processing from a single gene. The physiological importance of this type of RNA processing is not clear. It is possible that these two PTPases may possess different substrate specificities or enzyme kinetics. It is also possible that with different cytoplasmic structures, PTP-P1 and PTP-PS might interact with different sets of intracellular proteins to play different functional roles. To test these hypotheses, experiments can be designed to identify the functional differences between PTP-P1 and PTP-PS by testing substrate specificity and enzyme kinetics of recombinant proteins expressed in both bacteria (for example, GST-fusion proteins in E.coli.) and mammalian cells (for example, epitope tagged-fusion proteins expressed in CV1 cells) *in vitro*. Experiments can also be designed to identify the cellular proteins associated with the intracellular domain of PTP-P1 or PTP-PS using the biochemical method of coimmunoprecipitation. Other approaches to identify and isolate cDNAs that encode cellular proteins interacting with the intracellular domain of PTP-P1 or PTP-PS could be expression cloning using recombinant proteins of intracellular domains of PTP-P1 and PTP-PS as probes to hybridize a lambda expression library, or the two hybrid system using yeast S. cerevisiae strain that contains two integrated reporter constructs, the yeast *HIS3* gene and the bacterial lacZ gene. The two hybrid system has been successfully used to identify a number of important gene products (35-38). However, if homodimerization is a mechanical mechanism used by putative receptor PTPases following potential ligand activation or

inactivation, then it might be needed to express intracellular domains of PTP-P1 and PTP-PS as a dimer in mammalian cells for expression cloning, and that the two hybrid system might not be an appropriate approach, since that dimerized intracellular domains might be required for interacting with effectors in the cells.

I have shown that PTP-P1 and PTP-PS are primarily expressed within the brain and in some neuronal and endocrine cells, suggesting that these PTPases may be involved in neuronal functions. PTP-P1 is most abundantly expressed in the brain cortex. In addition, its mRNA levels are increased following NGF-induced PC12 cell differentiation but not by EGF, suggesting that PTP-P1 is involved in neuronal differentiation and that NGF and EGF can differentially regulate the expression of PTP-P1.

The hypothesis that PTP-P1 may be involved in neuronal differentiation is supported by several lines of evidence. First, PTP-NE3 (identical to PTP-P1) is expressed in olfactory neuroepithelium in a developmentally-specific manner with high expression in immature neurons and lower levels in mature neurons (39). Second, vanadate, a PTPase inhibitor, has been shown to be able to block neuronal differentiation of PC12 cells induced by both NGF and FGF (40). Third, PTPase activity of PC12 cells is elevated during the NGF-induced PC12 cell differentiation (41).

The PC12 cell line is an excellent model system for studying neuronal differentiation. This cell line was derived from rat pheochromocytoma cells. PC12 cells are induced by NGF to commit to neuronal differentiation but stimulated by EGF to proliferate. Both growth factors stimulate tyrosine

phorylation through their receptors which are receptor-tyrosine kinases. Both growth factors activate a very similar set of responses including activation of $P21^{ras}$, $P60^{src}$, MAP kinases, phospholipase C, and PI-3 kinase (42-48). It is still a mystery why NGF and EGF which share so many similar signaling transduction characteristics induce such different phenotypic changes (48). One hypothesis is that signals transduced by tyrosine phosphorylation in EGF receptor pathway may be sustained throughout the entire stimulation period, while tyrosine phosphorylation in NGF receptor pathway might be tightly modulated and maintained at a moderate level. The maintainance of this level might require one or more PTPases. The differential regulation of PTP-P1 by NGF and EGF provides an interesting clue that PTP-P1 may be a candidate PTPase to balance tyrosine phosphorylation in vivo in response to NGF induction of neuronal differentiation. It is possible that other PTPases are involved in this process. However, PTP-P1 is the only PTPase known to be regulated by NGF at present time. To obtain direct evidence that demonstrates the involvement of PTP-P1 in NGF induction of neuronal differentiation, we will require overexpression or elimination of PTP-P1 in PC12 cells and subsequent examination of NGF's actions in these cells. If PTP-P1 is a mediator of NGF induction, overexpression of PTP-P1 should stimulate neurite extension or potentiate NGF induction. An "anti-sense approach" using anti-sense oligonucleotides or anti-sense RNA expression or by microinjecting specific antibodies could be also useful. It might be expected that elimination of PTP-P1 expression could attenuate or diminish NGF induction of PC12 cell differentiation.

In summary, I have demonstrated that somatostatin, an anti-proliferative hormone, can stimulate a protein-tyrosine phosphatase activity in MIA

PaCa-2 cells, and this stimulation is mediated through a pertussis toxin-sensitive G protein coupling pathway. Somatostatin can also inhibit DNA synthesis in these cells, and this inhibition is independent of cAMP. I have also isolated two cDNAs encoding putative receptor protein-tyrosine phosphatases, PTP-P1 and PTP-PS, which are generated by RNA processing from a single gene. PTP-P1 contains two PTPase catalytic domains whereas PTP-PS contains only a single PTPase domain. PTP-P1 is primarily expressed in some neuronal tissues and cells and its expression is increased during NGF-induced PC12 cell differentiation, suggesting that PTP-P1 may be involved in neuronal differentiation.

References

1. Collett, M.S., Purchio, A.F. & Erikson, R.L. (1980) Avian sarcoma virus transforming protein, pp60^{src}, shows protein kinase activity specific for tyrosine. *Nature*. 285, 167-169

2. Tonks, N. K., Diltz, C.R. & Fischer, E.H. (1988) Purification of the major protein tyrosine phosphatase of human placenta. *J. Biol. Chem.* 263, 6722-6730

3. Tonks, N.K., Diltz, C.D. & Fischer, E.H. (1988) Characterization of the major tyrosine phosphatase of human placenta. *J. Biol. Chem.* 263, 6731-6737

4. Charbonneau, H., Tonks, N.K., Kumar, S., Diltz, C.D., Harrylock, M., Cool, D.G., Krebs, E.G., Fischer, E.H. & Walsh, K.A. (1989) Human placenta protein tyrosine phosphatase amino acid sequences and relationship to a family of receptor-like proteins. *Proc. Natl. Acd. Sci. USA* 86, 5252-5256

5. Fischer, E.H., Charbonneau, H. & Tonks, N.K. (1991) Protein tyrosine phosphatases: A diverse family of intracellular and transmembrane enzymes. *Science*, 253, 401-406

6. Millar, J.B.A. & Russel, P. (1992) The cdc25 M-phase inducer: an unconventional protein phosphatase. *Cell*, 68, 407-410

7. Pan, M., Florio, T. & Stork, P.J.S. (1992) G protein activation of a hormone-stimulated phosphatase in human tumor cells. *Science*, 256, 1215-1217

8. Pan, M., Rim, C., Lu, K., Florio, T. & Stork P. J.S. (1993) Expression and cloning of two structurally distinct receptor-linked protein-tyrosine phosphatases generated by RNA processing from a single gene. *J. Biol. Chem.*. 268, 19284-19291

9. Koch, B.D., Dorflinger, L.J. (1985) Pertussis toxin blocks both cyclic AMP-mediated and cAMP-independent actions of somatostatin: Evidence for coupling of Ni to decreases in intracellular free calcium. *J. Biol. Chem.* 260, 13138-13145

10. Ikeda, S.R. & Schofield,. G.G. (1989) Somatostatin blocks a calcium current in rat sympathetic ganglion neurons through a pertussis toxin-sensitive mechanism. *J. Physiol.* 407,221-240

11. Reisine, T, (1990) Cellular mechanisms of somatostatin inhibition of calcium influx in the anterior pituitary cell line AtT20. J. Pharm. and Exper. Therpeutics. 254, 646-651

12. Wang, H.L., Reisine, T. & Dichte, M. (1990) Somatostatin-14 and somatostatin-28 inhibit calcium currents in rat neocortical neorons. *J. Neuroscience* 38, 335-342

13. Wang, H.L., Bogen, C., Reisine, T. & Dichter, M. (1989) Somatostatin-14 and somatostatin-28 induce opposite effects on potassium currents in rat neocortical neurons. *Proc. Natl. Aca. Sci. USA* 86, 9616-9620

14. Inoue, M., Nakajima, S. & Nakajima, Y. (1988) Somatostatin induces an inward rectification in rat locus coeruleus neurons through a pertussis toxin-sensitive mechanism. *J. Physiol.* 407, 177-198

15. Jacqin ,T., Champagnat, J., Madamba, S., Denavit-Saubie, M. & Siggins, G.R. (1988) Somatostatin depresses excitability in neurons of the solitary tract complex through hyperpolarization and augmentation of IM, a non-activating voltage dependent outward current blocked by muscarinic agonists. *Proc. Natl .Aca. Sci. USA* 85:948-952

16. White, R.W., Schonbrunn, A. & Armstrong, D.L. (1991) Somatostatin stimulates $Ca2^{+}$-activated K^{+} channels through protein dephosphorylation. *Nature* 351, 570-573

17. Florio, T., Pan, M., Newman, B., Hershberg, R.E., Civelli, O. & Stork P.J.S. (1992) Dopaminergic inhibition of DNA synthesis in pituitary tumor sells is assodiated with phosphotyrosine phosphatase activity. *J. Biol. Chem.* 267, 24169-24172

18. Rijksen, G., Voller, M.C. & Van Zoelen, E.J. (1993) Orthovanadate both mimics and antogonize the transforming growth factor beta action on normal rat kidney cells. *J. Cell. Physiol.* 154, 393-401

19. Zafriri, D., Argaman, M., Canaani, E. & Kimchi, A. (1993) Induction of PTPase activity by interleukin-6 in M1 myeloblastic cells and analysis of possible counteraction by the BCR-ABL oncogene. *Proc. Natl. Aca. Sci. USA* 90, 477-481

20. Birnbaumer, L., Abramowitz, J. & Brown, A.M. (1990) Receptor-effector coupling by G proteins. *Biochimica et Biophysics Acta.* 1031, 163-224

21.Bourne, H.R., Sanders, D.A. & Mccormick, F. (1991) The GTPase superfamily: conserved structure and molecular mechanism. *Nature.* 349, 117127

22. Bourne, H.R., Sanders, D.A. & McCormick, F. (1990) The GTPase superfamily: a conserved switch for diverse cell functions. *Nature.* 348, 125132

23. Gilman, A.G. (1987) G proteins: transducers of receptor-generated signals. *Ann. Rev. Biochem.* 56, 615-649

24. Birnbaumer, L (1992) Receptor-to-effector signaling through G proteins: roles for ßg dimers as well as a subunits. *Cell.* 71, 10069-1072

25. Stephens, L., Eguinoa, A., Corey, S., Jackson, T. & Hawkins, P.T. (1993) Receptor stimulated accumulation of phosphatidylinositol (3,4,5)-triphosphate by G protein mediated pathways in human myeloid derived cells. *EMBO J.* 12, 2265-2273

26. Telfer, J.C. & Rudd, C.E. (1991) A 32 kD GTP-binding protein associated with the CD4-p56^{lck} and CD8-p56^{lck} T cell receptor complexes. *Science*. 254, 439-441

27. Yang, L., Baffy, G., Rhee, S. G., Manning, D., Hansen, C. & Williamson, J.R. (1991) Pertussis toxin-sensitive Gi protein involvement in epidermal growth factor-induced activation of phospholipase C-γ in rat hepatocytes. *J. Biol. Chem.* 266, 22451-22456

28. Teitebaum, I. (1990) The epidermal growth factor receptor is coupled to a phospholipase A2-specific pertussis toxin-inhibitable guanine nucleotide-binding regulatory protein in cultured rat medullary collecting tubule cells. *J. Biol. Chem.* 265, 4218-4222

29. Colas, B., Cambillau, C., Buscail, L., Zeggari, M., Esteve, J., Lautre, V., Thomas, F., Vaysse, N. & Susini, C. (1992) Stimulation of a membrane tyrosine phosphatase activity by somatostatin analogues in rat pancreatic acinar cells. *Eur. J. Biochem.* 207, 1017-1024

30. Ymada, Y., Post, S.R., Wang, K., Tager, H.S., Bell, G.I. & Seino, S. (1992) Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract, and kidney. *Proc .Natl. Aca. Sci. USA* 89:251-255

31. Yasuda, K., Rens-Domiano, S., Breder, C.D., Law, S.F., Saper, C.B/, Reisine, T. & Bell, G.I. (1992) Cloning of a novel somatostatin receptor, SSTR3, coupled to adenylyl cyclase. *J. Biol. Chem.* 267:20422-20428

32. O'Caroll, A., Lolait, S.J., Konig, M. & Mahan, L.C. (1992) Molecular cloning and expression of a pituitary somatostatin receptor with preferential affinity for somatostatin-28. *Mol. Pharmacol.* 42, 939-946

33. Bruno, J.F., Xu, Y., Song, J., & Berelowitz, M. (1992) Molecular cloning and functional expression of a brain-specific somatostatin receptor. *Proc. Natl. Aca. Sci. USA.* 89, 11151-11155

34. Hershberger, R.E., Newman, B.L., Florio, T., Bunzow, J., Civelli, O., Li, X., Forte, M. & Stork, P.J.S. (1993) The SSR1 and SSR2 are coupled to inhibition of adenylyl cyclase in CHO cells via pertussis toxin sensitive pathway. Endocrinol. (in press)

35. Fields, S & Song O. (1989) A novel genetic system to detect protein-protein interactions. *Nature*. 340, 245-248

36. Durfee, T., Becherer, K., Chen, P., Yeh, S., Yang, Y., Kilburn, A.E., Lee, W-H. & Elledge, S.J. (1993) The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Gene. Dev.* 7, 555-5692

37. Vojtek, A.B., Hollenberg, S.M. & Cooper, J.A. (1993) Mammalian Ras Interacts directly with the serine/threonine kinase raf. *Cell*, 74, 205-214

38. Zhang, X., Settleman, J., Kyriakis, J.M., Takeuchi-Suzuki, E., Elledge, S.J., Marshall, M.S., Bruder, J.T., Rapp, U.R. & Avruch, J. (1993) *Nature,* 364, 308-313

39. Walton, K., Martell, K.J., Kwak, S.P., Dixon, J.E. & Largent, B.L. (1993) A novel receptor-type protein tyrosine phosphatase is expressed during neurogenesis in the olfactory neuroepithelium. *Neuron,* 11, 387-400

40. Wu, Y.Y. & Bradshaw, R.A. (1993) Effect of nerve growth factor and fibroblast growth factor on PC12 cells: inhibition by orthovanadate. *J. Cell. Biol.* 121, 409-422

41. Aparicio, L.F., Ocrant, I., Boylan, J.M. & Gruppuso, P.A. (1992) Protein phosphatase activation during nerve growth factor-induced neuronal differentiation of PC12 cells. *Cell Grow. Differ.* 3, 363-367

42. Klein, R., Jing, S., Nanduri, V., O'Rourke, E. & Barbacid, M. (1991) The trk proto-oncogene encodes a receptor for nerver growth factor. *Cell*, 65, 189-197

43. Kaplan, D.R., Hempstead, B.L., Martin-Zanca, D., Chao, M. & Parada, L.F. (1991) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science* 252, 554-5558

44. Cordon-Cardo, C., Taoley, P., Jing, S., Nanduri, V., O'Rourke, E., Lamballe, F., Kovary, K., Klein, R., Jones, K.R., Reichardt, L.F. &

45. Hempstead, B.L., Martin-Zanca, D., Kaplan, D.R., Parada, L.F. & Chao, M.V. (1991) High-affinity NGF binding requires coexpression of the trk proto-oncogene and the low-affinity NGF receptor. *Nature*. 350, 678-683

46. Loeb, D.M., Maragos, J., Martin-Zanca, D., Chao, M., Parada, L.F. & Greene, L.A. The trk proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell line. *Cell*. 66, 961-966

47. Bothwell, M. (1991) Keeping track of neurotrophin receptors. *Cell*. 65, 915-918

48. Chao, M.V. (1992) Growth factor signaling: where is the specificity. *Cell*. 68, 995-997