Biochemical and genetic studies of the protein tyrosine phosphatase MPTP-PEST

by

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Abstract

Our search for protein tyrosine phosphatases (PTPases) that are involved in murine development led to the isolation of a novel PTPase called MPTP-PEST. To elucidate the function of MPTP-PEST, several biological aspects of the protein were investigated. It was determined that the MPTP-PEST enzyme is a stable cytosolic protein tyrosine phosphatase of 112 kDa that is ubiquitously expressed both in the adult and in the embryo. MPTP-PEST is composed of a single amino-terminus catalytic domain which is active against phosphorylated substrates *in vitro*. The gene structure and chromosomal localization of MPTP-PEST were determined using a series of λ phage clones isolated from a mouse genomic library. Analysis of the MPTP-PEST locus indicated that the gene spans over 90 kb of the mouse genome and is composed of 18 exons, 10 of which constitute the catalytic phosphatase domain. Fluorescence *in situ* hybridization with MPTP-PEST genomic DNA defines the map position of MPTP-PEST to mouse chromosome 5 region A3-B. To gain mechanistic insights into the function of MPTP-PEST, the association of proteins with MPTP-PEST was investigated using several different *in vitro* and *in vivo* binding assays. It was shown that the proto-oncoprotein SHC and the adaptor protein Grb2 associate with MPTP-PEST through specific carboxy-terminus sequences. In addition, the SHC/MPTP-PEST association was shown to be mediated by a novel type of protein-protein interaction. Both SHC and Grb2 function downstream of receptor type and cytoplasmic protein tyrosine kinases and have been shown to mediate several signal transduction events. The association of MPTP-PEST with these signaling proteins may therefore represent a function for MPTP-PEST in signaling events.

Résumé

Durant le cours de nos recherches sur les tyrosine phosphatases qui sont exprimées durant le development mammifère nous avons isolé une nouvelle tyrosine phosphatase qui fut nomée MPTP-PEST. En vue d'élucider la fonction de la MPTP-PEST, plusieurs aspects biologique de la protéine furent analysés. Tout d'abord, il fut déterminé que la protéine MPTP-PEST est une protéine cytoplasmic stable d'environ 112 kDa et qui est exprimée de façon ubiquiste autant chez l'adulte que chez l'embryon. La MPTP-PEST est composée d'un domaine catalytique actif simple qui est situé dans la région NH_2-terminale de la protéine. L'analyse de la struture du gène et de la localization chromosomique de la MPTP-PEST révèle que le gène est composé de 18 exons dont 10 formant le domaine catalytique et que le gène se situe dans la région A3-B du chromosome 5 chez la souris. En vue d' obtenir de plus ample informations pouvant ainsi mener a la charaterization de la (des) fonction(s) biologique(s) de la MPTP-PEST, une étude des protéines qui s'associent à MPTP-PEST fut menée. Il fut découvert que les protéines SHC et Grb2, deux protéines de type adapteur qui agissent sous les tyrosine kinases, s'associent à la portion COOH-terminale de la MPTP-PEST. Ces associations, en vertu des fonctions signalétiques démontrées par SHC et Grb2, suggèrent que la tyrosine phosphatase MPTP-PEST joue un role durant la transmission des signaux extracellulaires par les tyrosine kinases.

Preface

The work presented in Chapters 2, 3, and 4 of the thesis have been published in the following journals:

CHAPTER 2: Charest, A., Wagner, J., Shen, S.-H., and Tremblay, M. L. (1995) Biochem. J. 308: 425-432.

CHAPTER 3: Charest, A., Wagner, J., Muise, E. S., Heng, H. H. Q., and Tremblay, M. L. (1995) Genomics 28: 501-507.

CHAPTER 4: Charest, A., Wagner, J., Jacob, S., McGlade, C. J., and Tremblay, M. L. (1996) J. Biol. Chem. 271: 8424-8429.

The work presented in Chapter 5 has been submitted for publication.

The work described in Chapters 2, 3, 4, and 5 is mainly my own. In Chapter 2, John Wagner partially determined the sequence of the 5′ UTR of the MPTP-PEST cDNA. Shi-Hsiang Shen provided a uncharacterized cDNA partial sequence corresponding to human PTP-PEST that was used to derived MPTP-PEST specific oligonucleotides. In Chapter 3, John Wagner obtained a λ phage genomic clone from the genomic library. Eric S. Muise provided a partial map of the intron/exon boundaries for the protein tyrosine phosphatase MPTP and Henry H. Q. Heng performed the FISH analysis.

In Chapter 4, John Wagner created some of the GST fusion protein constructs. Sara Jacob and C. Jane McGlade provided the affinity purified anti SHC antibody, the human SHC cDNA and the MPTP-PEST peptides. In Chapter 5, John Wagner and Mei Kwan created some of the GST fusion protein constructs.

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of

manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

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List of Abbreviations

CA	Carbonic Anhydrase
CAM	Cell Adhesion Molecule
CAMs	Cell Adhesion Molecules
EC	Embryonal Carcinoma
EGF	Epidermal Growth Factor
EPO-R	Erythropoietin Receptor
FGF	Fibroblast Growth Factor
FNIII	Fibronectin type III
GPI	Glycosyl-Phosphatidyl Inositol
Grb2	Growth factor Receptor Binding protein 2
GST	Glutathione S-transferase
Ig	Immunoglobulin
IRS-1	Insulin Receptor Substrate-1
MAM	Meprin, A5, μ
MPTP	Murine Protein Tyrosine Phosphatase
NMR	Nuclear Magnetic Resonance
p.-c.	post-coitum
PDGF	Platelet-Derived Growth Factor
PEST	Proline, Glutamate, Serine, and Threonine
PH	Pleckstrin Homology
PI	Phosphotyrosine Interacting
PKA	cAMP-dependent Protein Kinase
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PPases	Phosphoprotein Phosphatases
PTB	Phosphotyrosine Binding
PTKs	Protein Tyrosine Kinases
PTPase	Protein Tyrosine Phosphatase
pTyr	phosphoTyrosine
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SHC	Src Homology, Collagen

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I am grateful to members, past and present, of Dr. Tremblay's laboratory for their encouragement and the occasional comic relief that they provided me with (sometimes unknowingly). I am indebted to Mr. John Wagner, who continuously provided me with moral and technical support throughout the course of my studies. I'm thankful towards Drs. Jerry Pelletier, Philippe Gros, and Morag Park for the scientific support that they gave me.

Finally, I would like to acknowledge my wife and family for their constant moral support which allowed me to achieved this work.

This thesis is dedicated to my wife, Rama
and to the memory of my grandfather, R. Blouin.

"Waiting for the winds of change
To sweep the clouds away
Waiting for the rainbow's end
To cast its gold your way
Countless ways
You pass the days...

...You don't get something for nothing
You don't get freedom for free
You won't get wise
With the sleep still in your eyes
No matter what your dreams might be

What you own is your own kingdom
What you do is your own glory
What you love is your own power
What you live is your own story
In your head is the answer
Let it guide you along
Let your heart be the anchor
And the beat of your own song..."

Neil Peart, 1976

CHAPTER 1

Introduction

Protein phosphorylation

Phosphate monoester formation (phosphorylation) and hydrolysis (dephosphorylation) of proteins are considered two of the most essential biochemical reactions carried out by cellular organisms (Stone and Dixon 1994). Reversible, enzyme-catalyzed protein phosphorylation and dephosphorylation are important post-translational regulatory modifications that cells use to regulate their response to external and internal stimuli. In eukaryotic cells, two types of phosphoamino acids have been reported; the N-phosphoamino acids (e.g. phosphohistidine, phosphoarginine, and phospholysine) and the O-phosphoamino acids (e.g. phosphoserine, phosphothreonine, and phosphotyrosine). In mammals, the quantities of the N-phosphoamino acids in proteins are comparable to the amounts of O-phosphoamino acids (phosphoserine and phosphothreonine) and far exceed the levels of phosphotyrosine (Chen et al. 1977; Zetterqvist 1967a; Zetterqvist 1967b).

The acid labile nature of the nitrogen-phosphorus (N-P) bond of the basic amino acid residues limits the use of conventional assays for protein kinases and phosphatases. Studies regarding the phosphorylation and dephosphorylation of basic amino acid residues are therefore scarce.

Histidine phosphorylation has been shown to be involved in prokaryotic cellular processes such as chemotaxis (Hess et al. 1988) and porin expression (Stock et al. 1990). In eukaryotes, phosphohistidine has been observed in proteins such as histone H4 (Chen et al. 1977; Fujitaki et al. 1981; Walinder 1968) and G-protein β subunits (Wieland et al.

1993). Eukaryotic histidine kinases and phosphatases have only been briefly characterized biochemically (Huang et al. 1991; Ohmori et al. 1993; Wei and Matthews 1990). Therefore, the function of histidine phosphorylation in eukaryotes remains unclear. However, the recent discovery of transient and inducible histidine phosphorylation on P-selectin upon stimulation of platelets with thrombin or collagen (Crovello et al. 1995) provides direct evidence for the induction of histidine phosphorylation in mammalian cell activation. This phenomenon may underlie a role for phosphohistidine in mammalian cell signaling mechanisms.

Much less is known regarding phospholysine and phosphoarginine. Various groups have reported the detection of lysine- (Smith et al. 1973; Smith et al. 1974) and arginine-specific (Levy-Favatier et al. 1987; Wakim et al. 1990) protein kinases and lysine- (Ohmori et al. 1993; Ohmori et al. 1994; Wong et al. 1993) and arginine-specific (Kuba et al. 1992; Yokoyama et al. 1993) protein phosphatases in eukaryotic cell extracts. However, the functions resulting from the phosphorylation of lysine and arginine residues in proteins remain largely unknown (Ohmori et al. 1993).

Most of the O-phosphate monoesters on proteins occur on seryl and threonyl residues (Sefton et al. 1980; Taborsky 1974). The regulation of many metabolic processes by serine and threonine protein phosphorylation has been well documented (reviewed in (Roach 1991)) and represents material which is beyond the scope of this introduction.

Tyrosine phosphorylation was discovered 16 years ago when the products of a unique catalytic activity were noticed while examining

retrovirus-mediated oncogenic transformation of eukaryotic cells. It was observed that the levels of phosphotyrosine, normally accounting for only 0.01-0.05% of the total phosphoamino acid content of the cell, rose to 1-3% upon retrovirus infection (Hunter et al. 1980; Martensen 1982; Sefton et al. 1981; Sefton et al. 1980). The correlation between the transforming activities of oncogenic retroviruses and the increase in cellular levels of phosphotyrosine suggested that phosphorylation of proteins on tyrosyl residues constitutes an essential component of cellular transformation (Collett and Erikson 1978; Cooper et al. 1983; Martensen 1982; Pawson et al. 1980; Sefton et al. 1981; Sefton et al. 1980; Witte et al. 1980). The potential role of tyrosine phosphorylation in cellular transformation ultimately led to intense research that focused around the tyrosyl phosphorylation of cellular proteins and the tyrosine kinases responsible for this phenomenon. Since then, the association of cellular activities such as proliferation, differentiation and transformation with phosphotyrosyl phosphorylation events has been well documented (Coughlin et al. 1988; Heldin and Westermark 1984; Sefton and Hunter 1984; Swarup et al. 1984). The correlation between cellular proliferation and tyrosyl phosphorylation was further supported by studies which showed that polypeptide growth factor receptors such as the epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor, and insulin display intrinsic tyrosine kinase activities (Cohen et al. 1980; Ek et al. 1982; Gammeltoft and Van Obberghen 1986; Jacobs et al. 1983; Reynolds et al. 1981; Sefton and Hunter 1984).

In addition, several oncogene products possess intrinsic tyrosine kinase activity and share extensive sequence similarity with several growth factor receptors. Experiments aimed at inhibiting the tyrosine

kinase activity of several growth factor receptors and oncogenes demonstrated that their proliferative actions and their transforming capabilities are dependent on their kinase activity (Chou et al. 1987; Morgan et al. 1986; Morgan and Roth 1987).

Pulse-chase experiments indicated that the turnover of phosphate on phosphotyrosine proteins is rapid (Beemon et al. 1982; Sefton et al. 1980), suggesting that phosphorylation on tyrosyl residues has a regulatory role. A dynamic balance exists therefore between phosphorylation and dephosphorylation processes. This balance is shifted by the introduction of oncogenes that code for tyrosine kinases, resulting in increased amounts of phosphotyrosine in cellular proteins.

Protein phosphatases

The levels of cellular protein phosphorylation are controlled by the activities of protein kinases and phosphatases. In eukaryotic cells, O-phospho protein kinases and phosphatases are generally categorized according to their substrate specificity. This classification system defines two groups for the protein kinases: the serine/threonine kinases and the tyrosine kinases. Despite the differences in substrate specificity, both types of kinases share amino acid sequence identity and three-dimensional structure similarities (Hunter 1987; Knighton et al. 1991). For the purposes of this introduction, I will focus on protein phosphatases rather than on protein kinases.

The protein phosphatases constitute a diverse family of biological catalysts. They can be typically categorized into three groups: nonspecific phosphatases, phosphoprotein (serine/threonine)

phosphatases (PPases), and protein tyrosine phosphatases (PTPases). Each of these groups, unlike their kinase counterparts, lack sequence and structural similarities with each other (Walton and Dixon 1993)).

The nonspecific phosphatases comprise enzymes such as the acid phosphatases and the alkaline phosphatases. These two groups are often capable of hydrolysing phosphate monoesters from both proteins and non proteinaceous material. The different members of the nonspecific phosphatases category do not share amino acid sequence similarities among them. The catalytic mechanisms for both alkaline and acid phosphatases have been elucidated (Coleman 1992; Kim and Wyckoff 1991; Van Etten 1982). The mechanism of dephosphorylation portrayed by alkaline phosphatases proceeds through a phosphoserine intermediate whereas phosphate ester hydrolysis by some acid phosphatases occurs through phosphohistidine intermediates (Stone and Dixon 1994).

Unlike the nonspecific phosphatases, the serine/threonine phosphatases (PPases) share amino acid sequence similarity with one another. Interestingly, the catalytic mechanism of the PPases does not seem to involve a phosphoenzyme intermediate (Martin and Graves 1986). A phosphoesterase signature motif found in all type 1, 2A and 2B PPases and in a variety of otherwise unrelated phosphoesterases has recently been described (Zhuo et al. 1994). This signature motif sequence could underlie a common catalytic mechanism (Zhuo et al. 1994).

Protein Tyrosine Phosphatases

Much of our understanding of tyrosine phosphorylation comes from unilateral progress in tyrosine kinase research. Until recently, the existence of enzymes responsible for the catalysis of phosphotyrosine esters was based on *in vitro* studies using cell membrane preparations (Brautigan et al. 1981; Carpenter et al. 1979; Ushiro and Cohen 1980) and *in vivo* studies using temperature-sensitive oncogenic retroviruses (Barbacid et al. 1980; Blomberg et al. 1980; Friis et al. 1980; Lee et al. 1981; Witt and Gordon 1980; Ziemiecki and Friis 1980). Progress similar to that made with tyrosine kinases was restricted by the lack of availability of pure preparations of PTPases and their corresponding amino acid sequences. (for a detailed review of the enzymology of PTPases before the isolation of the first PTPase see (Brautigan 1992; Lau et al. 1989).

The first purification of a PTPase (termed PTP1B) to homogeneity came in 1988 when Tonks *et al.*, (1988) developed a procedure to isolate PTPases from human placenta. The key feature of this procedure was the use of non-hydrolysable substrate affinity columns allowing for a high specificity purification (Tonks et al. 1988b). The amino acid sequence of a short segment of PTP1B was determined and surprisingly revealed no sequence similarity to any other known phosphatases (Charbonneau et al. 1988). Thus, PTP1B immediately defined a new family of phosphatases. Interestingly, databank searches for amino acid sequence similarity using this segment sequence revealed a significant homology to the intracellular portion of a single protein, CD45 (Charbonneau et al. 1988), suggesting a yet undescribed catalytic activity for this transmembrane protein. It was immediately demonstrated that

CD45 immunoprecipitates from human spleen had PTPase activity, indicating that indeed, CD45 functions as a PTPase (Tonks et al. 1988a). CD45 had been known to be a major lymphocyte transmembrane glycoprotein involved in early T- and B-cell activation (for a review see (Thomas 1989). Until this homology had been discovered, the mechanism of action of CD45 was unknown. The similarity between PTP1B and CD45 not only suggested a function for the cytoplasmic domain of CD45, but also implied that PTPases can possess a signaling function.

Eventually, the full amino acid sequence of PTP1B was determined (Charbonneau et al. 1989). Comparison of this amino acid sequence to those of the intracellular portions of CD45, LAR (a protein with sequence homology to PTP1B and CD45) and the T-cell-PTPase (a cDNA isolated from a human T-cell library using oligonucleotides encoding regions of PTP1B (Cool et al. 1989) defined a conserved region of ~250 amino acids displaying a high degree of homology (Charbonneau et al. 1989). Because of the full conservation of several residues, this region was postulated to act as a catalytic domain (a.k.a. the PTPase domain) (Charbonneau et al. 1989).

This initial identification of three members of the PTPase family led to the isolation of numerous other PTPases from many different sources. Using low stringency screening of cDNA libraries and RT-PCR with oligonucleotide primers corresponding to conserved amino acid sequences within the PTPase domain of several PTPases, a considerable number of novel PTPases were obtained by several groups. To this day, over 40 known PTPases have been isolated out of an estimated 500 different individual enzymes (Hunter 1995). Structural comparison

between the protein tyrosine phosphatase and tyrosine kinase families reveals an interesting similarity. Both types of enzymes parallel each other in that members of each family can be classified under the categories of either transmembrane/receptor-like or intracellular enzymes. The latter is further subdivided into two categories according to the nature of the substrates. Intracellular PTPases are classified as being tyrosine-specific or dual-specific (i.e. will dephosphorylate phosphoserine, phosphothreonine and phosphotyrosine).

Receptor-like PTPases

Members of the transmembrane receptor-like PTPase subfamily share several structural features such as an extracellular domain of variable length and composition, a single membrane-spanning region, and one or two intracellular catalytic domains and various amino- and carboxyl-terminal extensions (see figure 1). These PTPases can be subdivided into 5 types on the basis of their extracellular structures (for a recent review, see (Brady-Kalnay and Tonks 1995; Mourey and Dixon 1994).

The haematopoietic-specific PTPase CD45, defines a unique class of receptor-like enzymes (type I). The extracellular portion of the protein consists of an amino-terminal region enriched in O-linked carbohydrates and a 300 amino acid cysteine-rich domain with multiple N-linked glycosylation sites. The length of the O-linked carbohydrate region varies due to alternative splicing of 3 exons. Alternative exon usage is regulated in both lymphocyte differentiation and activation. The CD45 isoforms may associate with different cell surface proteins and therefore modulate the signaling properties of these complexes (for

detailed reviews of CD45 structure-function relationship see (McFarland et al. 1994; Trowbridge 1991; Trowbridge et al. 1991). It has been demonstrated that CD45 is an essential component of antigen-induced signaling events in lymphocytes. It acts as a positive regulator of signal transduction through T- and B-cell receptors (for a review on CD45 function see (Trowbridge and Thomas 1994).

Type II PTPases have extracellular sequence motifs that resemble those that define the cell adhesion molecules (CAMs). These transmembrane receptor-like PTPases possess a combination of extracellular immunoglobulin (Ig)-like domains and/or fibronectin type III repeats (FN III) as well as MAM (meprin, A5, and μ) motifs in certain members (PTP μ and PTP κ).

Ig domains are disulfide-bonded structures that are found in a variety of proteins such as cell surface receptors. These motifs contain a peptide sequence which is involved in homophilic binding in the neuronal cell adhesion molecule N-CAM (Rao et al. 1992). FN III motifs, originally observed in the extracellular matrix protein fibronectin, have been detected in more than 50 eukaryotic proteins. FN III motifs are made of ~100 amino acids and are characterized by highly conserved hydrophobic residues (for a review of fibronectin structure and assembly see (Potts and Campbell 1994). A specific amino acid sequence within one FN III repeat in fibronectin has been shown to mediate the binding of the latter to integrins, resulting in cell-cell interactions (Sastry and Horwitz 1993). MAM motifs have been recently identified in the extracellular portion of five proteins: PTP μ and PTP κ, meprin A and B, and the A5 glycoprotein (Beckmann and Bork 1993). The MAM domain is composed of ~170 amino acids containing four

conserved Cys residues and 2 specific structural and sequence motifs (Beckmann and Bork 1993). The role(s) that MAM motifs play in these proteins is not well established. However, Zondag *et al.*, (1995) have recently demonstrated that MAM motifs are involved in the PTP μ homophilic-mediated aggregation of insect cells (Zondag et al. 1995).

Ig-like, FN III, and MAM motifs have all been shown to mediate adherence among certain PTPases, resulting in cell-cell interactions (Brady-Kalnay et al. 1993; Eijgenraam 1993; Gebbink et al. 1991; Gebbink et al. 1993; Jiang et al. 1993). Cell-cell contact is intimately associated with cellular migration, cellular growth and cellular differentiation. The elucidation of the role(s) that CAM-like PTPases play in these cellular processes is clearly of importance.

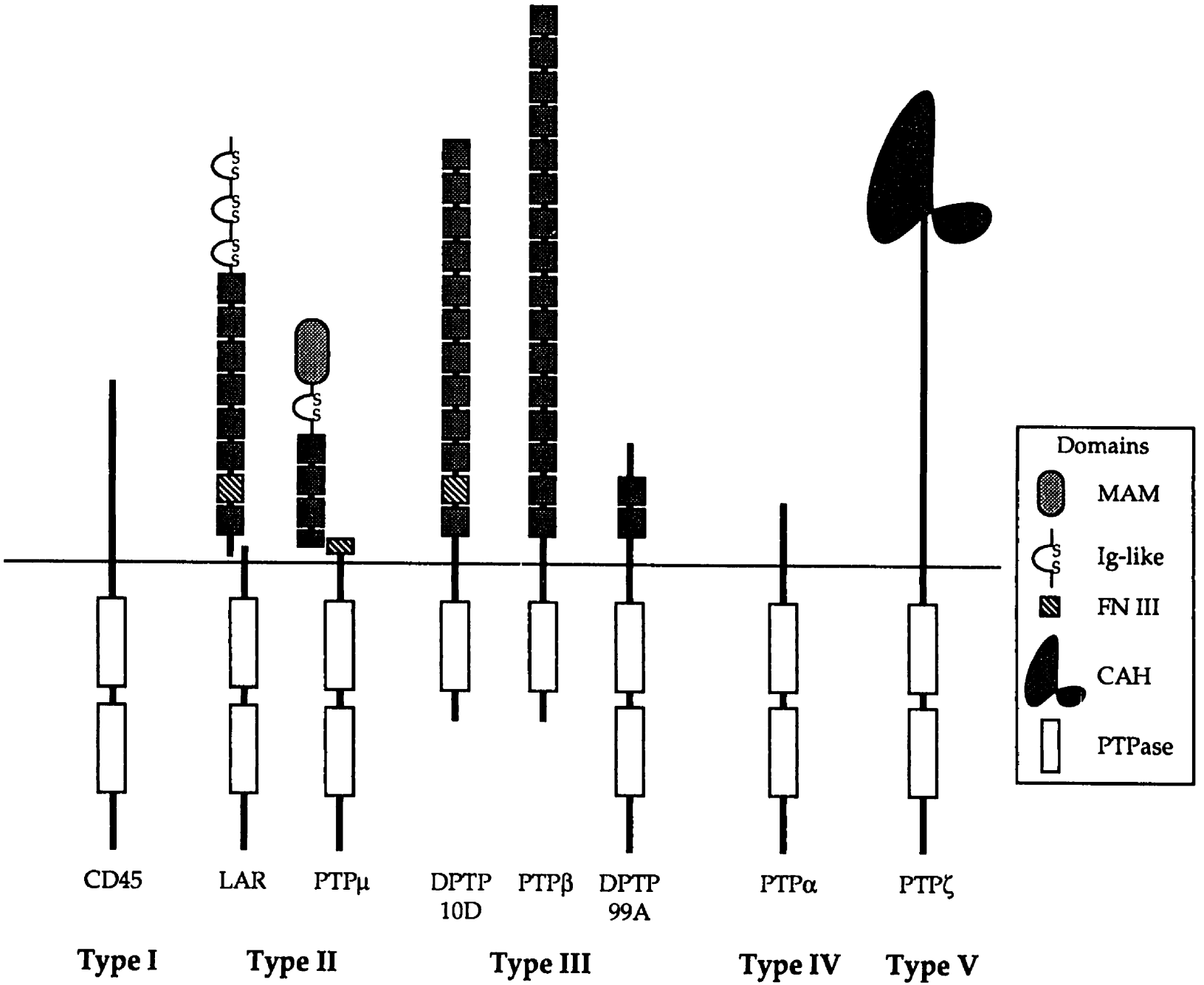
The type III receptor-like PTPases are characterized by the unique presence of FN III motifs in their extracellular portion and harbour no other known structural features. In *Drosophila,* this class of PTPase is involved in the processes by which growing axons arborify throughout their appropriate nervous tracks (for a review of PTPases in Drosophila, see (Zinn 1993). The signaling pathways mediating this axonal guidance phenomenon await characterization. However, this class of PTPases may provide valuable information regarding the mechanisms behind the transduction of these signals.

Table 1. Structural organization of receptor-like PTPases.

Type	Members	Structural features of extracellular portion
I	CD45[1]	Heavily glycosylated, cystein-rich segment
II	LAR[2], PTPδ[3], DPTP69D[4], CRYPα[5] RPTPσ[6]/PTPNU-3[7] PTPμ[8], and PTPκ[9]	Ig-like domains and FN III motifs
III	PTPβ[10], DEP-1[11], SAP-1[12], GLEPP1[13]/ PTPU2[14], DPTP10D[15], DPTP4E[16], DPTP99A[17]	FN III motifs alone
IV	PTPα[18] and PTPε[19]	Short highly glycosylated extracellular segments
V	PTPγ[20] and PTPζ[21]/β[22]	Amino-terminal CAH-like motifs

[1](Trowbridge and Thomas 1994), [2](Streuli et al. 1989), [3](Krueger et al. 1990), [4](Desai et al. 1994), [5](Stoker 1994), [6](Yan et al. 1993), [7](Wagner et al. 1994), [8](Gebbink et al. 1991), [9](Jiang et al. 1993), [10](Krueger et al. 1990), [11](Ostman et al. 1994), [12](Matozaki et al. 1994), [13](Thomas et al. 1994), [14](Seimiya et al. 1995), [15](Yang et al. 1991), [16](Oon et al. 1993), [17](Tian et al. 1991), [18](Daum et al. 1991), [19](Krueger et al. 1990), [20](Kaplan et al. 1990), [21](Krueger et al. 1990), [22](Levy et al. 1993).

Figure 1. Graphical representation of members of the receptor-like family of PTPases. Members of this family of PTPases are characterized by the presence of one or two catalytic domains, a single transmembrane domain and an extracellular portion of variable length and composition. These PTPases can be subdivided into five types on the basis of their extracellular structure. Prototype PTPases are represented for each type of enzyme.


Domains
MAM
Ig-like
FN III
CAH
PTPase
CD45
LAR
PTPμ
DPTP 10D
PTPβ
DPTP 99A
PTPα
PTPζ
Type I
Type II
Type III
Type IV
Type V

Only two members define the type IV receptor-like PTPases. PTPα and PTPε both have a short highly glycosylated extracellular domain with no known function. Overexpression of PTPα has been shown to cause oncogenic transformation of fibroblasts through its action on the downregulatory phosphorylated tyrosine residue of Src (Y^{527}) (Zheng et al. 1992). Like most PTPases, the role(s) that the members of this family perform on cellular function is largely unknown.

The enzymes RPTPβ (also known as RPTPζ) and RPTPγ represent another class of transmembrane PTPases (type V). The extracellular domain of these PTPases is characterized by the presence of an amino-terminal carbonic anhydrase (CAH)-like domain, a fibronectin type III repeat and a long cysteine-free (spacer) region (Barnea et al. 1993; Krueger and Saito 1992; Levy et al. 1993). The expression of RPTPβ/RPTPζ is restricted to the central and peripheral nervous systems (Krueger and Saito 1992; Levy et al. 1993), whereas RPTPγ is expressed both in the developing nervous system and in a variety of other tissues in the adult murine animal (Barnea et al. 1993; Canoll et al. 1993). Structural modelling of the CAH-like domain of RPTPγ based on the crystal structure of CAH suggests that the CAH-like domain of RPTPγ may have a function other than catalysis of hydration of metabolic CO_2 (Barnea et al. 1993). Recently, functional cloning experiments designed towards the identification and purification of proteins interacting with the CAH-like domain of RPTPβ resulted in the isolation of the protein contactin (Peles et al. 1995). It was shown that the CAH-like domain of RPTPβ act as a ligand for contactin.

Contactin is a GPI-anchored cell recognition glycoprotein expressed in a restricted manner on specific axons during development (Peles et al. 1995). The spatial and temporal expression patterns of both contactin and RPTPβ suggest that this kind of interaction may play an important role during development of the nervous system.

Intracellular PTPases

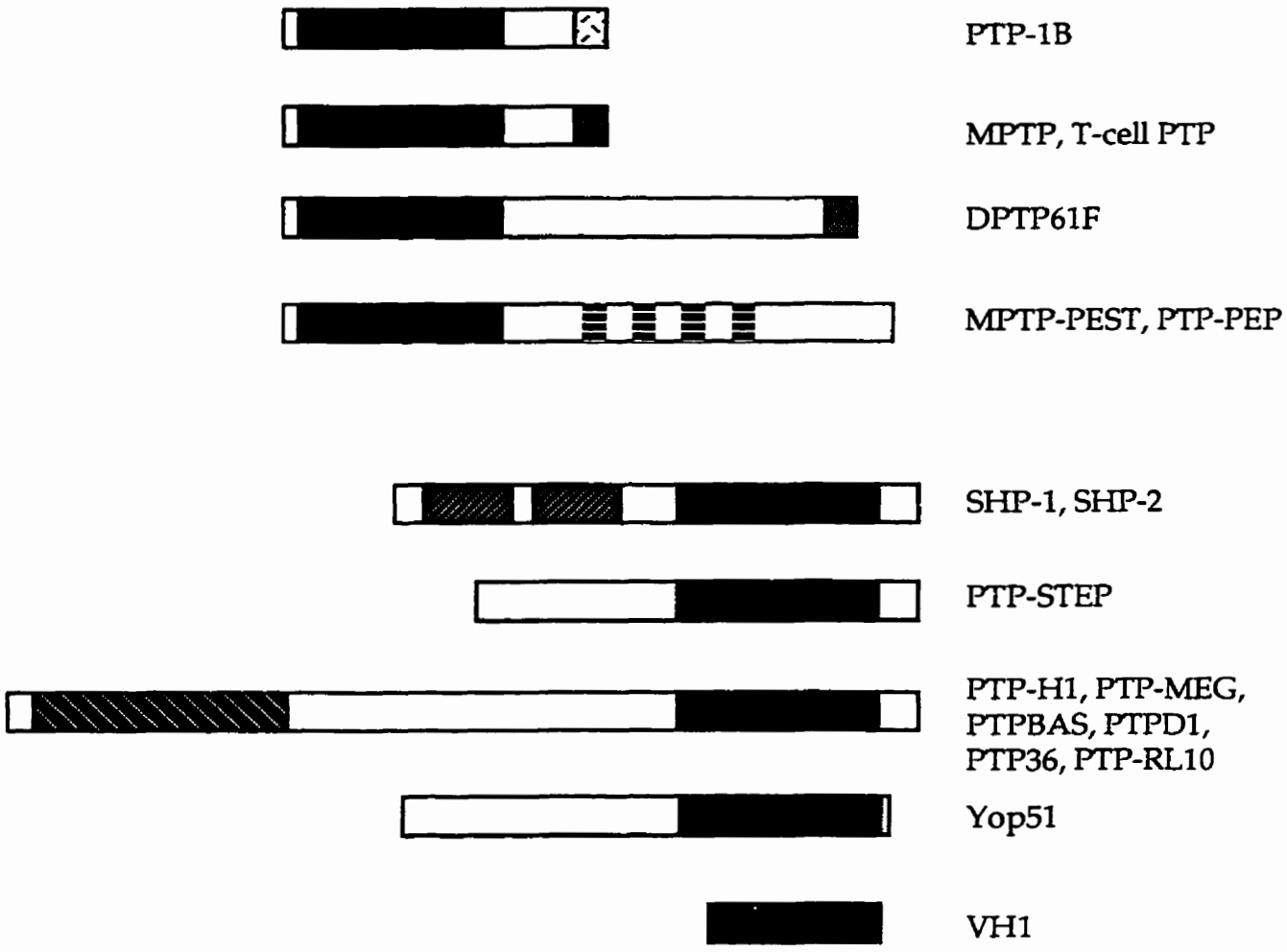
The dual specific PTPases are members of the intracellular family of PTPases. The first member of this class (termed VH1) was identified in vaccinia virus (Guan et al. 1991). VH1 was shown to be a small soluble phosphatase capable of dephosphorylating both phosphotyrosine- and phosphoserine-containing substrates. VH1-like phosphatases have also been identified in various viruses (Hakes et al. 1993) in yeast (Guan et al. 1992), and in mammals (Alessi et al. 1993; Charles et al. 1993; Rohan et al. 1993a). In mammals, the VH1-like phosphatase MKP-1 (also known as 3CH134) has been shown to dephosphorylate activated (i.e. phosphorylated on Thr183 and Tyr185 residues) mitogen-activated protein kinase (MAP kinase) resulting in a loss of MAP kinase activity (Sun et al. 1993a). MAP kinase has been shown to be a major component of the signaling pathway involved in transducing signal from activated protein tyrosine kinases to downstream signal-regulated gene expression (for reviews see (Crews and Erikson 1993; Pelech and Sanghera 1992). Modulation of these signal transduction events via the dephosphorylation of MAP kinase represents a potential function for MKP-1.

Table 2. Structural organization of intracellular PTPases.

Members	Comments	Structural features
MPTP-PEST/PTP-G1/ RK-PTP[1], PTP-PEP[2]	PEP family of PTPases	Contains PEST sequences
MPTP/ T-cell PTPase [3]		Nuclear PTPase
SHP-1[4], SHP-2[5]		SH2-containing PTPases
PTP-STEP[6]		Striatum-enriched PTPase
PTP-H1[7], PTP-MEG1[8] PTP-BAS[9], PTP-RL10[10] PTP-D1[11], PTP36[12]	Band 4.1-related family of PTPases	Cytoskeletal-associated protein band 4.1 homology
PTP1B[13]		Endoplasmic reticulum-associated PTPase
DPTP61F[14]		
Yop51[15], VH1[16], PAC-1[17], MKP-1[18], MKP-2[19], MKP-3[20]	Dual-specificity family of PTPases	Dephosphorylate phospho-serine, threonine, and tyrosine

[1](Charest et al. 1995a; Moriyama et al. 1994; Takekawa et al. 1992), [2](Matthews et al. 1992), [3](Cool et al. 1989; Tillmann et al. 1994), [4](Shen et al. 1991), [5](Freeman et al. 1992), [6](Li et al. 1995), [7](Yang and Tonks 1991), [8](Gu et al. 1991), [9](Maekawa et al. 1994), [10](Higashitsuji et al. 1995), [11](Moller et al. 1994), [12](Sawada et al. 1994), [13](Chernoff et al. 1990), [14](McLaughlin and Dixon 1993), [15](Guan and Dixon 1990a), [16](Guan et al. 1991), [19](Rohan et al. 1993b), [20](Sun et al. 1993a), [21](Chu et al. 1996), [22](Muda et al. 1996).

Figure 2. Graphical representation of members of the intracellular family of PTPases. Members of this family of PTPases possess a single catalytic domain with flanking regions composed of various structural features. Carboxy-terminal domains can target PTPases to subcellular compartments such as the endoplasmic reticulum (PTP1B) or the nucleus (DPTP61F and MPTP). Amino-terminal domains may direct PTPases to interact with cytoskeletal proteins (PTP-H1) or with phosphotyrosine-containing proteins via SH2 domains (SHP-1 and SHP-2). Prototype PTPases are represented for each type of enzyme.


PTP-1B
MPTP, T-cell PTP
DPTP61F
MPTP-PEST, PTP-PEP
SHP-1, SHP-2
PTP-STEP
PTP-H1, PTP-MEG,
PTPBAS, PTPD1,
PTP36, PTP-RL10
Yop51
VH1
PTPase
Endoplasmic reticulum targeting
Nuclear targeting
PEST sequences
SH2
Band 4.1-homology

The intracellular PTPases usually contain a single catalytic domain and various amino- and carboxyl-terminal extensions (see figure 2). Several lines of evidence suggest that the substrate specificity of PTPases is not solely governed by the primary amino acid sequence surrounding the target phosphotyrosine residue (Stone and Dixon 1994). An additional factor regulating the PTPase substrate specificity is localization of the catalyst by virtue of various amino- and carboxyl-terminal extensions. These flanking motifs are thought to confer regulatory information in the control of PTPase activity (Mourey and Dixon 1994).

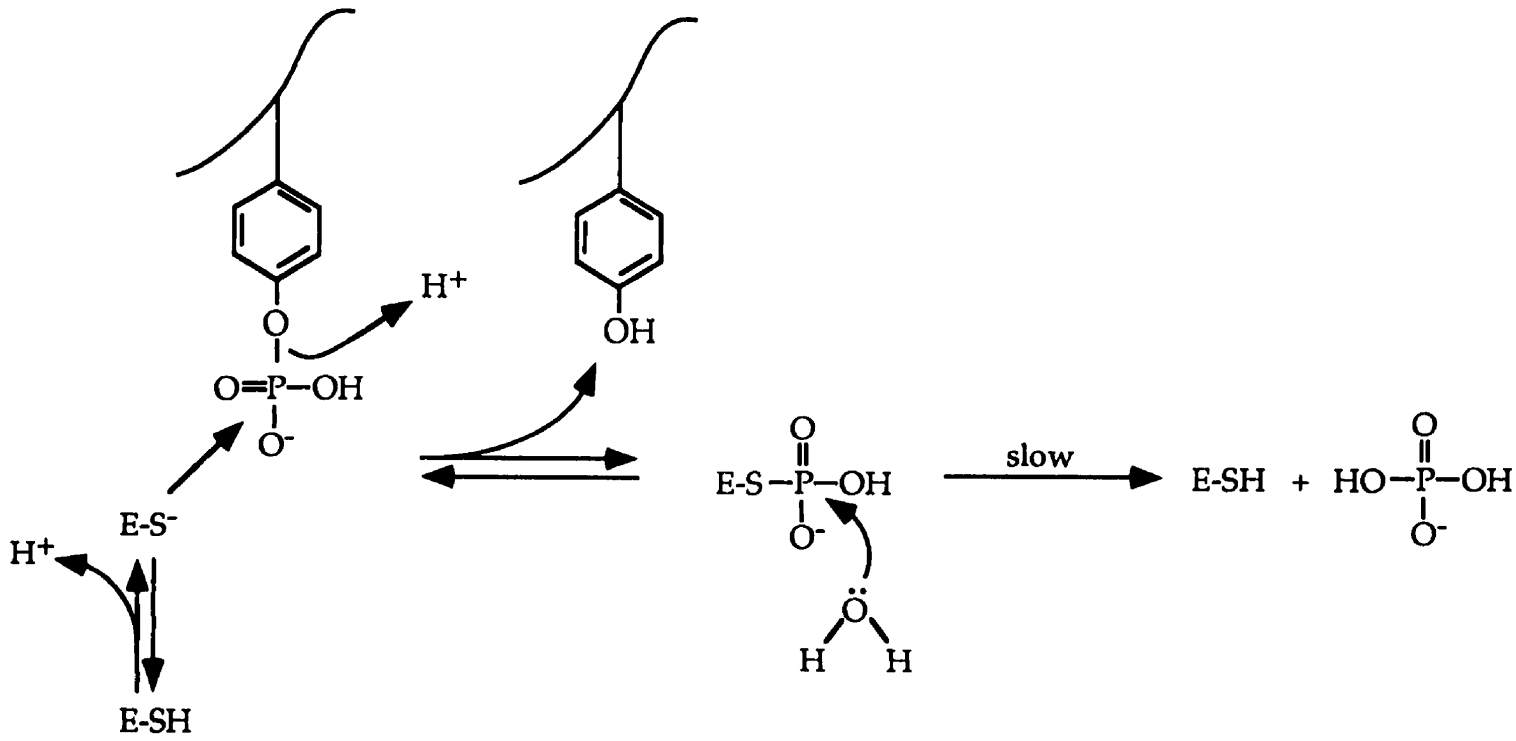
Subcellular localization experiments performed on PTP1B, T-cell PTPase, and *Drosophila* DPTP61F, demonstrate that the carboxyl-termini of these PTPases are involved in subcellular localization of the enzymes. PTP1B localizes to the cytoplasmic face of the endoplasmic reticulum via its terminal 35 amino acid residues (Frangioni et al. 1992). The T-cell PTPase and DPTP61F are both targeted to the nucleus by carboxyl-terminal sequences which closely resemble bipartite nuclear targeting sequences (McLaughlin and Dixon 1993; Tillmann et al. 1994). Interestingly, alternative splicing at the 3′ end of the DPTP61F message alters the carboxyl terminus of the protein and result in the relocation of the PTPase to the cytoplasmic portion of the cell (McLaughlin and Dixon 1993). This phenomenon underlies the potential for subcellular localization to restrict and partially define the substrate specificity of PTPases within the cell.

Several PTPases contain previously identified functional motifs that suggest localization to specific regions or subcellular compartments of the cell. For example, members of the band 4.1 subfamily of PTPases

including PTPMEG1, PTPH1, PTPBAS, PTP-RL10, PTP36, and PTPD1 all contain at their amino-terminus a region that is homologous to the cytoskeletal-associated band 4.1 protein (Gu et al. 1991; Higashitsuji et al. 1995; Maekawa et al. 1994; Moller et al. 1994; Sawada et al. 1994; Yang and Tonks 1991). The presence of such domains suggests that these PTPases may function in cytoskeletal rearrangement and/or reorganization by modulating cytoskeletal associated tyrosine phosphorylation (Yang and Tonks 1991).

Two PTPases have been shown to be targeted to tyrosine phosphorylated proteins. SHP-1 (also known as PTP1C (Shen et al. 1991), SH-PTP1 (Plutzky et al. 1992), HCP (Yi et al. 1992), and SHP (Matthews et al. 1992) and SHP-2 (also referred to SYP (Feng et al. 1993b), SH-PTP2 (Freeman et al. 1992), PTP2C (Ahmad et al. 1993), PTP1D (Vogel et al. 1993), and SH-PTP3 (Adachi et al. 1992) contain a tandem repeat of SH2 domains located at the amino terminal end of the proteins. SH2 domains are conserved modular domains of approximately 100 amino acids that recognize and bind in a sequence specific manner to tyrosine phosphorylated proteins. SH2 domains thus promote interactions between SH2-containing proteins, specific activated growth factor receptors and other tyrosine phosphorylated signaling molecules. The presence of such modular binding domains capable of targeting these PTPases to tyrosine phosphorylated proteins underlies a direct role for these PTPases in the regulation of signaling events.

Figure 3. Mechanism of action of PTPases. The protonated form of the enzyme (E-SH) becomes reduced to the thiol anion reactive form of the enzyme which attacks the phosphate of the phosphotyrosine substrate. This releases the dephosphorylated protein substrate and creates a thiolphosphate intermediate. In the second step of the reaction, a molecule of water attacks this intermediate, regenerating active enzyme and releasing inorganic phosphate.


H+
O
O=P–OH
O-
OH
E-S-
H+
E-SH
E-S–P–OH
O-
slow
E-SH + HO–P–OH
O-
H H

Detailed structural and functional descriptions of every member of the PTPase subfamilies are beyond the scope of this introduction. However, PTPases relevant to the work reported herein will be described in the appropriate sections below.

PTPase structure and mechanism of action

A common feature exhibited by all PTPases is their sensitivity to sulfhydryl reacting compounds. PTPases are irreversibly inhibited by alkylating compounds such as iodoacetate suggesting the presence of a reactive sulfhydryl residue essential for catalysis (Tonks et al. 1988b; Tonks et al. 1990). [^{14}C]-iodoacetate trapping experiments (Pot and Dixon 1992) and site-directed mutagenesis (Guan et al. 1990b; Streuli et al. 1989) experiments on various PTPases demonstrated that a single conserved cysteine residue is essential for PTPase activity. This cysteine residue is part of a PTPase active site signature sequence ((I/V)HCXAGXGR(S/T)G) which is conserved among all PTPases.

Using ^{32}P-labelled substrates and a technique of rapid denaturation, Dixon and coworkers were able to trap radioactively labelled phosphocysteine intermediates of PTPases (Guan and Dixon 1991; Pot et al. 1991). The formation of these intermediates was transient and was dependent on the presence of the catalytically essential cysteine residue. Together with kinetic data (Zhang and VanEtten 1991), these analyses have allowed for the definition of an enzymatic mechanism of dephosphorylation for PTPases (see figure 3). Briefly, ionic interactions provided from adjacent residues stabilize the

thiolate anion of the essential cysteine in its reduced state. This nucleophilic cysteine then attacks the phosphate group of the phosphotyrosine protein substrate, releasing the dephosphorylated substrate and forming a phosphothio-enzyme intermediate. Finally, water molecules hydrolyse this intermediate thereby releasing inorganic phosphate and the enzyme. The latter step appears to be the rate limiting step of the entire reaction (Pot and Dixon 1992).

Several elements of this catalytic mechanism can be explained by structural analysis of the catalytic domain of PTPases. Recently, the structures of the catalytic domains of two PTPases, PTP1B (Barford et al. 1994) and *Yersinia* Yop51 (Stuckey et al. 1994), were elucidated by X-ray crystallography using tungstate as a non-hydrolysable binding substrate. Even though both PTPases share only 20% sequence identity, comparison of their three-dimensional structure demonstrates striking similarities.

Both catalytic domains are composed of a twisted and mixed structure of β-sheets flanked by α-helices. The structural features within the molecular crevice that makes up the active site of each PTPase are remarkably similar. The base of the catalytic site is formed by the amino acids comprised between the His and Arg residues of the PTPase signature motif (I/V)HCXAGXGR(S/T)G. A phosphate binding site situated in the catalytic crevice occurs within a GxGxxG sequence motif. This motif is also found in many phosphate binding proteins (Barford et al. 1994). In PTP1B, the thiol anion of the invariant Cys residue is stabilized by a salt bridge to the Arg residue of the signature motif and by the helix dipole of an adjacent α-helix (Barford et al. 1994). In Yop51 PTPase, the invariable Cys^{403} residue seems to be stabilized by

an intricate and extensive network of hydrogen-bonds. This stability is considered to be responsible for the low pK_a (4.7) of this catalytically essential Cys residue (Zhang and Dixon 1993). The structure of the PTP1B-tungstate complex reveals that the Sγ of the Cys residue is ideally positioned to act as a nucleophile on a bound phosphotyrosine substrate. The Histidine residue that invariably precedes the essential Cys does not interact directly with either the phosphate ion or the side chain of the Cys residue. A direct role for this conserved His residue in catalysis is unlikely as it appears to play a structural function in defining the conformation of Cys and the phosphate binding loop (Barford et al. 1994). The sides of the catalytic cleft are lined with the side chains of several amino acids that form interactions with the tyrosine moiety of the substrate. These interactions are reminiscent of the mechanistic model of binding to phosphotyrosine peptides displayed by SH2 domains (Barford et al. 1994). Based on the conservation of amino acid sequence between the catalytic domains of PTP1B and Yop51 and those of other PTPases, the structures reported for PTP1B and Yop51 are most likely very similar to that of other PTPases.

Molecular signaling

The transduction of an extracellular signal into specific physiological intracellular responses usually involves the transmission of a complex series of controlled and amplified signals to specific targets. Growth factors that elicit their signaling through tyrosine kinases, can trigger a wide range of biochemical responses, ultimately leading to changes in the proliferation, architecture, differentiation, and metabolism of their target cells. The molecular basis underlying

specific signal transduction events remains largely unknown. With many transduction pathways known only in outline, finding the relevant units and understanding their role in the many specific pathways represents an exciting richness yet to be elucidated.

SH2 domains

Protein-protein interactions have long been studied, but the recent identification of defined binding modules illuminated the means by which receptor tyrosine kinases select their targets and thereby stimulate specific intracellular signaling pathways. The *src* oncogene from the Rous sarcoma virus, possesses, in addition to a tyrosine kinase catalytic domain, two domains that are also found in a remarkably diverse group of cytoplasmic proteins. The Src homology 2 (SH2) domain is a protein module of approximately 100 amino acids which directly recognizes and binds tightly to phosphotyrosine. The Src homology 3 (SH3) domain is a protein module of approximately 50 amino acids that has been recently shown to bind to proline-rich amino acid sequences. The discovery of these protein-binding modules represents a major breakthrough in the molecular elucidation of various signaling pathways.

SH2 and SH3 domains are true protein domains as they form compact units that maintain their structure in isolation. They are not restricted to certain type of proteins involved in signal transduction since they are found in numerous protein kinases, protein phosphatases, lipid kinases, phospholipases, proteins controlling RAS activity, and transcription factors. They are also found in adaptor proteins which are molecules devoid of enzymatic function that

mediates the aggregation of other proteins. SH3 domains are found in all eukaryotic organisms, including yeast, while SH2 domains are not found in yeast. Neither of these two domains has been observed in prokaryotes.

The transduction of extracellular signals into intracellular molecular changes using phosphotyrosine as a medium can be accomplished by two methods. For example, members of the cytokine receptor superfamily (which includes the receptors for granulocyte/macrophage colony-stimulating factor, the interleukins IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, and erythropoietin (EPO)) lack intrinsic protein tyrosine kinase activity. However, activation of these receptors has been shown to result in a rapid and transient tyrosine phosphorylation of a number of cellular proteins, including the receptors themselves. Generally, cytokine binding causes the receptor to dimerize which results in the recruitment and activation of the cytoplasmic Janus kinases (JAKs) that will interact noncovalently with the intracellular portion of the receptors (for reviews on cytokine signaling see (Ihle 1995; Ihle et al. 1994). Similarly, the engagement of the non-tyrosine kinase T cell receptor (TCR) by major histocompatibility complex-bound peptides leads to tyrosyl phosphorylation of various cellular proteins including several components of the receptor. The T cell receptor signals through cytoplasmic tyrosine kinases of the Src family that noncovalently associate to various receptor components (for a detailed review of lymphocyte signaling see (Perlmutter et al. 1993; Weiss and Littman 1994).

On the other hand, many growth factors mediate their biological

responses by binding to and activating cell-surface receptors with intrinsic tyrosine kinase activity. Here, binding of the growth factor causes a receptor dimerization which leads to the activation of their intrinsic kinase activity resulting in receptor autophosphorylation and phosphorylation of several cellular proteins (for review see (Lemmon and Schlessinger 1994). In both systems, receptor phosphorylation acts as a molecular switch to create high affinity binding sites for SH2 domains of various cytoplasmic signaling proteins. Binding of these SH2-containing signaling molecules to the phosphorylated receptors targets them for activation.

High affinity binding of an SH2 domain to a tyrosine phosphorylated residue requires the presence of specific amino acid sequences surrounding the phosphotyrosine. Mapping of SH2 binding sites revealed that the specificity is largely determined by the three residues immediately carboxyl to the pTyr (for a detailed review see (Cohen et al. 1995). In principle then, the ability of a given receptor to bind specific SH2-containing proteins and thereby activate the corresponding pathways, will depend on the sequence context of its phosphorylation sites.

The transmission of signals via SH2 domains can be described by the following molecular mechanisms. First, binding of an SH2 domain-containing protein to the appropriate pTyr binding site can alter the subcellular localization of the protein in question thereby relocating it closer to substrate(s) and/or effector protein(s). Secondly, binding of the SH2 domain to its pTyr target sequence can induce conformational changes that alter the catalytic activity of the interacting proteins. Numerous examples for both mechanisms have been documented

over the years and are well reviewed in Cohen *et al.*, (1995).

SH3 domains

While the knowledge concerning the biological function of SH2 domains has evolved quite rapidly, understanding the biology of SH3 domains has lagged behind until recently. Partial elucidation of the biological functions of SH3 domains arose when two proteins, termed 3BP-1 and 3BP-2, were shown to specifically interact with the SH3 domain of the tyrosine kinase Abl (Cicchetti et al. 1992). Detailed mapping of the SH3 binding sites on 3BP-1 and 3BP-2 revealed a sequence of ~10 amino acids unusually rich in proline residues (Ren et al. 1994). Since then, several other *in vivo* SH3 ligands have been identified. As well, all of the SH3 binding sites characterized so far are composed of similar Pro-rich sequences.

The biological relevance of the SH3 domain:proline-rich interaction was demonstrated when the SH3-SH2-SH3 adaptor protein Grb2 (also known as Sem-5 in *Caenorhabditis elegans*, and drk in *Drosophila*) was shown to mediate the activation of $p21^{ras}$ protein by receptor tyrosine kinases in mammals, flies and worms (Clark et al. 1992; Lowenstein et al. 1992; Olivier et al. 1993). The involvement of Ras in propagating signals downstream of tyrosine kinases was originally suggested by anti-Ras antibody microinjection studies which showed that by neutralizing Ras, one can block the ability of both normal growth factor receptors and oncogenic tyrosine kinases to induce DNA synthesis in fibroblasts and neurite outgrowth in PC12 cells (de Vries-Smits et al. 1992; Mulcahy et al. 1985; Smith et al. 1986; Thomas et al. 1992; Wood et al. 1992).

The role of Grb2 in recruiting a Ras activator to receptor tyrosine kinases to form stable complexes at the plasma membrane where Ras activation is taking place has been described by several groups (Buday and Downward 1993; Chardin et al. 1993; Egan et al. 1993; Gale et al. 1993; Li et al. 1993; Olivier et al. 1993; Skolnik et al. 1993). The Ras activator, known as Sos (Son of sevenless) in *Drosophila* and mSos1 in mammals, functions as a guanine-nucleotide-releasing protein converting inactive RasGDP to the active GTP-bound form of the protein by nucleotide exchange (Buday and Downward 1993; Chardin et al. 1993; Egan et al. 1993). The interaction between Grb2 and mSos1 was found to be mediated by the constitutive binding of the SH3 domains of Grb2 to several proline-rich sequences located at the COOH-terminus of mSos1 (Chardin et al. 1993; Egan et al. 1993; Li et al. 1993; Rozakis-Adcock et al. 1993). Upon growth factor stimulation, the Grb2:mSos1 complex is recruited to the tyrosine phosphorylated receptors via the SH2 domain of Grb2. Binding of the Grb2:mSos1 complex to autophosphorylated receptors relocates the guanine-nucleotide exchange activity from the cytoplasm to the plasma membrane where Ras protein is located.

The importance of the SH3 domain:proline-rich motif-mediated Grb2:mSos1 interaction in the activation of $p21^{ras}$ by growth factor receptors led to the discovery of several additional SH3 binding sites (for review see (Mayer and Eck 1995). As proline-rich regions are relatively common in proteins (for a review see (Williamson 1994), the establishment of specific rules defining what constitutes a high-affinity SH3 binding site and the structural requirements determining the specificity of binding among different SH3 domains was needed. To

address these issues, studies involving the selection of peptide ligands from random phage display libraries (Cheadle et al. 1994; Sparks et al. 1994) or biased combinatorial peptide library (Yu et al. 1994) in addition to the structural analysis of over 10 different SH3 domains by X-ray crystallography or NMR spectroscopy (Feng et al. 1994b; Goudreau et al. 1994; Lim et al. 1994; Musacchio et al. 1994; Wittekind et al. 1994; Wu et al. 1995) were performed.

The results of these studies demonstrated that proline-rich ligands, adopting a left-handed polyproline-II helix (PPII) conformation and fitting snugly in a set of shallow hydrophobic binding pockets on the surface of SH3 domains, can be classified into two classes. Class I proline-rich ligands follow the consensus binding sequence $_{NH2}$Arg-X-X-Pro-X-X-Pro$_{COOH}$ (where X represents any amino acid), whereas class II SH3 ligands display a $_{NH2}$Pro-X-X-Pro-X-Arg$_{COOH}$ consensus sequence and bind with opposite polarity to that seen for class I ligands. The left-handed polyproline-II helix nature of SH3 ligands entail that the two proline residues of the Pro-X-X-Pro core motif be on the same face of the helix. The proline residues at the base of the triangle intercalate with conserved hydrophobic residues of two binding sites on the surface of the SH3 domain. The terminal charged residue, usually an arginine, binds to an acidic residue on a third binding site. Because the amino acid side chains at the apex of the triangle extend away from the domain, any amino acid can be found at these positions. However, proline is commonly found as it probably stabilizes the PPII conformation (Mayer and Eck 1995). The selection for class I or class II binding modes lies in the amino- or carboxyl-terminal position of the arginine residue in addition to the subtle differences in van der Waal's bonding within binding sites (Mayer and Eck 1995).

Our present knowledge of the structures of several SH3 domains and the mechanism of binding of SH3 domains to proline-rich sequences will facilitate the search for new SH3 domain ligands. Finding novel proteins that interact with the SH3 domains of proteins involved in signal transduction will solidify the function of SH3 domains in signaling events and will deepen our understanding of signal transduction at the molecular level.

PTB domains

Shc was identified in a screen for human cDNAs encoding novel SH2-containing proteins using a cDNA fragment corresponding to the SH2 domain of c-fes as a probe (Pelicci et al. 1992). Shc proteins contain a carboxyl-terminal SH2 domain, an amino-terminal PTB domain and a central proline- and glycine-rich domain with some homology to α1 collagen (Kavanaugh and Williams 1994; Pelicci et al. 1992). Anti-Shc antisera specifically recognize three proteins of apparent molecular weight 46, 52 and 66 kDa from cell lysates. These three ubiquitously expressed proteins are generated from the same Shc mRNA by alternative splicing and alternative initiation of translation (O'Bryan et al. 1996; Pelicci et al. 1992) which make them different from each other only by their amino-terminal sequences.

Shc proteins are constitutively phosphorylated on serine residues in resting cells and become phosphorylated on tyrosine residues upon stimulation with a large number of growth factors and cytokines (Burns et al. 1993; Cutler et al. 1993; Kovacina and Roth 1993; Pronk et al. 1993; Rozakis-Adcock et al. 1992; Ruff-Jamison et al. 1993;

Segatto et al. 1993) as well as G-protein coupled receptors (Lev et al. 1995; van Biesen et al. 1995)). Shc proteins are also phosphorylated on tyrosine residues in cells transformed by oncogenic proteins such as v-Src, v-Fps, v-Sea and Bcr-Abl (Crowe et al. 1994; McGlade et al. 1992; Puil et al. 1994) and it has been proposed that this phosphorylation event was necessary for mediating the oncogenic properties of these proteins (Crowe et al. 1994; McGlade et al. 1992). The major site of tyrosine phosphorylation on Shc proteins is the tyrosine residue, Y^{317} which constitutes a high-affinity binding site for the SH2 domain of Grb2 (McGlade et al. 1992; Rozakis-Adcock et al. 1992; Salcini et al. 1994). Tyrosine phosphorylation of Shc mediates the activation of Ras signaling pathways following stimulation by growth factors (Crowe et al. 1994; Rozakis-Adcock et al. 1992; Salcini et al. 1994) and G-protein-coupled receptors (Lev et al. 1995; van Biesen et al. 1995) by its ability to bind to Grb2:Sos complexes. Consistent with this scenario, overexpression of Shc proteins induces transformation of rodent fibroblasts in a fashion that is dependent on Y^{317} (Salcini et al. 1994). Similarly, overexpression of Shc promotes neurite-outgrowth in PC12 cells in a Ras-dependent fashion (Rozakis-Adcock et al. 1992).

Investigation of proteins that coimmunoprecipitate with Shc in growth factor-stimulated cells revealed the presence of a tyrosine phosphorylated protein of ~145 kDa (Kavanaugh and Williams 1994). *In vitro* mapping experiments demonstrated that the amino-terminal region of Shc is responsible for this interaction and that tyrosine phosphorylation of the pp145 was required for binding (Kavanaugh and Williams 1994). These results led to the definition of a novel phosphotyrosine binding domain (designated PTB domain) and assigned a function to the amino-terminal region of Shc. Subsequent

work by several other groups corroborated the identity of the PTB domain in Shc (Blaikie et al. 1994; van der Geer et al. 1995). cDNA library screening using the amino-terminal PTB domain of Shc as a probe resulted in the isolation of a novel Shc-related protein called Sck (Kavanaugh and Williams 1994). Recently, low stringency screenings of mouse cDNA libraries using Shc cDNAs as probes resulted in the isolation of an additional member of the Shc family of proteins called Shc C (O'Bryan et al. 1996). Interestingly, Shc C is specifically expressed in brain tissues (O'Bryan et al. 1996). Both Sck and Shc C contain an amino-terminal PTB domain (Kavanaugh and Williams 1994; O'Bryan et al. 1996). The Shc C PTB domain has been shown to specifically associate *in vitro* with the autophosphorylated receptors for NGF and EGF (O'Bryan et al. 1996). These results indicate that the PTB domain represent a novel phosphotyrosine recognition module.

The PTB domain was defined as the region between amino acids 46-209 of the Shc protein. This region is structurally unrelated to SH2 domains and was shown to bind with high affinity to phosphotyrosine residues found on several growth factor receptors. Searches of various sequence data bases geared towards the identification of proteins with sequences analogous to the PTB domain of Shc revealed several putative PTB domain-containing proteins (Bork and Margolis 1995; Yajnik et al. 1996). These proteins are unrelated to each other, suggesting a general role for the PTB domain in protein-protein interactions (Bork and Margolis 1995; Yajnik et al. 1996). In addition, this demonstrates that the PTB domain is not unique to Shc proteins but rather represents a modular domain that can be found in various proteins and is somewhat analogous to SH2 and SH3 domains.

The PTB domain of Shc has been shown to bind to the autophosphorylation sites of TrkA Tyr^{490} (Blaikie et al. 1994; Dikic et al. 1995; van der Geer et al. 1995), c-Erb B2 Tyr^{1222} (Kavanaugh et al. 1995; Kavanaugh and Williams 1994), EGF receptor Tyr^{1148} (Batzer et al. 1995; Blaikie et al. 1994; Prigent et al. 1995; van der Geer et al. 1995), c-Erb B3 Tyr^{1309} (Prigent et al. 1995), insulin-like growth factor 1 receptor Tyr^{950} (Craparo et al. 1995), insulin receptor Tyr^{960} (He et al. 1995; Isakoff et al. 1996), and the phosphorylated Tyr^{250} residue of polyoma middle T antigen (Trub et al. 1995). Sequence analysis of the growth factor receptors binding sites, peptide competition assays, phosphotyrosine peptide library screenings and isothermal titration calorimetric studies have demonstrated that the PTB domain of Shc preferentially binds to the sequence motif ΦXNPXpY with high affinity (single letter amino acid code, pY represents phosphotyrosine, X represents any amino acid and Φ represents a hydrophobic residue) (Batzer et al. 1995; Kavanaugh et al. 1995; Mandiyan et al. 1996; Prigent et al. 1995; van der Geer and Pawson 1995). It is proposed that the PTB domain recognizes the β-turn that is found in NPXpY-containing peptides and that this β-turn may be required for the simultaneous interaction of the PTB domain with both the amino-terminal residues of the peptide and the phosphotyrosine residue (Trub et al. 1995; Zhou et al. 1995a; Zhou et al. 1995b).

Independent work in the insulin receptor signaling field has revealed the presence of PTB domains in the insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) proteins (He et al. 1995; Sun et al. 1995; Wolf et al. 1995). IRS proteins are cytoplasmic docking proteins that serve as a link between various activated receptors (such as insulin receptor, insulin-like growth factor receptor, IL-4, -9 and -13 and other cytokine

receptors) and SH2-containing effector proteins (Sun et al. 1995). The PTB domain of IRS-1 has been shown to mediate the recruitment of the latter to the insulin receptor by binding to an autophosphorylated juxtamembrane NPXpY sequence motif found in the insulin receptor β-subunit (O'Neill et al. 1994; White et al. 1988). This recruitment facilitates the phosphorylation of IRS-1 on several tyrosine residues by activated insulin receptors. These tyrosine phosphorylation sites on IRS-1 now serve as binding sites for SH2-containing proteins such as SHP-2, p85 subunit of PI3-K and Grb2. Binding of these effector proteins mediates the activation of the corresponding second messenger pathways (for reviews on insulin signaling see (Myers and White 1993; White and Kahn 1994).

Interestingly, the PTB domains of Shc and IRS-1 and -2 share no sequence similarity except for three fully conserved amino acid residues (G^{58}, S^{151}, and H^{187} of $p52^{Shc}$). The PTB domains of IRS-1 and Shc demonstrate distinct but overlapping interactions with ΦXNPXpY containing sequence motifs (Wolf et al. 1995). Despite the lack of sequence similarity, the PTB domain of IRS-1 and Shc perform analogous functions. This contradictory relationship has recently been resolved by studies of the 3-dimensional structures of both IRS-1 (Eck et al. 1996) and Shc (Zhou et al. 1995a) PTB domains by X-ray crystallography and NMR spectroscopy analyses respectively. Both PTB domains share a core pleckstrin homology domain (PH) fold and a common overall mode of NPXpY peptide binding (Eck et al. 1996; Zhou et al. 1995a).

The PH domain was originally identified as a region of homology (~120 amino acids) duplicated in the protein pleckstrin.

Sequence analysis has allowed the identification of over 90 different putative PH domains in proteins involved in cellular signaling or cytoskeletal functions (for a review on PH domains see (Gibson et al. 1994; Lemmon et al. 1996). PH domains have been suggested to mediate protein-protein interactions in signaling processes.

The mechanism of phosphotyrosine recognition displayed by both IRS-1 and Shc PTB domains diverge quite considerably. The residues that coordinate the phosphotyrosine do not appear to be conserved between the two PTB domains (Eck et al. 1996). Nevertheless, the PTB domains of IRS-1 and Shc bind similar ligands in a closely related general mode. Both PTB domains use divergent sets of residues to accomplish this act.

The PTB domain represents an additional mechanism whereby signaling proteins can interact with tyrosine-phosphorylated protein targets. The identification and characterization of this novel phosphotyrosine binding domain furthers our understanding of the mechanistic details entailed to signaling events.

PTPases in signal transduction events

Signaling events mediated by tyrosine phosphorylation necessitate the actions of tyrosine kinases and tyrosine phosphatases. The role of tyrosine phosphatases as regulators of phosphotyrosine-dependent signaling processes is readily apparent. However, there are only a few examples of the involvement of PTPases in signaling events.

CD45

The function of CD45 in signal transduction has been studied by the generation of CD45 deficient cell lines. CD45-deficient mouse T cell clones fail to proliferate or produce cytokines in response to TCR stimuli such as antigen or anti-CD3 crosslinking (Pingel and Thomas 1989; Weaver et al. 1991). The absence of CD45 appears to uncouple the TCR from the intracellular signal transduction machinery. Several reports demonstrated that the engagement of TCR on different CD45-deficient cell lines is blocked at initial signaling events such as the induction of protein tyrosine kinase activity (Koretzky et al. 1991; Koretzky et al. 1990). These data suggested that CD45 may regulate the Src family member tyrosine kinases p56*lck* and p59*fyn* (Mustelin et al. 1989; Ostergaard et al. 1989). The regulation of the activity of Lck and Fyn tyrosine kinases by CD45 has been well documented (for review see (McFarland et al. 1994). CD45 modulates the activities of Lck and Fyn by specifically dephosphorylating the COOH-terminal inhibitory phosphotyrosine residues of Lck and Fyn (Y^{505} in Lck and Y^{531} in Fyn) (McFarland et al. 1993; Mustelin and Altman 1990; Mustelin et al. 1992). This dephosphorylation event represents a mechanism by which a tyrosine phosphatase indirectly regulates signaling events. Coverage of the signaling processes ensuing from the activities of Lck and Fyn in lymphoid cells (reviewed in (McFarland et al. 1994) is beyond the scope of the present introduction and therefore will not be discussed.

SH2-containing PTPases

Perhaps the strongest example of the involvement of PTPases in signal transduction comes from the SH2-containing PTPases SHP-1 and

SHP-2. The first evidence of these two PTPases playing a role in signal transduction came from the molecular characterization of two developmentally affected animal mutants: *motheaten* in mice and *corkscrew* in *Drosophila*.

The *motheaten* (*me*) mouse come from a spontaneous mutation in a C57BL/6J production colony at Jackson Laboratories in 1965 (Green and Shultz 1975). Its milder allele, *viable motheaten* (me^v), also arose spontaneously in the same genetic background in 1980 (Shultz et al. 1984). Mice homozygous for the autosomal recessive allelic mutation *me* or me^v on chromosome 6 develop severe combined immunodeficiency and systemic autoimmunity and die at the age of 3 and 9 weeks, respectively. Motheaten mice express multiple haematopoietic abnormalities including developmental or functional defects in macrophages, granulocytes, T and B cells, and natural killer cells (Clark et al. 1981; Davidson et al. 1979; Shultz 1988; Shultz 1991; Shultz et al. 1984; Shultz and Green 1976; Sidman et al. 1978). Focal abscesses in the skin of these mice result in patchy alopecia which gives the mutant mice a motheaten appearance. For a detailed review of the haematopoietic defects associated with the *me* mutation consult McFarland *et al.*, (1994).

The motheaten mutations were recently localized to the SHP-1 gene, which is expressed almost exclusively in all haematopoietic lineages (Shultz et al. 1993; Tsui et al. 1993). The me^v mutation consists of a single base pair change in the SHP-1 gene resulting in aberrant RNA splicing (Shultz et al. 1993; Tsui et al. 1993). This mutation affects the catalytic domain of the enzyme which results in a substantial decrease in PTPase activity (Kozlowski et al. 1993). The more drastic *m e*

mutation consists of a single base pair deletion which creates an abnormal splice site resulting in prematurely truncated SHP-1 proteins (Shultz et al. 1993; Tsui et al. 1993). In order to understand the molecular mechanisms underlying the catastrophic effects imparted on haematopoiesis and immune effector function by the loss of SHP-1, signaling pathways involving SHP-1 need to be characterized. The presence of SH2 domains in SHP-1 suggests that SHP-1 might function by associating with and/or modulating the activities of haematopoietic growth factor receptors.

Few haematopoietic growth factor receptors (c-Kit and c-Fms) are receptor tyrosine kinases. The majority of the haematopoietic growth factor receptors belong to a large superfamily of cytokine receptors that do not have intrinsic tyrosine kinase activity. Nevertheless, stimulation of cytokine receptors induces rapid tyrosine phosphorylation of cellular substrates as well as portions of the receptors (for a review on cytokine signaling see (Ihle 1995). The JAK family of protein tyrosine kinases (Jak1, Jak2, Jak3, Tyk2) have been strongly implicated in signal transduction by a number of cytokine receptors (Argetsinger et al. 1993; Ihle and Kerr 1995; Witthuhn et al. 1993). Members of the JAK family of PTKs are composed of a carboxyl-terminal kinase domain, a kinase-like domain of unknown function, and an amino-terminal portion of ~60 kDa (Firmbach-Kraft et al. 1990; Takahashi and Shirasawa 1994; Wilks et al. 1991). With the exception of Jak3, which is primarily expressed in haematopoietic cells, JAKs are ubiquitously expressed. There are several patterns by which JAK kinases associate with receptors (reviewed in (Ihle 1995). In all cases, ligand-mediated activation will trigger receptor aggregation and a concomitant homo- or heterotypic dimerization of JAKs. This leads to

the activation of JAK kinases by auto- or trans-phosphorylation which results in phosphorylation of tyrosine residues of several cellular proteins and receptor components.

Like the receptor tyrosine kinases, cytokine receptors activate many signaling pathways. Most cytokines activate Ras by triggering the phosphorylation of the adaptor protein Shc (Schlessinger 1993). This creates a Grb2 SH2 binding site and leads to the recruitment of Grb2:Sos complexes to the plasma membrane. Other common targets of cytokine activation include the p85 subunit of PI3-K and pLC-γ1 which are both recruited (like Shc) to the tyrosine phosphorylated receptors by virtue of their SH2 domains.

Molecular characterization of SHP-1 function in different signaling pathways has revealed that SHP-1 is an important negative regulator of cytokine signaling (Klingmuller et al. 1995; Yi and Ihle 1993; Yi et al. 1993). Binding of SHP-1 via its SH2 domains to phosphorylated cytokine receptors such as c-Kit, IL-3, Erythropoietin and Interferon-α/β complex has been reported (Klingmuller et al. 1995; Yi and Ihle 1993; Yi et al. 1993). For example, recruitment and binding of SHP-1 to the tyrosine phosphorylated erythropoietin receptor via its SH2 domains allows for the specific dephosphorylation of EPO-R-bound Jak2 tyrosine kinase resulting in the termination of its signaling capability (Klingmuller et al. 1995). It has been suggested that binding of SHP-1 to other cytokine receptors would have similar consequences (Klingmuller et al. 1995). Additional characterization of similar SHP-1-mediated molecular interactions and their resulting functions in haematopoietic signaling will lead to a better understanding of the *motheaten* phenotype.

The participation of SHP-2 in signaling processes was demonstrated by genetic studies in *Drosophila*. The gene *corkscrew* (*csw*) which encodes a homolog of the ubiquitously expressed SHP-2, has been shown to function in signal transduction pathways essential for normal development of anterior and posterior segments of the *Drosophila* embryo (Perkins et al. 1992). *Csw* acts positively in conjunction with D-Raf to transduce signals from the *torso* receptor tyrosine kinase (a PDGF receptor homolog) (Perkins et al. 1992).

Biochemical evidence for the involvement of SHP-2 in signal transduction events comes from studies showing that SHP-2 binds through its SH2 domains to phosphorylated sites in PDGF, EGF, and insulin receptors as well as to IRS-1 (Bennett et al. 1994; Feng et al. 1993b; Kuhne et al. 1993; Lechleider et al. 1993b; Sugimoto et al. 1993; Vogel et al. 1993). Specific binding sequences for SHP-2 SH2 domains have been found in the PDGF receptor (Feng et al. 1994a; Kazlauskas et al. 1993; Lechleider et al. 1993a) and IRS-1 (Case et al. 1994; Sun et al. 1993b), and a consensus binding sequence has been elucidated (Case et al. 1994; Songyang et al. 1993). In addition, SHP-2 becomes tyrosine phosphorylated within its carboxyl-terminus upon PDGF and EGF treatment of cells (Feng et al. 1993b; Lechleider et al. 1993b; Vogel et al. 1993). These phosphorylation sites represent SH2 domain binding sites for the adaptor protein Grb2. It has been demonstrated that tyrosine phosphorylated SHP-2, bound to activated PDGF receptors through its SH2 domains, recruits Grb2:Sos complexes to the receptor thereby activating Ras (Bennett et al. 1994; Li et al. 1994).

SHP-2 is considered to be a positive regulator of growth factor

signaling. Neutralizing the action of SHP-2 by microinjecting anti-SHP-2 antibodies or a recombinant SH2 domain alone blocked insulin- and EGF-induced mitogenesis in cells (Xiao et al. 1994). Expression of a dominant-negative catalytically inactive SHP-2 in cells also diminished mitogenic signaling in response to insulin (Milarski and Saltiel 1994). In addition, expression of a dominant negative SHP-2 mutant in *Xenopus* embryos caused severe posterior truncations and impaired mesodermal induction upon FGF and activin treatment (Tang et al. 1995). These phenotypes resemble those created by the expression of dominant negative mutants of the FGF receptor (Tang et al. 1995), suggesting that SHP-2 act as a positive component in FGF signaling.

It has been demonstrated that SHP-2 can be activated by removal of its SH2 domains or by occupying the SH2 domains with specific phosphopeptides (Dechert et al. 1994; Lechleider et al. 1993a). This suggests that binding of SHP-2 to activated growth factor receptors through its SH2 domains stimulates its phosphatase activity and thus promotes signaling. However, the positive signaling regulation displayed by SHP-2 remains to be assessed mechanistically. Specifically, the effects of SHP-2 enzymatic activity on relevant substrates and the signaling ensuing from such modulation in phosphorylation are yet to be determined.

HPTPα

HPTPα is a transmembrane receptor-like PTPase that exhibits a widespread pattern of expression suggesting an important role in cellular physiology (Jirik et al. 1990; Kaplan et al. 1990; Krueger et al. 1990; Matthews et al. 1990; Sap et al. 1990). It is composed of a short,

heavily glycosylated, extracellular domain of 142 amino acids, a single transmembrane domain, and two intracellular catalytic domains. Lack of enzymatic activity in the second catalytic domain is common to all dual-domain receptor PTPases with the exception of CD45 (Tan et al. 1993) and HPTPα (Wang and Pallen 1991). Both catalytic domains of HPTPα are active but display distinct substrate specificities (Wang and Pallen 1991). However, the functional and physiological significance of this difference has not been determined.

The evidence for the involvement of HPTPα in signal transduction events is not as direct as that described for CD45 and the SH2-containing PTPases. For example, overexpression of HPTPα in Fisher rat embryo fibroblasts (REF) causes cellular transformation and tumorigenesis (Zheng et al. 1992) whereas in the pluripotent P19 embryonic carcinoma cell line, overexpression of HPTPα facilitates neuronal differentiation (den Hertog et al. 1993). It has been suggested that these phenomenon are the result of the activity of HPTPα on an inhibitory phosphotyrosine residue (Y^{527}) of the tyrosine kinase $pp60^{c\text{-}src}$ (den Hertog et al. 1993; Zheng et al. 1992). This dephosphorylation event which is similar to the activation of Lck and Fyn tyrosine kinases by CD45 in lymphocytes, results in the persistent activation of $pp60^{c\text{-}src}$. $pp60^{c\text{-}src}$ kinase activity is normally regulated in a cell cycle-dependent fashion by the phosphorylation of the regulatory tyrosine residue (Y^{527}) (Bagrodia et al. 1991; Cartwright et al. 1987; Cooper et al. 1986; Kaech et al. 1991; Kmiecik and Shalloway 1987; Piwnica-Worms et al. 1987) and has been shown to be enhanced in a number of cell lines during neuronal differentiation (Bjelfman et al. 1990; Boulter and Wagner 1988; Lynch et al. 1986). Deregulation of $pp60^{c\text{-}src}$ activity by

overexpression of HPTPα in different cellular backgrounds results in different phenotypes suggesting that HPTPα actions are mediated by specific sets of proteins.

It has recently been demonstrated that overexpression of HPTPα in REFs result in the activation of c-Jun and MAP kinase concomitant with an elevation in the phosphotyrosine levels of c-Jun. In addition, overexpression studies in transformed cell lines (U-937 and Jurkat) and primary fibroblasts (MRC-5 and REFs) reveals that HPTPα is involved in the activation of NF-kappa B by tumor necrosis factor (Menon et al. 1995). These results indirectly suggest that HPTPα is involved in the regulation of cell proliferation and differentiation, exerting some of its effects through pp60$^{c\text{-}src}$, c-Jun and MAP kinase.

The participation of HPTPα in signal transduction events has been recently strengthened by the description of a molecular interaction between the adaptor molecule Grb2 and HPTPα (den Hertog et al. 1994; Su et al. 1994). It was demonstrated that HPTPα is constitutively phosphorylated *in vivo* on tyrosine 789, and that this phosphotyrosine residue constitutes a binding site for the SH2 domain of Grb2 (den Hertog et al. 1994). Binding of Grb2 to HPTPα is not only dependent on the SH2 domain of Grb2, but also on the carboxyl-terminal SH3 domain of Grb2 (den Hertog 1996). Binding of the carboxyl-terminal SH3 domain of Grb2 to HPTPα was mapped to an 18 residue portion which is located in the carboxyl-terminal region of the amino-terminal PTPase domain (or D1) (den Hertog 1996). Interestingly, this potential SH3 recognition binding site does not conform to either class I or II SH3

domain binding consensus motifs. In fact, it lacks proline residues altogether (den Hertog 1996). This suggests the existence of an intermediary protein between the carboxyl-terminal SH3 domain of Grb2 and HPTPα. The physiological significance of this interaction remains to be determined but certainly represents a link between a receptor-like tyrosine phosphatase and a key component of a central cellular signaling pathway.

MPTP-PEST

Using the polymerase chain reaction to amplify PTPase-related cDNAs from different sources, six groups independently isolated portions of the MPTP-PEST message from human and murine origins (Charest et al. 1995a; den Hertog et al. 1992; Moriyama et al. 1994; Takekawa et al. 1992; Yang et al. 1993; Yi et al. 1991) (Chapter2). Using these fragments as probes, full length cDNAs were obtained from various cDNA libraries and analyzed (Charest et al. 1995a; den Hertog et al. 1992; Moriyama et al. 1994; Takekawa et al. 1992; Yang et al. 1993) (Chapter2).

MPTP-PEST is also known as PTPTy43 (Yi et al. 1991) and P19-PTP (den Hertog et al. 1992) in mouse, RKPTP in rat (Moriyama et al. 1994) and PTPG1 (Takekawa et al. 1992) and PTP-PEST (Yang et al. 1993) in human. Several structural and functional aspects of this enzyme have been characterized and are described in this section.

The MPTP-PEST cDNA was isolated from differentiated P19 embryonal carcinoma (EC) cells under the name of P19-PTP (den Hertog et al. 1992). P19 EC cells are pluripotent cells resembling the

totipotent stem cells of the inner cell mass of a blastocyst (Jones-Villeneuve et al. 1982; McBurney et al. 1982). P19 EC cells can be induced to differentiate *in vitro* into representatives of the three germ layers (Jones-Villeneuve et al. 1982; McBurney et al. 1982). Analysis of the expression of MPTP-PEST (P19-PTP) during *in vitro* differentiation of P19 cells demonstrated that MPTP-PEST (P19-PTP) transcripts were only detected in embryoid bodies, (which contain mesodermal-like tissues) suggesting that MPTP-PEST (P19-PTP) is expressed upon mesodermal induction (den Hertog et al. 1992). In addition, Northern blot analysis of murine adult tissues as well as human and murine cell lines revealed that MPTP-PEST is expressed ubiquitously. Moreover, in situ hybridization performed on 17 day p.-c. rat embryos using MPTP-PEST specific probes suggests that MPTP-PEST is expressed throughout the embryo at this stage of development (Sahin et al. 1995; Sahin and Hockfield 1993).

Sequence analysis of both the human and murine enzymes revealed that MPTP-PEST is composed of a single amino-terminal catalytic domain of ~244 amino acids and a long carboxyl-terminal tail of ~475 residues rich in proline, serine, threonine and aspartate residues otherwise known as PEST sequences. The catalytic domain of human PTP-PEST, when expressed as a GST fusion protein in *E. coli.*, was shown to readily dephosphorylate phosphotyrosine-containing proteinaceous substrates but not serine-phosphorylated proteins (Yang et al. 1993), thus demonstrating that PTP-PEST is a genuine protein tyrosine phosphatase.

Certain features of the amino acid primary sequence of proteins play important roles in determining the half life of a protein. For

example, the presence of arginine, lysine and several other amino acids bearing charged or bulky side-chains at the amino-terminus of a protein has been shown to promote a rapid ubiquitin-dependent degradation of certain proteins (Bachmair et al. 1986; Dice 1987; Rechsteiner 1991). In addition, lysosomal targeting of cytoplasmic proteins via specific peptide sequences has been reported to lead to their destruction (Chiang and Dice 1988; Dice 1987). PEST sequences have also been proposed to act as a signal for rapid intracellular breakdown of proteins (Olson and Dice 1989; Rechsteiner 1987; Rogers et al. 1986). The PEST domain hypothesis arose from the observation that several proteins displaying short intracellular half lives have stretches of sequence unusually rich in proline, glutamate, serine and threonine (Rogers et al. 1986). This observation led to the development of an algorithm (called PEST-FIND) which allows for the automatic search of PEST regions in any given protein (Rogers et al. 1986). The strength of this correlation is mostly limited to nuclear proteins. In addition, only two reports demonstrated the biochemical effects of PEST sequences on protein stability (Loetscher et al. 1991; Salama et al. 1994). The mechanism by which PEST sequences convey instability to the proteins that harbour them has yet to be determined.

Because of the potential role that protein tyrosine phosphatases may play in carcinogenesis, Takekawa *et al.*, (1994) screened by RT-PCR several human cancer cell lines for the integrity of the PTP-PEST message. In the human DLD-1 colon cancer cell line, two aberrant PTP-PEST transcripts were detected. DNA sequence analysis of these atypical transcripts revealed that the two messages correspond to deletions within the catalytic domain of PTP-PEST (Takekawa et al. 1994). These deletions would result in shifts of the reading frame which would

ultimately lead to premature termination of translation giving rise to truncated PTP-PEST proteins. It has been postulated that these alterations might lead to the observed phenotype of the cell line (Takekawa et al. 1994).

Several PTPases including PTP1B (Flint et al. 1993; Schievella et al. 1993), cdc25 (Moreno et al. 1990; Strausfeld et al. 1994), CD45 (Autero et al. 1994; Ostergaard and Trowbridge 1991; Valentine et al. 1991), HPTPα (Tracy et al. 1995), and both SH2 domain-containing enzymes (Feng et al. 1993a) are phosphoproteins *in vivo*, suggesting that the activities of these enzymes can be potentially modulated by reversible phosphorylation. The possibility of modulating the function of PTP-PEST by phosphorylation was investigated by Garton and Tonks (1994). Human PTP-PEST was shown to be serine-phosphorylated *in vitro* and *in vivo* on Ser^{39} and Ser^{435} residues by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). Phosphorylation of Ser^{39} *in vitro* decreased the activity of PTP-PEST by reducing its affinity for substrate. In addition, phosphorylated PTP-PEST immunoprecipitated from cells that had been treated with PKC activators displayed decreased activity when compared to PTP-PEST enzymes obtained from untreated cells. These observations offered a mechanism whereby signal transduction pathways acting through PKA or PKC may directly influence the activity of PTP-PEST resulting in modulation of cellular processes involving reversible tyrosine phosphorylation.

Finally, it has recently been demonstrated that the adaptor onco-protein $p52^{Shc}$ interacts with human PTP-PEST *in vivo* and *in vitro* (Habib et al. 1994). This interaction was discovered using a yeast two-hybrid screen for proteins that interact with $p52^{Shc}$. Partial *in vitro*

mapping of the sites of interaction between these two proteins revealed that the amino-terminal portion of p52^{Shc} binds to the carboxyl-terminal half of PTP-PEST. Interestingly, stimulation of cells with PKC activators induced a 6-8 fold increase in complex formation of p52^{Shc}:PTP-PEST (Habib et al. 1994). However, the molecular mechanisms underlying this phenomenon are still uncharacterized. This interaction suggests that PTP-PEST, by virtue of its catalytic activity and its ability to interact with signal transduction molecules, is involved in the regulation of signaling events where tyrosine phosphorylation is a key mediator.

The characterization of the PTP-PEST enzyme is still in its infancy. Little is known about the function(s) that PTP-PEST imparts on cellular processes such as proliferation and differentiation. This Ph. D. thesis partly addresses these questions. It describes the isolation and initial characterization of the murine homologue of PTP-PEST. It also analyzes the details of several MPTP-PEST interactions with proteins involved in signaling events. These results constitute an underlying foundation upon which theories regarding the cellular function(s) of MPTP-PEST can be built.

CHAPTER 2

Murine Protein Tyrosine Phosphatase-PEST, a Stable Cytosolic Protein Tyrosine Phosphatase.

Murine protein tyrosine phosphatase-PEST, a stable cytosolic protein tyrosine phosphatase

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We have isolated the murine cDNA homologue of the human protein tyrosine phosphatase PTP-PEST (MPTP-PEST) from an 18.5-day mouse embryonic kidney library. The cDNA isolated has a single open reading frame predicting a protein of 775 amino acids. When expressed *in vitro* as a glutathione S-transferase fusion protein, the catalytic domain (residues 1–453) shows intrinsic phosphatase activity. Reverse transcriptase PCR and Northern-blot analysis show that MPTP-PEST mRNA is expressed throughout murine development. Indirect immunofluorescence in COS-1 cells against a heterologous epitope tag attached to the N-terminus of MPTP-PEST, together with cellular fractionation and Western-blot experiments from different murine cell lines, indicate that MPTP-PEST is a free cytosolic protein of 112 kDa. Finally, sequence analysis indicates that the C-terminal portion of the protein contains four regions rich in proline, glutamate, serine and threonine, otherwise known as PEST sequences. These are characteristic of proteins that display very short intracellular half-lives. Despite the presence of these motifs, pulse–chase labelling experiments demonstrate that MPTP-PEST has a half-life of more than 4 h.

INTRODUCTION

Protein tyrosine phosphorylation is an important phenomenon by which a wide variety of environmental signals modulate cellular homoeostasis. Phosphorylation on tyrosine residues is the result of a highly regulated balance of activity between two antithetic enzymes, protein tyrosine kinases and protein tyrosine phosphatases (PTPases). The latter can be categorized into two different groups on the basis of their substrate specificity [1]. The first group is known as the VH1 subfamily. Members of this dual-specificity subfamily are capable of removing phosphate moieties from phosphoserine, phosphothreonine and phosphotyrosine residues. The second group comprises PTPases that display a unique specificity toward phosphotyrosine residues. PTPases of this group show a broad structural diversity and have been subclassified into either intracellular or transmembrane subfamilies (reviewed in [2]).

Our search for PTPases involved in murine embryogenesis led us to the isolation of a member of the intracellular subfamily of PTPases. This enzyme, which we named MPTP-PEST, has been reported by several groups during the course of our investigation. It has been isolated from murine tissue under the name of P19-PTP [3] or PTPTY43 [4] and from human sources as PTPG1 [5] or PTP-PEST [6]. This enzyme displays a ubiquitous pattern of expression in both human and murine tissues and cell lines [3–6]. It is characterized by the presence of a single catalytic domain linked to several PEST motifs. These motifs represent clusters of sequence rich in proline, glutamate, serine and threonine residues (PEST) and were first identified in rapidly degraded proteins [7].

The cellular function of MPTP-PEST has yet to be identified. However, several lines of evidence suggest that it may play an important role in cell growth and differentiation. First, the human gene has recently been mapped to chromosome 7q11.23 which is a common site of chromosomal rearrangement in some malignant melanomas [8]. Second, aberrant transcripts of PTPG1 have been isolated from a human colon cancer cell line [8]. Third, its broad range of expression is also indicative of a fundamental role in cellular processes. Finally, PTP-PEST was recently shown to be associated with the multifaceted adaptor molecules SHC [9] (A. Charest and M. L. Tremblay, unpublished work). The SHC proteins perform an important function in signalling from various receptor tyrosine kinases to the Ras pathway. Also, SHC has recently been shown to contain a phosphotyrosine-binding domain, termed PTB domain [10], that is different from the conventional SH2 domain. These results demonstrate that PTP-PEST molecules are directly associated with a class of molecules that employ tyrosine phosphorylation as a means of transducing signals and therefore emphasize the possible role of PTP-PEST in signal transduction. To gain further insight into the function and regulation of MPTP-PEST, we have characterized various features of this enzyme. We show that it is an active PTPase that is expressed throughout embryonic development. We also demonstrate that it is a stable protein and that most of it is located in the soluble fraction of the cytoplasm.

MATERIALS AND METHODS

Cell culture and transient transfection

COS-1, Ltk$^-$, NIH 3T3, NIH 3T6 and P19 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum at 37 °C in 5% CO_2. COS-1 cells (between 10^6 and 10^7) were electroporated in cold PBS with 20 μg/ml plasmid DNA at 1.2 kV and 25 μF using a Bio-Rad gene pulser.

Oligonucleotides, reverse transcription and PCR

First-strand synthesis reverse transcription of total RNA from whole mouse embryo was performed using Superscript RNase H(−) reverse transcriptase (RT; Gibco–BRL) and random oligomers p(dN)$_6$ (Pharmacia) or oligod(T)$_{12}$ (Gibco–BRL) as described by the manufacturer. Subsequent amplification was performed using Vent DNA polymerase (NEB) on a Perkin–

Abbreviations used: PTPase, protein tyrosine phosphatase; pNPP, *p*-nitrophenol phosphate; RT-PCR, reverse transcriptase PCR; RCM-lysozyme, reduced, carboxyamidomethylated and maleylated lysozyme; UTR, untranslated region; ECL, enhanced chemiluminescence; GST, glutathione S-transferase; PDGF, platelet-derived growth factor.

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Elmer Cetus thermal cycler. Oligonucleotides PEST-878s (5'-GCAGTACAAACAAAGGAGCA-3') and PEST-1400 as (5'-AGTGTGTTCCCATCAAAAC-3') were derived from the human PTP-PEST sequence (S.-H. Shen, unpublished work). Oligonucleotides PEST-3 (5'-AGGATGGAGCAAGTGGAG-A-3') and PEST-4 (5'-TGTCCTTTCACTCCCTGCAT-3') were derived from the P19 PTPase sequence [3]. Oligonucleotides PEST-1 (5'-ACTGCCATTTGATCACAGCC-3') and PEST-708 (5'GGCACATCTTCATGTTCTTGG-3') were derived from the MPTP-PEST sequence.

Molecular cloning and cDNA sequencing

The 18.5-day murine embryonic kidney library was generously provided by Dr. G. Dressler (Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor, MI, U.S.A.). Two partial cDNAs (clones 1.2 and 2.8) were isolated using an RT-PCR-generated probe from whole mouse embryo RNA. Full-length cDNAs were reconstituted using RT-PCR methodology as described above. DNA sequencing of both strands was performed with Sequenase (USB) as described by the manufacturer.

Glutathione S-transferase (GST) fusion protein

The phosphatase domain was amplified by Vent DNA polymerase using the cDNA as a template and oligonucleotides PEST-3 and PEST-1400as. The resulting fragment (corresponding to amino acids 1–453) was then subcloned into *Sma*I-digested pGEX-RC (provided by Dr. P. E. Branton, McGill University). In-frame ligations were verified by sequencing. GST fusion proteins were extracted and affinity-purified on glutathione–Sepharose beads (Pharmacia) according to the manufacturer's protocol.

Enzyme assays

For *p*-nitrophenol phosphate (pNPP)-dephosphorylation assays the fusion proteins were incubated in P buffer (25 mM Hepes, pH 7.3, 5 mM EDTA, 10 mM 2-mercaptoethanol) in the presence of 10 mM pNPP at 37 °C. Reactions were terminated at the indicated time points by the addition of an equal volume of 2 M Na_2CO_3; the mixtures were then diluted 5-fold with water, and absorbance was measured at 410 nm. For tyrosine-dephosphorylation experiments, reduced, carboxyamidomethylated and maleylated lysozyme (RCM-lysozyme; provided by Dr. P. E. Branton) was phosphorylated on tyrosine residues as follows: 10 μg of RCM-lysozyme was labelled with 150 μCi of [γ-^{32}P]ATP (Dupont–NEN) by recombinant Lck tyrosine kinase (provided by Dr. J. Bolen, Bristol-Myers Squibb, Princeton, NJ, U.S.A.) in buffer (50 mM Hepes, pH 7.3, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.06 % 2-mercaptoethanol and 30 μg/ml BSA) at 30 °C overnight. The phosphorylated RCM-lysozyme was separated from unincorporated [γ-^{32}P]ATP by trichloroacetate precipitation (final concentration 25 %) in the presence of 0.2 % BSA and 5 mM NaH_2PO_4. After centrifugation (16000 *g*), the pellet was washed several times with cold 20 % trichloroacetate/20 mM NaH_2PO_4 and resuspended in a PTPase assay buffer (25 mM Hepes, pH 7.3, 5 mM EDTA, 10 mM dithiothreitol). Dephosphorylation was assayed by mixing a given amount of radiolabelled substrate with the recombinant MPTP-PEST in the PTPase assay buffer, incubated at 25 °C for the indicated time periods and stopped by trichloroacetate precipitation (as described above), and the supernatant (containing released [^{32}P]P_i) was assayed for radioactivity by liquid-scintillation counting.

HA tag plasmid construct

Plasmid pSM491 (provided by Dr. N. Sonenberg, McGill University) consists of a 9-amino acid triplet repeat HA epitope (YPYDVPDYA) derived from the influenza virus haemagglutinin protein [11–13] inserted into the *Vor*I site of pBluescriptIISK (Stratagene). The three HA tags were amplified by PCR with Vent DNA polymerase using pSM491 as template and oligonucleotides H3-ATG (5'-TATCACAAGCTTCCACCATGAT-CTTTTACCCATACGATGTTCCT-3') and the T7 sequencing primer. The resulting fragment was then digested with *Hin*dIII and ligated into *Hin*dIII-digested pRC/CMV (Invitrogen), giving rise to pACTAG-1. pACTAG-2 was derived from the latter simply by digesting with *Xba*I and religating the plasmid on to itself. Inserting the full-length MPTP-PEST cDNA into pACTAG-2 was achieved using recombinant DNA technology. All products were verified by restriction digest pattern and/or DNA sequencing.

Indirect immunofluorescence

pACTAG-2-MPTP-PEST plasmid was transfected by electroporation and transiently expressed in COS-1 cells as described above. At 48 h after transfection, cells were subjected to immunofluorescence microscopy as described elsewhere [14] using the monoclonal antibody 12CA5 as a primary antibody and rhodamine-conjugated goat anti-mouse IgG antibody (Pierce) as a secondary antibody.

Generation of MPTP-PEST antibodies

Polyclonal antibodies were generated in New Zealand White rabbits by the method of Harlow and Lane [15] using MPTP-PEST residues 276–453 fused to GST as the antigen. The bleeding regimen and antibody purification were as described by Harlow and Lane [15]. Immune serum no. 1075 contained a high titre of anti-MPTP-PEST antibodies and was used throughout the experiments.

mRNA detection

Total RNA was isolated from different embryonic stages and Northern-blot analyses was performed as described previously [16]. RT-PCR protocols are described above. ^{32}P-labelled DNA fragments were prepared using a T7 DNA polymerase random-primed labelling kit (T7 Quick Prime; Pharmacia) according to the instructions of the manufacturer.

Immunoprecipitation of MPTP-PEST

Cells (transfected and non-transfected) were washed several times with PBS and harvested in PBS, centrifuged at 2000 *g* for 5 min at 4 °C and lysed by resuspension in 10 ml of RIPA buffer [50 mM Tris/HCl, pH 7.2, 150 mM NaCl, 0.1 % (w/v) SDS, 0.5 % (w/v) sodium deoxycholate, 1 % (v/v) Nonidet P40). In addition, phosphatase and protease inhibitors (5 mM EDTA, 1 mM NaF, 2 mM sodium orthovanadate, 2 mM PMSF, 20 μg/ml aprotinin and 20 μg/ml leupeptin) were added to the RIPA buffer. The lysates were incubated for 10 min on ice and the cell debris was pelleted by centrifugation at 12500 *g* for 20 min at 4 °C. To the supernatants were added 12CA5 monoclonal antibody and protein G–agarose beads (Gibco–BRL), and binding was allowed to proceed for 16 h at 4 °C. The immunoprecipitated protein complexes were washed three times with RIPA buffer and three times in 100 mM Tris/HCl, pH 7.0, containing 200 mM LiCl and 0.1 % (v/v) 2-mercaptoethanol. Samples were subsequently subjected to SDS/PAGE.

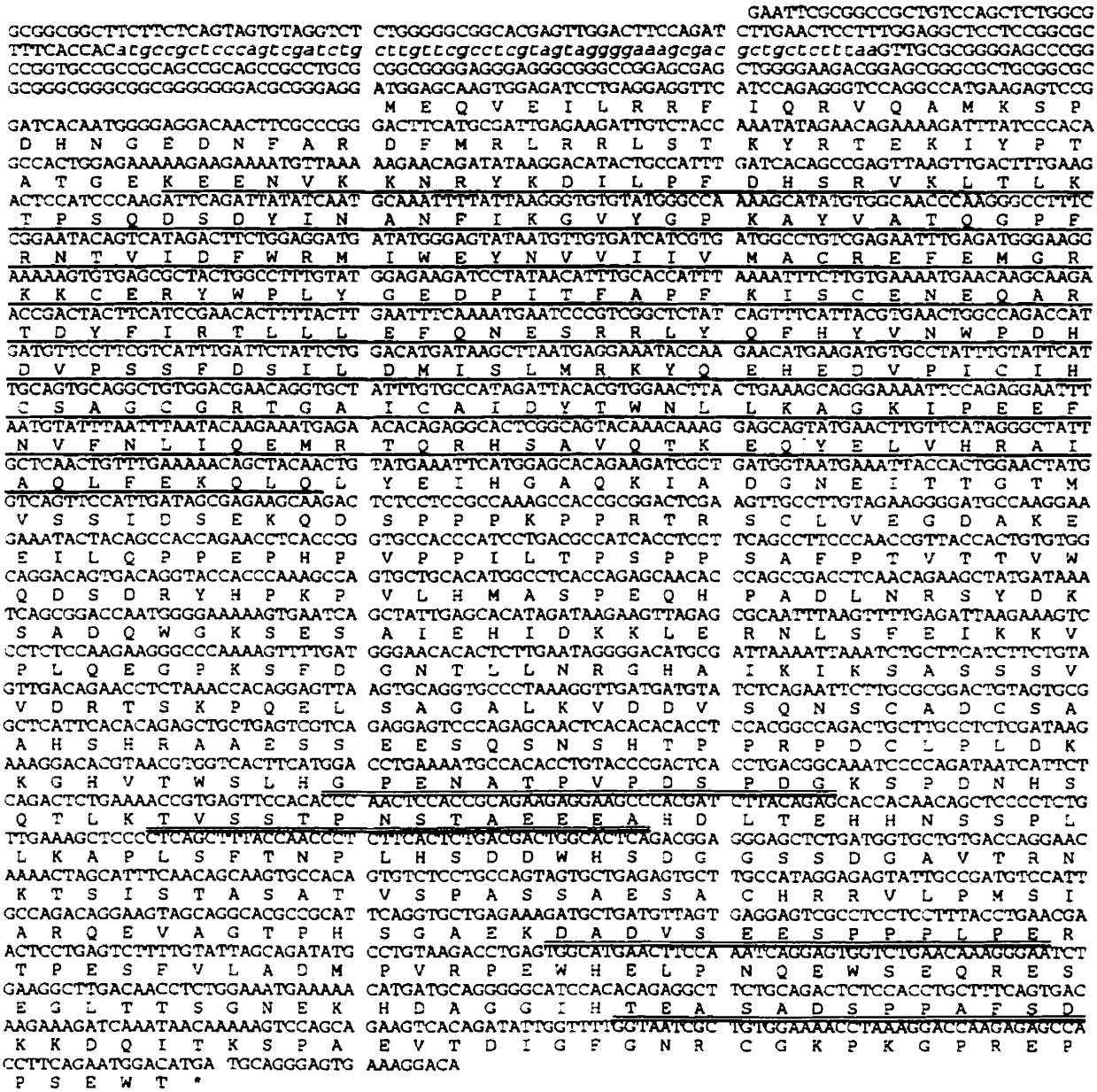


Figure 1 MPTP-PEST cDNA and amino acid sequence

The nucleotide and predicted amino acid sequence of MPTP-PEST are shown. The underlined sequence represents the phosphatase domain. Double-underlined sequences represent PEST motifs. Italicized lower-case letters indicate an open reading frame.

Pulse–chase labelling of MPTP-PEST

Cell monolayers (either Ltk⁻ or transfected COS-1 cells) were washed several times with PBS and starved in methionine/cysteine-free medium (ICN) for 2 h. The cells were then labelled for an additional 3 h with [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-label; ICN). Labelled cells were then washed several times with PBS and chased with normal medium for the indicated times after which they were lysed, and MPTP-PEST was immunoprecipitated as described above. MPTP-PEST immunoprecipitates were subjected to SDS/PAGE and transferred to nitrocellulose membranes. Before exposure of the blot to X-ray film to visualize the metabolically labelled MPTP-PEST proteins, the amount of MPTP-PEST was quantified by Western blotting using the 1075 polyclonal antibody at a dilution of 1:500, followed by enhanced chemiluminescence (ECL) detection (Amersham).

Subcellular fractionation

Ltk⁻, NIH 3T3 and pACTAG2-MPTP-PEST-transfected COS-1 cells were fractionated as described previously [17], and P19, Ltk⁻ and NIH 3T6 cells were subjected to Triton X-100 extraction [18]. Equal amounts of protein from each fraction were subjected to SDS/PAGE and immunoblotted with the 1075 polyclonal antibody. Anti-[platelet-derived growth factor (PDGF) receptor] antibody was obtained from UBI.

RESULTS

Isolation and structure of the MPTP-PEST

PTP-PEST-specific oligonucleotides, derived from the human PTP-PEST cDNA sequence (S.-H. Shen, unpublished work), were used in an RT-PCR-based approach to isolate a cDNA fragment from 14.5- and 15.5-day mouse embryo mRNA. This 522 bp fragment was then used to probe an 18.5-day embryonic kidney library. Two overlapping clones, coding for the entire cDNA, were isolated. The complete nucleotide and predicted amino acid sequence of MPTP-PEST is depicted in Figure 1. The full MPTP-PEST cDNA sequence contains a single open reading

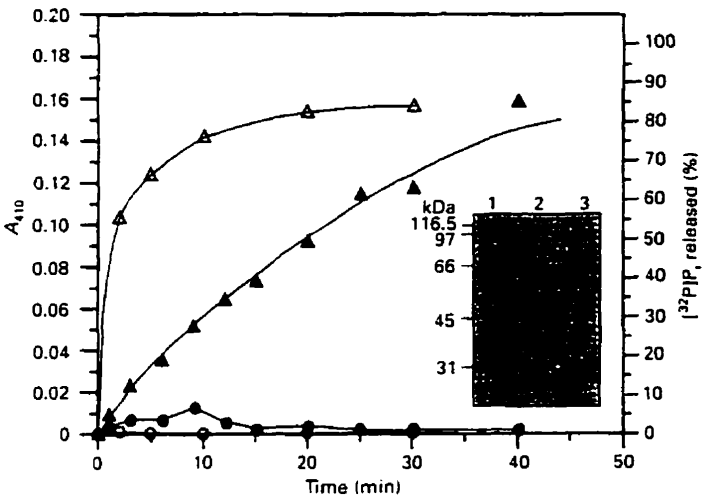


Figure 2 Demonstration of the intrinsic enzyme activity of the phosphatase domain of MPTP-PEST

Dephosphorylation curves of two substrates, pNPP and RCM-lysozyme, by GST (●, ○), and GST–MPTP-PEST (▲, △) are shown. ▲, ●, A_{410}. △, ○, [^{32}P]P released. Inset, Coomassie-Blue-stained gels after SDS/PAGE of the proteins used in the experiment. Lane 1, molecular-mass standards; lane 2, GST; lane 3, GST–MPTP-PEST. The results are representative of at least three different experiments.

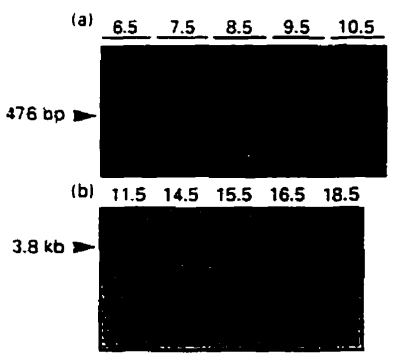
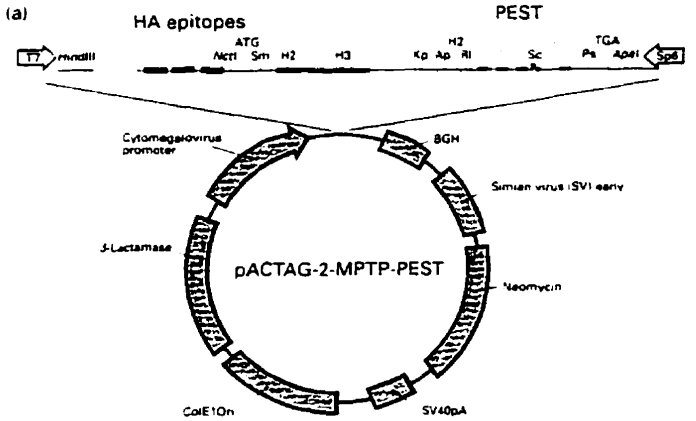


Figure 3 Onset of MPTP-PEST gene expression during murine development

(**a**) RT-PCR of total RNA isolated from embryonic stages (6.5–10.5 days *post coitum*) using oligonucleotides PEST-1 and PEST-708. RNA from each embryonic stage was reverse-transcribed with either random p(dN)$_6$ oligonucleotides (first lane) or oligo d(T)$_{12}$ primer (second lane). A control without RT was in the third lane. The arrow indicates the expected size of the PCR product. (**b**) Northern-blot analysis of MPTP-PEST from later stages of embryonic development (11.5–18.5 days *post coitum*). The arrow indicates the MPTP-PEST message, which is 3.8 kb.



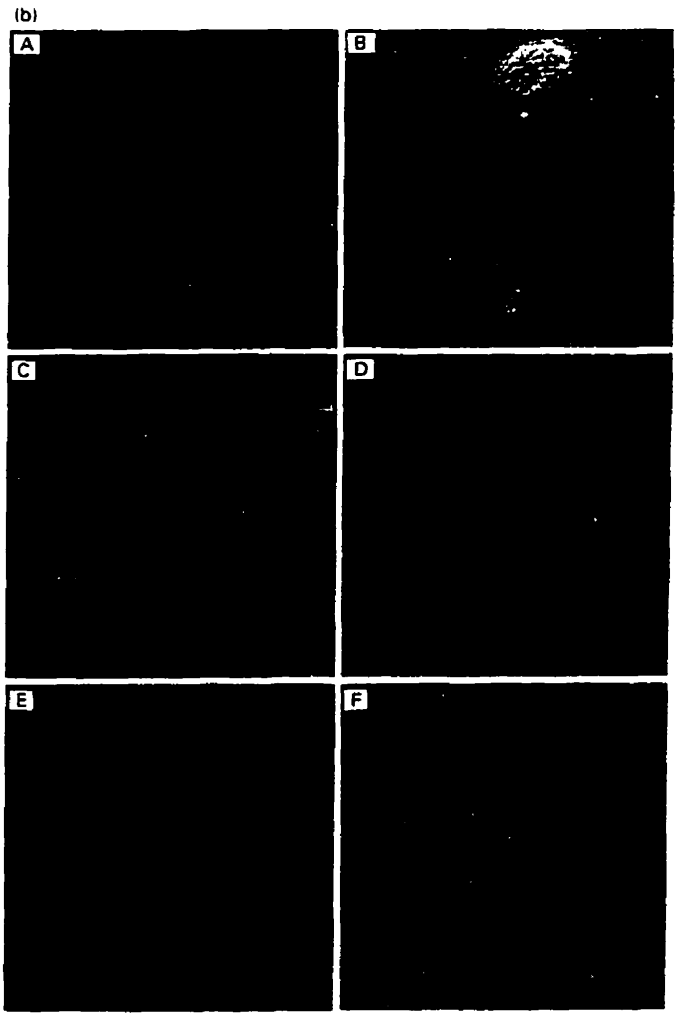


Figure 4 Cellular localization of MPTP-PEST by indirect immunofluorescence

(**a**) Schematic representation of the triple HA-tagged MPTP-PEST cDNA used to direct transient expression of the MPTP-PEST protein in COS-1 cells. (**b**) Photomicrographs of COS-1 cells transfected and treated as described in the Materials and methods section. A, C and E, Immunofluorescence microscopy; B, D and F, phase-contrast microscopy. Arrowheads indicate cells that fluoresce in corresponding A, C and E. Photomicrographs in (**b**) are representative of three different experiments.

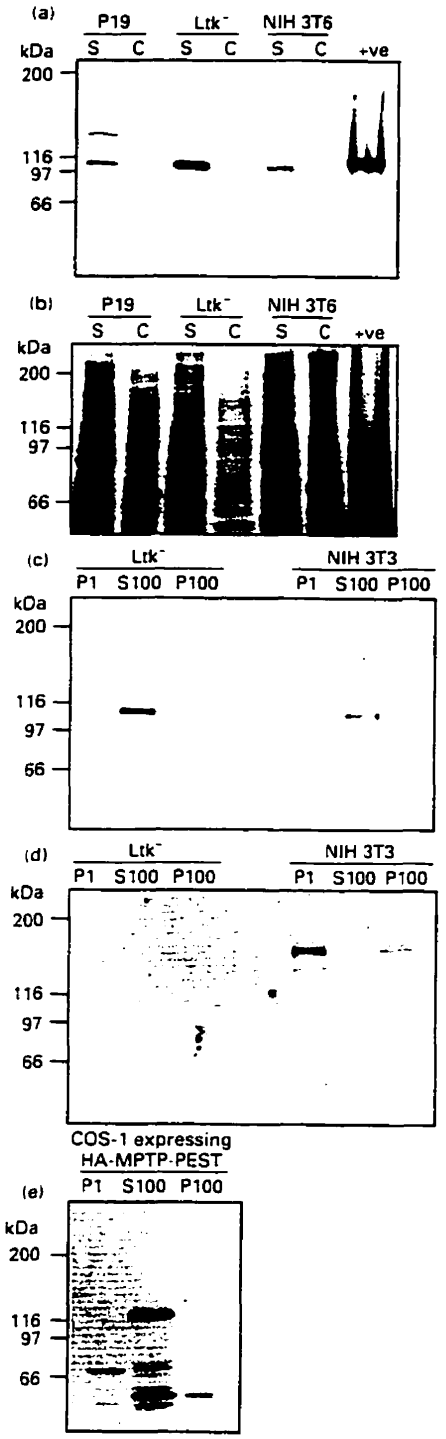


Figure 5 Localization of MPTP-PEST by subcellular fractionation studies

(**a**) Immunoblot analysis of Triton X-100 extraction of P19, Ltk⁻ and NIH 3T6 cells. Cellular components were extracted in a soluble (S) and cytoskeletal fraction (C) as described in the Materials and methods section. Each extract (30 μg) was separated by SDS/PAGE and immunoblotted with anti-MPTP-PEST polyclonal antibody no. 1075. (**b**) Coomassie-Blue-stained gel control showing similar amounts of protein in each fraction. The positive (+ve) control refers to 5 μg of protein extracts from COS-1 cells transiently expressing HA-MPTP-PEST. (**c**), (**d**) and (**e**) Immunoblot analysis of cellular fractions from different sources. Cells were fractionated as described in the Materials and methods section. Protein from each fraction (30 μg) was subjected to SDS/PAGE and immunoblotted with the anti-MPTP-PEST antibody 1075 (**c**), anti-(PDGF receptor) antibody (**d**) and anti-(HA tag) antibody 12CA5 (**e**). P1, Plasma-membrane and nuclear fraction; S100, cytosolic fraction; P100, particulate fraction. Immunoblot analyses in (**c**, **d** and **e**) are representative of two different experiments.

frame (from nucleotide 330 to 2699) that encodes a protein of 775 amino acids of predicted molecular mass 87 kDa. One of the cDNA clones isolated contains 315 bp of 5′-untranslated region (UTR). This sequence is rich in guanine and cytosine nucleotides and can potentially form secondary structures known to be involved in translational control [19,20]. The 5′-UTR also contains a small open reading frame of 60 nucleotides (Figure 1; lower-case italicized sequence). The N-terminal region of the deduced MPTP-PEST amino acid sequence (from amino acid 55 to 299) contains the catalytic domain. MPTP-PEST catalytic domain contains all of the conserved amino acids found among all tyrosine phosphatases. Overall, the homology between the phosphatase domain of MPTP-PEST and that of other PTPases varies between 30 and 62%.

Activity of the MPTP-PEST phosphatase domain

To evaluate whether the region defined as the phosphatase domain was capable of intrinsic catalytic activity, a GST fusion protein containing residues 1–453 of the MPTP-PEST protein was constructed (see the Materials and methods section). GST and the GST–MPTP-PEST fusion proteins were affinity-purified on a glutathione–Sepharose matrix, quantified and equal amounts subjected to SDS/PAGE and assayed for enzyme activity (Figure 2). The GST–MPTP-PEST fusion protein readily dephosphorylated the general substrates pNPP and tyrosine ^{32}P-labelled RCM-lysozyme. As expected, the GST protein was completely devoid of PTPase activity. However, the same fusion protein was not able to dephosphorylate a synthetic tyrosine-phosphorylated peptide derived from an N-terminal region of p34^{cdc2} [21,22] (results not shown).

Expression of MPTP-PEST throughout development

MPTP-PEST has been shown to be ubiquitously expressed [4]. However, its expression during murine development has never been ascertained. Using RT-PCR (Figure 3a) and Northern-blot analysis (Figure 3b), we detected the presence of MPTP-PEST mRNA from day 6.5 *post coitum* onwards. In addition, RNase-protection assays performed on total RNA from embryonic stem cells suggest that MPTP-PEST is expressed as early as the blastocyst stage, i.e. day 3.5 *post coitum* (results not shown).

Cellular localization of MPTP-PEST

In order to narrow the field of potential *in vivo* substrates, we examined the cellular localization of MPTP-PEST by several techniques. The HA epitope (YPYDVPDYA) derived from the haemagglutinin protein of the influenza virus was attached as a three-tandem repeat to the N-terminus of the MPTP-PEST cDNA resulting in the pACTAG-2-MPTP-PEST vector (Figure 4a) (see the Materials and methods section). This vector, when expressed in COS-1 cells, allows the subcellular localization of the HA-MPTP-PEST protein to be tracked by indirect immuno-fluorescence. At 48 h after transfection, cells were fixed and incubated with the monoclonal antibody 12CA5 which recognizes the HA epitope. Three different fields are shown in Figure 4(b). Neighbouring cells, which are presumably not transfected, display low to relatively non-existent background fluorescence. The micrographs clearly show that the fluorescence staining from the transfected cells, representing the localization of HA-MPTP-PEST, is cytoplasmic. However, the punctate nature of the staining suggests that HA-MPTP-PEST associates or is an integral component of either the cytoskeleton or intracellular membranes such as the endoplasmic reticulum.

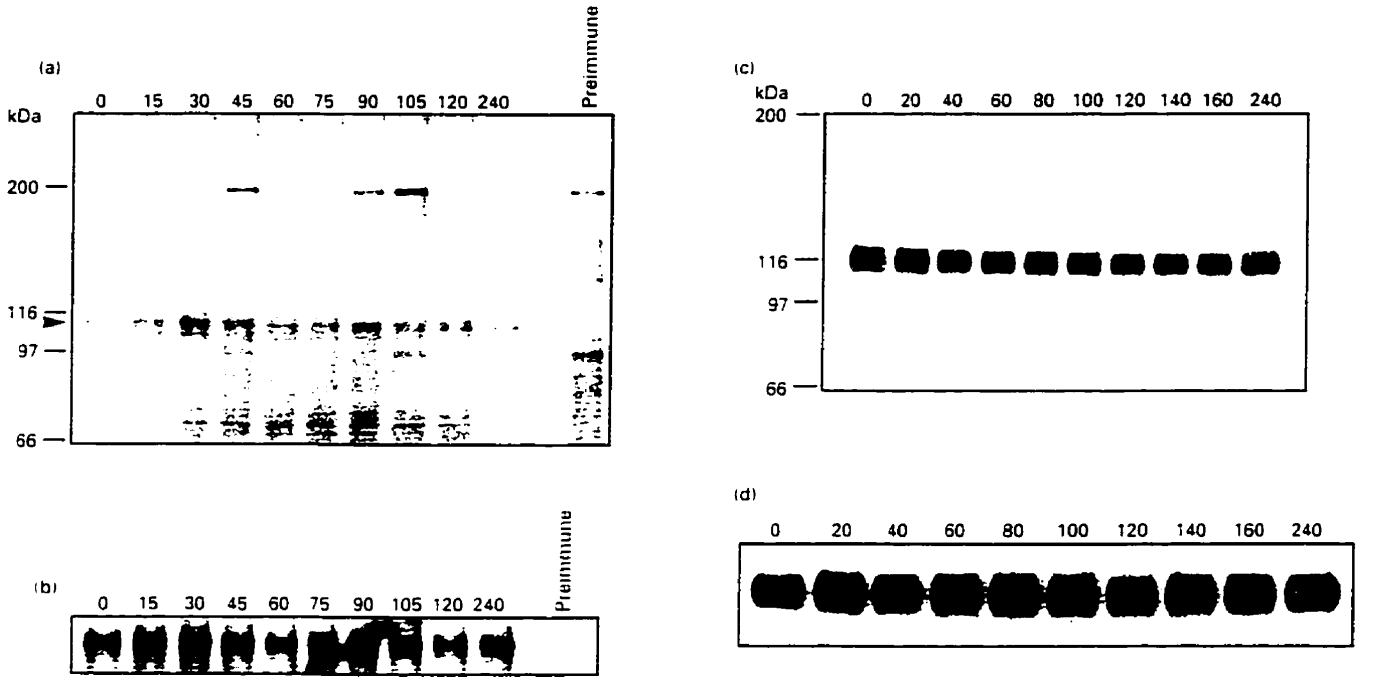


Figure 6 Pulse–chase analysis of MPTP-PEST revealing that MPTP-PEST is not rapidly degraded

a) and **b**) Endogenous MPTP-PEST proteins were immunoprecipitated from [^{35}S]-labelled Ltk$^-$ cells after a chase with unlabelled methionine and cysteine for the indicated time (min). Immunoprecipitates were separated by SDS-PAGE and immunoblotted as described in the Materials and methods section. Western blot **b**) allows the quantification of MPTP-PEST proteins present in each sample. **a**) Level of [^{35}S]-labelled MPTP-PEST immunoprecipitates. The arrowhead indicates MPTP-PEST. Results shown are representative of three different experiments. **c**) and **d**) COS-1 cells transfected with pACTAG-2 MPTP-PEST and labelled with [^{35}S]methionine/[^{35}S]cysteine were treated as in **a**) and **b**) except that HA-MPTP-PEST was immunoprecipitated using the 12CA5 monoclonal antibody. Results shown are representative of two different experiments.

To verify the potential association of MPTP-PEST with subcellular structures, a series of subcellular fractionation studies was conducted. First, to determine if MPTP-PEST associates with the cytoskeleton, a differential extraction technique was employed [18]. Adherent cell monolayers were extracted with a buffer that separates cellular components into a Triton X-100-soluble fraction and an insoluble cytoskeleton component. Triton X-100 treatment typically solubilizes membranes allowing the isolation of membrane-bound and cytosolic proteins, leaving the cytoskeleton intact. The latter can therefore be isolated, washed and solubilized. Figure 5(a) clearly shows that MPTP-PEST is present in the soluble (S) fraction of three different murine cell lines but not in the cytoskeletal fraction (C). This suggests that MPTP-PEST is not an integral component of, or does not strongly associate with, the cytoskeleton. As a control for the purity of the fractions, an equal amount of each extract was subjected to SDS-PAGE and then stained with Coomassie Blue. Figure 5(b) shows that each fraction consists of different sets of proteins.

The results obtained from the non-ionic-detergent extraction do not exclude the possibility that MPTP-PEST associates with intracellular membrane structures. To test this, we isolated different cellular fractions and subjected them to Western-blot analysis in order to localize MPTP-PEST. Identical results were obtained with both Ltk$^-$ and NIH 3T3 fibroblasts, both of which endogenously express MPTP-PEST proteins (Figure 5c), and COS-1 cells, which transiently express the HA-tagged MPTP-PEST (Figure 5e). Figures 5(c) and 5(e) show that MPTP-PEST is present in the S100 fraction which represents the cytosolic content of the cell. In order to monitor the purity of our preparations, the Ltk$^-$ NIH 3T3 blot was stripped and incubated with an anti-(mouse PDGF receptor) antibody. As shown in Figure 5(d) most of the PDGF receptor immunoreactivity is present in the P1 fraction which is composed of nuclei and plasma membrane. Notice that there is no PDGF receptor immunoreactivity in Ltk$^-$ cells. The non-specific bands seen in all three fractions of Figure 5(e) are due to 12CA5 immunoreactive proteins which are detected when large amounts of protein (30 μg) are assayed.

Pulse–chase analysis of MPTP-PEST

As described for the human homologue PTPG1, MPTP-PEST also contains an unusually high content of proline, serine, threonine and glutamate residues within the C-terminal region of the protein. The preponderance of these amino acids is reminiscent of a structure known as PEST motifs [7]. On the basis of the observations of Takekawa et al. [5], we subjected the C-terminal sequence of MPTP-PEST to a PESTFIND algorithm (PC/GENE; Intelligenetics) in order to localize defined regions of PEST sequences. Four regions gave high PEST scores (a score higher than 5 is indicative of potential rapid degradation [7]). Sequence clusters comprised of amino acids 540–553, 565–578, 666–679 and 728–740 (Figure 1, double-underlined sequences) gave PEST scores of 9.78, 11.67, 16.2 and 6.98 respectively. Comparison between the human and mouse PEST motifs demonstrates that only two (565–578 and 666–679) of the four PEST motifs are conserved. Because of the presence of PEST sequences within the C-terminal portion of MPTP-PEST and because proteins harbouring PEST sequences are often thought to be

rapidly degraded [7], we performed pulse–chase experiments to examine the half-life of MPTP-PEST.

Pulse–chase analysis was performed on Ltk$^-$ cells and COS-1 cells transiently transfected with pACTAG-2-MPTP-PEST. Cells were starved in methionine- and cysteine-free media, labelled with [^{35}S]methionine/[^{35}S]cysteine, and chased with unlabelled methionine and cysteine for the indicated periods of time at which point MPTP-PEST proteins were immunoprecipitated using anti-MPTP-PEST antibody (Figures 6a and 6b) or anti-(HA tag) antibody (Figures 6c and 6d). The [^{35}S]methionine/[^{35}S]cysteine-labelled immunoprecipitates were separated by SDS/PAGE and transferred to nitrocellulose membranes. The amount of MPTP-PEST protein present in each lane was quantified by Western-blot analysis using the anti-MPTP-PEST antibody 1075 (Figures 6b and 6d). After ECL detection, the blots were completely stripped allowing efficient detection of the ^{35}S-labelled MPTP-PEST proteins using a FUJIX BAS 2000 detection system (Figures 6a and 6c). On densitometric quantification (results not shown) no fluctuation in the level of ^{35}S-labelled MPTP-PEST proteins was observed in immunoprecipitates of Ltk$^-$ cells during a chase period of 4 h (Figure 6a). Similarly, 12CA5 immunoprecipitates of COS-1 cells transiently expressing HA-MPTP-PEST also failed to demonstrate that MPTP-PEST is a short-lived protein (Figure 6c). These results indicate that MPTP-PEST is a very stable protein with a half-life of over 4 h.

DISCUSSION

We have isolated the murine homologue of the human PTP-PEST cDNA [6] from an 18.5-day embryonic kidney cDNA library. Alignment of the predicted amino acid sequences of MPTP-PEST with the previously reported murine P19-PTP [3] reveals several discrepancies. One of the major differences lies in a stretch of 118 amino acids contained within residues 296–414 of MPTP-PEST. Nucleotide alignment between MPTP-PEST and P19-PTP cDNAs demonstrates that additional nucleotides in the P19-PTP cDNA sequence are responsible for these observed in-frame shifts (results not shown). DNA sequencing of the regions encompassing the discrepancies at the genomic level (A. Charest, J. Wagner and M. L. Tremblay, unpublished work) indicates that both the nucleotide and amino acid sequences reported in Figure 1 represent the genuine murine PTP-PEST. The overall amino acid sequence identity between MPTP-PEST and its human counterpart is 82.6% (97.9% within the catalytic domain but only 73.8% within the PEST-containing C-terminal domain). Examination of the 5′-UTR sequence (Figure 1) indicates that it is rich in guanine and cytosine nucleotides and thus has the potential of forming stable secondary structures. A second feature of this 315 bp 5′-UTR is the presence of a small open reading frame that may encode a 20-amino acid peptide (Figure 1, nucleotides 129–189). The presence of GC-rich segments and small open reading frames within 5′-UTRs has been shown to influence the efficiency of translation initiation in eukaryotic systems [23]. This represents an additional mechanism through which the function of MPTP-PEST could be regulated.

The predicted molecular mass of MPTP-PEST is 88 kDa. However, Western-blot analysis and immunoprecipitation studies on different murine cell lines indicate that MPTP-PEST migrates as a single 112 kDa band. This molecular mass was confirmed by transient transfection studies of COS-1 cells with the pACTAG-2-MPTP-PEST vector. As expected, the HA-MPTP-PEST protein migrates slightly more slowly than MPTP-PEST protein [Figure 5a, compare murine cell lines with positive control (+ve)] because of the presence of HA tags in the former. The difference between the predicted and actual molecular mass of MPTP-PEST can probably be attributed to its high proline content which represents 11.1% of the total amino acid content of the protein. Proteins with high proline content are known to display retarded migration on SDS/PAGE. The extensive phosphorylation of MPTP-PEST could also contribute to the retardation of its migration on SDS/PAGE (A. Charest and M. L. Tremblay, unpublished work). Interestingly, Takekawa et al. [8] recently reported that the molecular mass of the human, simian and murine PTP-PEST protein is 88 kDa. This is contradictory to what we have observed. The nature of the phenomenon underlying this inconsistency remains unknown.

When expressed *in vitro* as a GST fusion protein, the phosphatase domain of MPTP-PEST readily dephosphorylated pNPP and tyrosine-phosphorylated RCM-lysozyme (Figure 2) but not a tyrosine-phosphorylated p34^{cdc2} synthetic peptide (results not shown). These results demonstrate that a bacterially expressed MPTP-PEST–GST fusion protein shows specificity in its phosphatase activity towards tyrosine-phosphorylated substrates.

It has been reported that MPTP-PEST is broadly expressed in adult tissues [4]. However, the expression of MPTP-PEST during embryogenesis has not previously been studied. Using a combination of RT-PCR (Figure 3a) and Northern-blot analysis (Figure 3b), we showed that MPTP-PEST mRNA is present as early as day 6.5 *post coitum* of the gestation period. Moreover, RNase-protection experiments, carried out on total RNA isolated from embryonic stem cells which represent embryonic day 3.5 *post coitum*, indicated that MPTP-PEST gene expression can be detected as early as the blastocyst stage of development. These observations, combined with the ubiquitous nature of MPTP-PEST expression in the adult, suggest that MPTP-PEST performs a basic function in mammalian cells.

Because the antibodies available against MPTP-PEST are not sufficiently sensitive to detect the protein by immunofluorescence in non-transfected cells, we utilized the expression system described in Figure 4 to study its cellular localization. Insertion of three HA epitope tags at the N-terminus of MPTP-PEST allowed us to visualize HA-MPTP-PEST by indirect immunofluorescence. Transfected COS-1 cells have been employed extensively in immunofluorescence studies. It has been shown for many different proteins that localization to the appropriate compartments is not altered because of the ectopic expression [14,24,25]. The results from the immunofluorescence (Figure 4b) and cellular fractionation experiments (Figure 5) clearly indicate that MPTP-PEST is a cytoplasmic protein.

Interestingly, Flores et al. [26] recently demonstrated that PTP-PEP, a PTPase with sequence similarity to MPTP-PEST, is located in the nucleus. These authors identified a region of 12 amino acids [^{788}SKPKGPRNPPSA^{799}) located toward the C-terminus as being responsible for this nuclear translocation. As a similar sequence (^{762}GKPKGPREPPSE^{773}) is also present in MPTP-PEST, they proposed that this PTPase will also localize to the nucleus. However, our data do not agree with this hypothesis. A possible explanation is the presence of two glutamate residues at positions 769 and 773 of MPTP-PEST. Negatively charged amino acids in the nuclear localization signal are very infrequent, and it has been proposed that such acidic residues counteract the basic amino acid motif present in all nuclear localization signals [27]. Thus these subtle differences could account for the disparity in targeting between MPTP-PEST and PTP-PEP.

PEST sequences were originally defined as being protein motifs that promote rapid degradation [7]. Having localized four of these clusters within the C-terminal region of MPTP-PEST, we

performed pulse–chase experiments to determine if MPTP-PEST, according to the model proposed by Rogers et al. [7], was also a protein with a short half-life. Surprisingly both endogenous MPTP-PEST from Ltk⁻ cells and HA-MPTP-PEST from COS-1 cells transiently transfected with the pACTAG2-MPTP-PEST vector demonstrated very stable half-lives of over 4 h. The lack of conservation of PEST sequences between human and mouse forms suggests that PEST motifs may not be involved in rapid protein degradation for this enzyme. Except for one report by Loetscher et al. [28], the PEST-motif model is solely based on amino acid comparison. In addition, recent data from Pakdel et al. [29] tend to suggest that the PEST hypothesis lacks universality. Interestingly, Flores et al. [26] also demonstrated that the presence of PEST sequences within PTP-PEP does not confer rapid degradation.

The presence of a stable MPTP-PEST free in the cytosol indicates that it could potentially act on any tyrosine-phosphorylated cytoplasmic substrates. We have demonstrated that, although this PTPase contains many PEST motifs, rapid protein degradation does not appear to be a means by which it is regulated. Thus it is likely that the activity of this enzyme is regulated by other mechanisms, such as post-translational modification. Indeed, phosphorylation has been identified in PTP-PEST [30] (A. Charest and M. L. Tremblay, unpublished work) and in several other PTPases [31–33]. The recent observation that MPTP-PEST interacts with SHC proteins suggests another method of regulation of this enzyme [9].

This MPTP-PEST-SHC interaction provides clues to the function of MPTP-PEST. It is possible that, after receptor tyrosine kinase activation, the recruitment of SHC proteins to these receptors may also relocalize a fraction of the MPTP-PEST proteins towards tyrosine-phosphorylated signalling molecules and therefore modulate the extent of tyrosine phosphorylation. Under these conditions, MPTP-PEST could act as a modulator of downstream targets such as the Ras pathway. In order to understand the roles and regulation of MPTP-PEST, potential substrates and additional associated proteins of MPTP-PEST will have to be identified.

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REFERENCES

1 Pot, D. A. and Dixon, J. E. (1992) Biochim. Biophys. Acta **1136**, 35–43
2 Mourey, R. J. and Dixon, J. (1994) Curr. Opin. Genet. Dev. **4**, 31–39
3 den Hertog, J., Pals, C. E. G. M., Jonk, L. J. C. and Kruijer, W. (1992) Biochim. Biophys. Res. Commun. **184**, 1241–1249
4 Yi, T., Cleveland, J. L. and Ihle, J. N. (1991) Blood **78**, 2222–2228
5 Takekawa, M., Itoh, F., Hinoda, Y. et al. (1992) Biochim. Biophys. Res. Commun. **189**, 1223–1230
6 Yang, Q., Co, D., Sommercorn, J. and Tonks, N. (1993) J. Biol. Chem. **268**, 6622–6628
7 Rogers, S., Wells, R. and Rechsteiner, M. (1986) Science **234**, 364–368
8 Takekawa, M., Itoh, F., Hinoda, Y. et al. (1994) FEBS Lett. **339**, 222–228
9 Habib, T., Herrera, R. and Williams, S. J. (1994) J. Biol. Chem. **269**, 25243–25246
10 Kavanaugh, W. M. and Williams, L. T. (1994) Science **266**, 1862–1865
11 Field, J., Nikawa, J. I., Broek, D. et al. (1988) Mol. Cell. Biol. **8**, 2159–2165
12 Kolodziej, P. and Young, R. A. (1989) Mol. Cell. Biol. **9**, 5387–5394
13 Pelletier, J., Schalling, M., Buckler, A. J., Rogers, A., Haber, D. A. and Houseman, D. (1991) Genes Dev. **5**, 1345–1356
14 Lejbkowicz, F., Goyer, C., Darveau, A., Neron, S., Lemieux, R. and Sonenberg, N. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 9612–9616
15 Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
16 Sambrook, K. J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
17 Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A. and Neel, B. G. (1992) Cell **68**, 545–560
18 Wilson, R. and Bropny, P. J. (1989) J. Neurosci. Res. **22**, 439–448
19 Hanford, J. B. and Klausner, R. D. (1990) Enzyme **44**, 28–41
20 Mathews, M. B. (1990) Enzyme **44**, 250–264
21 Cheng, H.-C., Litwin, C. M. E., Hwang, D. M. and Wang, J. H. (1991) J. Biol. Chem. **266**, 17919–17925
22 Cheng, H. C., Nishio, H., Hatase, O., Ralph, S. and Wang, J. H. (1992) J. Biol. Chem. **267**, 9248–9256
23 Hinnebusch, A. G. (1988) Trends Genet. **4**, 169–174
24 Tillman, U., Wagner, J., Boerboom, D., Westphal, H. and Tremblay, M. L. (1994) Mol. Cell. Biol. **14**, 3030–3040
25 Herscovics, A., Schneikert, J., Athanassiadis, A. and Moremen, K. W. (1994) J. Biol. Chem. **269**, 9864–9871
26 Flores, E., Roy, G., Patel, D., Shaw, A. and Thomas, M. L. (1994) Mol. Cell. Biol. **14**, 4938–4946
27 Boulikas, T. (1994) J. Cell. Biochem. **55**, 32–58
28 Loetscher, P., Pratt, G. and Rechsteiner, M. (1991) J. Biol. Chem. **266**, 11213–11220
29 Pakdel, F., Le Goff, P. and Katzenellenbogen, B. S. (1993) J. Ster. Biochem. Mol. Biol. **46**, 663–672
30 Flint, A. J., Gebbink, M. F. G. B., Franza, B. R. Jr., Hill, D. E. and Tonks, N. K. (1993) EMBO J. **12**, 1937–1946
31 Vogel, W., Lammers, R., Huang, J. and Ullrich, A. (1993) Science **259**, 1611–1614
32 Ostergaard, H. L. and Trowbridge, I. S. (1991) Science **253**, 1423–1426
33 Garton, A. J. and Tonks, N. K. (1994) EMBO J. **13**, 3763–3771

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The work presented in Chapter 2 constitutes a basic characterization of the tyrosine phosphatase MPTP-PEST at different levels. The MPTP-PEST cDNA was isolated from an 18 day old mouse embryonic kidney library and sequenced in its entirety. The catalytic activity of the MPTP-PEST enzyme was assayed *in vitro* and its cellular localization demonstrated using indirect immunofluorescence microscopy and biochemical approaches. The expression of the MPTP-PEST gene during murine development was assayed by RT-PCR and Northern blot analysis. Finally, the stability of the MPTP-PEST protein *in vivo* was determined using pulse-chase analysis. The characterization of the latter feature demonstrated that the presence of PEST sequences within MPTP-PEST do not mediate a rapid degradation.

The information gathered by such general initial characterization of any given protein helps to direct the subsequent research towards the design of experiments that will ultimately lead to a better understanding of the function of the protein in question. The delineation of the molecular basis behind the different messages observed by Takekawa *et al.*, (1994) and denHertog *et al.*, (1992) and their potential role(s) in processes such as cell transformation and differentiation will further enhance our understanding of the function of MPTP-PEST. To address this issue, the genomic structure of MPTP-PEST was characterized. This work constitutes part of Chapter 3.

CHAPTER 3

Structure of the Murine MPTP-PEST Gene: Genomic Organization and Chromosomal Mapping.

GENOMICS 28, 501–507 (1995)

Structure of the Murine MPTP-PEST Gene: Genomic Organization and Chromosomal Mapping

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Protein tyrosine phosphatases comprise a large family of enzymes that are involved in the control of cellular tyrosine phosphorylation. We have used λ phage analysis to elucidate the complete genomic structure of an intracellular member of this family, the murine MPTP-PEST gene. Eight overlapping λ phage clones representing the MPTP-PEST locus were isolated from a 129/*sv* mouse genomic library. The gene spans over 90 kb of the mouse genome and is composed of 18 exons, 10 of which constitute the catalytic phosphatase domain. Detailed comparison of the position of intron/exon boundaries of the phosphatase domain of MPTP-PEST to those of several other protein tyrosine phosphatases indicates that the MPTP-PEST catalytic domain contains additional exons as a consequence of the insertion of novel introns. In addition, this analysis reveals a strong conservation of the genomic organization within the catalytic domain of the protein tyrosine phosphatase gene family. Finally, fluorescence *in situ* hybridization with MPTP-PEST genomic DNA refines the map position of MPTP-PEST to mouse chromosome 5A3 to B. This result is in agreement with the previous mapping of the human PEST gene to chromosome 7q11.23, a region of synteny with the centromeric portion of mouse chromosome 5.

INTRODUCTION

Protein tyrosine phosphatases (PTPases[2]) and kinases are directly responsible for the regulation of the level of tyrosine phosphorylation in the cell. Tyrosine phosphorylation has been shown to be an important regulatory mechanism by which many different types of signaling cascades are controlled (for review see Schlessinger and Ullrich, 1992). Several reports directly implicate PTPases in the modulation of cell growth (Mishra and Hamburger, 1993; Noguchi *et al.*, 1993), cellular differentiation (den Hertog *et al.*, 1993), and transformation (Zheng *et al.*, 1992; LaForgia *et al.*, 1991). However, little is known about the mechanisms and controls by which PTPases modulate these processes.

We and others have recently isolated and characterized the cDNA of a ubiquitously expressed member of the PTPase supergene family, the murine MPTP-PEST (Charest *et al.*, 1995; EMBL Accession No. X86781). Also known as PTPG1, P19-PTP, and TY43 (Takekawa *et al.*, 1992; den Hertog *et al.*, 1992; Yi *et al.*, 1991; Yang *et al.*, 1993), this cytosolic PTPase of 112 kDa (775 amino acids) is phosphorylated on serine and threonine residues *in vivo*. Interestingly, phosphorylation on a single serine residue (ser 39) decreases its enzymatic activity (Garton and Tonks, 1994). As its name implies, MPTP-PEST contains PEST motifs. Located within the C-terminal portion of the enzyme, these motifs are stretches of amino acids rich in proline, glutamate, serine, and threonine that are thought to confer rapid degradation to the proteins that harbor them (Rogers *et al.*, 1986). We have determined that the half-life of MPTP-PEST is over 4 h, thus indicating that in this case, the PEST sequences found within MPTP-PEST are not involved in rapid protein degradation processes (Charest *et al.*, 1995).

Recent findings suggest that PTP-PEST may be involved in cellular transformation. First, aberrant PTP-PEST transcripts were found in a human colon cancer cell line (Takekawa *et al.*, 1994). Second, the PTP-PEST protein was shown to bind to the adaptor molecule SHC both *in vitro* and *in vivo* (Habib *et al.*, 1994; A. Charest and M. L. Tremblay, unpublished results). This interaction suggests that the PTP-PEST enzyme might play an important role in signal transduction events related to oncogenesis. Since a strong correlation between the accumulation of genetic abberations and the process of tumorigenicity exists (Fearon and Vogelstein, 1990), the presence of mutated PTPases in specific signal

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[2] Abbreviations used: PTPases, protein tyrosine phosphatases; FISH, fluorescence *in situ* hybridization; SSCP, single-strand conformatical polymorphism; DAPI, 4,6-diamidino-2-phenylindole; PEST, proline, glutamate, serine, and threonine; UTR, untranslated region.

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transduction pathways controlled by tyrosine phosphorylation may represent a key step in the course leading to cellular transformation. Thus, the determination of the gene structure and chromosomal position of MPTP-PEST is essential to understanding the mechanisms involved in the production of mutated forms of this enzyme.

Here we report that the murine MPTP-PEST gene comprises 18 exons and spans a region of over 90 kb of the genome. In addition, the map of the intron/exon junctions of MPTP-PEST was used to compare the structural features of the catalytic domains of different PTPases. Finally, fluorescence *in situ* hybridization (FISH) studies reveal that the MPTP-PEST gene maps to chromosome 5A3–B.

MATERIALS AND METHODS

Genomic library screening. A Lambda DASH II (Stratagene) phage genomic library made from 129/*sv* mouse female kidney DNA (a kind gift of Dr. Janet Rossant, Mt. Sinai Hospital, Toronto, Canada) was screened by standard plaque-hybridization techniques using ^{32}P-labeled MPTP-PEST cDNA as a probe. One million plaques were transferred to nylon membrane (Hybond-N$^+$, Amersham) and prehybridized for 2 h at 42°C in 50% formamide, 3.4× SSC (1× SSC: 0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.4), 10 m*M* Tris–Cl, pH 7.5, 1% SDS, 10% dextran sulfate, and 1× Denhardt's solution (0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin) in the presence of 100 μg/ml of nonhomologous DNA (from salmon testes, Pharmacia). The heat-denatured ^{32}P-radiolabeled MPTP-PEST cDNA probe was added at 10^6 cpm/ml and hybridized at 42°C for 16–18 h. Filters were washed three times with 2× SSC–0.1% SDS at 42°C and three times at 65°C with 0.2× SSC–0.1% SDS. Positive plaques underwent three more cycles of purification, and phage DNA was isolated using a standard liquid lysate procedure (Davis *et al.*, 1987).

Radiolabeling of cDNA and oligonucleotides probes. The MPTP-PEST cDNA was radiolabeled to a specific activity >10^9 cpm/μg DNA by [α-^{32}P]dCTP (3000 Ci/mmol, DuPont–NEN) incorporation with a T$_7$ DNA polymerase random-primed DNA labeling kit (T$_7$ Quick Prime, Pharmacia). Synthetic oligonucleotides were 5′-end-labeled with [γ-^{32}P]ATP (6000 Ci/mmol, DuPont–NEN) and T$_4$ polynucleotide kinase (New England Biolabs) to a specific activity of 10^7 cpm/pmol. Unincorporated nucleotides were removed by centrifugation through Sephadex G-50 (Pharmacia) spin columns.

Southern blotting, subcloning, and sequence analysis. λ phage DNAs (1–5 μg) were digested with a series of restriction enzymes, electrophoresed through an 0.8% agarose gel, and capillary transferred in 0.4 *N* NaOH to positively charged nylon membranes (Hybond-N$^+$). Membranes were analyzed by Southern blot hybridization with ^{32}P-labeled cDNA and oligonucleotide probes according to the manufacturer's protocol. Selected fragments obtained by preparative digestion of the appropriate λ phage DNA were separated by electrophoresis on 1% agarose gels, isolated by GeneClean II kit (Bio 101), and subcloned into pBluescript II KS(+) vector (Stratagene). DNA sequences of MPTP-PEST exons and intron/exon boundaries were determined by the dideoxynucleotide chain termination method using either (1) a Sequenase kit (U.S.B.) with alkali-denatured double-stranded plasmid DNA templates or (2) a Circumvent thermal cycle DNA sequencing kit (New England Biolabs) and ^{32}P 5′-end-labeled primers.

Fluorescence in situ hybridization. Mouse chromosomes were prepared according to the procedure described in Feng *et al.* (1994). A 19-kb phage DNA probe was labeled with biotinylated dATP using the BioNick labeling kit (Gibco–BRL). The denaturation of slides and probes, hybridization, and signal detection and amplification were performed according to Heng *et al.* (1992) and Heng and Tsui (1993).

RESULTS

Organization of the MPTP–PEST Gene

Using the MPTP-PEST cDNA (Charest *et al.*, 1995), we have isolated eight λ phage clones representing the entire MPTP-PEST gene locus from a 129/*sv* mouse genomic library. The sizes of the inserts ranged between 15 and 20 kb. The eight overlapping λ phage genomic clones (Fig. 1) were used to analyze the MPTP-PEST gene structure. The intron/exon boundaries were mapped by sequencing the exons in their totality and portions of the adjacent introns using oligonucleotides derived from the cDNA sequence. A graphical representation of the MPTP-PEST gene is shown in Fig. 1. The 2.3-kb open reading frame coding for MPTP-PEST is distributed over 18 exons that range in size from 31 bp (exon 16) to 974 bp (exon 13) (see Table 1). The sizes of the introns varied between 0.7 and >14 kb in size. Exon 1 contains the initiating methionine codon as well as 315 bp of 5′ UTR (Charest *et al.*, 1995). The catalytic domain of MPTP-PEST is partitioned over 10 exons (exons 2 to 11). The C-terminal region of MPTP-PEST is encoded by 8 exons (exons 11 to 18). The PEST motifs, as defined by the PESTFIND algorithm (PCGENE, Intelligenetics) (PEST I, amino acids 540–553; II, 565–578; III, 666–679; and IV, 728–740), found within the C-terminal portion of the protein are encoded by exons 13 (I and II), 14 (III), and 17 (IV). Exon 18 contains the translation termination codon and sequences that are part of the 3′ UTR. Analysis of the DNA sequence spanning the intron/exon boundaries (Table 1) showed that all exons had splice donor and splice acceptor sites conforming to the 5′ gt. . . .ag 3′ splice junction consensus (Breathnach and Chambon, 1981; Shapiro and Senapathy, 1987).

Conservation of Intron/Exon Boundaries among PTPases

The catalytic unit of PTPases is composed of approximately 300 amino acids and contains residues that are conserved among all known PTPases. We compared the genomic structure of the MPTP-PEST PTPase domain to those of human and murine receptor type protein tyrosine phosphatases, CD45 (Hall *et al.*, 1988; Saga *et al.*, 1988; Johnson *et al.*, 1989), LAR (O'Grady *et al.*, 1994), and LRP (Wong *et al.*, 1993), and to that of the intracellular PTPase, MPTP (Mosinger *et al.*, 1992), which was recently mapped in our laboratory. The alignment is shown in Fig. 2. The phosphatase domain of MPTP-PEST is encoded by 10 exons (exons 2 to 11), whereas all other enzymes are encoded by no more than 8 exons. Seven of the nine intron/exon splice site positions found in the MPTP-PEST PTPase domain are also found in other protein tyrosine phosphatases; two of the nine positions are unique to MPTP-PEST. The

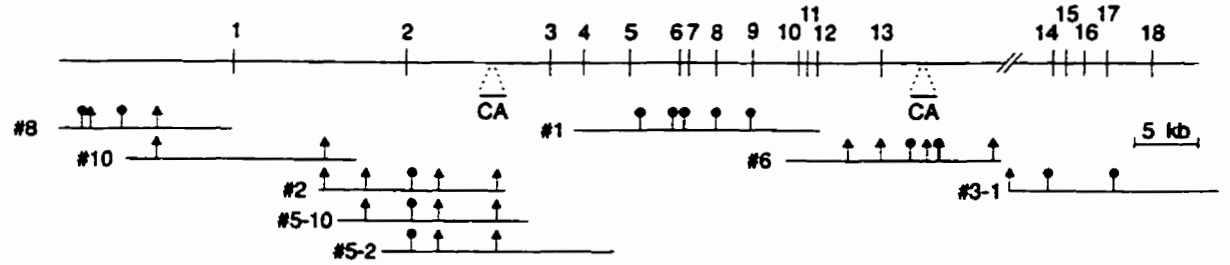

FIG. 1. Schematic representation of the organization of the murine MPTP-PEST gene. The upper portion of the figure shows the arrangement of the 18 exons (depicted by vertical lines and numbers). Two $(CA)_n$ repeats (within introns 2 and 13) are shown. The eight overlapping λ phage clones are depicted in the bottom part of the figure. *Hin*dIII and *Kpn*I restriction endonuclease sites are represented by circles and triangles, respectively. The figure is drawn to scale.

intron present in the DNA sequences encoding the essential catalytic motif (VHCSAG) is found in all PTPases analyzed (Fig. 2).

Chromosomal Localization of MPTP-PEST

Insert DNA isolated from λ phage clone 1 (19 kb in length) was used to determine the regional location of the MPTP-PEST gene in the genome by FISH (Figs. 3A and 3B). FISH was performed on mouse metaphase chromosomes. One hundred mitotic figures were analyzed, and 90 displayed a positive signal (indicated by double staining) on chromosome 5 near the centromere (Fig. 3A). There were no detectable signals on other chromosomes. A total of 10 mitotic figures were photographed, and the data are summarized in Fig. 3C. By comparison to DAPI banding patterns, seven chromosomes displayed positive signals on chromosome 5 to a region equivalent to band A3 and three chromosomes to band B (Fig. 3C). Therefore, the location of the murine MPTP-PEST gene on chromosome 5 was assigned to the region A3 to B.

DISCUSSION

To gain insight into the function of MPTP-PEST, we have isolated and characterized the gene coding for this enzyme. A contig of eight λ phage genomic clones was assembled and used to determine the genomic structure of the MPTP-PEST gene (Fig. 1). The gene consists of 18 exons distributed over 90 kb of genomic DNA. The amino acid sequence deduced from the exon nucleotide sequence perfectly matched that of the MPTP-PEST cDNA (data not shown). The catalytic domain of MPTP-PEST (amino acids 58–465) is encoded by 10 exons (exons 2 to 11), whereas its C-terminal portion is distributed over 8 exons (11 to 18). The four PEST motifs of MPTP-PEST are encoded by exons 13 (PEST motifs I and II), 14 (III), and 17 (IV). All intron/exon splice junctions followed exactly the 5′ gt. . . .ag 3′ rule of donor and acceptor sites (Breathnach and Chambon, 1981; Shapiro and Senapathy, 1987).

Northern blot analysis of MPTP-PEST from different sources reveals a single mRNA species of 3.8 kb (Charest *et al.*, 1995; Yi *et al.*, 1991). The presence of a single transcript suggests that the MPTP-PEST message is not subject to alternative splicing. However, due to the relatively small size of several exons (see Table 1) and the inherently weak resolving properties of Northern blots, we cannot exclude the possibility of alternative splicing.

Comparison of the genomic structure of the MPTP-PEST catalytic domain to those of other protein tyrosine phosphatases indicates that the 10 exons encoding the phosphatase domain of MPTP-PEST represent the most intricate intron/exon organization of all PTPases characterized so far. For instance, two intron/exon junctions (between exons 4 and 5 and exons 6 and 7) are absent from the other PTPases described. The remaining seven of the nine intron/exon boundary positions of the MPTP-PEST phosphatase domain are present in most (and in certain cases, in all) of the other PTPases examined (Fig. 2). The unique presence of two additional introns within the catalytic domain of MPTP-PEST suggests that the MPTP-PEST gene is the product of a different branch of the PTPase phylogenetic tree. The catalytic domains of MPTP-PEST and that of another PEST-containing enzyme, PTP-PEP (Matthews *et al.*, 1992; Flores *et al.*, 1994), share a 62% sequence identity at the amino acid level (data not shown). This makes the PTP-PEP gene the closest known relative of the MPTP-PEST gene. It will be interesting to compare the genomic organization of the MPTP-PEST phosphatase domain to that of the closely related PTP-PEP enzyme.

Using FISH, we have determined the location of the MPTP-PEST gene in the genome. The gene was shown to map to chromosome 5A3–B. The mapping of the MPTP-PEST gene to the centromeric portion of mouse chromosome 5 is in accordance with results obtained for the human homologue. The human PTP-PEST gene has been shown to reside on the proximal portion of the long arm of human chromosome 7, more precisely to 7q11.23 (Takekawa *et al.*, 1994). This region is syntenic to the centromeric portion of mouse chromosome 5 where the murine MPTP-PEST gene has been located by our FISH analysis. Based on the mapping of the human PTP-PEST homologue, our results extend the proximal limit of this syntenic region from 7q21 to 7q11.

In an attempt to define the chromosomal location of the MPTP-PEST gene more precisely, we screened, by

TABLE 1

Splice Junction Sequences, Exons Sizes, and Estimated Intron Sizes of the Murine MPTP-PEST Gene

Exon	3′-Splice site		5′-Splice site		Exon size (bp)	Intron size (kb)
		33				
1	.....	GAC TTC ATG		gtgagtgtcg	>518	~14.0
		D F M				
	34	69				
2	gtattttaag	CGA TTG AGA	ATA CTG CCA	T gtaagttga	108	~11.5
		R L R	I L P			
	70	95				
3	tcttttgtag	TT GAT CAC	TTT ATT AAG	gtgcgtatga	77	~2.8
		F D H	F I K			
	96	127				
4	actctttcag	GGT GTG TAT	AAT GTT GTG	gtgagtgact	96	~3.5
		G V Y	N V V			
	128	148				
5	tccttcttag	ATC ATC GTG	ATG GGA AGG	gtacgttaac	39	~3.8
		I I V	M G R			
	141	164				
6	tgcacaacag	AAA AAG TGT	ATT TCT TGT	gtaagtacct	72	~0.7
		K K C	I S C			
	165	184				
7	atctttttag	GAA AAT GAA	TTT CAA AAT	gtaagtaatt	60	~2.0
		E N E	F Q N			
	185	232				
8	tttcatgtag	GAA TCC CGT	CAT TGC AG	gtacaaaata	143	~4.0
		E S R	H C S			
	233	254				
9	gttcccttag T	GCA GGC TGT	AAA GCA GGG	gtaagaatgc	67	~2.8
		A G C	K A G			
	255	280				
10	tttgttttag	AAA ATT CCA	CAA ACA AAG	gtagagttgg	78	~0.9
		K I P	Q T K			
	281	312				
11	tttataatag	GAG CAG TAT	GCT GAT GGT	gtaagtgttc	96	~1.0
		E Q Y	A D G			
	313	341				
12	cccttggcag	AAT GAA ATT	ACT CGA AG	gtactctgtg	86	~4.8
		N E I	T T S			
	342	665				
13	tcctcctcag T	TGC CTT GTA	GCT GAG AAA	G gtatacggtg	974	>12.5
		C L V	A E K			
	666	688				
14	tttaattcag	AT GCT GAT	GTA TTA GCA	G gtgagcgtaa	69	~1.1
		D A D	V L A			
	689	711				
15	tgttccccag	AT ATG CCT	GAA TCT GAA	gtgagtcgtg	68	~1.4
		D M P	E S E			
	712	731				
16	ctctttgcag	GGC TTG ACA	GAA AAA CAT	G gtagttattt	31	~1.9
		G L T	E K H			
	732	755				
17	tttatcttag	AT GCA GGG	ACA GAT ATT	G gtaacttact	102	~3.6
		D A G	T D I			
	756	775				
18	ttggcaatag	GT TTT GGT	TGG ACA TGA	TGCAGGGAGTGAA		
		G F G	W T *			

single-strand conformational polymorphism (SSCP), many progenitor strains of recombinant inbred (RI) panels for polymorphism in several exons as well as in two $(CA)_n$ repeats located within introns 2 and 13 (Fig. 1). Using SSCP analysis, no polymorphisms were detected in any of the RI progenitor strains analyzed (A/J, AKR/J, C57BL/6J, C3H/HeJ, DBA/2J) (data not shown).

The location of the MPTP-PEST gene to the centromeric portion of chromosome 5 maps in the vicinity of the reeler (*rl*) mutation. Interestingly, the human PTP-PEST gene maps to the same locus as Zellweger syndrome (Takekawa *et al.*, 1994), a human counterpart of reeler. However, a candidate gene responsible for the reeler phenotype that encodes a gene product related to

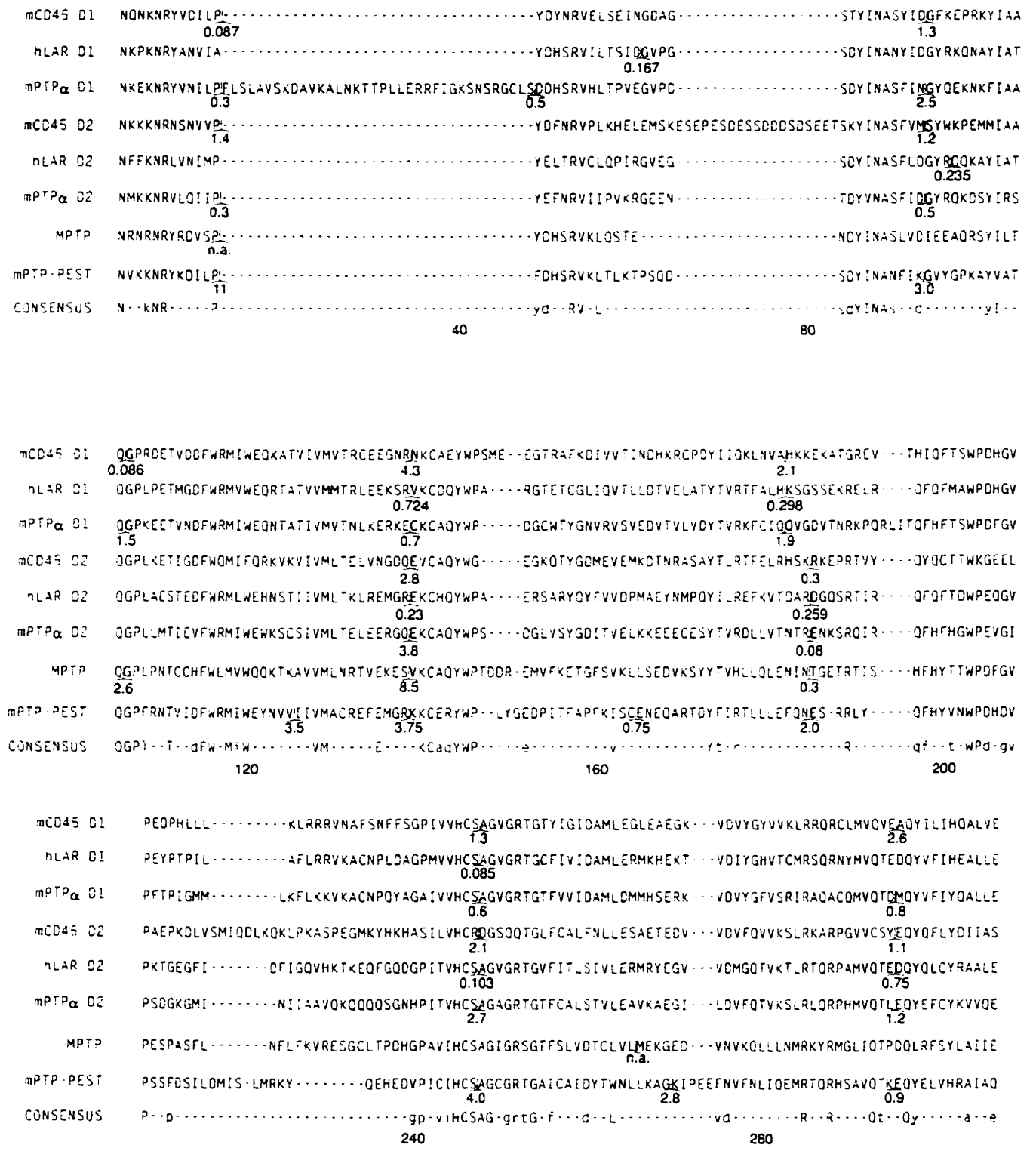

FIG. 2. Comparison of the intron/exon boundaries of different PTPase domains. The amino acid sequences of the catalytic subunit(s) of mCD45, hLAR, mPTPα, MPTP, and MPTP-PEST were aligned using a previously established consensus sequence (Matthews *et al.*, 1990). The capital letters of the consensus sequence represent amino acid residues that are fully conserved among the five different PTPases examined. Lowercase letters depict residues that are less conserved. The intron/exon boundaries are represented by an inverted T with the size of the intron (in kb) indicated below. n.a., not available.

extracellular matrix proteins has recently been identified (d'Arcangelo *et al.*, 1995).

Human PTP-PEST transcripts are genetically modified in a human colon cancer cell line by what appears to be aberrant splicing (Takekawa *et al.*, 1994). Using the intron/exon boundaries of the murine MPTP-PEST

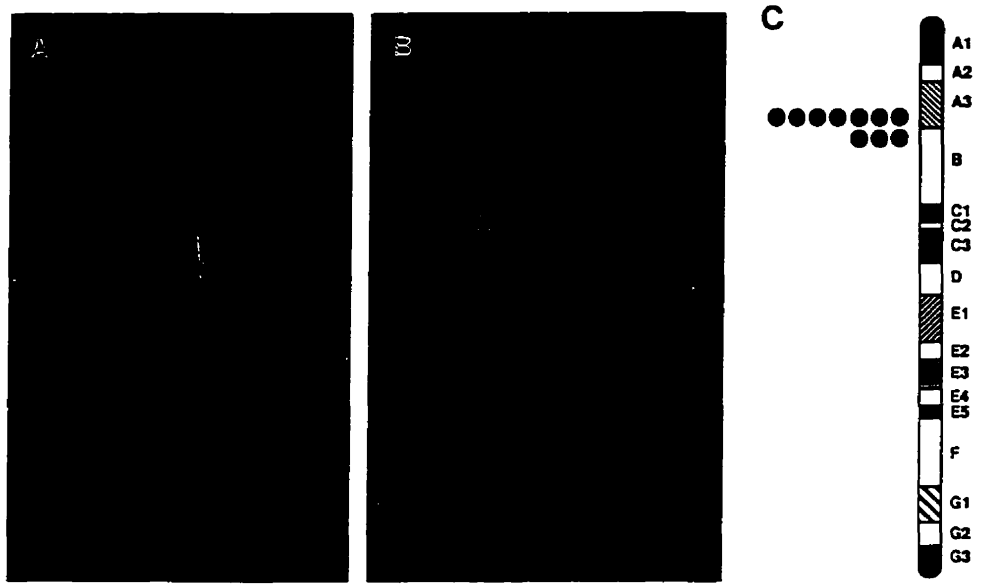

FIG. 3. Localization of the murine MPTP-PEST gene to mouse chromosome 5A3–B. (**A**) A biotinylated insert of λ phage clone 1 was hybridized to mouse metaphase chromosomes and detected according to Heng *et al.* (1992) and Heng and Tsui, (1993). (**B**) Chromosomes were counterstained with DAPI and the hybridization signal was assigned to chromosome 5. (**C**) Idiogram of mouse chromosome 5 summarizing the results of 10 different photographs. Each dot represents a double fluorescent signal on chromosome 5.

gene as a reference, these atypical PTP-PEST messages observed by Takekawa *et al.* (1994) can be explained. The two abnormal mRNAs isolated would be the result of the removal of exon 3 (the DC2 form) and exons 3 and 4 (the DC3 form) during splicing of the messages. In both cases this alternative splicing would disrupt the reading frame, giving rise to prematurely terminated inactive enzymes. The circumstances under which such splicing phenomena would occur and the biological consequences in relation to signal transduction and carcinogenesis are yet to be determined.

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REFERENCES

Breathnach, R., and Chambon, P. (1981). Organization and expression of eukaryotic split genes coding for proteins. *Annu. Rev. Biochem.* **50:** 349–383.

Charest, A., Wagner, J., Shen, S.-H., and Tremblay, M. L. (1995). Murine protein tyrosine phosphatase–PEST, a stable cytosolic protein tyrosine phosphatase. *Biochem. J.* **308**(2): 425–432.

d'Arcangelo, G., Miao, G. G., Chen, S.-C., Soares, H. D., Morgan, J. I., and Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature* **374:** 719–723.

Davis, L. G., Dibner, M. D., and Batley, J. F. (1987). Preparation of DNA from bacteriophage clones. *In* "Basic Methods in Molecular Biology," pp. 158–165, Elsevier, New York.

den Hertog, J., Pals, C. E. G. M., Jonk, L. J. C., and Kruijer, W. (1992). Differential expression of a novel murine non-receptor protein tyrosine phosphatase during differentiation of P19 embryonal carcinoma cells. *Biochem. Biophys. Res. Commun.* **184:** 1241–1249.

den Hertog, J., Pals, C. E. G. M., Peppelenbosh, M. P., Tertoolen, L. G. J., de Laat, S. W., and Kruijer, W. (1993). Receptor protein tyrosine phosphatase α activates $pp60^{c\text{-}src}$ and is involved in neuronal differentiation. *EMBO J.* **12:** 3789–3798.

Fearon, E. R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell* **61:** 759–767.

Feng, G. S., Shen, R., Heng, H. H. Q., Tsui, L.-C., Kazlauskas, A., and Pawson, T. (1994). Receptor-binding, tyrosine phosphorylation and chromosome localization of the mouse SH2-containing phosphotyrosine phosphatase Syp. *Oncogene* **9:** 1545–1550.

Flores, E., Roy, G., Patel, D., Shaw, A., and Thomas, M. L. (1994). Nuclear localization of the PEP protein tyrosine phosphatase. *Mol. Cell. Biol.* **14:** 4939–4946.

Garton, A. J., and Tonks, N. (1994). PTP-PEST: A protein tyrosine phosphatase regulated by serine phosphorylation. *EMBO J.* **13:** 3763–3771.

Habib, T., Herrera, R., and Decker, S. J. (1994). Activators of protein kinase C stimulate association of Shc and the PEST tyrosine phosphatase. *J. Biol. Chem.* **269:** 25243–25246.

Hall, L. R., Streuli, M., Schlossman, S. F., and Saito, H. (1988). Complete exon–intron organization of the leukocyte common antigen (CD45) gene. *J. Immunol.* **141:** 2781–2787.

Heng, H. H. Q., Squire, J., and Tsui, L.-C. (1992). High-resolution mapping of mammalian genes by in situ hybridization to free chromatin. *Proc. Natl. Acad. Sci. USA* **89:** 9509–9513.

Heng, H. H. Q., and Tsui, L.-C. (1993). Modes of DAPI banding and simultaneous in situ hybridization. *Chromosoma* **102:** 325–332.

Johnson, N. A., Meyer, C. M., Pingel, J. T., and Thomas, M. L. (1989). Sequence conservation in potential regulatory regions of the mouse

and human leucocyte common antigen gene. *J. Biol. Chem.* **264:** 6220–6229.

LaForgia, S., Morse, B., Levy, J., Barnea, G., Cannizzaro, L. A., Li, F., Nowell, P. C., Boghosian-Sell, L., Glick, J., Weston, A., Harris, C. C., Drabkin, H., Patterson, D., Croce, C. M., Schlessinger, J., and Huebner, K. (1991). Receptor protein-tyrosine phosphatase gamma is a candidate tumor suppressor gene at human chromosome region 3p21. *Proc. Natl. Acad. Sci. USA* **88:** 5036–5040.

Matthews, R. J., Cahir, E. D., and Thomas, M. L. (1990). Identification of an additional member of the protein-tyrosine-phosphatase family: Evidence for alternative splicing in the tyrosine phosphatase domain. *Proc. Natl. Acad. Sci. USA* **87:** 4444–4448.

Matthews, R. J., Bowne, D. B., Flores, E., and Thomas, M. T. (1992). Characterization of hematopoetic intracellular protein tyrosine phosphatases: Description of a phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine-rich sequences. *Mol. Cell. Biol.* **12:** 2396–2405.

Mishra, S., and Hamburger, A. W. (1993). *O*-Phospho-L-tyrosine inhibits cellular growth by activating protein tyrosine phosphatases. *Cancer Res.* **53:** 557–563.

Mosinger, B., Jr., Tillmann, U., Westphal, H., and Tremblay, M. L. (1992). Cloning and characterization of a cDNA encoding a cytoplasmic protein-tyrosine-phosphatase. *Proc. Natl. Acad. Sci. USA* **89:** 499–503.

Noguchi, T., Metz, R., Chen, L., Mattei, M.-G., Carrasco, D., and Bravo, R. (1993). Structure, mapping and expression of erp, a growth factor-inducible gene encoding a nontransmembrane protein tyrosine phosphatase, and effect of ERP on cell growth. *Mol. Cell. Biol.* **13:** 5195–5205.

O'Grady, P., Krueger, N. X., Streuli, M., and Saito, H. (1994). Genomic organization of the human LAR protein tyrosine phosphatase gene and alternative splicing in the extracellular fibronectin type-III domains. *J. Biol. Chem.* **269:** 25193–25199.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. *Science* **234:** 364–368.

Saga, Y., Tung, J.-S., Shen, F.-W., Pancost, T. C., and Boyse, E. A. (1988). Organization of the Ly-5 gene. *Mol. Cell. Biol.* **8:** 4889–4895.

Schlessinger, J., and Ullrich, A. (1992). Growth factor signaling by receptor tyrosine kinases. *Neuron* **9:** 383–391.

Shapiro, M. B., and Senapathy, P. (1987). RNA splice junctions of different classes of eukaryotes: Sequence statistics and functional implications in gene expression. *Nucleic Acids Res.* **15:** 7155–7174.

Takekawa, M., Itoh, F., Hinoda, Y., Arimura, Y., Toyota, M., Sekiya, M., Adachi, M., Imai, K., and Yachi, A. (1992). Cloning and characterization of a human cDNA encoding a novel putative cytoplasmic protein-tyrosine-phosphatase. *Biochem. Biophys. Res. Commun.* **189:** 1223–1230.

Takekawa, M., Itoh, F., Hinoda, Y., Adachi, M., Ariyama, T., Inazawa, J., Imai, K., and Yachi, A. (1994). Chromosomal localization of the protein tyrosine phosphatase G1 gene and characterization of the aberrant transcripts in human colon cancer cells. *FEBS Lett.* **339:** 222–228.

Yang, Q., Co, D., Sommercorn, J., and Tonks, N. (1993). Cloning and expression of PTP-PEST: A novel, human nontransmembrane protein tyrosine phosphatase. *J. Biol. Chem.* **268:** 6622–6628.

Yi, T., Cleveland, J. L., and Ihle, J. N. (1991). Identification of novel protein tyrosine phosphatases of hematopoetic cells by polymerase chain reaction amplification. *Blood* **78:** 2222–2228.

Wong, E. C. C., Mullersman, J. E., and Thomas, M. L. (1993). Leukocyte common antigen-related phosphatase (LRP) gene structure: Conservation of the genomic organization of transmembrane protein tyrosine phosphatases. *Genomics* **17:** 33–38.

Zheng, X. M., Wang, Y., and Pallen, C. J. (1992). Cell transformation and activation of $pp60^{c\text{-}src}$ by overexpression of a protein tyrosine phosphatase. *Nature* **359:** 336–338.

Chapter 3 describes the gene structure and the chromosomal localization of MPTP-PEST. MPTP-PEST is composed of 18 exons of variable sizes spread over 90 kb of genomic DNA. Alignment of the intron/exon boundaries of the catalytic domain of several PTPases including MPTP-PEST, revealed that PTPases originated from a common ancestral gene. Interestingly, the genomic structure of the catalytic domain of MPTP-PEST is different from the other PTPases analyzed. It contains additional introns in comparison to the other PTPases, suggesting that during evolution, the MPTP-PEST gene branched off at a different time from the other PTPases.

By localizing the chromosomal region to which a gene is situated, one might uncover a link between the gene in question and a previously established mutant phenotype. The information generated by this kind of analysis usually allows one to study the potential role(s) that a gene might play in the etiology of a given mutant phenotype.

In order to verify if the gene for MPTP-PEST localizes to a chromosomal region shown to be involved in the generation of mutant phenotypes, Fluorescence *In Situ* Hybridization (FISH) was performed using MPTP-PEST DNA as a probe. It was shown that the MPTP-PEST gene localizes to mouse chromosome 5, region A3-B, a region adjacent to the *reeler* locus. However, a candidate gene responsible for the *reeler* phenotype has recently been isolated (Goffinet 1995). The chromosomal mapping of MPTP-PEST to mouse chromosome 5A3-B is in accordance with a previous report indicating that the human PTP-PEST gene is situated on human chromosome 7q11.23, a region syntenic to the proximal portion of mouse chromosome 5. Localizing the MPTP-PEST gene to mouse chromosome

5 extended the proximal boundary of this synteny between human and mouse.

In addition to the gene structure and chromosomal localization, the identification of proteins that associate with MPTP-PEST may uncover important aspects of the function of MPTP-PEST. The next chapter describes the characterization of the association between MPTP-PEST and Shc proteins.

CHAPTER 4

Phosphotyrosine-independent Binding of SHC to the NPLH Sequence of Murine Protein-tyrosine Phosphatase-PEST. Evidence for Extended PTB/PI Domain Recognition Specificity.

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Phosphotyrosine-independent Binding of SHC to the NPLH Sequence of Murine Protein-tyrosine Phosphatase-PEST

EVIDENCE FOR EXTENDED PHOSPHOTYROSINE BINDING/PHOSPHOTYROSINE INTERACTION DOMAIN RECOGNITION SPECIFICITY*

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The phosphotyrosine binding (PTB) or phosphotyrosine interaction (PI) domain of the proto-oncoprotein p52^{SHC} binds to an NPXpY consensus sequence found in several growth factor receptors (Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1994) *Science* 268, 1177–1179). The amino-terminal region of p52^{SHC}, which includes the PTB/PI domain, has been previously shown to associate with protein-tyrosine phosphatase-PEST (PTP-PEST) *in vivo* (Habib, T., Herrera, R., and Decker, S. J. (1994) *J. Biol. Chem.* 269, 25243–25246). We report here the detailed mapping of this interaction in a murine context using glutathione S-transferase fusion protein binding studies and peptide competition assays. We show that the interaction between murine SHC and murine PTP-PEST is mediated through the PTB/PI domain of murine SHC and an NPLH sequence found in the carboxyl terminus of murine PTP-PEST. Since this interaction is not dependent on the presence of a tyrosine-phosphorylated residue in the target sequence, this reveals that the PTB/PI domain of SHC can recognize both tyrosine-phosphorylated sequences and non-tyrosine-based recognition motifs.

External stimuli are often transduced into intracellular events via specific cascades of protein tyrosine phosphorylation and dephosphorylation, which are modulated by the presence and availability of adaptor molecules, protein-tyrosine kinases, and PTPases.[1] The cytoplasmic adaptor molecule SHC is among many mediators that act downstream of receptor type and cytoplasmic protein-tyrosine kinases. SHC can transform fibroblasts and differentiate PC12 cells in a Ras-dependent manner. SHC regulates these signaling events through its own tyrosine phosphorylation on residue Tyr^{317} and by mediating the assembly of tyrosine-phosphorylated signaling complexes via its SH2 and phosphotyrosine binding/phosphotyrosine interaction (PTB/PI) domains (1–3). The p52^{SHC} NH_2-terminal PTB/PI domain is a novel phosphotyrosine recognition motif that is structurally unrelated to SH2 domains and that was shown to bind with high affinity to the autophosphorylation sites of c-Erb B2 Tyr^{1222} (4, 5), TrkA Tyr^{490} (6–8), EGF receptor Tyr^{1148} (6, 7, 9, 10), c-Erb B3 Tyr^{1309} (9), insulin-like growth factor 1 receptor Tyr^{950} (11), insulin receptor Tyr^{960} (12), and the phosphorylated Tyr^{250} residue of polyoma middle T antigen (13). Peptide competition assays and screening of phosphotyrosine peptide libraries have demonstrated that the PTB/PI domain of p52^{SHC} preferentially binds to the sequence NPXpY (where X represents any amino acid and pY indicates a phosphotyrosine residue) with high affinity (5, 9, 10, 13, 14). The PTB/PI domain represents a novel mechanism whereby signaling proteins can interact with tyrosine-phosphorylated protein targets.

MPTP-PEST is a ubiquitously expressed, stable, cytosolic PTPase of 112 kDa that is characterized by the presence of four Pro, Glu, Ser, and Thr-rich PEST domains within the COOH terminus (15). MPTP-PEST is heavily phosphorylated on serine and threonine residues,[2] and the enzymatic activity for the human homologue can be modulated by phosphorylation on specific serine residues (16). Another possible mode of regulation by which the activities of protein-tyrosine phosphatases might be controlled is via association with other proteins (for review see Ref. 17). Recently, it has been demonstrated that the human PTP-PEST protein interacts with the human p52^{SHC} protein *in vivo* (18). In this report we demonstrate that the murine p52^{SHC} protein binds to MPTP-PEST via its PTB/PI domain and that this binding is not dependent on the presence of a phosphotyrosine residue in the target sequence.

MATERIALS AND METHODS

MPTP-PEST and mSHC GST Fusion Proteins—GST fusion proteins were constructed using standard recombinant DNA technology. The constructs created by polymerase chain reaction were sequenced using Sequenase version 2.0 (Amersham Corp.).

Cell Culture, Immunoprecipitation, and Immunoblotting—NIH 3T3 and COS-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Transient transfection of COS-1 cells with hp52^{SHC} cDNA and HA-MPTP-PEST cDNAs was achieved by electroporation as described previously (15). Cell lysate preparations and immunoprecipitation experiments were performed according to previously published protocols (19). Immunoblotting on polyvinylidene difluoride membranes (Millipore) using antibodies described below and horseradish peroxidase-conjugated secondary antibodies was performed with chemiluminescence reagents (DuPont NEN or Amersham) according to the manufacturer's protocol.

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[1] The abbreviations used are: PTPase, protein-tyrosine phosphatase; SH2, Src homology 2; EGF, epidermal growth factor; EGFR, EGF receptor; GST, glutathione S-transferase; PTB/PI domain, phosphotyrosine-binding/phosphotyrosine interaction domain; HA, hemagglutinin antigen; PTP-PEST, protein-tyrosine phosphatase-PEST; MPTP-PEST, murine PTP-PEST; mSHC, murine SHC.

[2] A. Charest and M. L. Tremblay, unpublished results.

FIG. 1. **SHC interacts with MPTP-PEST *in vivo*.** 500 μg of NIH 3T3 cell lysates were immunoprecipitated using an affinity-purified anti-SHC polyclonal antibody. The immunoprecipitates (*IP*) and 10 μg of NIH 3T3 cell lysates were subjected to Western blot and analyzed for the presence of bound MPTP-PEST proteins (*arrow*) using an anti-MPTP-PEST antibody.

Antibodies—The anti-SHC antibodies used in this study were a monoclonal antibody, S14620 (Transduction Laboratories), directed against residues 359–473 of human SHC, and a rabbit polyclonal antibody raised against a GST-SH2 domain fusion protein of human $p52^{SHC}$ (1). The anti-MPTP-PEST polyclonal antibody and the anti-HA tag monoclonal antibody 12CA5 have been described elsewhere (15). These antibodies were used for immunoprecipitation and immunoblotting procedures as described in the figure legends.

Binding Studies—The GST fusion proteins were expressed in bacteria and affinity-purified on glutathione-Sepharose beads (Pharmacia Biotech Inc.) according to published protocols (20). Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad) according to the manufacturer's protocol. Peptide concentrations were determined spectrophotometrically at A_{205} (21). Human SHC or HA-MPTP-PEST-transfected COS-1 cell lysates were incubated with the appropriate GST fusion proteins bound to glutathione-Sepharose beads for 90 min at 4 °C in HNMETG buffer (19) supplemented with protease and phosphatase inhibitors. The beads were then washed 4 times in HNTG buffer (19), separated by SDS-polyacrylamide gel electrophoresis, and immunoblotted with the appropriate antibody as described above. Peptide competition studies were performed as described above except that the peptides were preincubated with the GST fusion proteins for 30 min at 4 °C prior to the addition of cell lysates.

RESULTS

Murine $p52^{SHC}$ Binds to MPTP-PEST in Vivo—A recent report demonstrated the *in vivo* interaction between the NH_2 terminus of the human $p52^{SHC}$ protein with the COOH terminus of the human PTP-PEST PTPase using a yeast two-hybrid system and co-immunoprecipitation studies (18). We sought to determine if this interaction also exists in a murine context since the COOH termini of human and murine PTP-PEST enzymes display weak (74%) sequence identity relative to the highly conserved NH_2-terminal catalytic domains (98% identity).[3]

Using an affinity-purified antibody that recognizes SHC proteins, we immunoprecipitated endogenous mSHC from NIH 3T3 cells under conditions where protein complexes have been shown to co-immunoprecipitate (19). The anti-mSHC immunoprecipitates were screened for the presence of endogenous MPTP-PEST by Western blotting using an anti-MPTP-PEST antibody (Fig. 1). An immunoreactive band of 112 kDa (corresponding to the electrophoretic size of MPTP-PEST) is present in the anti-mSHC immunoprecipitates. In addition, screenings of a mouse embryonic cDNA library in a yeast two-hybrid system using $p52^{SHC}$ as a probe resulted in the isolation of cDNA clones coding for MPTP-PEST (data not shown). These results indicate that SHC proteins interact with MPTP-PEST *in vivo*.

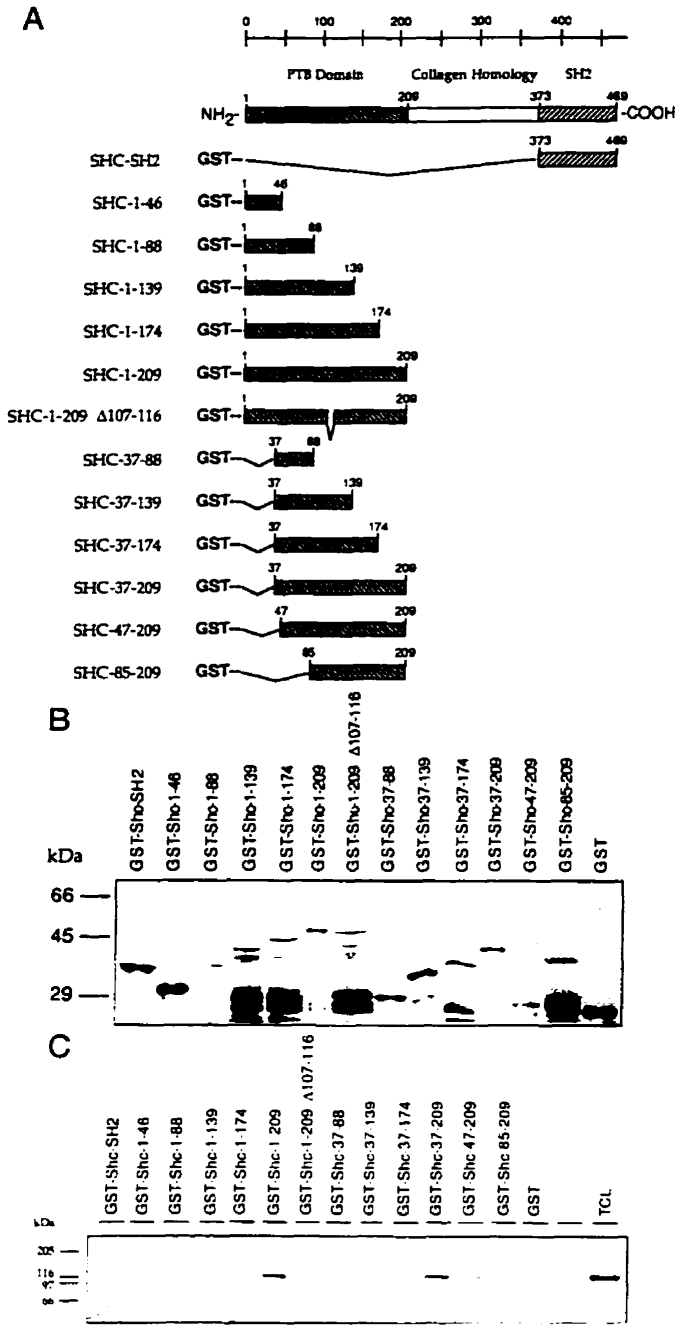

FIG. 2. **MPTP-PEST associates with the PTB domain of mSHC.** Schematic representation (*A*) and Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis (*B*) of the different GST-SHC fusion proteins used in the MPTP-PEST/SHC binding studies are shown. *C*, *in vitro* binding of HA-MPTP-PEST proteins to various GST-SHC fusion proteins. Identical amounts (100 ng) of the different fusion proteins shown in *A* and *B* were incubated with COS-1 cell lysate (200 μg) transiently expressing HA-MPTP-PEST proteins (see "Materials and Methods") Bound HA-MPTP-PEST proteins were detected using the anti-HA tag antibody 12CA5. The *schematic* in *A* is drawn to scale. Some of the GST-Shc fusion proteins shown in *B* display inherent proteolytic degradation associated with bacterial expression of SHC proteins. Immunoblot analysis in *C* is representative of three different experiments.

SHC Binds to MPTP-PEST via Its NH_2-terminal PTB/PI Domain—In order to delineate the minimal binding require-

[3] A. Charest and M. L. Tremblay, unpublished observation.

ments that are involved in the interaction between mSHC and MPTP-PEST, GST-mSHC fusion protein constructs were used in an *in vitro* binding assay. Using these GST fusion proteins representing different regions of mSHC (Fig. 2, *A* and *B*), we identified the portion of mSHC that binds to HA-MPTP-PEST (Fig. 2*C*). The segment of mSHC that lies between amino acids 47 and 209 represents the minimal region required for binding to HA-MPTP-PEST *in vitro*. Since the boundaries of this region in $p52^{SHC}$ coincide with those of the recently characterized PTB/PI domain (4, 6, 7), we used a mutated mSHC PTB/PI domain GST fusion protein that is defective in binding tyrosine-phosphorylated residues (6) in order to determine if the binding of $p52^{SHC}$ to MPTP-PEST is mediated through the PTB/PI domain. As shown in Fig. 2*C*, the mutated mSHC PTB/PI domain GST fusion protein does not bind to HA-MPTP-PEST (compare the *lanes* labeled *GST-Shc-1–209* with *GST-Shc-1–209 Δ107–116*). These results represent evidence that the PTB/PI domain of $p52^{SHC}$ interacts with MPTP-PEST.

The PTB/PI Domain of SHC Binds to MPTP-PEST via an NPLH Sequence—It has been previously shown that the binding of human SHC to the COOH terminus of human PTP-PEST occurs through a region situated between amino acids 416 and 775 of human PTP-PEST (18). We found that the mSHC/MPTP-PEST interaction is mediated by the PTB/PI domain of mSHC, a domain that was shown to be dependent on the presence of a phosphotyrosine residue on the target sequence. However, since no tyrosine residues are present in the equivalent sequence of MPTP-PEST, this interaction raises the possibility of a novel, tyrosine-independent target sequence binding.

Using a series of GST-MPTP-PEST fusion proteins representing different portions of the MPTP-PEST COOH terminus (Fig. 3, *A* and *B*), the region of MPTP-PEST required for binding to $p52^{SHC}$ was determined by an *in vitro* binding assay. The segment located between amino acids 576 and 613 of MPTP-PEST is sufficient for binding to $p52^{SHC}$ protein (Fig. 3*C*). Interestingly, this region contains a sequence (^{599}NPLH^{602}) that closely resembles the $p52^{SHC}$ PTB/PI domain binding consensus motif (NPXpY). In order to verify if the ^{599}NPLH^{602} sequence of MPTP-PEST is involved in the interaction with mSHC, a triple point mutant (N599I,P600A,H602L) was created in the GST-MPTP-PEST-471–613 fusion protein background (Fig. 3, *A–C*, *lanes* labeled *NPXH mut*) and analyzed for binding to $p52^{SHC}$ *in vitro*. By mutating all three amino acids (N599I, P600A, and H602L), the binding of GST-MPTP-PEST-471–613 fusion protein to $p52^{SHC}$ was completely abolished (Fig. 3*C*). These results indicate that the sequence NPXH, found in the COOH terminus of MPTP-PEST, is required for binding to SHC *in vitro*.

The same mutations (N599I, P600A, and H602L) were recreated in a full-length HA-MPTP-PEST protein, and the effects of these substitutions were studied *in vivo*. Immunoprecipitation of SHC proteins in COS-1 cells transfected with either wild type or NPXH mutant HA-MPTP-PEST cDNAs were probed for the presence of associated HA-MPTP-PEST molecules by Western blot analysis (Fig. 4). An immunoreactive band of 120 kDa corresponding to HA-MPTP-PEST co-immunoprecipitated with SHC in cells transfected with the wild type HA-MPTP-PEST cDNA but not in the NPXH mutant cDNA transfectants despite an equal amount of both HA-MPTP-PEST proteins present in the lysates (Fig. 4*B*). This demonstrates that mutating all three amino acids (N599I, P600A, and H602L) in the context of a full-length HA-MPTP-PEST protein eliminates binding to SHC proteins *in vivo*.

Residues Asn^{599} and Pro^{600} of the ^{599}NPLH^{602} Sequence of MPTP-PEST Are Essential for Binding in Vitro—In order to

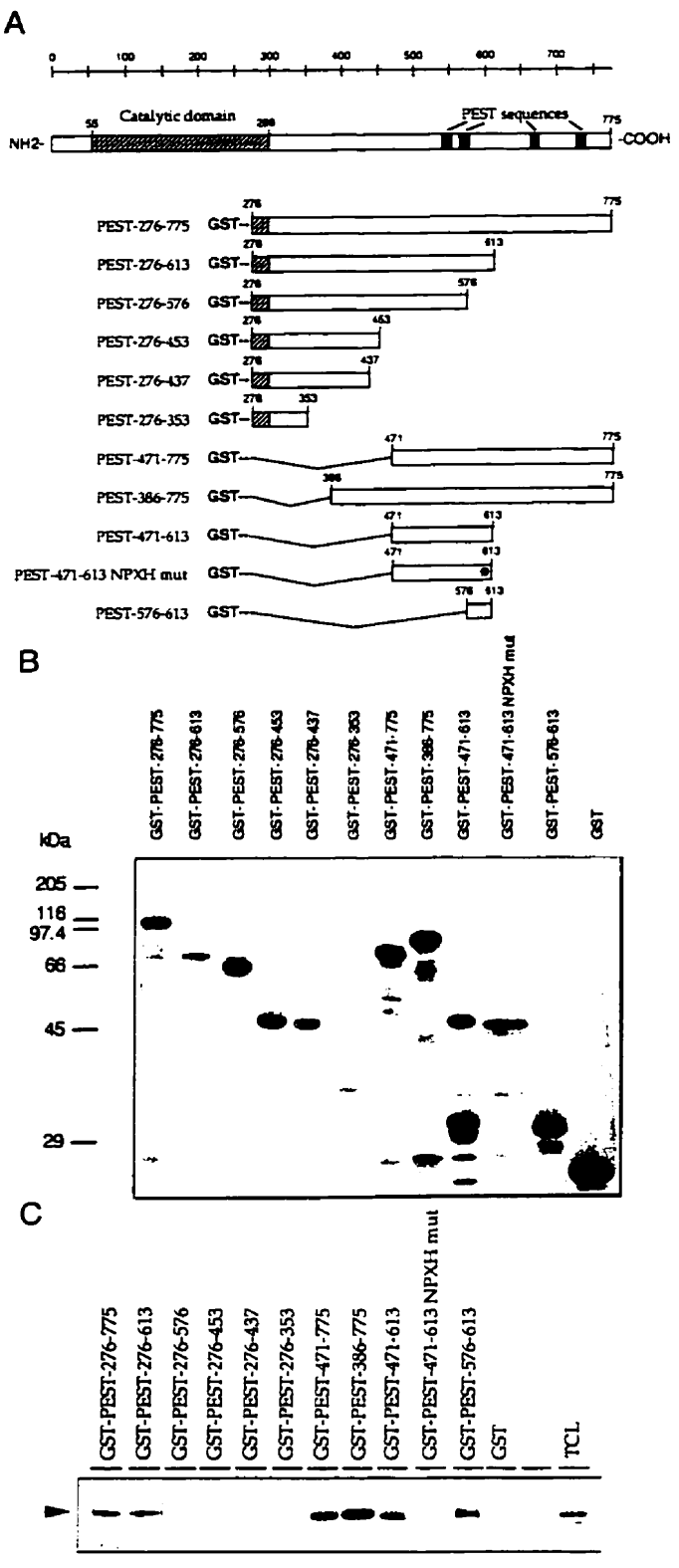

FIG. 3. **SHC binds to a region of MPTP-PEST containing an NPXH sequence.** Schematic representation (*A*) and Coomassie Blue-stained SDS-polyacrylamide gel electrophoresis (*B*) of the GST-MPTP-PEST fusion proteins used in the SHC/MPTP-PEST binding studies are shown. *C*, *in vitro* binding of human $p52^{SHC}$ proteins to various GST-MPTP-PEST fusion proteins. Identical amounts (100 ng) of the GST fusion proteins shown in *A* and *B* were incubated with 200 μg of extracts from COS-1 cells transiently expressing human $p52^{SHC}$ proteins as described under "Materials and Methods." Bound human $p52^{SHC}$ proteins (*arrow*) were detected using an anti-SHC monoclonal antibody. The *schematic* in *A* is drawn to scale. Results shown in *C* are representative of three different experiments.

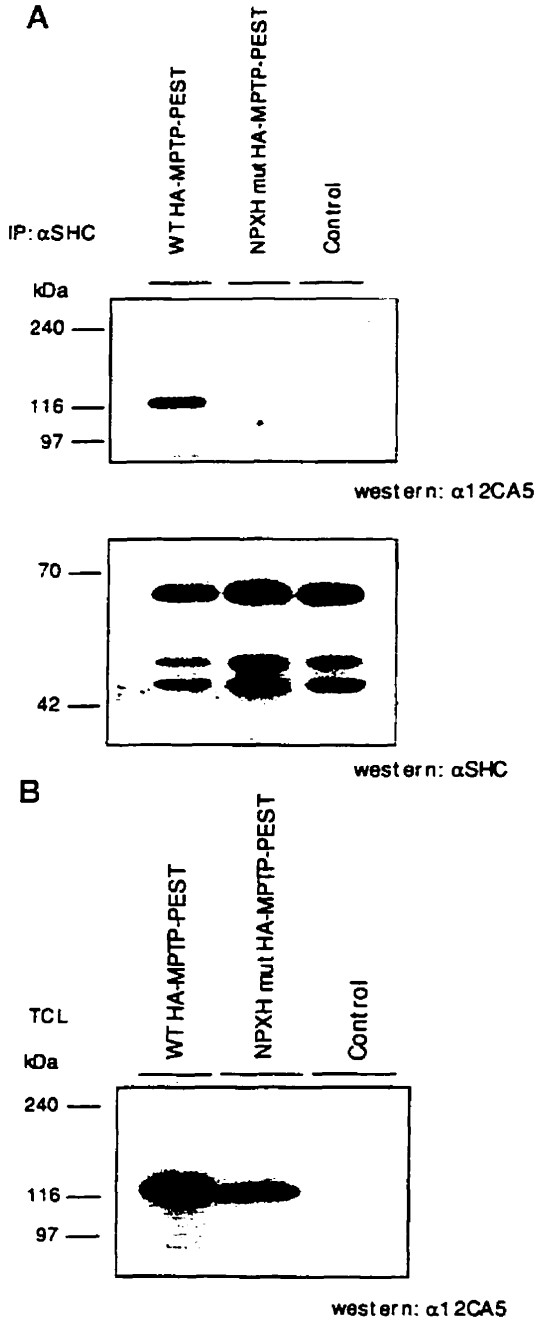

FIG. 4. **The NPXH sequence of MPTP-PEST is required for binding to SHC proteins *in vivo*.** *A*, SHC proteins were immunoprecipitated from identical amounts of extracts derived from COS-1 cells transiently expressing either wild type HA-MPTP-PEST (*WT HA-MPTP-PEST*) or HA-MPTP-PEST with ^{599}NPLH^{602} mutated to ^{599}IALL^{602} (*NPXH mut HA-MPTP-PEST*) and untransfected cells as a control. SHC immunoprecipitates were analyzed for the presence of bound HA-MPTP-PEST proteins (*upper panel*) and for p46^{SHC}, p52^{SHC}, and p66^{SHC} proteins (*lower panel*) using the anti-HA tag antibody 12CA5 and an anti SHC monoclonal antibody, respectively. *B*, 40 μg of total cell lysate (*TCL*) from the different transfectants were analyzed for the presence of HA-MPTP-PEST proteins (wild type and NPXH mutant) by Western blot analysis using the anti-HA tag 12CA5 antibody as described under "Materials and Methods." The results shown in *A* and *B* are representative of two independent experiments.

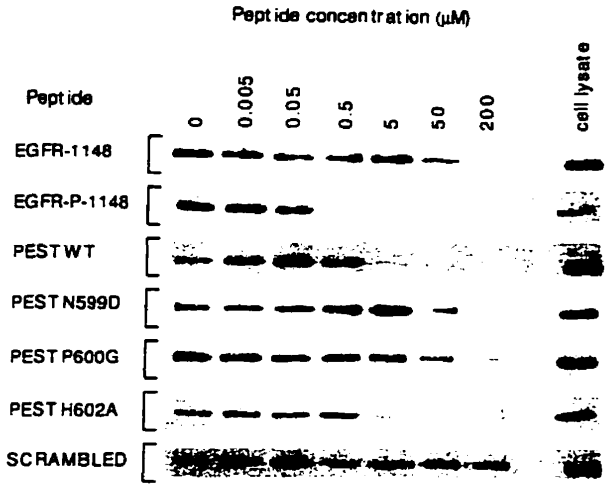

FIG. 5. **Competition by peptides for HA-MPTP-PEST binding to the PTB/PI domain of SHC.** 200 μg of total cell lysate from COS-1 cells transiently expressing HA-MPTP-PEST proteins were incubated with 100 ng of GST-SHC-1–209 fusion protein in the presence of the indicated concentrations of peptides (see "Materials and Methods"). The noncompeted, bound HA-MPTP-PEST was visualized by Western blot analysis using the anti-HA tag antibody 12CA5. The blots were then stripped and reprobed with an anti-GST antibody to assess loading (data not shown). The amino acid sequences of the synthetic peptides are as follows: EGFR-1148, ^{1140}QISLDNPDYQQDF^{1152}; EGFR-P-1148, ^{1140}QISLDNPDpYQQDF^{1152}; PEST-WT, ^{594}PLSFTNPLHSDDWH^{607}; and Scrambled, DPSTHSHPLWLNFD. The results shown are representative of at least three independent experiments.

determine which residues within the NPXH sequence are necessary for binding, we studied the ability of synthetic peptides (corresponding to the amino acid sequence of MPTP-PEST located between residues 594 and 607) to compete for binding of the mSHC PTB/PI domain to full-length HA-MPTP-PEST *in vitro*. Tyrosine-phosphorylated and nonphosphorylated peptides corresponding to a previously characterized SHC PTB/PI domain recognition site on the EGF receptor (10) were used as controls in the same assay. The phosphorylated EGF receptor peptide (EGFR-P-1148) is capable of competing with full-length HA-MPTP-PEST for binding to the PTB/PI domain of mSHC (Fig. 5) at concentrations (between 50 and 500 nM) similar to those reported in other systems (10). Accordingly, its nonphosphorylated counterpart (EGFR-1148) is inefficient at competing with HA-MPTP-PEST for binding. A peptide representing the MPTP-PEST wild type sequence (PEST-WT) competes but at concentrations that are intermediate to those of the phosphorylated and nonphosphorylated EGFR peptides (Fig. 5).

Similar peptide competition assays (10, 13) and phosphopeptide library screenings (14) have previously demonstrated that the −3 Asn, −2 Pro, and the phosphorylated tyrosine residue in the NPXpY motif are essential for SHC PTB/PI domain interaction with the autophosphorylated sites of growth factor receptors. To determine the contribution of these residues in the NPXH-mediated binding of SHC to MPTP-PEST, mutant peptides were used in the same *in vitro* competition binding assays. An MPTP-PEST mutant peptide bearing a N599D substitution is incapable of competition (Fig. 5). A peptide with a P600G mutation is also inefficient at competing for binding even at the highest concentration (Fig. 5). This suggests that both the Asn^{599} and Pro^{600} residues are involved in the binding of HA-MPTP-PEST to the PTB/PI domain of SHC *in vitro*. However, substituting the His^{602} for an Ala residue did not alter the competing potential of the peptide when compared with the wild type peptide. Finally, a peptide with a scrambled amino acid sequence is incapable of competing for binding. These results indirectly demonstrate that the binding of the PTB/PI domain of SHC to the ^{599}NPLH^{602} sequence of MPTP-PEST *in vitro* is dependent on both the Asn^{599} and Pro^{600} amino acid residues.

Residue His^{602} Is Necessary for Binding in Vivo—The affinities with which the WT and H602A MPTP-PEST peptides bind to the PTB/PI domain of SHC *in vitro* are intermediate to those

displayed by the phosphorylated and nonphosphorylated EGFR peptides (Fig. 5). This difference could represent inherent variations in the affinities of the SHC PTB/PI domain for phosphorylated EGF receptor and MPTP-PEST target sequences, in which case the His^{602} residue would not be an essential binding component *in vitro*. Conversely, this difference could reflect a situation where absent histidine post-translational modification is necessary for high affinity binding. In this case, the presence of a histidine residue at amino acid position 602 would be essential for binding.

In order to determine the importance of the His^{602} residue for binding to SHC proteins *in vivo*, we mutated the histidine to an alanine residue in the context of a full-length HA-MPTP-PEST. This H602A mutant was assayed for binding to SHC proteins *in vivo* under the same conditions described in the legend to Fig. 4. Anti-HA-MPTP-PEST immunoblot analysis of SHC immunoprecipitates reveals that the wild type but not the H602A mutant co-immunoprecipitated with SHC proteins (Fig. 6). Upon longer exposure of Fig. 6 (*upper panel*), less than 5% of the H602A HA-MPTP-PEST co-immunoprecipitated with SHC proteins (data not shown). This demonstrates that changing the histidine 602 to an alanine residue drastically reduces (>95%) the binding of SHC proteins to HA-MPTP-PEST *in vivo*, suggesting that the His^{602} residue is essential for binding to SHC proteins *in vivo*.

DISCUSSION

Our data show that the PTB/PI domain of SHC binds to the murine protein-tyrosine phosphatase-PEST via an NPXH sequence. Computer aided searches of sequence data bases have revealed that several proteins contain regions that have sequence similarity to the PTB/PI domain of $p52^{SHC}$ (22). However, their ability to bind to tyrosine-phosphorylated residues in a sequence-specific manner and/or to non-phosphotyrosine-containing recognition sequences remains to be determined. The amino acids immediately surrounding the NPLH sequence in human and murine PTP-PEST proteins are identical (data not shown) and could underscore the importance of this region given the relatively low overall identity between the two COOH termini.

Previous peptide competition assays reveal that distinct amino acids located at different positions in the SHC PTB/PI domain binding sites of the EGF receptor, polyoma middle T antigen, and c-Erb B2 proteins are essential for binding (5, 10, 13). In all three examples, the Asn at position −3 and the phosphorylated tyrosine residue are absolutely essential for high affinity binding, but the location of other required amino acids seems to be protein-specific (data not shown). Although these experiments were performed by different groups and under slightly different conditions, the results nevertheless demonstrate that the modality of binding for the PTB/PI domain of SHC does not appear to be universal. This diversity and the recent NMR studies of both the PTB/PI domain of SHC (23) and of a PTB/PI domain recognition binding sequence (13) suggest that structural components of the SHC PTB/PI domain and its recognition binding site are critical. Therefore, a detailed mutagenic analysis of the surrounding amino acids of the $^{599}NPLH^{602}$ sequence of MPTP-PEST could define the requirements for binding of the SHC PTB/PI domain to the nonphosphotyrosine target sequence of MPTP-PEST. Such analysis could unmask unique residues required for binding in a nonphosphotyrosine-dependent manner.

The necessity for the His^{602} residue in MPTP-PEST for binding to SHC proteins *in vivo*, in addition to the lower binding affinities displayed by the nonmodified wild type and H602A mutated MPTP-PEST peptides *in vitro*, suggest that the His^{602} could be post-translationally modified. Phosphohistidine is an

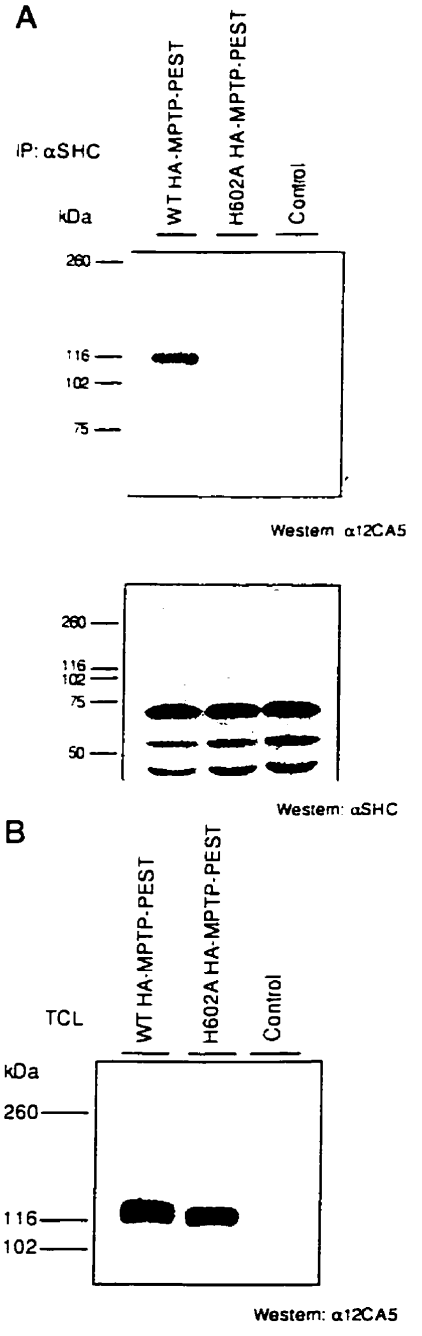

FIG. 6. **The His^{602} residue of MPTP-PEST is necessary for binding to SHC proteins *in vivo*.** *A*, SHC proteins were immunoprecipitated from equal amounts of lysates derived from COS-1 cells transiently expressing either wild type (*WT*) HA-MPTP-PEST or HA-MPTP-PEST with an H602A mutation and untransfected cells as a control. SHC immunoprecipitates were probed for the presence of bound HA-MPTP-PEST proteins (*upper panel*, *Western:αHA*) and for SHC proteins (*lower panel*, *Western:αSHC*) as described under "Materials and Methods." *B*, 20 μg of total cell lysate (*TCL*) from the different transfectants were probed for the presence of HA-MPTP-PEST proteins (wild type and H602A mutant) by immunoblot analysis as described above.

important intermediate in prokaryotic signal transduction pathways involved in processes such as chemotaxis (24) and porin expression (25). Phosphohistidine has been found in many eukaryotic proteins and has recently been implicated in eukaryotic signal transduction in platelets (26). The cytoplasmic tail of P-selectin (a leukocyte adhesion molecule) undergoes rapid and transient histidine phosphorylation on the peptide sequence $^{768}NPHSH^{772}$, where both histidine residues are po-

tential sites of phosphorylation following thrombin or collagen stimulation of platelets (26). It is therefore possible that the sequence ^{599}NPLH^{602} of MPTP-PEST could be phosphorylated on the His^{602} residue and that this post-translational modification is required to achieve high affinity binding to SHC proteins much in the same way phosphorylation of tyrosine residues leads to an increase in binding affinity. Phosphorylation, be it on histidine or other phosphorylatable amino acids, may be an essential recognition parameter by which PTB/PI domain binding occurs in a tyrosine-independent manner. Our data demonstrating a phosphotyrosine-independent binding for this domain serves to underscore the need to re-evaluate the specificity of this interaction.

In conclusion, we demonstrate that the PTB/PI domain of SHC interacts with the non-phosphotyrosine-based NPLH sequence of MPTP-PEST. *In vivo* and *in vitro* studies established that the Asn and Pro residues at positions −3 and −2, respectively, are essential for binding and that the histidine residue at position 0 may require post-translational modification for high affinity binding to SHC proteins.

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REFERENCES

1. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T., and Pelicci, P. G. (1992) *Cell* **70**, 93–104
2. Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G., Schlessinger, J., and Pawson, T. (1992) *Nature* **360**, 689–692
3. Cutler, R. L., Liu, L., Damen, J. E., and Krystal, G. (1993) *J. Biol. Chem.* **268**, 21463–21465
4. Kavanaugh, W. M., and Williams, L. T. (1994) *Science* **266**, 1862–1865
5. Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1995) *Science* **268**, 1177–1179
6. Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V., and Margolis, B. (1994) *J. Biol. Chem.* **269**, 32031–32034
7. van der Geer, P., Wiley, S., Ka-Man Lai, V., Olivier, J. P., Gish, G. D., Stephens, R., Kaplan, D., Shoelson, S., and Pawson, T. (1995) *Curr. Biol.* **5**, 404–412
8. Dikic, I., Batzer, A. G., Blaikie, P., Obermeier, A., Ullrich, A., Schlessinger, J., and Margolis, B. (1995) *J. Biol. Chem.* **270**, 15125–15129
9. Prigent, S. A., Pillay, T. S., Ravichandran, K. S., and Gullick, W. J. (1995) *J. Biol. Chem.* **270**, 22097–22100
10. Batzer, A. G., Blaikie, P., Nelson, K., Schlessinger, J., and Margolis, B. (1995) *Mol. Cell. Biol.* **15**, 4403–4409
11. Craparo, A., O'Neill, T. J., and Gustafson, T. A. (1995) *J. Biol. Chem.* **270**, 15639–15643
12. He, W., O'Neill, J. O., and Gustafson, T. A. (1995) *J. Biol. Chem.* **270**, 23258–23262
13. Trub, T., Choi, W. E., Wolf, G., Ottinger, E., Chen, Y., Weiss, M., and Shoelson, S. E. (1995) *J. Biol. Chem.* **270**, 18205–18208
14. Songyang, Z., Margolis, B., Chaudhuri, M., Shoelson, S. E., and Cantley, L. E. (1995) *J. Biol. Chem.* **270**, 14863–14866
15. Charest, A., Wagner, J., Shen, S.-H., and Tremblay, M. L. (1995) *Biochem. J.* **308**, 425–432
16. Garton, A. J., and Tonks, N. K. (1994) *EMBO J.* **13**, 3763–3771
17. Mauro, L. J., and Dixon, J. E. (1994) *Trends Biochem. Sci.* **19**, 151–155
18. Habib, T., Herrera, R., and Decker, S. J. (1994) *J. Biol. Chem.* **269**, 25243–25246
19. Rotin, D., Bar-Sagi, D., O'Brodovich, H., Merilainen, J., Lehto, V. P., Canessa, C. M., Rossier, B. C., and Downey, G. P. (1994) *EMBO J.* **13**, 4440–4450
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995) in *Current Protocols in Molecular Biology*, 1st Ed., John Wiley & Sons, Inc., New York
21. Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W. and Wingfield, P. T. (1995) in *Current Protocols in Protein Science*, 1st Ed., John Wiley & Sons, Inc., New York
22. Bork, P., and Margolis, B. (1995) *Cell* **80**, 693–694
23. Zhou, M.-M., Ravichandran, K. S., Olejniczak, E. T., Petros, A. M., Meadows, R. P., Sattler, M., Harlan, J. E., Wade, W. S., Burakoff, S. J., and Fesik, S. W. (1995) *Nature* **378**, 584–592
24. Hess, J. F., Bourret, R. B., and Simon, M. I. (1988) *Nature* **336**, 139–143
25. Stock, J. B., Stock, A. M., and Mottonen, J. M. (1990) *Nature* **344**, 395–400
26. Crovello, C. S., Furie, B. C., and Furie, B. (1995) *Cell* **82**, 279–286

The work presented in Chapter 4 describes in detail, the interaction between the adaptor protein Shc and MPTP-PEST. It was discovered that the amino-terminal PTB domain of Shc binds to a core sequence found in the carboxy-terminal portion of MPTP-PEST. Detailed analysis of the modalities of binding revealed that the PTB domain of Shc binds to an NPLH sequence within MPTP-PEST. This data represents the first reported example of a PTB domain interacting with a protein in a non-phosphotyrosine dependent fashion.

The association between Shc proteins and MPTP-PEST imply that MPTP-PEST may play a role in Shc-mediated signaling events. This suggests that the function of MPTP-PEST is to regulate phosphotyrosine-mediated signal transduction pathways.

The carboxy-terminal region of MPTP-PEST contains several proline-rich sequences similar to those that have been shown to act as binding sites for SH3 domains of many different signaling proteins. The next chapter describes the propensity of these proline-rich sequences to act as binding sites for SH3 domains and the functional significance of this kind of binding. The results reported in Chapter 5 further our knowledge of the function of MPTP-PEST in signal transduction events.

CHAPTER 5

Coupling of the Murine Protein Tyrosine Phosphatase PEST to the Epidermal Growth Factor (EGF) Receptor through a Src Homology 3 (SH3) Domain-Mediated Association with Grb2.

ABSTRACT

The involvement of murine protein tyrosine phosphatase-PEST (MPTP-PEST) in signal transduction pathways is suggested by its interaction with the adaptor protein SHC and by the presence of five proline-rich stretches in its non-catalytic carboxyl terminus. Proline-rich sequences have been identified as binding sites for Src homology 3 (SH3) domains found in proteins associated with signal transduction events. The ability of these sequences to act as SH3 domain recognition motifs was investigated using bacterially expressed SH3 domains derived from several different signalling proteins. *In vitro* binding assays indicate that four of these proline-rich sequences constitute specific binding sites for both SH3 domains of the adaptor molecule Grb2. Wild type Grb2, but not Grb2 proteins corresponding to loss-of-function mutants in the *Caenorhabditis elegans sem-5* protein, associate with MPTP-PEST *in vivo*. Experiments in EGF receptor expressing cells show that the interaction between MPTP-PEST and Grb2 results in the binding of this complex to activated EGF receptors. In addition, identification of putative substrate(s) of MPTP-PEST have revealed a candidate protein of 120 kDa which is tyrosine phosphorylated upon EGF stimulation. Together, these results describe a novel SH3 domain-dependent recruitment of a protein tyrosine phosphatase to an activated receptor tyrosine kinase and establish a potential role for MPTP-PEST in signalling pathways at the molecular level.

INTRODUCTION

Src homology 2 (SH2) and Src homology 3 (SH3[1]) domains are

conserved structural motifs mediating protein-protein interactions between effector proteins that are responsible for transmitting various regulatory cascades from cell surface receptors (for review see (1)). These protein modules are often the unique constituents of a growing group of signal transduction proteins know as "adaptor" proteins which appear to function as "connectors", integrating several extracellular signals into multiple intracellular signalling pathways. One of the strongest evidence implicating adaptor molecules as critical components in signal transduction pathways arose from studies of the Grb2/Sem-5/Drk protein.

Grb2 is a ubiquitously expressed protein of 25 kDa that is composed of a single SH2 domain and two flanking SH3 domains. Grb2 is functionally and structurally homologous to the product of the *C. elegans* gene *sem-5* (2, 3) and to the *Drosophila* gene *drk* (4, 5). Genetic and biochemical studies have demonstrated that one of the functions of Grb2 is to link tyrosine-phosphorylated receptors to Ras activation via the SH3 domain-mediated binding of Grb2 to Sos, a guanine nucleotide exchange factor for Ras (2, 4-10). In addition to its role in the Ras signalling pathway, Grb2 appears to be involved in other pathways, including the regulation of membrane ruffles formation, possibly through its interaction with the cytoskeleton (11, 12).

MPTP-PEST is a ubiquitously expressed protein tyrosine phosphatase (PTPase) that is composed of a single NH_2-terminal phosphatase domain and a non-catalytic COOH-terminal tail. The latter has been shown recently to interact with the phosphotyrosine binding (PTB) domain of the adaptor oncoprotein SHC in a phosphotyrosine-independent manner (13) thus implicating MPTP-

PEST in SHC mediated cellular functions. In addition to this SHC PTB domain binding site, the COOH-terminus of MPTP-PEST contains several proline-rich sequences (14). Proline-rich motifs have recently been demonstrated to serve as binding sites for SH3 domains which are present in several signalling proteins (11, 15-18). Structural and binding studies using many different SH3 domains have led to the generation of models for SH3 domain-ligand interactions and to the characterization of two classes of SH3 domain binding sites. Class I sites display a R-X-X-P-X-X-P consensus sequence (single letter amino acid code, where X represents any amino acid residue) whereas class II sites consist of a P-X-X-P-X-R consensus sequence (for review see (19)).

In this report we demonstrate that the proline-rich sequences of MPTP-PEST constitute specific binding sites for the SH3 domains of the adaptor protein Grb2. We also show that this SH3 domain-mediated interaction serves as the molecular basis for the recruitment of MPTP-PEST to activated EGF receptors. Finally, we reveal the existence of a potential tyrosine phosphorylated substrate of 120 kDa for MPTP-PEST. The data presented here suggest that the MPTP-PEST/Grb2 interaction may play a role in growth factor receptor-mediated signal transduction events.

MATERIALS AND METHODS

Cell culture and Transient Transfections. NIH 3T3, COS-1, 293, and HER14 cells were maintained in Dubelcco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Sf9 cells were maintained at 25 °C in complete Grace's insect cell medium (Gibco-BRL) supplemented with 10% fetal bovine serum and

antibiotics. Electroporation of COS-1 cells was performed as previously described (14). 293 cells were transiently transfected using the calcium phosphate coprecipitation method (20) using 50 μg/mL of plasmid DNA. 48 hours post-transfection, cellular proteins were harvested as described (13) and analyzed by western blot and/or immunoprecipitation as described below.

Recombinant Proteins, Binding Studies and Protein Overlay (FarWestern) Assays. The GST fusion proteins described herein were prepared from their respective cDNAs by polymerase chain reaction (PCR) using oligonucleotide primers flanking the appropriate domain boundaries. Mutation of the catalytically essential cysteine residue (Cys231) of MPTP-PEST to a serine residue in a GST fusion protein consisting of the catalytic domain of MPTP-PEST (GST-PEST-1-453aa) was generated by PCR. The recombinant polyhistidine HA-tagged MPTP-PEST protein (6XHis-HA-MPTP-PEST) was constructed in the baculovirus transfer vector pP10 (21) using standard recombinant DNA technology. Recombinant baculoviruses were then generated using a linear AcMNPV DNA Transfection Module (Invitrogen) and used to infect Sf9 insect cells. Cells were harvested 4 days after infection and the 6XHis-HA-MPTP-PEST recombinant proteins were affinity purified from cell lysates on a Ni^{2+} -NTA-agarose column (Qiagen) according to previously described protocols (22, 23). GST fusion proteins derived from the pGEX-2TK vector were expressed in *E. coli* and affinity purified on glutathione-Sepharose beads (Pharmacia Biotech Inc.) according to established protocols (24). The purified GST-SH3 domains (NH_2- and COOH-) of Grb2 were labelled with [γ-^{32}P]ATP (NEN) using protein kinase A (Sigma) according to the manufacturer's protocol and thrombin cleaved to remove the GST moiety. Protein concentration

and *in vitro* binding studies using GST fusion proteins were performed as described elsewhere (13). Overlay assays using ^{32}P-labelled Grb2 SH3 domains as probes were performed according to established protocols (25).

Antibodies, Immunoprecipitation, and Immunoblotting. The anti-human Grb2, the anti-human EGF receptor LA22 (Upstate Biotechnology Inc.), the anti- Phosphotyrosine PY20, and the anti-human EGF receptor L2 (Transduction Laboratories) are monoclonal antibodies. The anti-human Grb2 C-23 (Santa Cruz Biotechnology) is a polyclonal antibody. The rabbit anti-MPTP-PEST polyclonal and the anti-HA tag monoclonal antibodies have been described elsewhere (14). These antibodies were used for immunoprecipitation and immunoblotting procedures as described in the figure legends. Cell lysate preparation, immunoprecipitation and immunoblotting experiments were performed as previously described (13).

RESULTS AND DISCUSSION

The capacity of MPTP-PEST to regulate tyrosine phosphorylation by virtue of its catalytic nature, its ability to bind to the adaptor protein SHC (13) and the presence of proline-rich sequences in the carboxyl terminus suggest potential role(s) for MPTP-PEST in signal transduction events. These proline rich stretches may serve as binding sites for SH3 domains found in proteins involved in signalling cascades. In order to verify the ability of the proline-rich sequences within the MPTP-PEST enzyme to act as SH3 binding sites, *in vitro* binding studies were conducted using a total of eleven GST-SH3

domain fusion proteins derived from different signalling molecules (Fig. 1a). The SH3 domain of the viral oncoprotein v-Src and the full length SH3-SH2-SH3 adaptor protein Grb2 specifically bound to HA-MPTP-PEST proteins *in vitro* (Fig. 1a). To corroborate these results under *in vivo* conditions, MPTP-PEST immunoprecipitates from NIH 3T3 cells were examined by immunoblot analysis for the presence of endogenous c-Src and Grb2 proteins. Immunoblotting of MPTP-PEST immunoprecipitates using an anti-Src antibody failed to detect the presence of Src proteins (data not shown) suggesting that specific *in vivo* interactions between Src and MPTP-PEST do not exist under these conditions. However, the presence of immunoreactive Grb2 proteins in endogenous and HA-tagged MPTP-PEST immunoprecipitates using anti-Grb2 antibodies (Fig. 1b) demonstrates that MPTP-PEST interacts with Grb2 *in vivo* thus validating the results obtained from the GST fusion protein binding studies.

A series of GST fusion proteins derived from Grb2 (Fig. 2a, upper panel) were generated in order to identify the necessary domain(s) (i.e. $SH3_{(N)}$, SH2, and $SH3_{(C)}$) required for binding to MPTP-PEST. Both SH3 domains ($SH3_{(N)}$ and $SH3_{(C)}$) but not the SH2 domain are capable of binding individually to recombinant purified MPTP-PEST proteins *in vitro* (Fig. 2a, lower panel). Interestingly, GST fusion proteins containing both SH3 domains (i.e Grb2-FL and $SH3_{(N)}$-$SH3_{(C)}$), displayed a higher degree of binding to MPTP-PEST. Similar synergistic binding have previously been reported for interactions described between Grb2 and Sos proteins (26). These results demonstrate that the adaptor molecule Grb2 associates with the tyrosine phosphatase MPTP-PEST through either or both SH3 domains.

Figure 1. GRB2 interacts with MPTP-PEST *in vitro* and *in vivo*. A) Binding of SH3 domains to MPTP-PEST *in vitro*. Identical amounts (100 ng) of the indicated GST-SH3 domain fusion proteins (in addition to the SH3 domains of RasGAP, Nck and Crk, data not shown) were incubated with cell lysates (200 µg) of COS-1 cells transiently expressing HA-MPTP-PEST proteins. Bound HA-MPTP-PEST proteins were detected by western blot analysis using the anti-HA tag antibody. B) Interaction between MPTP-PEST and Grb2 *in vivo*. 1 mg of cell lysates from NIH 3T3 cells (upper panel) or COS-1 cells transiently expressing the indicated proteins (lower panel) were immunoprecipitated with anti-MPTP-PEST and anti-HA tag antibodies respectively. The immune complexes were subjected to western blot and analyzed for the presence of bound Grb2 proteins using monoclonal (upper panel) and polyclonal (lower panel) anti-Grb2 antibodies respectively. TCL represents 10 µg of total cell lysate used as a control.

Figure 1a

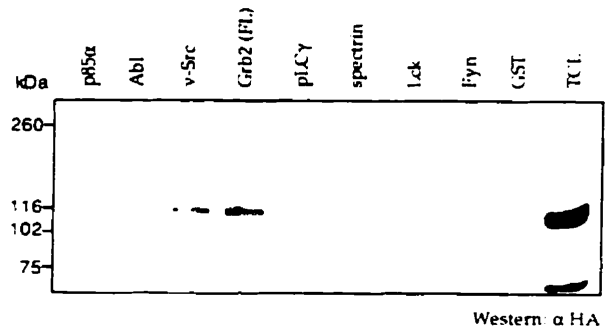


FIgure 1b, upper panel

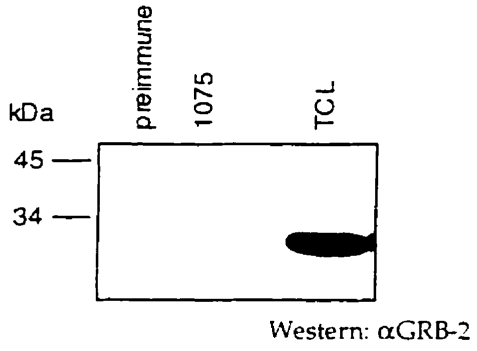
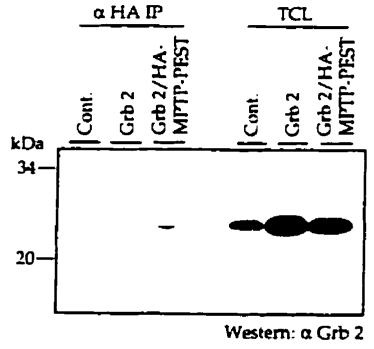


Figure 1b, lower panel



The binding preferences of both SH3 domains in relation to the five proline-rich sequences of MPTP-PEST (Pro 1-5) was determined using GST fusion proteins containing each of the five proline-rich domains (Fig. 2c). These GST fusion proteins (Fig. 2d, upper panel) were subjected to protein overlay analysis using ^{32}P-labelled Grb2 $SH3_{(N)}$ and $SH3_{(C)}$ domains (Fig. 2d, middle and lower panels) as probes. The results from the overlay assays demonstrate that the $SH3_{(N)}$ domain preferentially binds to the GST-Pro 1 and GST-Pro 4 constructs. Interestingly, both Pro 1 (PPPKPPR) and Pro 4 (PPPLPER) sequences belong to class II SH3 domain binding consensus sequence (PXXPXR). The $SH3_{(N)}$ domain of Grb2 has been shown to bind to proline-rich motifs belonging to class II consensus sequences such as those found on the hSos1 (PPPVPPR, PPAIPPR and PPLLPPR), hSos2 (PPPLPPR, PPPPPPR, PPPVPLR and APPVPPR) and Cbl (PPVPPPR) proteins (26, 27). Sequence comparison between these Grb2 $SH3_{(N)}$ binding sites and MPTP-PEST Pro 1 and Pro 4 reveals a high degree of identity. The binding affinities for each of these proline-rich sequences of MPTP-PEST, hSos1-2 and Cbl need to be determined. The observation that these effector molecules bind to an adaptor protein via the same SH3 domain raises the possibility of competitive mechanisms thus creating additional regulatory processes.

The $SH3_{(C)}$ domain of Grb2 preferentially binds to the GST-Pro 3, GST-Pro 4, and GST-Pro 5 constructs (Fig. 2d, lower panel). Pro 3 (PPRPDCLP) and Pro 5 (PKGPREPP) sequences do not conform to any canonical SH3 binding consensus sequences and therefore might represent novel SH3 binding motifs. Detailed mutagenic analyses of these sites could reveal variations on established SH3 binding sites.

The fact that the SH3 domains of Grb2 bind to different subsets of proline-rich sequences on MPTP-PEST (with the exception of Pro 4) suggests that both SH3 domains of a Grb2 molecule could simultaneously bind to different sites on MPTP-PEST. This bipartite interacting phenomenon could in part be responsible for the synergistic binding effect observed when both SH3 domains are present on the same molecule (Fig. 2a, lower panel). A detailed three-dimensional structure analysis of the spatial orientations of the proline-rich sequences in relation to each other and to their binding SH3 domain counterparts is essential to study the molecular basis underlying such a synergistic effect.

The adaptor protein Grb2 links proteins containing phosphorylated tyrosine residues to proteins with proline-rich sequences via its SH2 and SH3 domains respectively. Grb2 has been shown to play an essential role in several signal transduction pathways including the activation of $p21^{ras}$ by growth factor receptors (for review see (28)). The SH3 domain-mediated Grb2/MPTP-PEST complex may participate in growth factor receptor signalling cascades analogous to the $p21^{ras}$ activation pathways. To test this hypothesis, *in vivo* reconstitution experiments were performed by transient co-expression of human EGF receptor, MPTP-PEST, and Grb2 proteins in 293 cells. MPTP-PEST immunoprecipitates from different combinatorial transfections (Fig. 3a) were analyzed for the presence of activated EGF receptors by western blot using an anti-phosphotyrosine antibody. Tyrosine phosphorylated EGF receptors were detected in MPTP-PEST immunoprecipitates from Grb2 co-transfectants. However, activated EGF receptors were not detected in control transfectants lacking Grb2 (Fig. 3a, upper panel) suggesting that the presence of Grb2 is required for this *in vivo*

Figure 2. Domain analysis of Grb2/MPTP-PEST interaction. A) Schematic representation of the different GST-Grb2 fusion proteins used in this study. B) Identical amounts (100 ng) of the different GST-Grb2 fusion proteins shown in (A) were incubated with purified baculovirus produced 6XHis-HA-MPTP-PEST (~10 μg) proteins. 1 μg of purified 6XHis-HA-MPTP-PEST protein was used as a control (cont.). Bound recombinant MPTP-PEST proteins were visualized by western blot analysis using the anti-HA tag antibody. C) Schematic representation of the different proline-rich sequences located at the COOH-terminus of MPTP-PEST and the corresponding GST fusion proteins used in the overlay (FarWestern) assay. The sequence of the proline-rich motifs contained within the GST fusion proteins is as follow: Pro 1, ^{332}PPPKPPR^{338}; Pro 2, ^{355}PPEPHPVPPILTPSPPSAFP^{374}; Pro 3, ^{519}PPRPDCLP^{527}; Pro 4, ^{672}ESPPPLPER^{682}; and Pro 5, ^{764}PKGPREPP^{771}. D) The different GST-Pro-rich fusion proteins represented in (C) were separated on SDS-PAGE and Coomassie Blue-stained (upper panel) or transferred to nitrocellulose membranes and probed with purified ^{32}P-labelled NH_2-terminus (middle panel, $SH3_{(N)}$) or COOH-terminus (lower panel, $SH3_{(C)}$) SH3 domains of Grb2 as described under "Materials and Methods".

Figure 2a

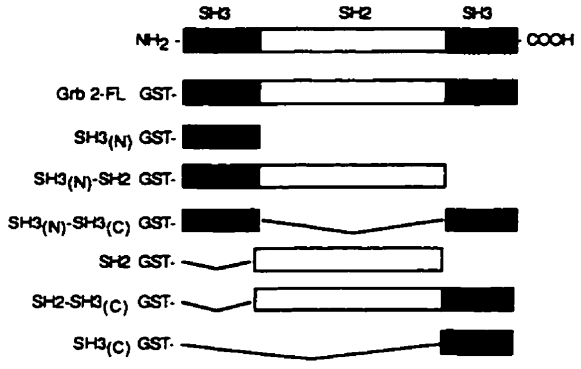


Figure 2b

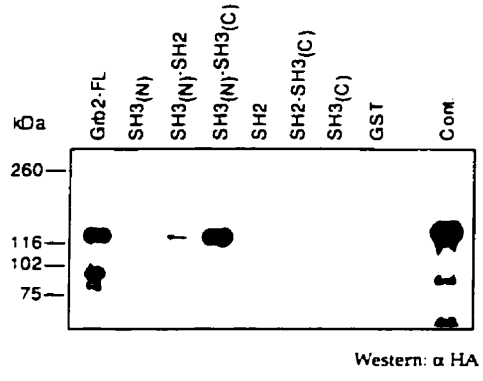
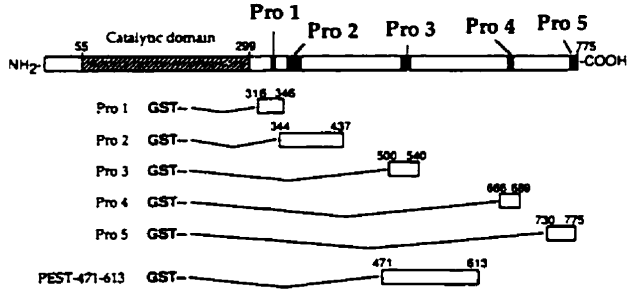


Figure 2c



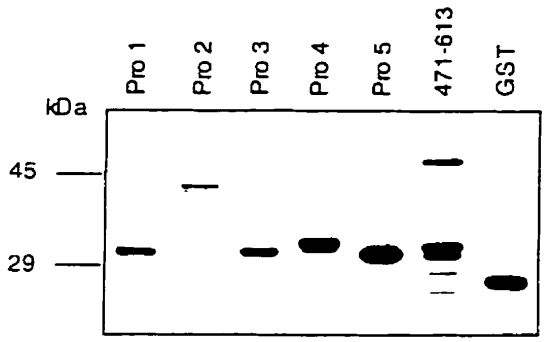


Figure 2d upper panel

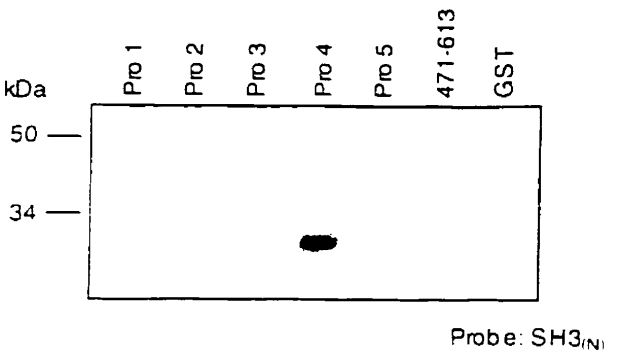


Figure 2d middle panel

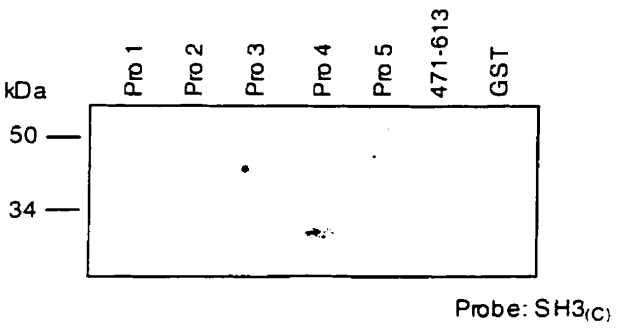


Figure 2d lower panel

interaction. The equivalent amounts of MPTP-PEST/Grb2 protein complexes in both EGF treated and non-stimulated cells (Fig. 3a, upper panel) suggest that this constitutive *in vivo* interaction does not seem to be modulated by EGF receptor activity.

Mutant Grb2 proteins (P49L and G203R) corresponding to the loss of function mutants alleles n1619 and n2195 of the Grb2 *C. elegans* homologue *sem-5* protein (2) were used to determine the involvement of the SH3 domains of Grb2 in the MPTP-PEST/Grb2/EGF receptor complex. These mutants were expressed either individually or as a double mutant and analyzed for their ability to mediate the binding of MPTP-PEST to activated EGF receptors in the *in vivo* reconstitution assay system described above. The failure of all three Grb2 mutants (P49L, G203R and P49L/G203R) to mediate this molecular interaction when compared to the wild type Grb2 protein (Fig. 3a, upper panel) despite equivalent expression levels (Fig. 3a, middle panel) correlate with their reduced ability to interact with MPTP-PEST proteins (Fig. 3a, lower panel). Control immunoblot analysis of cellular lysates using anti-EGF receptor and anti-MPTP-PEST antibodies indicates identical amounts of EGF receptor and MPTP-PEST proteins in each transfectant (data not shown). The low levels of bound G203R Grb2 mutant proteins remaining in the MPTP-PEST immunoprecipitates (Fig. 3a, upper panel) appear to be insufficient in mediating a trimolecular interaction with activated EGF receptors *in vivo*. Threshold levels of MPTP-PEST-bound Grb2 complexes may have to be attained in order for this tri-molecular interactions to be detected. The low amounts of phosphorylated EGF receptors seen in the MPTP-PEST immunoprecipitates of the mutant Grb2 transfectants may represent residual binding from the Grb2 mutants and/or by endogenous (i.e.

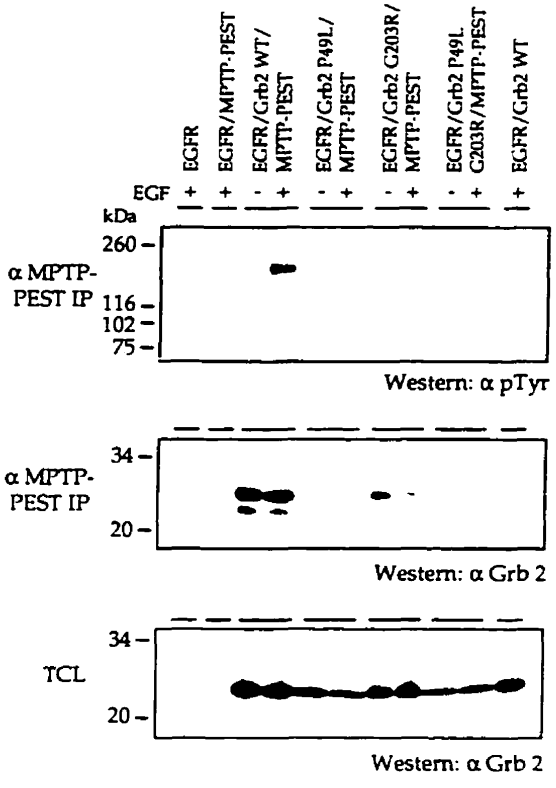
wild type) Grb2 proteins and/or by SHC/MPTP-PEST complexes (13). Nevertheless, these results constitute evidence that the binding of the SH3 domains of Grb2 to the MPTP-PEST protein represents a principle requirement in the recruitment of MPTP-PEST to the activated EGF receptor.

These results were substantiated in a physiologically relevant context by analyzing immunoprecipitates of endogenous MPTP-PEST proteins from quiescent and EGF stimulated cells expressing the human EGF receptor (HER14) (29) for the presence of activated EGF receptors. MPTP-PEST immunoprecipitates analyzed by immunoblot analysis using either anti-phosphotyrosine (Fig. 3b, upper panel) or anti-human EGF receptor (Fig. 3b, lower panel) antibodies reveal the presence of activated EGF receptors and that this phenomenon is dependent on tyrosine phosphorylation of the receptor.

The Grb2 SH3 domain-mediated recruitment of cytoplasmic MPTP-PEST to membrane-bound EGF receptors might serve to relocalize the MPTP-PEST enzyme where it could fulfil its function by acting upon its substrate(s). In an attempt to identify substrate(s) of MPTP-PEST we exploited the observation that mutation of the conserved catalytically essential cysteine residue to a serine renders tyrosine phosphatases inactive but still permits substrate binding (30). This mutation maintains the three-dimensional structure of the catalytic domain (31) and allows for the trapping and identification of potential substrate(s). A mutated MPTP-PEST^{C231S} was expressed as a bacterial GST fusion protein (aa 1-453) which comprises the catalytic domain of MPTP-PEST (14) and used in *in vitro* binding experiments to specifically detect tyrosine phosphorylated substrate(s) of MPTP-PEST.

Figure 3. The activated human EGF receptor is found in a molecular complex with MPTP-PEST *in vivo*. A) 500 μg of cell lysates from untreated (-EGF) or EGF stimulated (+EGF) 293 cells transiently expressing the indicated proteins were immunoprecipitated with an anti-MPTP-PEST polyclonal antibody. MPTP-PEST immunoprecipitates were probed for the presence of bound activated EGF receptors (upper panel, Western: α pTyr) and Grb2 proteins (middle panel, Western: α Grb2) using anti-phosphotyrosine and anti-Grb2 antibodies respectively. 10 μg of total cell lysate (TCL) from each fraction was analyzed by immunoblot for the presence of equivalent amount of Grb2 proteins (lower panel, Western: α Grb2). B) 1 mg of cell lysates from untreated (-EGF) or EGF stimulated (+EGF) HER14 cells were immunoprecipitated with preimmune antiserum (PI) or with anti-MPTP-PEST antiserum (1075). The immune complexes were separated by SDS-PAGE and analyzed by western blot using anti-phosphotyrosine (upper panel, Western: αpTyr) or anti-human EGF receptor (lower panel, Western: αEGFR) antibodies. 20 μg of total cell lysate (TCL) were used as internal controls on the immunoblots for the presence and state of activation of the EGF receptor.

Figure 3a


EGFR
EGFR/MPTP-PEST
EGFR/Grb2 WT/ MPTP-PEST
EGFR/Grb2 P49L/ MPTP-PEST
EGFR/Grb2 G203R/ MPTP-PEST
EGFR/Grb2 P49L G203R/MPTP-PEST
EGFR/Grb2 WT
EGF + + - + - + - + - + + +
kDa
260
116
102
75
α MPTP-PEST IP
Western: α pTyr
34
20
α MPTP-PEST IP
Western: α Grb 2
34
20
TCL
Western: α Grb 2

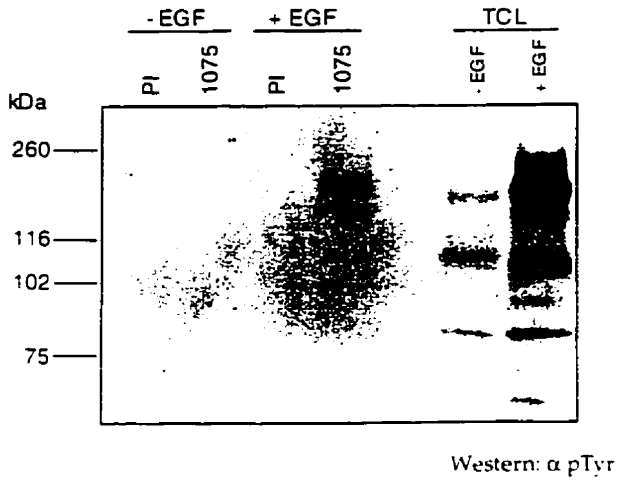


Figure 3b, upper panel

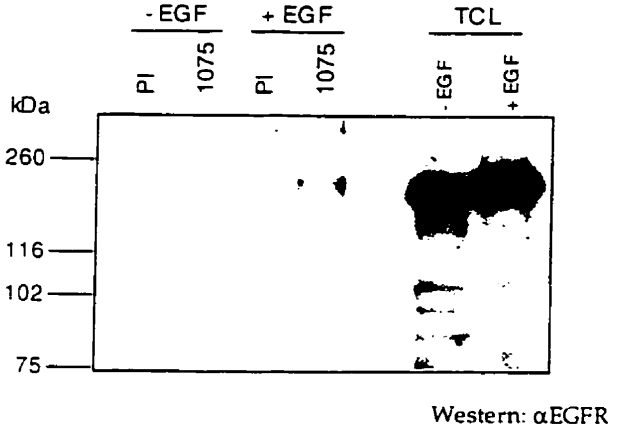


Figure 3b, lower panel

Figure 4. MPTP-PEST recognizes a single tyrosine phosphorylated protein as a potential substrate *in vitro*. 1 mg of cell lysates from untreated (-EGF) or EGF stimulated (+EGF) Her14 cells were incubated with purified wild type (WT) or cysteine 231 to serine mutant (C231S) GST-PEST-1-453aa fusion proteins. Tyrosine phosphorylated proteins recognized by the catalytic domain of MPTP-PEST were detected by western blot analysis using an anti-phosphotyrosine antibody. 25 μg of total cell lysates (TCL) were used to control for the level of tyrosine phosphorylation in the protein extracts.

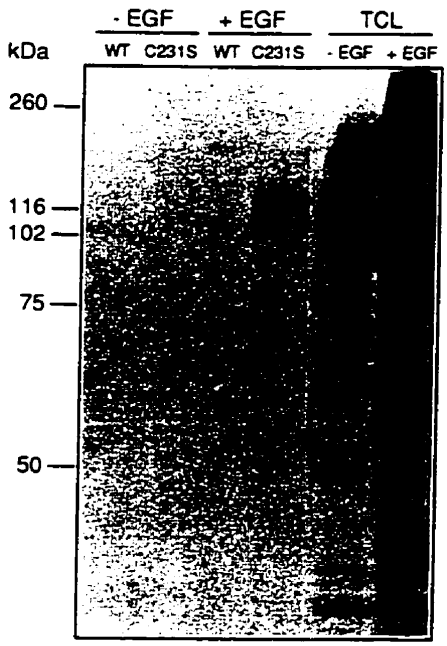


Figure 4

Affinity purified MPTP-PEST^{C231S} and its wild type (WT) counterpart were incubated with cell lysates derived from quiescent (-EGF) or EGF stimulated (+EGF) HER14 cells. The protein complexes were then subjected to western blot analysis using anti-phosphotyrosine antibodies. A single tyrosine phosphorylated protein migrating on an SDS-PAGE with an apparent molecular weight of 120 kDa (pp120) is specifically recognized by the mutant MPTP-PEST^{C231S} protein (Fig. 4). Phosphorylation of the p120 is dependent on stimulation of the cells with EGF. This tyrosine phosphorylation event could be the result of a direct kinase activity of the EGF receptor on the p120 or simply represents secondary phosphorylation events triggered by EGF receptor stimulation. This *in vitro* assay does not distinguish between these two possible mechanisms of action. Binding of this pp120 to MPTP-PEST^{C231S} proteins could conceivably be the result of a substrate-independent protein-protein association where the MPTP-PEST^{C231S}-bound p120 would remain phosphorylated given the inactive state of the enzyme. To address this issue, an inactive splice variant deletion of the MPTP-PEST enzyme lacking amino acids 141-164 which is the result of exon VI (32) being spliced out (Charest and Tremblay, unpublished results) was used in similar trapping experiments. This mutant MPTP-PEST protein was unable to interact with the phosphorylated p120 (data not shown) suggesting that the p120 is not a tyrosine phosphorylated protein associated to MPTP-PEST but rather is a genuine substrate for MPTP-PEST.

To determine the identity of the pp120, several antibodies directed against candidate proteins exhibiting a similar size and phosphorylation profile to this pp120 were tested by western blot analysis. Antibodies directed against p120^{RasGAP} (33), p120^{cbl} (34), Gab-1

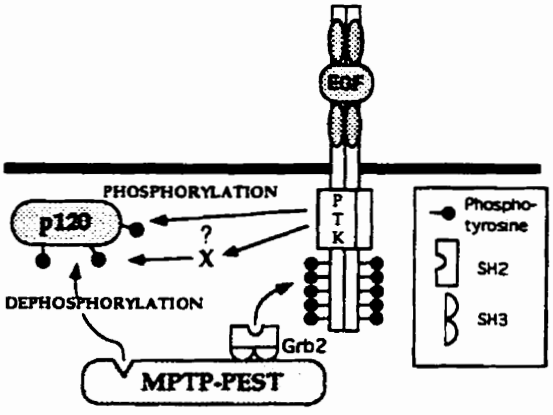
(35) and p120^{CAS} (36) failed to recognize pp120 suggesting that it does not correspond to any of the proteins tested (data not shown). Additional experiments indicate that this pp120 is not MPTP-PEST (data not shown) despite the close similarity in size. The identification of this p120 substrate in addition to the function(s) arising from its tyrosine phosphorylation levels represent essential elements in determining crucial aspects of MPTP-PEST function at the molecular level.

The results described here enable the formulation of a model (Fig. 5) whereby the MPTP-PEST enzyme, by virtue of its constitutive association with the adaptor molecule Grb2 through an SH3-proline-rich domain interaction, can form molecular complexes with activated EGF receptors. This interaction occurs presumably via the SH2 domain of bound Grb2 to phosphorylated tyrosine residues on the EGF receptor (7). This complex can be the result of a direct trimolecular interaction (MPTP-PEST/Grb2/EGF receptor) or could be mediated by other yet unidentified component(s). In either case, autophosphorylation of the activated EGF receptor is essential for the formation of the complex. The potential mobilization of MPTP-PEST proteins towards activated EGF receptors might serve to bring MPTP-PEST in close proximity to the pp120 substrate which is itself dependent on EGF for its phosphorylation. The consequences of this functional recruitment may reside in downstream events triggered by the modulation of tyrosine phosphorylation of the p120.

Other previously characterized interactions between cytoplasmic protein tyrosine phosphatases and activated growth factor receptors include the SH2-containing tyrosine phosphatases SHP-1 (also known as SHPTP-1, SHP, HCP, and PTP1C) and SHP-2 (also referred to as

Figure 5. Molecular model of the function of MPTP-PEST. Specific SH3-mediated interactions between the adaptor molecule Grb2 and proline-rich sequences of the tyrosine phosphatase MPTP-PEST allows for the recruitment of MTPT-PEST to the activated EGF receptor. This may result in a relocalization phenomenon whereby MPTP-PEST is recruited towards a potential substrate of 120 kDa (p120) which becomes tyrosine phosphorylated upon EGF stimulation of cells.

Figure 5


EGF
PHOSPHORYLATION
p120
?
X
DEPHOSPHORYLATION
P
T
K
Grb2
MPTP-PEST
Phospho-
tyrosine
SH2
SH3

SHPTP-2, SHPTP-3, Syp, PTP-2C, and PTP1D). The ubiquitously expressed SHP-2 has been shown to directly bind via its SH2 domains to the phosphorylated EGF and PDGF receptor tyrosine kinases (37-39) as well as to the Tpr-Met oncoprotein (40) and the insulin signalling docking protein IRS-1 (41). Conversely, the hematopoietic specific SHP-1 interacts with the phosphorylated c-Kit (42) and erythropoetin receptors (43) in addition to the IL-3 β chain (44). The presence of these PTPases in phosphotyrosine signalling events ascribes a regulatory function for these enzymes. Here we show that another cytoplasmic protein tyrosine phosphatase, MPTP-PEST, can indirectly associate to the activated EGF receptor via a novel SH3 domain-mediated binding to Grb2 thus potentially attributing analogous regulatory functions to MPTP-PEST in signalling cascades.

The bimolecular and modular interaction of Grb2 and MPTP-PEST may represent a prototype mechanism of recruitment given the ubiquitous pattern of expression of both of these proteins. Tissue specific related members of each of these components may participate interchangeably in similar interactions thereby conferring tissue and/or induction specificity by exposing such complexes to specialized tyrosine kinases and/or signalling cascades. For example, the closely related hematopoetic-specific PTP-PEP (45) and the more distant brain-specific striatum enriched PTPase STEP (46) also contain several putative SH3 binding sites. The hematopoetic specific SH3-SH2-SH3 adaptor Grap1 protein (47) together with these PTPases may participate in the regulation of signalling events via similar SH3 domain-mediated interactions.

The work presented here assigns a potential role for MPTP-PEST in the context of signalling cascades involving the EGF receptor tyrosine kinase and the adaptor molecule Grb2. The identification of the pp120 and the discovery of signalling systems which recruit MPTP-PEST in an analogous fashion are required to address the function of MPTP-PEST in signal transduction.

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REFERENCES

1. Mayer, B. J., Ren, R., Clark, K. L. & Baltimore, D. (1993) *Cell* **73,** 629-30.

2. Clark, S. G., Stern, M. J. & Horvitz, H. R. (1992) *Nature* **356,** 340-4.

3. Stern, M. J., Marengere, L. E., Daly, R. J., Lowenstein, E. J., Kokel, M., Batzer, A., Olivier, P., Pawson, T. & Schlessinger, J. (1993) *Molecular Biology of the Cell* **4,** 1175-88.

4. Olivier, J. P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E. & Pawson, T. (1993) *Cell* **73,** 179-91.

5. Simon, M. A., Dodson, G. S. & Rubin, G. M. (1993) *Cell* **73,** 169-77.

6. Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B. & Schlessinger, J. (1993) *Nature* **363,** 85-8.

7. Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. & Bowtell, D. (1993) *Nature* **363,** 83-5.

8. Buday, L. & Downward, J. (1993) *Cell* **73,** 611-20.

9. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M. & Weinberg, R. A. (1993) *Nature* **363,** 45-51.

10. Gale, N. W., Kaplan, S., Lowenstein, E. J., Schlessinger, J. & Bar-Sagi, D. (1993) *Nature* **363,** 88-92.

11. Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V. & Schlessinger, J. (1993) *Cell* **74,** 83-91.

12. Matuoka, K., Shibasaki, F., Shibata, M. & Takenawa, T. (1993) *EMBO Journal* **12,** 3467-73.

13. Charest, A., Wagner, J., Jacob, S., McGlade, C. J. & Tremblay, M. L. (1996) *Journal of Biological Chemistry* **271,** 8424-8429.

14. Charest, A., Wagner, J., Shen, S. H. & Tremblay, M. L. (1995) *Biochemical Journal* **308,** 425-32.

15. Fantl, W. J., Escobedo, J. A., Martin, G. A., Turck, C. W., del Rosario, M., McCormick, F. & Williams, L. T. (1992) *Cell* **69,** 413-23.

16. 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) *Cell* **70,** 431-42.

17. Ren, R., Mayer, B. J., Cicchetti, P. & Baltimore, D. (1993) *Science* **259,** 1157-61.

18. Viguera, A. R., Arrondo, J. L., Musacchio, A., Saraste, M. & Serrano, L. (1994) *Biochemistry* **33,** 10925-33.

19. Mayer, B. J. & Eck, M. J. (1995) *Current Biology* **5,** 364-7.

20. Sambrook, K. J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Labiratiry Press, Cold Spring Harbor, NY).

21. Vialard, J., Lalumiere, M., Vernet, T., Briedis, D., Alkhatib, G., Henning, D., Levin, D. & Richardson, C. (1990) *Journal of Virology* **64,** 37-50.

22. Amarneh, B. & Simpson, E. R. (1995) *Molecular & Cellular Endocrinology* **109,** R1-5.

23. Horstman, D. A., Ball, R. & Carpenter, G. (1995) *Protein Expression & Purification* **6,** 278-83.

24. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1995) *Current Protocols in Molecular Biology* (John Wiley & Sons, Inc., New York).

25. Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G. & Campbell, K. P. (1995) *Journal of Biological Chemistry* **270,** 11711-4.

26. Yang, S. S., Van Aelst, L. & Bar-Sagi, D. (1995) *Journal of Biological Chemistry* **270,** 18212-5.

27. Meisner, H., Conway, B. R., Hartley, D. & Czech, M. P. (1995) *Molecular & Cellular Biology* **15,** 3571-8.

28. Chardin, P., Cussac, D., Maignan, S. & Ducruix, A. (1995) *FEBS Letters* **369,** 47-51.

29. Honegger, A. M., Dull, T. J., Felder, S., Van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A. & Schlessinger, J. (1987) *Cell* **51,** 199-209.

30. Milarski, K. L., Zhu, G., Pearl, C. G., McNamara, D. J., Dobrusin, E. M., MacLean, D., Thieme-Sefler, A., Zhang, Z. Y., Sawyer, T., Decker, S. J. & et al. (1993) *Journal of Biological Chemistry* **268,** 23634-9.

31. Jia, Z., Barford, D., Flint, A. J. & Tonks, N. K. (1995) *Science* **268,** 1754-8.

32. Charest, A., Wagner, J., Muise, E. S., Heng, H. H. & Tremblay, M. L. (1995) *Genomics* **28,** 501-7.

33. Serth, J., Weber, W., Frech, M., Wittinghofer, A. & Pingoud, A. (1992) *Biochemistry* **31,** 6361-5.

34. Meisner, H. & Czech, M. P. (1995) *Journal of Biological Chemistry* **270,** 25332-5.

35. Holgadomadruga, M., Emlet, D. R., Moscatello, D. K., Godwin, A. K. & Wong, A. J. (1996) *Nature* **379,** 560-564.

36. Reynolds, A. B., Daniel, J., McCrea, P. D., Wheelock, M. J., Wu, J. & Zhang, Z. (1994) *Molecular & Cellular Biology* **14,** 8333-42.

37. Feng, G. S., Hui, C. C. & Pawson, T. (1993) *Science* **259,** 1607-11.

38. Lechleider, R. J., Freeman, R. M., Jr. & Neel, B. G. (1993) *Journal of Biological Chemistry* **268,** 13434-8.

39. Vogel, W., Lammers, R., Huang, J. & Ullrich, A. (1993) *Science* **259,** 1611-4.

40. Fixman, E. D., Fournier, T. M., Kamikura, D. M., Naujokas, M. A. & Park, M. (1996) *Journal of Biological Chemistry* **271,** 13116-13122.

41. Kuhne, M. R., Pawson, T., Lienhard, G. E. & Feng, G. S. (1993) *Journal of Biological Chemistry* **268,** 11479-81.

42. Yi, T. & Ihle, J. N. (1993) *Molecular & Cellular Biology* **13,** 3350-8.

43. Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G. & Lodish, H. F. (1995) *Cell* **80,** 729-38.

44. Yi, T., Mui, A. L., Krystal, G. & Ihle, J. N. (1993) *Molecular & Cellular Biology* **13,** 7577-86.

45. Matthews, R. J., Bowne, D. B., Flores, E. & Thomas, M. L. (1992) *Molecular & Cellular Biology* **12,** 2396-405.

46. Li, X., Luna, J., Lombroso, P. J. & Francke, U. (1995) *Genomics* **28,** 442-9.

47. Feng, G. S., Ouyang, Y. B., Hu, D. P., Shi, Z. Q., Gentz, R. & Ni, J. A. (1996) *Journal of Biological Chemistry* **271,** 12129-12132.

CHAPTER 6

Discussion

Cloning of MPTP-PEST

During our search for PTPases expressed during murine embryogenesis, we isolated by RT-PCR and cDNA library screening, a novel member of the intracellular family of protein tyrosine phosphatases. The cDNA for this novel PTPase contains a single open reading frame of 2325 base pairs coding for a protein of 775 amino acids. Sequence analysis demonstrates that this protein is composed of a single catalytic domain located at the amino-terminus of the protein and a long carboxyl-terminal end rich in PEST sequences. The presence of this architectural signature motif in the human homologue prompted Yang *et al.*, (1993) to name this enzyme protein tyrosine phosphatase (PTP)-PEST.

PTPase domains are conserved polypeptides of ~250 amino acids found in every PTPase identified to date. They are responsible for the intrinsic catalytic activity displayed by PTPases. They represent genuine protein domains since they can function adequately outside the context of a full PTPase protein. This feature has enabled large scale expression and purification of several PTPase domains. The availability of high amounts of purified material has allowed for detailed enzymatic studies which ultimately lead to the elucidation of a mechanism for dephosphorylation.

The activity of the catalytic domain of human PTP-PEST has been analyzed *in vitro* (Yang et al. 1993). It was found that bacterially expressed PTP-PEST PTPase domain can readily dephosphorylate tyrosine phosphorylated proteins and that several compounds can modulate the activity of the catalytic domain (Yang et al. 1993). We

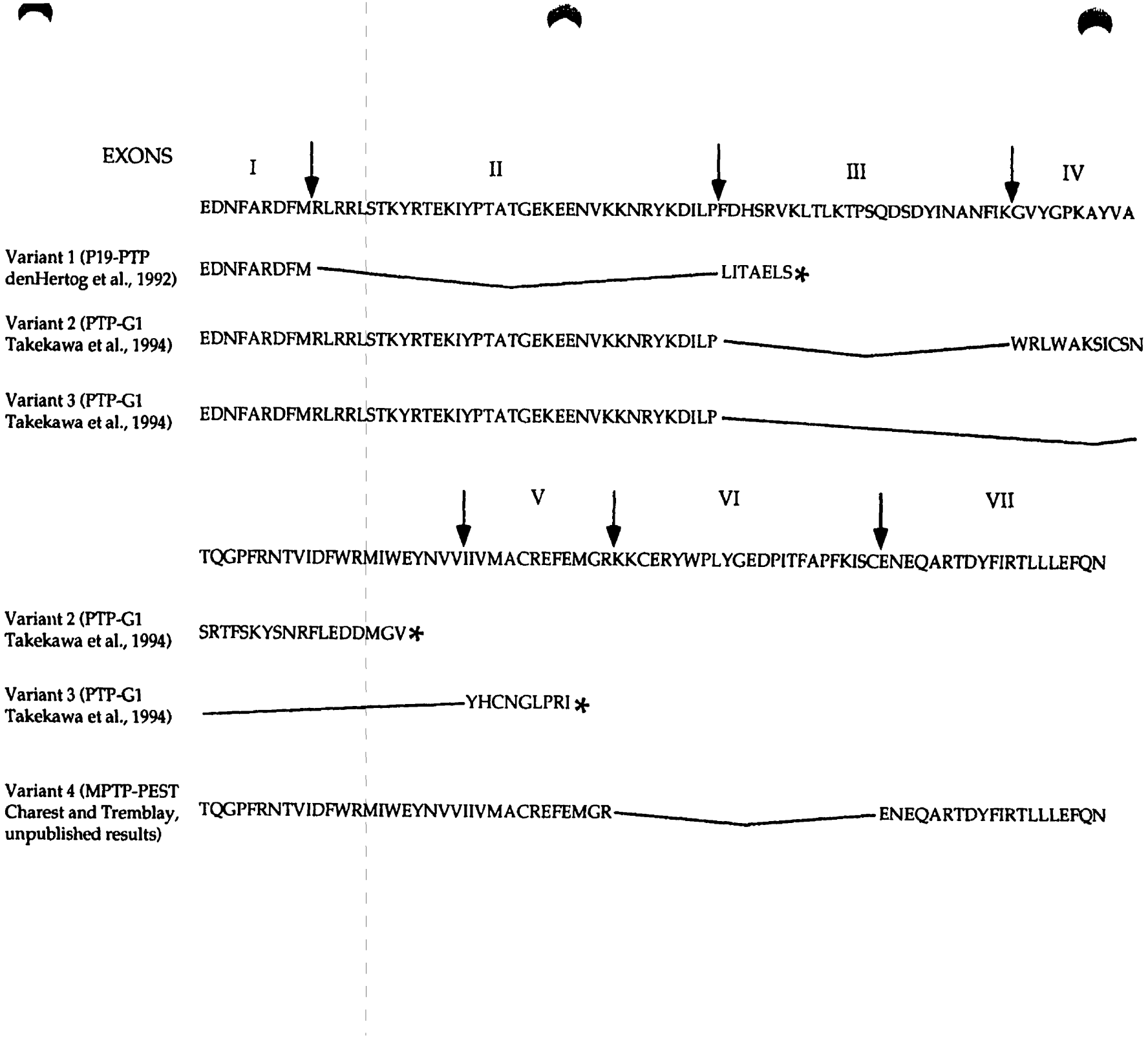
performed similar experiments and demonstrated that the PTPase domain of MPTP-PEST also displays intrinsic catalytic activity (Charest et al. 1995a) (Chapter 2). Interestingly, we discovered that the PTPase domain of MPTP-PEST shows specificity among phosphotyrosine-containing proteins. We found that a tyrosine phosphorylated synthetic peptide derived from the sequence surrounding a phosphorylated tyrosine residue on $p34^{cdc2}$ was not dephosphorylated by MPTP-PEST. This demonstrates that the PTPase domain of MPTP-PEST can discriminate between substrates and tyrosine-phosphorylated proteins. This specificity suggests an inherent mode of regulation for the catalytic activity of MPTP-PEST.

The catalytic domains of PTPases display various degrees of identity when compared to each other. Members of the same PTPase subfamily tend to have a higher degree of identity. Comparison of the PTPase domain of MPTP-PEST to those of other PTPases reveals overall identities ranging between 30 and 70%. All but one PTPase domains share ~30% identities with the catalytic domain of MPTP-PEST (A. Charest, M. L. Tremblay, unpublished observation), suggesting that MPTP-PEST is not related to any of these PTPases. However, the high identity (67%) between the enzymes PTP-PEP (Matthews et al. 1992) and MPTP-PEST may indicate an evolutionary relationship between these two proteins. The resemblance between MPTP-PEST and PTP-PEP, (PEST Enriched Phosphatase), goes further than the high degree of homology between their catalytic domains. PTP-PEP also has a similar overall architecture to MPTP-PEST. It is composed of an amino-terminal catalytic domain and carboxyl-terminal PEST sequences. This overall structural composition defines a novel subfamily of intracellular PTPases.

The tissue distribution of these two PTPases is remarkably different. PTP-PEP is expressed primarily in cells of haematopoietic origin (Matthews et al. 1992), whereas PTP-PEST is expressed ubiquitously. This difference might reflect the possibility that both PTP-PEST and PTP-PEP serve similar functions, but in different systems given their pattern of expression. This is somewhat analogous to the Src family of tyrosine kinases, where structurally related members fulfil similar functions but in different systems due to their restricted expression patterns. Further insights into the functions of PTP-PEST and PTP-PEP, in addition to the discovery and characterization of related PTPases, will provide a framework upon which a general mechanism of action for members of this novel family of PTPases can be built.

PEST sequences are thought to confer rapid degradation to the proteins which harbour them. The molecular mechanism upon which PEST sequences provide a rapid intracellular half life to proteins has yet to be established. In fact, with the exception of only two examples, the effect(s) of PEST sequences on protein half life have never been examined and is simply based on circumstantial observations. We have determined by pulse-chase analysis that the half life of MPTP-PEST is over 4 hours (Charest et al. 1995a) (Chapter 2). In addition, Flores *et al.*, (1995) have shown by similar means that the PTP-PEP protein has a long intracellular half life (> 6 hours). This suggests that, under the conditions in which the experiments were performed, the PEST sequences found in MPTP-PEST and PTP-PEP do not provide a short half life to these enzymes. However, these PEST sequences might play a role in regulating protein stability under specific circumstances such as during the cell cycle or as the result of inducible subcellular relocations.

Figure 1. Molecular rationale behind the splicing variants of MPTP-PEST. The amino acid sequence of the three splice variants of murine and human PTP-PEST enzymes are aligned with the genomic structure of the MPTP-PEST gene. Downward arrows indicate the intron/exon boundaries as determined in Charest *et al.*, (1995). If we assume a conservation in the genomic structure of the human and mouse PTP-PEST genes, we can address the splice variants of PTP-PEST observed by Takekawa *et al.*, (1994).


EXONS
I
II
III
IV
EDNFARDFMRLRRLSTKYRTEKIYPTATGEKEENVKKNRYKDILPFDHSRVKLTLKTPSQDSDYINANFIKGVYGPKAYVA
Variant 1 (P19-PTP denHertog et al., 1992)
EDNFARDFM
LITAELS*
Variant 2 (PTP-G1 Takekawa et al., 1994)
EDNFARDFMRLRRLSTKYRTEKIYPTATGEKEENVKKNRYKDILP
WRLWAKSICSN
Variant 3 (PTP-G1 Takekawa et al., 1994)
EDNFARDFMRLRRLSTKYRTEKIYPTATGEKEENVKKNRYKDILP
V
VI
VII
TQGPFRNTVIDFWRMIWEYNVVIIVMACREFEMGRKKCERYWPLYGEDPITFAPFKISCENEQARTDYFIRTLLLEFQN
Variant 2 (PTP-G1 Takekawa et al., 1994)
SRTFSKYSNRFLEDDMGV*
Variant 3 (PTP-G1 Takekawa et al., 1994)
YHCNGLPRI*
Variant 4 (MPTP-PEST Charest and Tremblay, unpublished results)
TQGPFRNTVIDFWRMIWEYNVVIIVMACREFEMGR
ENEQARTDYFIRTLLLEFQN

MPTP-PEST RNA processing

During the initial characterization of MPTP-PEST, we isolated two different forms of the MPTP-PEST cDNA from an 18 day old embryonic kidney library. One form corresponded to the full length cDNA sequence already described by denHertog *et al.*, (1992) whereas the other form represented a novel uncharacterized cDNA. This cDNA consisted of an in frame deletion of 72 base pairs within the PTPase domain, corresponding to amino acids 141-164 of MPTP-PEST. Partial characterization of this alternative form of MPTP-PEST revealed that this deletion completely abrogates the enzymatic activity of MPTP-PEST *in vitro* (Charest and Tremblay, unpublished observations). The molecular events triggering the production of such an alternate MPTP-PEST message are currently being investigated.

The initial report describing the molecular cloning of the cDNA for MPTP-PEST (P19-PTP) outlined a similar deletion in one of the MPTP-PEST lambda phage cDNA clone (den Hertog et al. 1992). A deletion of 108 base pairs at the 5′ end of the cDNA corresponding to amino acids 34-70 of MPTP-PEST (P19-PTP) was identified (see figure 4). This deletion results in a shift in the reading frame which ultimately produces a premature stop codon 8 amino acids downstream of the deleted sequence boundary. The effects of this deletion on the corresponding protein structure and function were never addressed. Additional aberrant transcripts of PTP-PEST (PTPG1) have also been observed by Takekawa *et al.*, (1994) during the screening of several human cancer cell lines for the integrity of the PTP-PEST message. Using RT-PCR, two shorter messages for PTP-PEST were found in the human colon cancer cell line DLD-1 (Takekawa et al. 1994). Sequencing

analysis of these messages demonstrated that they represented deletions of 77 and 173 base pairs in the PTPase domain corresponding to amino acids 70-95 and 70-127 respectively. Each of these deletions resulted in frame-shifts which ultimately gave rise to termination codons 29 and 9 amino acids downstream of the respective deletions (see figure 4). The effects that these truncated PTP-PEST proteins might have on cellular function remain to be determined.

Elucidation of the *mptp-pest* locus

Differential splicing may be responsible for the origin of these different murine and human PTP-PEST messages. In order to determine the nature of the molecular mechanism underlying the production of these aberrant messages we studied the gene structure of the MPTP-PEST enzyme. As such, we isolated and analyzed, from a 129/sv genomic library, the complete genomic structure of the MPTP-PEST gene. The MPTP-PEST locus spans over 90 kb and the gene is composed of 18 exons ranging in size between 31 bp to 974 bp (Charest et al. 1995b) (Chapter 3).

Comparison of the genomic structure of the MPTP-PEST PTPase domain to those of several other PTPases indicates that the catalytic domain of MPTP-PEST is composed of 10 exons, whereas all other PTPase domains are encoded by no more than 8 exons. Seven of the nine intron/exon splice site positions found in the MPTP-PEST PTPase domain are also found in other PTPases; two of the nine positions are unique to MPTP-PEST (Charest et al. 1995b) (Chapter 3). This demonstrates that the genomic organization of the MPTP-PEST gene is the most intricate of all PTPases characterized so far. The unique

presence of two additional introns within the catalytic domain of MPTP-PEST suggests that the MPTP-PEST gene is the product of a different branch of the PTPase phylogenetic tree.

Mapping of the intron/exon boundaries allowed for a complete analysis of the deletions corresponding to the afore-mentioned aberrant messages (see figure 4). The 72 bp in frame deletion within the PTPase domain that we observed (variant 4) corresponds to the removal of exon 6 from the MPTP-PEST message whereas the 108 bp deletion at the 5′ end of the MPTP-PEST message detected by denHertog *et al.*, (1992) (variant 1), corresponds to the deletion of exon 2 (see figure 4). The shift in frame resulting from the exclusion of exon 2 would produce a truncated protein due to the presence of a newly formed stop codon 8 amino acids downstream of the aberrant junction. The atypical PTP-PEST messages observed by Takekawa *et al.*, (1994) can be explained if we assume a conservation in the genomic structure of the PTP-PEST gene between mouse and human. The two abnormal mRNAs isolated would correspond to the removal of exon 3 (the 77 bp deletion) (variant 2) and exons 3 and 4 (the 173 bp deletion) (variant 3) during splicing of the messages (see figure 4).

Alternative RNA splice variants have been reported for several PTPases including mPTPα (Matthews et al. 1990), DPTP10D and DPTP99A (Tian et al. 1991; Yang et al. 1991), DPTP 4E (Oon et al. 1993), PTP-S (Reddy and Swarup 1995), DPTP61F (McLaughlin and Dixon 1993) and SHP-2 (Mei et al. 1994). There are only two examples where RNA splicing variations affect the catalytic domains of PTPases. Matthews *et al.*, (1990) isolated a PTPα cDNA variant from a mouse pre-B-cell library which contains an insert generated by the presence of an additional

exon. While preserving the reading frame, this exon disrupts the first catalytic domain of the protein. The functional significance of this insertion is not yet clear.

Recently, Mei *et al.*, (1994) isolated an RNA splice variant of the rat SHP-2 enzyme. This cDNA variant is characterized by the presence of four additional amino acid residues in the catalytic domain of the enzyme. Interestingly, this insertion results in a 8-20 fold decrease in the rate of dephosphorylation when compared to the normal enzyme (Mei et al. 1994). To verify the specificity of this splicing-mediated modulation in the catalytic activity of this PTPase, the same additional sequence was inserted in the related PTPase SHP-1. Similarly, the enzyme activity of the modified SHP-1 was only 11-24% of that of normal SHP-1 (Mei et al. 1994). These results demonstrate that the presence of a four amino acid insert in the catalytic domains of SHP-1 and -2 down-regulates their catalytic activity. Together, these results suggest that RNA splicing may serve as a regulatory mechanism of PTPase activity.

The circumstances under which the differentially spliced MPTP-PEST messages arise are yet to be elucidated. However, the production of a catalytically inactive version of MPTP-PEST through the alternative removal of a single exon (exon 6, amino acids 141-164) from the MPTP-PEST message might represent a mechanism by which the activity of this enzyme is regulated. This potential mode of regulation is somewhat similar to the RNA splicing that regulates the activity of SHP-2 (Mei et al. 1994). Regulation of PTPase function through the modulation of RNA splicing might represent an additional mechanism by which PTPases exert their tasks. In addition, the presence of

aberrantly spliced PTP-PEST messages in colon cancer cells may indicate a function for PTP-PEST in carcinogenesis. The truncated PTP-PEST proteins resulting from these splicing events could be endowed with oncogenic properties. On the other hand, removal of the wild type PTP-PEST could be responsible for the cancerous phenotype of the cell in which case PTP-PEST would function as a tumor suppressor protein. The biological consequences emanating from these splicing events in relation to signal transduction and carcinogenesis await further investigation.

The genetic mapping of a gene might unveil links between the location of the gene in question and a previously localized locus responsible for a given phenotype. For example, the discovery that mutations in SHP-1 are responsible for the *motheaten* phenotype in mice came from the observation that the gene for SHP-1 maps to the same locus that encompasses *motheaten*. By localizing a gene to a locus of a well characterized phenotype, one can readily assess the function of the gene in the whole animal. We have mapped the chromosomal localization of MPTP-PEST to mouse chromosome 5 region A3-B by Fluorescence *In Situ* Hybridization (FISH) (Charest et al., 1995b) (Chapter 3). This portion of mouse chromosome 5 does not correspond to any previously characterized region. Similarly, mapping of the human PTP-PEST to human chromosome 7q11.23 does not localize the gene to a region known to be involved in any phenotype. Mapping of the MPTP-PEST gene is in accordance with mapping of the human PTP-PEST gene. The region surrounding human chromosome 7q11 is syntenic to the proximal portion of mouse chromosome 5. Mapping MPTP-PEST to this region extends the proximal limit of synteny between mouse and human.

MPTP-PEST interacts with SHC proteins

Using the yeast two hybrid screening system, Habib *et al.*, (1994) isolated cDNA clones encoding proteins that interact with the adaptor protein p52^{Shc} from a library derived from HeLa cells. One of these clones encoded the non-catalytic carboxyl-terminal portion of human PTP-PEST (amino acids 416-780). Primary mapping experiments in yeast using p52^{Shc} deletion mutants demonstrated that the binding of p52^{Shc} to PTP-PEST occurred through the amino-terminal portion of p52^{Shc} (amino acids 1-232). *In vivo* studies conducted in HeLa and SH-SY5Y neuroblastoma cells demonstrated that the binding of PTP-PEST to p52^{Shc} could be increased by treating the cells with activators of PKC. In fact, treatment of HeLa cells with PMA (a direct activator of PKC) or SH-SY5Y cells with carbachol (a muscarinic Ach receptor agonist, an indirect activator of PKC) resulted in an increase in complex formation of p52^{Shc}:PTP-PEST of 6-8 fold and 3-5 fold respectively. A concomitant increase in the phosphorylation of p52^{Shc} and PTP-PEST was also apparent in PMA treated cells.

The carboxyl-termini of murine and human PTP-PEST enzymes display a weak (74%) sequence identity relative to the highly conserved amino-terminal catalytic domains (98% identity). This observation prompted us to determine if the p52^{Shc}:PTP-PEST interaction also exists in a murine context. *In vivo* co-immunoprecipitation and *in vitro* binding studies revealed that mShc proteins interact with MPTP-PEST (Charest et al. 1996) (Chapter 4). Detailed mapping analyses allowed for the delineation of the minimal binding requirements that are involved in this interaction between mShc and MPTP-PEST. The amino-terminal

PTB domain of Shc was found to recognize and bind to the phosphotyrosine-devoid NPXH sequence motif found within the carboxyl-terminal region of MPTP-PEST (Charest et al. 1996) (Chapter 4). This phosphotyrosine-independent binding of the PTB domain of Shc to the NPLH sequence of MPTP-PEST represents evidence for an extended PTB recognition specificity. The Shc PTB domain, previously thought to interact solely with tyrosine phosphorylated proteins through the core motif NPXpY, can now interact with proteins in a phosphotyrosine independent manner, but still through a core sequence motif resembling the NPXpY.

Various experimental results tend to suggest that post-translational modification of the His residue of the NPXH core motif of MPTP-PEST is required in order for the NPXH sequence motif to act as a high affinity PTB domain recognition binding site (Charest et al. 1996) (Chapter 4). Phosphorylation of the tyrosine residue in the PTB domain NPXpY binding sequence found in several growth factor receptors, has been shown to be an essential requirement for high affinity binding. Similarly, phosphorylation of the His residue in the MPTP-PEST NPXH sequence would represent an analogous situation where such modification is necessary in order to achieve high affinity binding.

Phosphohistidine has been found to play a major role in several prokaryotic signaling systems. Autophosphorylating histidine kinases and response-regulator domains constitute core elements of various two-component signaling systems in bacteria. These systems use a unique phosphotransfer chemistry (involving phosphohistidine) to regulate many aspects of bacterial physiology such as carbohydrate uptake (Jia et al. 1994), osmotic (Roberts et al. 1994) and nitrate (Walker

and DeMoss 1993) sensing systems. Phosphohistidine is found in many eukaryotic proteins and has recently been implicated in signal transduction pathways in platelets (Crovello et al. 1995). The cytoplasmic tail of P-selectin, a platelet and endothelial cells adhesion receptor involved in leukocyte adhesion, undergoes rapid and transient phosphorylation on His residues. The P-selectin sequence NPHSH, where both His residues are potential sites of phosphorylation, becomes heavily Histidine-phosphorylated following thrombin or collagen stimulation of platelets (Crovello et al. 1995). Although the histidine kinase(s) and phosphatase(s) responsible for this phosphorylation event have not been identified, these results represent evidence that His phosphorylation is an inducible and reversible phenomenon that constitutes a distinct means by which signals can be transduced (for a thorough review on histidine phosphorylation see (Matthews 1995).

Histidine phosphorylation of the NPLH sequence of MPTP-PEST represents a plausible mechanism by which binding of the PTB domain of Shc to MPTP-PEST can be regulated. The observed stimulation of Shc:PTP-PEST complex formation upon treatment of cells with PMA is concomitant with an increase in PTP-PEST phosphorylation. PMA treatment of cells might possibly lead to an increase in His phosphorylation of the NPLH sequence of PTP-PEST, resulting in the observed increase in binding between Shc and PTP-PEST (Habib et al. 1994). However, phorbol ester-induced stimulation of His phosphorylation has never been demonstrated. Direct evidence linking phorbol ester treatment to an increase in cellular phosphohistidine levels remains to be established in order to solidify the aforementioned theory. Alternatively, phosphorylation of serine and threonine residues surrounding the NPLH sequence of PTP-PEST may also facilitate the

binding of Shc to PTP-PEST. Analysis of the structural modalities required for the binding of the PTB domain of Shc to the phosphotyrosine-devoid NPLH sequence of MPTP-PEST would allow for the assessment of phosphorylation or any other post-translational modifications on the modulation of binding.

The recent identification of several different PTB domains in a variety of unrelated proteins (Bork and Margolis 1995; Yajnik et al. 1996) raises the potential for interactions between MPTP-PEST and these putative PTB domains. In addition, the isolation of Shc-related proteins (Sck and Shc C) both containing PTB domains, opens the possibility of different Shc:MPTP-PEST interactions. Given the restricted tissue distribution of Shc C, complex formation between MPTP-PEST and Shc C could ascribe a function for MPTP-PEST in signaling events specific to nervous tissues.

Finally, the interaction between the adaptor oncoprotein Shc and the tyrosine phosphatase MPTP-PEST suggests that MPTP-PEST might function in Shc-mediated signaling events. The involvement of Shc proteins in various signal transduction pathways has been reported by several groups (for brief review see (van der Geer and Pawson 1995)). Any regulation that MPTP-PEST might confer to these phosphotyrosine-dependent pathways remains to be determined.

MPTP-PEST interacts with Grb2

The involvement of MPTP-PEST in phosphotyrosine-mediated signal transduction pathways is supported by its intrinsic catalytic activity, its interaction with Shc proteins, and by the presence of five

proline-rich stretches in its non-catalytic carboxyl terminus. Similar proline-rich sequences have been shown to act as high affinity binding sites for SH3 domains which are generally found in proteins associated with phosphotyrosine-dependent signal transduction events. The extent to which these MPTP-PEST pro-rich sequences act as SH3 domain binding sites was investigated using several *in vitro* and *in vivo* screening methods (Chapter 5).

We demonstrated that four of the MPTP-PEST pro-rich sequences constitute specific binding sites for both SH3 domains of the adaptor molecule Grb2. The binding preferences of Grb2 SH3 domains in relation to MPTP-PEST pro-rich sequences were determined by *in vitro* binding experiments (Chapter 5). The amino-terminal SH3 domain of Grb2 binds to two pro-rich sequences on MPTP-PEST which belong to class II of SH3 domain binding consensus sequence (Chapter 5). This is not surprising since the amino-terminal SH3 domain of Grb2 has been shown to bind to class II pro-rich motifs such as those found on hSos1 and hSos2. Sequence comparison between the MPTP-PEST pro-rich sequences and these known Grb2 amino SH3 binding sites reveals a high degree of identity (Chapter 5). However, the binding affinities for each of these pro-rich binding sites need to be determined in order to assess the possibility of competitive mechanisms of binding. Interestingly, the carboxyl SH3 domain preferentially binds to MPTP-PEST pro-rich sequences which do not conform to any canonical SH3 domain binding consensus sequences (Chapter 5). These might represent novel binding sites for the carboxyl SH3 domain of Grb2. Detailed structural and mutagenic analyses of these binding sites could reveal the modalities with which the carboxyl SH3 domain of Grb2 binds to these pro-rich sequences and possibly uncover a novel mode of

binding for SH3 domains.

The SH3 domains of Grb2 bind to different subsets of pro-rich sequences suggesting that both SH3 domains of a Grb2 molecule could simultaneously bind to different sites on MPTP-PEST. Detailed 3-dimensional structural analyses of the spatial orientations of the pro-rich sequences of MPTP-PEST in relation to each other and to the bound SH3 domains of Grb2 would allow for the study of the molecular basis responsible for such binding.

The function of Grb2 in linking tyrosine-phosphorylated proteins (such as activated growth factor receptors) to proteins that contain pro-rich sequences via its SH2 and SH3 domains respectively has been well documented (for review see (Chardin et al. 1995; Downward 1994). To test whether the SH3 domain-mediated Grb2:MPTP-PEST complex participates in growth factor receptor signaling cascades, *in vivo* binding experiments were performed by co-expressing the EGF receptor, MPTP-PEST and Grb2 proteins in mammalian cells (Chapter 5). The EGF receptor represents a powerful growth factor receptor model since several signaling pathways initiated by the activation of the receptor have been established and are now well characterized. Analysis of MPTP-PEST immunoprecipitates from different combinatorial transfections demonstrate that MPTP-PEST, Grb2 and activated EGF receptors form a trimolecular complex, and that the binding of the SH3 domains of Grb2 to MPTP-PEST represents an essential requirement in the formation of this complex (Chapter 5). These results suggest that the Grb2:MPTP-PEST complex, upon activation of the EGF receptor, is recruited to the membrane.

The recruitment of the Grb2:MPTP-PEST complex to other growth factor receptors upon ligand stimulation remains to be determined. However, it is likely that these observations are not restricted to the EGF receptor. Theoretically, growth factor receptors that are capable of recruiting Grb2 through its SH2 domain should bind Grb2:MPTP-PEST complexes. Additional experiments addressing this issue are required in order to validate the commonality of this interaction. SH3 domain-dependent recruitment of tyrosine phosphatases to activated growth factor receptors is a concept that has never been described before. It represents a novel means by which phosphotyrosine signaling events might be regulated. The results described in Chapter 5 unequivocally demonstrate a potential involvement for MPTP-PEST in tyrosine kinase signaling events.

It has been demonstrated that the activity of phosphatidyl inositol 3′ kinase (PI-3 kinase) is stimulated by the binding of Src family kinase SH3 domains to a pro-rich sequence located within the 85 kDa subunit (p85) of PI-3 kinase (Pleiman et al. 1994). However, the mechanistic details responsible for this activation remain unaddressed. In view of these observations, it is possible that the binding of the SH3 domains of Grb2 to MPTP-PEST might affect MPTP-PEST catalytic activity and/or substrate specificity. This would represent an additional means by which the function of MPTP-PEST can be regulated.

The physiological significance behind this Grb:SH3 domain-mediated recruitment of cytoplasmic MPTP-PEST to membrane-bound EGF receptors might be to relocalize the MPTP-PEST enzyme where it could fulfil its function by acting upon its substrate(s). In order to identify potential substrate(s) of MPTP-PEST, we exploited the

observation that mutating the catalytically essential Cys residue renders tyrosine phosphatases inactive but still permits substrate binding (Milarski et al. 1993). Since this mutation maintains the three-dimensional structure of the catalytic domain (Jia et al. 1995), it allows for the proper recognition and binding of natural substrates. Given the absence of the essential nucleophilic Cys residue, substrate hydrolysis is prevented. This, in addition to the intrinsic specificity displayed by the PTPase domain of MPTP-PEST will result in the specific trapping of tyrosine-phosphorylated substrates which can then be visualized by antiphosphotyrosine blots.

A mutated MPTP-PEST^{C231S} phosphatase domain was used to trap specific substrate(s) from lysates of cells that had been stimulated with EGF (Chapter 5). We found that a single tyrosine-phosphorylated protein with an apparent molecular weight of 120 kDa on SDS-PAGE is specifically trapped by this method (Chapter 5). The discovery of this potential substrate (pp120) whose tyrosine phosphorylation is dependent on stimulation of cells with EGF, allows for the formulation of a putative model describing a function for MPTP-PEST (see figure 5-Chapter 5). The MPTP-PEST enzyme, by virtue of its constitutive association with the adaptor protein Grb2 through an SH3 domain pro-rich interaction, can form molecular complexes with activated EGF receptors. This mobilization of MPTP-PEST from the cytoplasm towards activated EGF receptors brings MPTP-PEST in close proximity to the pp120 substrate which is itself dependent on EGF stimulation for its phosphorylation. The consequences of this functional recruitment reside in the downstream events triggered by the modulation of tyrosine phosphorylation of the pp120.

Analysis of the spatio-temporal relationship between the activation of growth factor receptors and the recruitment of Grb2:MPTP-PEST complexes in relation to the phosphorylation profile of the pp120 need to be performed in order to fully delineate the function of MPTP-PEST in signaling events. Recent studies of analogous systems involving the Grb2-mediated recruitment of the guanine nucleotide exchange factor Sos to activated growth factor receptors indicate that the coupling and uncoupling of Grb2:Sos complexes to and from the EGF and insulin receptors is a highly dynamic process (Buday et al. 1995).

Insulin stimulates the Ras/Raf/MEK/ERK pathway which lead to feed back serine/threonine phosphorylation of Sos, resulting in a dissociation of Grb2 from Sos (Cherniack et al. 1994; Waters et al. 1995a). Biochemical means aimed at blocking ERK activation were shown to prevent Sos phosphorylation, dissociation of Grb2:Sos complexes and prolonged the GTP-bound state of Ras (Langlois et al. 1995; Waters et al. 1995b). Together, these results suggest that insulin-stimulated feedback uncoupling of Grb2 from Sos may contribute to Ras downregulation.

Interestingly, it has been demonstrated that EGF stimulation lead to a similar ERK-mediated feedback serine/threonine phosphorylation of Sos (Porfiri and McCormick 1996). However, this EGF-triggered phosphorylation of Sos does not result in the dissociation of Grb2 from Sos but rather in a decreased ability for Grb2:Sos complexes to interact with EGF receptors or Shc proteins (Porfiri and McCormick 1996). The details of the molecular mechanistics underlying both of these phenomenon remain to be addressed. However, Holt *et al.*, (1996) recently showed that the Sos phosphorylation-triggered uncoupling of Grb2 from Sos (as seen in the insulin receptor system) can be prevented

by inducing a persistent plasma membrane receptor targeting of the Grb2:Sos complex (Holt et al. 1996). These negative feedback mechanisms represent an interesting method by which phosphorylation-dependent pathways auto-regulate themselves. Similarly, the time course of MPTP-PEST recruitment and the potential events ensuing from its relocalization and activity towards the pp120 represent an interesting challenge to resolve.

Conclusion

Other previously characterized interactions between cytoplasmic PTPases and activated growth factor receptors include the SH2-containing PTPases SHP-1 and SHP-2. The presence of these PTPases in phosphotyrosine signaling events ascribes a regulatory function for these enzymes. Here, we were able to show that another cytoplasmic PTPase, MPTP-PEST, is also present in phosphotyrosine-mediated signaling events. MPTP-PEST's novel associations with the adaptor proteins Shc and Grb2 strongly suggest a potential role for this PTPase in the context of signaling cascades involving tyrosine kinases.

Preliminary analysis of mice carrying a genetically targeted disruption of the MPTP-PEST gene indicate that MPTP-PEST is necessary for proper embryonic development (Charest and Tremblay, unpublished results). Homozygous mice for the disrupted MPTP-PEST gene failed to develop past 8.0 day p.-c. In view of the potential role of MPTP-PEST in tyrosine kinase-mediated signaling events and given the role of tyrosine kinases and their ensuing signaling pathways in processes such as cellular proliferation and differentiation during development, one can postulate that the observed MPTP-PEST null

phenotype is probably the result of aberrant signaling pathways brought about by the absence of MPTP-PEST. Analysis of the downstream events resulting from the deregulation in the phosphorylation status of MPTP-PEST's pp120 represent a key element in the elucidation of the observed phenotype at the molecular level.

This thesis describes several structural and functional aspects of the protein tyrosine phosphatase MPTP-PEST. The information provided herein represents a comprehensive initial study towards the elucidation of the functions of MPTP-PEST in cellular processes such as growth, differentiation and transformation.

Bibliography

Adachi, M., Sekiya, M., Miyachi, T., Matsuno, K., Hinoda, Y., Imai, K., and Yachi, A. (1992). "Molecular cloning of a novel protein-tyrosine phosphatase SH-PTP3 with sequence similarity to the src-homology region 2." *FEBS Letters*, 314(3), 335-9.

Ahmad, S., Banville, D., Zhao, Z., Fischer, E. H., and Shen, S. H. (1993). "A widely expressed human protein-tyrosine phosphatase containing src homology 2 domains." *Proceedings of the National Academy of Sciences of the United States of America*, 90(6), 2197-201.

Alessi, D. R., Smythe, C., and Keyse, S. M. (1993). "The human CL100 gene encodes a Tyr/Thr-protein phosphatase which potently and specifically inactivates MAP kinase and suppresses its activation by oncogenic ras in Xenopus oocyte extracts." *Oncogene*, 8(7), 2015-20.

Argetsinger, L. S., Campbell, G. S., Yang, X., Witthuhn, B. A., Silvennoinen, O., Ihle, J. N., and Carter-Su, C. (1993). "Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase." *Cell*, 74(2), 237-44.

Autero, M., Saharinen, J., Pessa-Morikawa, T., Soula-Rothhut, M., Oetken, C., Gassmann, M., Bergman, M., Alitalo, K., Burn, P., Gahmberg, C. G., and et al. (1994). "Tyrosine phosphorylation of CD45

phosphotyrosine phosphatase by p50csk kinase creates a binding site for p56lck tyrosine kinase and activates the phosphatase." *Molecular & Cellular Biology*, 14(2), 1308-21.

Bachmair, A., Finley, D., and Varshavsky, A. (1986). "In vivo half-life of a protein is a function of its amino-terminal residue." *Science*, 234(4773), 179-86.

Bagrodia, S., Chackalaparampil, I., Kmiecik, T. E., and Shalloway, D. (1991). "Altered tyrosine 527 phosphorylation and mitotic activation of p60c-src." *Nature*, 349(6305), 172-5.

Barbacid, M., Beemon, K., and Devare, S. G. (1980). "Origin and functional properties of the major gene product of the Snyder-Theilen strain of feline sarcoma virus." *Proceedings of the National Academy of Sciences of the United States of America*, 77(9), 5158-62.

Barford, D., Flint, A. J., and Tonks, N. K. (1994). "Crystal structure of human protein tyrosine phosphatase 1B [see comments]." *Science*, 263(5152), 1397-404.

Barnea, G., Silvennoinen, O., Shaanan, B., Honegger, A. M., Canoll, P. D., D'Eustachio, P., Morse, B., Levy, J. B., Laforgia, S., Huebner, K., and et al. (1993). "Identification of a carbonic anhydrase-like domain in the extracellular region of RPTP gamma defines a new subfamily of receptor tyrosine phosphatases." *Molecular & Cellular Biology*, 13(3), 1497-506.

Batzer, A. G., Blaikie, P., Nelson, K., Schlessinger, J., and Margolis, B. (1995). "The phosphotyrosine interaction domain of Shc binds an LXNPXY motif on the epidermal growth factor receptor." *Molecular & Cellular Biology*, 15(8), 4403-9.

Beckmann, G., and Bork, P. (1993). "An adhesive domain detected in functionally diverse receptors." *Trends in Biochemical Sciences*, 18(2), 40-1.

Beemon, K., Ryden, T., and McNelly, E. A. (1982). "Transformation by avian sarcoma viruses leads to phosphorylation of multiple cellular proteins on tyrosine residues." *Journal of Virology*, 42(2), 742-7.

Bennett, A. M., Tang, T. L., Sugimoto, S., Walsh, C. T., and Neel, B. G. (1994). "Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor beta to Ras." *Proceedings of the National*

Academy of Sciences of the United States of America, 91(15), 7335-9.

Bjelfman, C., Meyerson, G., Cartwright, C. A., Mellstrom, K., Hammerling, U., and Pahlman, S. (1990). "Early activation of endogenous pp60src kinase activity during neuronal differentiation of cultured human neuroblastoma cells." *Molecular & Cellular Biology,* 10(1), 361-70.

Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V., and Margolis, B. (1994). "A region in Shc distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptors." *Journal of Biological Chemistry,* 269(51), 32031-4.

Blomberg, J., Reynolds, F. H., Jr., Van de Ven, W. J., and Stephenson, J. R. (1980). "Abelson murine leukaemia virus transformation involves loss of epidermal growth factor-binding sites." *Nature,* 286(5772), 504-7.

Bork, P., and Margolis, B. (1995). "A phosphotyrosine interaction domain [letter]." *Cell,* 80(5), 693-4.

Boulter, C. A., and Wagner, E. F. (1988). "Expression of c-src and c-abl in embryonal carcinoma cells and adult mouse tissues." *Experimental Cell Research,* 179(1), 214-21.

Brady-Kalnay, S. M., Flint, A. J., and Tonks, N. K. (1993). "Homophilic binding of PTP mu, a receptor-type protein tyrosine phosphatase, can mediate cell-cell aggregation." *Journal of Cell Biology,* 122(4), 961-72.

Brady-Kalnay, S. M., and Tonks, N. K. (1995). "Protein tyrosine phosphatases as adhesion receptors." *Current Opinion in Cell Biology,* 7(5), 650-7.

Brautigan, D. L. (1992). "Great expectations: protein tyrosine phosphatases in cell regulation." *Biochimica et Biophysica Acta,* 1114(1), 63-77.

Brautigan, D. L., Bornstein, P., and Gallis, B. (1981). "Phosphotyrosyl-protein phosphatase. Specific inhibition by Zn." *Journal of Biological Chemistry,* 256(13), 6519-22.

Buday, L., and Downward, J. (1993). "Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor." *Cell,* 73(3), 611-20.

Buday, L., Warne, P. H., and Downward, J. (1995). "Downregulation of the Ras activation pathway by MAP kinase phosphorylation of Sos." *Oncogene*, 11(7), 1327-31.

Burns, L. A., Karnitz, L. M., Sutor, S. L., and Abraham, R. T. (1993). "Interleukin-2-induced tyrosine phosphorylation of p52shc in T lymphocytes." *Journal of Biological Chemistry*, 268(24), 17659-61.

Canoll, P. D., Barnea, G., Levy, J. B., Sap, J., Ehrlich, M., Silvennoinen, O., Schlessinger, J., and Musacchio, J. M. (1993). "The expression of a novel receptor-type tyrosine phosphatase suggests a role in morphogenesis and plasticity of the nervous system." *Brain Research. Developmental Brain Research*, 75(2), 293-8.

Carpenter, G., King, L., Jr., and Cohen, S. (1979). "Rapid enhancement of protein phosphorylation in A-431 cell membrane preparations by epidermal growth factor." *Journal of Biological Chemistry*, 254(11), 4884-91.

Cartwright, C. A., Eckhart, W., Simon, S., and Kaplan, P. L. (1987). "Cell transformation by pp60c-src mutated in the carboxy-terminal regulatory domain." *Cell*, 49(1), 83-91.

Case, R. D., Piccione, E., Wolf, G., Benett, A. M., Lechleider, R. J., Neel, B. G., and Shoelson, S. E. (1994). "SH-PTP2/Syp SH2 domain binding specificity is defined by direct interactions with platelet-derived growth factor beta-receptor, epidermal growth factor receptor, and insulin receptor substrate-1-derived phosphopeptides." *Journal of Biological Chemistry*, 269(14), 10467-74.

Charbonneau, H., Tonks, N. K., Kumar, S., Diltz, C. D., Harrylock, M., Cool, D. E., Krebs, E. G., Fischer, E. H., and Walsh, K. A. (1989). "Human placenta protein-tyrosine-phosphatase: amino acid sequence and relationship to a family of receptor-like proteins." *Proceedings of the National Academy of Sciences of the United States of America*, 86(14), 5252-6.

Charbonneau, H., Tonks, N. K., Walsh, K. A., and Fischer, E. H. (1988). "The leukocyte common antigen (CD45): a putative receptor-linked protein tyrosine phosphatase." *Proceedings of the National Academy of Sciences of the United States of America*, 85(19), 7182-6.

Chardin, P., Camonis, J. H., Gale, N. W., van Aelst, L., Schlessinger, J., Wigler, M. H., and Bar-Sagi, D. (1993). "Human Sos1: a guanine

nucleotide exchange factor for Ras that binds to GRB2." *Science*, 260(5112), 1338-43.

Chardin, P., Cussac, D., Maignan, S., and Ducruix, A. (1995). "The Grb2 adaptor." *FEBS Letters*, 369(1), 47-51.

Charest, A., Wagner, J., Jacob, S., McGlade, C. J., and Tremblay, M. L. (1996). "Phosphotyrosine-independent binding of SHC to the NPLH sequence of murine protein-tyrosine phosphatase-PEST. Evidence for extended phosphotyrosine binding/phosphotyrosine interaction domain recognition specificity." *Journal of Biological Chemistry*, 271(14), 8424-9.

Charest, A., Wagner, J., Muise, E. S., Heng, H. H., and Tremblay, M. L. (1995b). "Structure of the murine MPTP-PEST gene: genomic organization and chromosomal mapping." *Genomics*, 28(3), 501-7.

Charest, A., Wagner, J., Shen, S. H., and Tremblay, M. L. (1995a). "Murine protein tyrosine phosphatase-PEST, a stable cytosolic protein tyrosine phosphatase." *Biochemical Journal*, 308(Pt 2), 425-32.

Charles, C. H., Sun, H., Lau, L. F., and Tonks, N. K. (1993). "The growth factor-inducible immediate-early gene 3CH134 encodes a protein-tyrosine-phosphatase." *Proceedings of the National Academy of Sciences of the United States of America*, 90(11), 5292-6.

Cheadle, C., Ivashchenko, Y., South, V., Searfoss, G. H., French, S., Howk, R., Ricca, G. A., and Jaye, M. (1994). "Identification of a Src SH3 domain binding motif by screening a random phage display library." *Journal of Biological Chemistry*, 269(39), 24034-9.

Chen, C. C., Bruegger, B. B., Kern, C. W., Lin, Y. C., Halpern, R. M., and Smith, R. A. (1977). "Phosphorylation of nuclear proteins in rat regenerating liver." *Biochemistry*, 16(22), 4852-5.

Cherniack, A. D., Klarlund, J. K., and Czech, M. P. (1994). "Phosphorylation of the Ras nucleotide exchange factor son of sevenless by mitogen-activated protein kinase." *Journal of Biological Chemistry*, 269(7), 4717-20.

Chernoff, J., Schievella, A. R., Jost, C. A., Erikson, R. L., and Neel, B. G. (1990). "Cloning of a cDNA for a major human protein-tyrosine-phosphatase." *Proceedings of the National Academy of Sciences of the United States of America*, 87(7), 2735-9.

Chiang, H. L., and Dice, J. F. (1988). "Peptide sequences that target proteins for enhanced degradation during serum withdrawal." *Journal of Biological Chemistry*, 263(14), 6797-805.

Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A., and Rosen, O. M. (1987). "Human insulin receptors mutated at the ATP-binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin." *Journal of Biological Chemistry*, 262(4), 1842-7.

Chu, Y., Solski, P. A., Khosravi-Far, R., Der, C. J., and Kelly, K. (1996). "The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation." *Journal of Biological Chemistry*, 271(11), 6497-591.

Cicchetti, P., Mayer, B. J., Thiel, G., and Baltimore, D. (1992). "Identification of a protein that binds to the SH3 region of Abl and is similar to Bcr and GAP-rho." *Science*, 257(5071), 803-6.

Clark, E. A., Shultz, L. D., and Pollack, S. B. (1981). "Mutations in mice that influence natural killer (NK) cell activity." *Immunogenetics*, 12(5-6), 601-13.

Clark, S. G., Stern, M. J., and Horvitz, H. R. (1992). "C. elegans cell-signalling gene sem-5 encodes a protein with SH2 and SH3 domains [see comments]." *Nature*, 356(6367), 340-4.

Cohen, G. B., Ren, R., and Baltimore, D. (1995). "Modular binding domains in signal transduction proteins." *Cell*, 80(2), 237-48.

Cohen, S., Carpenter, G., and King, L., Jr. (1980). "Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity." *Journal of Biological Chemistry*, 255(10), 4834-42.

Coleman, J. E. (1992). "Structure and mechanism of alkaline phosphatase." *Annual Review of Biophysics & Biomolecular Structure*, 21, 441-83.

Collett, M. S., and Erikson, R. L. (1978). "Protein kinase activity associated with the avian sarcoma virus src gene product." *Proceedings of the National Academy of Sciences of the United States of America*,

75(4), 2021-4.

Cool, D. E., Tonks, N. K., Charbonneau, H., Walsh, K. A., Fischer, E. H., and Krebs, E. G. (1989). "cDNA isolated from a human T-cell library encodes a member of the protein-tyrosine-phosphatase family." *Proceedings of the National Academy of Sciences of the United States of America*, 86(14), 5257-61.

Cooper, J., Nakamura, K. D., Hunter, T., and Weber, M. J. (1983). "Phosphotyrosine-containing proteins and expression of transformation parameters in cells infected with partial transformation mutants of Rous sarcoma virus." *Journal of Virology*, 46(1), 15-28.

Cooper, J. A., Gould, K. L., Cartwright, C. A., and Hunter, T. (1986). "Tyr527 is phosphorylated in pp60c-src: implications for regulation." *Science*, 231(4744), 1431-4.

Coughlin, S. R., Barr, P. J., Cousens, L. S., Fretto, L. J., and Williams, L. T. (1988). "Acidic and basic fibroblast growth factors stimulate tyrosine kinase activity in vivo." *Journal of Biological Chemistry*, 263(2), 988-93.

Craparo, A., O'Neill, T. J., and Gustafson, T. A. (1995). "Non-SH2 domains within insulin receptor substrate-1 and SHC mediate their phosphotyrosine-dependent interaction with the NPEY motif of the insulin-like growth factor I receptor." *Journal of Biological Chemistry*, 270(26), 15639-43.

Crews, C. M., and Erikson, R. L. (1993). "Extracellular signals and reversible protein phosphorylation: what to Mek of it all." *Cell*, 74(2), 215-7.

Crovello, C. S., Furie, B. C., and Furie, B. (1995). "Histidine phosphorylation of P-selectin upon stimulation of human platelets: a novel pathway for activation-dependent signal transduction." *Cell*, 82(2), 279-86.

Crowe, A. J., McGlade, J., Pawson, T., and Hayman, M. J. (1994). "Phosphorylation of the SHC proteins on tyrosine correlates with the transformation of fibroblasts and erythroblasts by the v-sea tyrosine kinase." *Oncogene*, 9(2), 537-44.

Cutler, R. L., Liu, L., Damen, J. E., and Krystal, G. (1993). "Multiple cytokines induce the tyrosine phosphorylation of Shc and its association with Grb2 in hemopoietic cells." *Journal of Biological Chemistry*,

268(29), 21463-5.

Daum, G., Zander, N. F., Morse, B., Hurwitz, D., Schlessinger, J., and Fischer, E. H. (1991). "Characterization of a human recombinant receptor-linked protein tyrosine phosphatase." *Journal of Biological Chemistry*, 266(19), 12211-5.

Davidson, W. F., Morse, H. C. d., Sharrow, S. O., and Chused, T. M. (1979). "Phenotypic and functional effects of the motheaten gene on murine B and T lymphocytes." *Journal of Immunology*, 122(3), 884-91.

de Vries-Smits, A. M., Burgering, B. M., Leevers, S. J., Marshall, C. J., and Bos, J. L. (1992). "Involvement of p21ras in activation of extracellular signal-regulated kinase 2 [see comments]." *Nature*, 357(6379), 602-4.

Dechert, U., Adam, M., Harder, K. W., Clark-Lewis, I., and Jirik, F. (1994). "Characterization of protein tyrosine phosphatase SH-PTP2. Study of phosphopeptide substrates and possible regulatory role of SH2 domains." *Journal of Biological Chemistry*, 269(8), 5602-11.

den Hertog, J., and Hunter, T. (1996). "Tight association of GRB2 with receptor protein-tyrosine phosphatase alpha is mediated by the SH2 and C-terminal SH3 domains." *EMBO Journal*, 15(12), 3016-3027.

den Hertog, J., Pals, C. E., Jonk, L. J., and Kruijer, W. (1992). "Differential expression of a novel murine non-receptor protein tyrosine phosphatase during differentiation of P19 embryonal carcinoma cells." *Biochemical & Biophysical Research Communications*, 184(3), 1241-9.

den Hertog, J., Pals, C. E., Peppelenbosch, M. P., Tertoolen, L. G., de Laat, S. W., and Kruijer, W. (1993). "Receptor protein tyrosine phosphatase alpha activates pp60c-src and is involved in neuronal differentiation." *EMBO Journal*, 12(10), 3789-98.

den Hertog, J., Tracy, S., and Hunter, T. (1994). "Phosphorylation of receptor protein-tyrosine phosphatase alpha on Tyr789, a binding site for the SH3-SH2-SH3 adaptor protein GRB-2 in vivo." *EMBO Journal*, 13(13), 3020-32.

Desai, C. J., Popova, E., and Zinn, K. (1994). "A Drosophila receptor tyrosine phosphatase expressed in the embryonic CNS and larval optic lobes is a member of the set of proteins bearing the "HRP" carbohydrate epitope." *Journal of Neuroscience*, 14(12), 7272-83.

Dice, J. F. (1987). "Molecular determinants of protein half-lives in eukaryotic cells [published erratum appears in FASEB J 1988 Apr;2(7):2262]." *FASEB Journal*, 1(5), 349-57.

Dikic, I., Batzer, A. G., Blaikie, P., Obermeier, A., Ullrich, A., Schlessinger, J., and Margolis, B. (1995). "Shc binding to nerve growth factor receptor is mediated by the phosphotyrosine interaction domain." *Journal of Biological Chemistry*, 270(25), 15125-9.

Downward, J. (1994). "The GRB2/Sem-5 adaptor protein." *FEBS Letters*, 338(2), 113-7.

Eck, M. J., Dhe-Paganon, S., Trub, T., Nolte, R. T., and Shoelson, S. E. (1996). "Structure of the IRS-1 PTB domain bound to the juxtamembrane region of the insulin receptor." *Cell*, 85(5), 695-705.

Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993). "Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation [see comments]." *Nature*, 363(6424), 45-51.

Eijgenraam, F. (1993). "Things start getting sticky for a cell surface enzyme [news]." *Science*, 261(5123), 833.

Ek, B., Westermark, B., Wasteson, A., and Heldin, C. H. (1982). "Stimulation of tyrosine-specific phosphorylation by platelet-derived growth factor." *Nature*, 295(5848), 419-20.

Feng, G. S., Hui, C. C., and Pawson, T. (1993a). "SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases." *Science*, 259(5101), 1607-11.

Feng, G. S., Hui, C. C., and Pawson, T. (1993b). "SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases." *Science*, 259(5101), 1607-11.

Feng, G. S., Shen, R., Heng, H. H., Tsui, L. C., Kazlauskas, A., and Pawson, T. (1994a). "Receptor-binding, tyrosine phosphorylation and chromosome localization of the mouse SH2-containing phosphotyrosine phosphatase Syp." *Oncogene*, 9(6), 1545-50.

Feng, S., Chen, J. K., Yu, H., Simon, J. A., and Schreiber, S. L. (1994b). "Two binding orientations for peptides to the Src SH3 domain:

development of a general model for SH3-ligand interactions." *Science*, 266(5188), 1241-7.

Firmbach-Kraft, I., Byers, M., Shows, T., Dalla-Favera, R., and Krolewski, J. J. (1990). "tyk2, prototype of a novel class of non-receptor tyrosine kinase genes." *Oncogene*, 5(9), 1329-36.

Flint, A. J., Gebbink, M. F., Franza, B. R., Jr., Hill, D. E., and Tonks, N. K. (1993). "Multi-site phosphorylation of the protein tyrosine phosphatase, PTP1B: identification of cell cycle regulated and phorbol ester stimulated sites of phosphorylation." *EMBO Journal*, 12(5), 1937-46.

Frangioni, J. V., Beahm, P. H., Shifrin, V., Jost, C. A., and Neel, B. G. (1992). "The nontransmembrane tyrosine phosphatase PTP-1B localizes to the endoplasmic reticulum via its 35 amino acid C-terminal sequence." *Cell*, 68(3), 545-60.

Freeman, R. M., Jr., Plutzky, J., and Neel, B. G. (1992). "Identification of a human src homology 2-containing protein-tyrosine-phosphatase: a putative homolog of Drosophila corkscrew." *Proceedings of the National Academy of Sciences of the United States of America*, 89(23), 11239-43.

Friis, R. R., Jockusch, B. M., Boschek, C. B., Ziemiecki, A., Rubsamen, H., and Bauer, H. (1980). "Transformation-defective, temperature-sensitive mutants of Rous sarcoma virus have a reversibly defective src-gene product." *Cold Spring Harbor Symposia on Quantitative Biology*, 44(Pt 2,), 1007-12.

Fujitaki, J. M., Fung, G., Oh, E. Y., and Smith, R. A. (1981). "Characterization of chemical and enzymatic acid-labile phosphorylation of histone H4 using phosphorus-31 nuclear magnetic resonance." *Biochemistry*, 20(12), 3658-64.

Gale, N. W., Kaplan, S., Lowenstein, E. J., Schlessinger, J., and Bar-Sagi, D. (1993). "Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras [see comments]." *Nature*, 363(6424), 88-92.

Gammeltoft, S., and Van Obberghen, E. (1986). "Protein kinase activity of the insulin receptor." *Biochemical Journal*, 235(1), 1-11.

Gebbink, M. F., van Etten, I., Hateboer, G., Suijkerbuijk, R., Beijersbergen, R. L., Geurts van Kessel, A., and Moolenaar, W. H. (1991). "Cloning, expression and chromosomal localization of a new putative

receptor-like protein tyrosine phosphatase." *FEBS Letters*, 290(1-2), 123-30.

Gebbink, M. F., Zondag, G. C., Wubbolts, R. W., Beijersbergen, R. L., van Etten, I., and Moolenaar, W. H. (1993). "Cell-cell adhesion mediated by a receptor-like protein tyrosine phosphatase." *Journal of Biological Chemistry*, 268(22), 16101-4.

Gibson, T. J., Hyvonen, M., Musacchio, A., Saraste, M., and Birney, E. (1994). "PH domain: the first anniversary." *Trends in Biochemical Sciences*, 19(9), 349-53.

Goffinet, A. M. (1995). "Developmental neurobiology. A real gene for reeler [news; comment]." *Nature*, 374(6524), 675-6.

Goudreau, N., Cornille, F., Duchesne, M., Parker, F., Tocque, B., Garbay, C., and Roques, B. P. (1994). "NMR structure of the N-terminal SH3 domain of GRB2 and its complex with a proline-rich peptide from Sos." *Nature Structural Biology*, 1(12), 898-907.

Green, M. C., and Shultz, L. D. (1975). "Motheaten, an immunodeficient mutant of the mouse. I. Genetics and pathology." *Journal of Heredity*, 66(5), 250-8.

Gu, M. X., York, J. D., Warshawsky, I., and Majerus, P. W. (1991). "Identification, cloning, and expression of a cytosolic megakaryocyte protein-tyrosine-phosphatase with sequence homology to cytoskeletal protein 4.1." *Proceedings of the National Academy of Sciences of the United States of America*, 88(13), 5867-71.

Guan, K., Hakes, D. J., Wang, Y., Park, H. D., Cooper, T. G., and Dixon, J. E. (1992). "A yeast protein phosphatase related to the vaccinia virus VH1 phosphatase is induced by nitrogen starvation [published erratum appears in Proc Natl Acad Sci U S A 1993 Feb 1;90(3):1137]." *Proceedings of the National Academy of Sciences of the United States of America*, 89(24), 12175-9.

Guan, K. L., Broyles, S. S., and Dixon, J. E. (1991). "A Tyr/Ser protein phosphatase encoded by vaccinia virus." *Nature*, 350(6316), 359-62.

Guan, K. L., and Dixon, J. E. (1990a). "Protein tyrosine phosphatase activity of an essential virulence determinant in Yersinia." *Science*, 249(4968), 553-6.

Guan, K. L., and Dixon, J. E. (1991). "Evidence for protein-tyrosine-phosphatase catalysis proceeding via a cysteine-phosphate intermediate." *Journal of Biological Chemistry*, 266(26), 17026-30.

Guan, K. L., Haun, R. S., Watson, S. J., Geahlen, R. L., and Dixon, J. E. (1990b). "Cloning and expression of a protein-tyrosine-phosphatase." *Proceedings of the National Academy of Sciences of the United States of America*, 87(4), 1501-5.

Habib, T., Herrera, R., and Decker, S. J. (1994). "Activators of protein kinase C stimulate association of Shc and the PEST tyrosine phosphatase." *Journal of Biological Chemistry*, 269(41), 25243-6.

Hakes, D. J., Martell, K. J., Zhao, W. G., Massung, R. F., Esposito, J. J., and Dixon, J. E. (1993). "A protein phosphatase related to the vaccinia virus VH1 is encoded in the genomes of several orthopoxviruses and a baculovirus." *Proceedings of the National Academy of Sciences of the United States of America*, 90(9), 4017-21.

He, W., O'Neill, T. J., and Gustafson, T. A. (1995). "Distinct modes of interaction of SHC and insulin receptor substrate-1 with the insulin receptor NPEY region via non-SH2 domains." *Journal of Biological Chemistry*, 270(40), 23258-62.

Heldin, C. H., and Westermark, B. (1984). "Growth factors: mechanism of action and relation to oncogenes." *Cell*, 37(1), 9-20.

Hess, J. F., Bourret, R. B., and Simon, M. I. (1988). "Histidine phosphorylation and phosphoryl group transfer in bacterial chemotaxis." *Nature*, 336(6195), 139-43.

Higashitsuji, H., Arii, S., Furutani, M., Imamura, M., Kaneko, Y., Takenawa, J., Nakayama, H., and Fujita, J. (1995). "Enhanced expression of multiple protein tyrosine phosphatases in the regenerating mouse liver: isolation of PTP-RL10, a novel cytoplasmic-type phosphatase with sequence homology to cytoskeletal protein 4.1." *Oncogene*, 10(2), 407-14.

Holt, K. H., Waters, S. B., Okada, S., Yamauchi, K., Decker, S. J., Saltiel, A. R., Motto, D. G., Koretzky, G. A., and Pessin, J. E. (1996). "Epidermal growth factor receptor targeting prevents uncoupling of the Grb2-SOS complex." *Journal of Biological Chemistry*, 271(14), 8300-6.

Huang, J. M., Wei, Y. F., Kim, Y. H., Osterberg, L., and Matthews, H. R. (1991). "Purification of a protein histidine kinase from the yeast

Saccharomyces cerevisiae. The first member of this class of protein kinases." *Journal of Biological Chemistry*, 266(14), 9023-31.

Hunter, T. (1987). "A thousand and one protein kinases." *Cell*, 50(6), 823-9.

Hunter, T. (1995). "Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling." *Cell*, 80(2), 225-36.

Hunter, T., Sefton, B. M., and Beemon, K. (1980). "Studies on the structure and function of the avian sarcoma virus transforming-gene product." *Cold Spring Harbor Symposia on Quantitative Biology*, 44(Pt 2,), 931-41.

Ihle, J. N. (1995). "Cytokine receptor signalling." *Nature*, 377(6550), 591-4.

Ihle, J. N., and Kerr, I. M. (1995). "Jaks and Stats in signaling by the cytokine receptor superfamily." *Trends in Genetics*, 11(2), 69-74.

Ihle, J. N., Witthuhn, B. A., Quelle, F. W., Yamamoto, K., Thierfelder, W. E., Kreider, B., and Silvennoinen, O. (1994). "Signaling by the cytokine receptor superfamily: JAKs and STATs." *Trends in Biochemical Sciences*, 19(5), 222-7.

Isakoff, S. J., Yu, Y. P., Su, Y. C., Blaikie, P., Yajnik, V., Rose, E., Weidner, K. M., Sachs, M., Margolis, B., and Skolnik, E. Y. (1996). "Interaction between the phosphotyrosine binding domain of Shc and the insulin receptor is required for Shc phosphorylation by insulin in vivo." *Journal of Biological Chemistry*, 271(8), 3959-62.

Jacobs, S., Kull, F. C., Jr., Earp, H. S., Svoboda, M. E., Van Wyk, J. J., and Cuatrecasas, P. (1983). "Somatomedin-C stimulates the phosphorylation of the beta-subunit of its own receptor." *Journal of Biological Chemistry*, 258(16), 9581-4.

Jia, Z., Barford, D., Flint, A. J., and Tonks, N. K. (1995). "Structural basis for phosphotyrosine peptide recognition by protein tyrosine phosphatase 1B." *Science*, 268(5218), 1754-8.

Jia, Z., Quail, J. W., Delbaere, L. T., and Waygood, E. B. (1994). "Structural comparison of the histidine-containing phosphocarrier protein HPr." *Biochemistry & Cell Biology*, 72(5-6), 202-17.

Jiang, Y. P., Wang, H., D'Eustachio, P., Musacchio, J. M., Schlessinger, J., and Sap, J. (1993). "Cloning and characterization of R-PTP-kappa, a new member of the receptor protein tyrosine phosphatase family with a proteolytically cleaved cellular adhesion molecule-like extracellular region." *Molecular & Cellular Biology*, 13(5), 2942-51.

Jirik, F. R., Janzen, N. M., Melhado, I. G., and Harder, K. W. (1990). "Cloning and chromosomal assignment of a widely expressed human receptor-like protein-tyrosine phosphatase." *FEBS Letters*, 273(1-2), 239-42.

Jones-Villeneuve, E. M., McBurney, M. W., Rogers, K. A., and Kalnins, V. I. (1982). "Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells." *Journal of Cell Biology*, 94(2), 253-62.

Kaech, S., Covic, L., Wyss, A., and Ballmer-Hofer, K. (1991). "Association of p60c-src with polyoma virus middle-T antigen abrogating mitosis-specific activation." *Nature*, 350(6317), 431-3.

Kaplan, R., Morse, B., Huebner, K., Croce, C., Howk, R., Ravera, M., Ricca, G., Jaye, M., and Schlessinger, J. (1990). "Cloning of three human tyrosine phosphatases reveals a multigene family of receptor-linked protein-tyrosine-phosphatases expressed in brain." *Proceedings of the National Academy of Sciences of the United States of America*, 87(18), 7000-4.

Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1995). "PTB domain binding to signaling proteins through a sequence motif containing phosphotyrosine." *Science*, 268(5214), 1177-9.

Kavanaugh, W. M., and Williams, L. T. (1994). "An alternative to SH2 domains for binding tyrosine-phosphorylated proteins." *Science*, 266(5192), 1862-5.

Kazlauskas, A., Feng, G. S., Pawson, T., and Valius, M. (1993). "The 64-kDa protein that associates with the platelet-derived growth factor receptor beta subunit via Tyr-1009 is the SH2-containing phosphotyrosine phosphatase Syp." *Proceedings of the National Academy of Sciences of the United States of America*, 90(15), 6939-43.

Kim, E. E., and Wyckoff, H. W. (1991). "Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis." *Journal of Molecular Biology*, 218(2), 449-64.

Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995). "Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals." *Cell*, 80(5), 729-38.

Kmiecik, T. E., and Shalloway, D. (1987). "Activation and suppression of pp60c-src transforming ability by mutation of its primary sites of tyrosine phosphorylation." *Cell*, 49(1), 65-73.

Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S., and Sowadski, J. M. (1991). "Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase [see comments]." *Science*, 253(5018), 407-14.

Koretzky, G. A., Picus, J., Schultz, T., and Weiss, A. (1991). "Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production." *Proceedings of the National Academy of Sciences of the United States of America*, 88(6), 2037-41.

Koretzky, G. A., Picus, J., Thomas, M. L., and Weiss, A. (1990). "Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway." *Nature*, 346(6279), 66-8.

Kovacina, K. S., and Roth, R. A. (1993). "Identification of SHC as a substrate of the insulin receptor kinase distinct from the GAP-associated 62 kDa tyrosine phosphoprotein." *Biochemical & Biophysical Research Communications*, 192(3), 1303-11.

Kozlowski, M., Mlinaric-Rascan, I., Feng, G. S., Shen, R., Pawson, T., and Siminovitch, K. A. (1993). "Expression and catalytic activity of the tyrosine phosphatase PTP1C is severely impaired in motheaten and viable motheaten mice." *Journal of Experimental Medicine*, 178(6), 2157-63.

Krueger, N. X., and Saito, H. (1992). "A human transmembrane protein-tyrosine-phosphatase, PTP zeta, is expressed in brain and has an N-terminal receptor domain homologous to carbonic anhydrases." *Proceedings of the National Academy of Sciences of the United States of America*, 89(16), 7417-21.

Krueger, N. X., Streuli, M., and Saito, H. (1990). "Structural diversity and evolution of human receptor-like protein tyrosine phosphatases."

EMBO Journal, 9(10), 3241-52.

Kuba, M., Ohmori, H., and Kumon, A. (1992). "Characterization of N omega-phosphoarginine hydrolase from rat liver." *European Journal of Biochemistry*, 208(3), 747-52.

Kuhne, M. R., Pawson, T., Lienhard, G. E., and Feng, G. S. (1993). "The insulin receptor substrate 1 associates with the SH2-containing phosphotyrosine phosphatase Syp." *Journal of Biological Chemistry*, 268(16), 11479-81.

Langlois, W. J., Sasaoka, T., Saltiel, A. R., and Olefsky, J. M. (1995). "Negative feedback regulation and desensitization of insulin- and epidermal growth factor-stimulated p21ras activation." *Journal of Biological Chemistry*, 270(43), 25320-3.

Lau, K. H., Farley, J. R., and Baylink, D. J. (1989). "Phosphotyrosyl protein phosphatases." *Biochemical Journal*, 257(1), 23-36.

Lechleider, R. J., Freeman, R. M., Jr., and Neel, B. G. (1993a). "Tyrosyl phosphorylation and growth factor receptor association of the human corkscrew homologue, SH-PTP2." *Journal of Biological Chemistry*, 268(18), 13434-8.

Lechleider, R. J., Sugimoto, S., Bennett, A. M., Kashishian, A. S., Cooper, J. A., Shoelson, S. E., Walsh, C. T., and Neel, B. G. (1993b). "Activation of the SH2-containing phosphotyrosine phosphatase SH-PTP2 by its binding site, phosphotyrosine 1009, on the human platelet-derived growth factor receptor." *Journal of Biological Chemistry*, 268(29), 21478-81.

Lee, W. H., Bister, K., Moscovici, C., and Duesberg, P. H. (1981). "Temperature-sensitive mutants of Fujinami sarcoma virus: tumorigenicity and reversible phosphorylation of the transforming p140 protein." *Journal of Virology*, 38(3), 1064-76.

Lemmon, M. A., Ferguson, K. M., and Schlessinger, J. (1996). "PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface." *Cell*, 85(5), 621-4.

Lemmon, M. A., and Schlessinger, J. (1994). "Regulation of signal transduction and signal diversity by receptor oligomerization." *Trends in Biochemical Sciences*, 19(11), 459-63.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995). "Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions [see comments]." *Nature*, 376(6543), 737-45.

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., and et al. (1993). "The cloning of a receptor-type protein tyrosine phosphatase expressed in the central nervous system." *Journal of Biological Chemistry*, 268(14), 10573-81.

Levy-Favatier, F., Delpech, M., and Kruh, J. (1987). "Characterization of an arginine-specific protein kinase tightly bound to rat liver DNA." *European Journal of Biochemistry*, 166(3), 617-21.

Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B., and Schlessinger, J. (1993). "Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling [see comments]." *Nature*, 363(6424), 85-8.

Li, W., Nishimura, R., Kashishian, A., Batzer, A. G., Kim, W. J., Cooper, J. A., and Schlessinger, J. (1994). "A new function for a phosphotyrosine phosphatase: linking GRB2-Sos to a receptor tyrosine kinase." *Molecular & Cellular Biology*, 14(1), 509-17.

Li, X., Luna, J., Lombroso, P. J., and Francke, U. (1995). "Molecular cloning of the human homolog of a striatum-enriched phosphatase (STEP) gene and chromosomal mapping of the human and murine loci." *Genomics*, 28(3), 442-9.

Lim, W. A., Richards, F. M., and Fox, R. O. (1994). "Structural determinants of peptide-binding orientation and of sequence specificity in SH3 domains [see comments] [published erratum appears in Nature 1995 Mar 2;374(6517):94]." *Nature*, 372(6504), 375-9.

Loetscher, P., Pratt, G., and Rechsteiner, M. (1991). "The C terminus of mouse ornithine decarboxylase confers rapid degradation on dihydrofolate reductase. Support for the pest hypothesis." *Journal of Biological Chemistry*, 266(17), 11213-20.

Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E. Y., Bar-Sagi, D., and Schlessinger, J. (1992). "The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling." *Cell*, 70(3), 431-42.

Lynch, S. A., Brugge, J. S., and Levine, J. M. (1986). "Induction of altered c-src product during neural differentiation of embryonal carcinoma cells." *Science*, 234(4778), 873-6.

Maekawa, K., Imagawa, N., Nagamatsu, M., and Harada, S. (1994). "Molecular cloning of a novel protein-tyrosine phosphatase containing a membrane-binding domain and GLGF repeats." *FEBS Letters*, 337(2), 200-6.

Mandiyan, V., O'Brien, R., Zhou, M., Margolis, B., Lemmon, M. A., Sturtevant, J. M., and Schlessinger, J. (1996). "Thermodynamic studies of SHC phosphotyrosine interaction domain recognition of the NPXpY motif." *Journal of Biological Chemistry*, 271(9), 4770-5.

Martensen, T. M. (1982). "Phosphotyrosine in proteins. Stability and quantification." *Journal of Biological Chemistry*, 257(16), 9648-52.

Martin, B. L., and Graves, D. J. (1986). "Mechanistic aspects of the low-molecular-weight phosphatase activity of the calmodulin-activated phosphatase, calcineurin." *Journal of Biological Chemistry*, 261(31), 14545-50.

Matozaki, T., Suzuki, T., Uchida, T., Inazawa, J., Ariyama, T., Matsuda, K., Horita, K., Noguchi, H., Mizuno, H., Sakamoto, C., and et al. (1994). "Molecular cloning of a human transmembrane-type protein tyrosine phosphatase and its expression in gastrointestinal cancers." *Journal of Biological Chemistry*, 269(3), 2075-81.

Matthews, H. R. (1995). "Protein kinases and phosphatases that act on histidine, lysine, or arginine residues in eukaryotic proteins: a possible regulator of the mitogen-activated protein kinase cascade." *Pharmacology & Therapeutics*, 67(3), 323-50.

Matthews, R. J., Bowne, D. B., Flores, E., and Thomas, M. L. (1992). "Characterization of hematopoietic intracellular protein tyrosine phosphatases: description of a phosphatase containing an SH2 domain and another enriched in proline-, glutamic acid-, serine-, and threonine-rich sequences." *Molecular & Cellular Biology*, 12(5), 2396-405.

Matthews, R. J., Cahir, E. D., and Thomas, M. L. (1990). "Identification of an additional member of the protein-tyrosine-phosphatase family: evidence for alternative splicing in the tyrosine phosphatase domain."

Proceedings of the National Academy of Sciences of the United States of America, 87(12), 4444-8.

Mayer, B. J., and Eck, M. J. (1995). "SH3 domains. Minding your p's and q's." *Current Biology*, 5(4), 364-7.

McBurney, M. W., Jones-Villeneuve, E. M., Edwards, M. K., and Anderson, P. J. (1982). "Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line." *Nature*, 299(5879), 165-7.

McFarland, E. C., Flores, E., Matthews, R. J., and Thomas, M. L. (1994). "Protein tyrosine phosphatases involved in lymphocyte signal transduction." *Chemical Immunology*, 59, 40-61.

McFarland, E. D., Hurley, T. R., Pingel, J. T., Sefton, B. M., Shaw, A., and Thomas, M. L. (1993). "Correlation between Src family member regulation by the protein-tyrosine-phosphatase CD45 and transmembrane signaling through the T-cell receptor." *Proceedings of the National Academy of Sciences of the United States of America*, 90(4), 1402-6.

McGlade, J., Cheng, A., Pelicci, G., Pelicci, P. G., and Pawson, T. (1992). "Shc proteins are phosphorylated and regulated by the v-Src and v-Fps protein-tyrosine kinases." *Proceedings of the National Academy of Sciences of the United States of America*, 89(19), 8869-73.

McLaughlin, S., and Dixon, J. E. (1993). "Alternative splicing gives rise to a nuclear protein tyrosine phosphatase in Drosophila." *Journal of Biological Chemistry*, 268(10), 6839-42.

Mei, L., Doherty, C. A., and Huganir, R. L. (1994). "RNA splicing regulates the activity of a SH2 domain-containing protein tyrosine phosphatase." *Journal of Biological Chemistry*, 269(16), 12254-62.

Menon, S. D., Guy, G. R., and Tan, Y. H. (1995). "Involvement of a putative protein-tyrosine phosphatase and I kappa B-alpha serine phosphorylation in nuclear factor kappa B activation by tumor necrosis factor." *Journal of Biological Chemistry*, 270(32), 18881-7.

Milarski, K. L., and Saltiel, A. R. (1994). "Expression of catalytically inactive Syp phosphatase in 3T3 cells blocks stimulation of mitogen-activated protein kinase by insulin." *Journal of Biological Chemistry*, 269(33), 21239-43.

Milarski, K. L., Zhu, G., Pearl, C. G., McNamara, D. J., Dobrusin, E. M., MacLean, D., Thieme-Sefler, A., Zhang, Z. Y., Sawyer, T., Decker, S. J., and et al. (1993). "Sequence specificity in recognition of the epidermal growth factor receptor by protein tyrosine phosphatase 1B." *Journal of Biological Chemistry*, 268(31), 23634-9.

Moller, N. P., Moller, K. B., Lammers, R., Kharitonenkov, A., Sures, I., and Ullrich, A. (1994). "Src kinase associates with a member of a distinct subfamily of protein-tyrosine phosphatases containing an ezrin-like domain." *Proceedings of the National Academy of Sciences of the United States of America*, 91(16), 7477-81.

Moreno, S., Nurse, P., and Russell, P. (1990). "Regulation of mitosis by cyclic accumulation of p80cdc25 mitotic inducer in fission yeast." *Nature*, 344(6266), 549-52.

Morgan, D. O., Ho, L., Korn, L. J., and Roth, R. A. (1986). "Insulin action is blocked by a monoclonal antibody that inhibits the insulin receptor kinase." *Proceedings of the National Academy of Sciences of the United States of America*, 83(2), 328-32.

Morgan, D. O., and Roth, R. A. (1987). "Acute insulin action requires insulin receptor kinase activity: introduction of an inhibitory monoclonal antibody into mammalian cells blocks the rapid effects of insulin." *Proceedings of the National Academy of Sciences of the United States of America*, 84(1), 41-5.

Moriyama, T., Kawanishi, S., Inoue, T., Imai, E., Kaneko, T., Xia, C., Takenaka, M., Noguchi, T., Kamada, T., and Ueda, N. (1994). "cDNA cloning of a cytosolic protein tyrosine phosphatase (RKPTP) from rat kidney." *FEBS Letters*, 353(3), 305-8.

Mourey, R. J., and Dixon, J. E. (1994). "Protein tyrosine phosphatases: characterization of extracellular and intracellular domains." *Current Opinion in Genetics & Development*, 4(1), 31-9.

Muda, M., Boschert, U., Dickinson, R., Martinou, J. C., Martinou, I., Camps, M., Schlegel, W., and Arkinstall, S. (1996). "MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase." *Journal of Biological Chemistry*, 271(8), 4319-26.

Mulcahy, L. S., Smith, M. R., and Stacey, D. W. (1985). "Requirement for ras proto-oncogene function during serum-stimulated growth of NIH

3T3 cells." *Nature*, 313(5999), 241-3.

Musacchio, A., Saraste, M., and Wilmanns, M. (1994). "High-resolution crystal structures of tyrosine kinase SH3 domains complexed with proline-rich peptides [see comments]." *Nature Structural Biology*, 1(8), 546-51.

Mustelin, T., and Altman, A. (1990). "Dephosphorylation and activation of the T cell tyrosine kinase pp56lck by the leukocyte common antigen (CD45)." *Oncogene*, 5(6), 809-13.

Mustelin, T., Coggeshall, K. M., and Altman, A. (1989). "Rapid activation of the T-cell tyrosine protein kinase pp56lck by the CD45 phosphotyrosine phosphatase." *Proceedings of the National Academy of Sciences of the United States of America*, 86(16), 6302-6.

Mustelin, T., Pessa-Morikawa, T., Autero, M., Gassmann, M., Andersson, L. C., Gahmberg, C. G., and Burn, P. (1992). "Regulation of the p59fyn protein tyrosine kinase by the CD45 phosphotyrosine phosphatase." *European Journal of Immunology*, 22(5), 1173-8.

Myers, M. G., Jr., and White, M. F. (1993). "The new elements of insulin signaling. Insulin receptor substrate-1 and proteins with SH2 domains." *Diabetes*, 42(5), 643-50.

O'Bryan, J. P., Songyang, Z., Cantley, L., Der, C. J., and Pawson, T. (1996). "A mammalian adaptor protein with conserved Src homology 2 and phosphotyrosine-binding domains is related to Shc and is specifically expressed in the brain." *Proceedings of the National Academy of Sciences of the United States of America*, 93(7), 2729-34.

O'Neill, T. J., Craparo, A., and Gustafson, T. A. (1994). "Characterization of an interaction between insulin receptor substrate 1 and the insulin receptor by using the two-hybrid system." *Molecular & Cellular Biology*, 14(10), 6433-42.

Ohmori, H., Kuba, M., and Kumon, A. (1993). "Two phosphatases for 6-phospholysine and 3-phosphohistidine from rat brain." *Journal of Biological Chemistry*, 268(11), 7625-7.

Ohmori, H., Kuba, M., and Kumon, A. (1994). "3-Phosphohistidine/6-phospholysine phosphatase from rat brain as acid phosphatase." *Journal of Biochemistry*, 116(2), 380-5.

Olivier, J. P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E., and Pawson, T. (1993). "A Drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos." *Cell*, 73(1), 179-91.

Olson, T. S., and Dice, J. F. (1989). "Regulation of protein degradation rates in eukaryotes." *Current Opinion in Cell Biology*, 1(6), 1194-200.

Oon, S. H., Hong, A., Yang, X., and Chia, W. (1993). "Alternative splicing in a novel tyrosine phosphatase gene (DPTP4E) of Drosophila melanogaster generates two large receptor-like proteins which differ in their carboxyl termini." *Journal of Biological Chemistry*, 268(32), 23964-71.

Ostergaard, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Sefton, B. M., and Trowbridge, I. S. (1989). "Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma T-cell lines." *Proceedings of the National Academy of Sciences of the United States of America*, 86(22), 8959-63.

Ostergaard, H. L., and Trowbridge, I. S. (1991). "Negative regulation of CD45 protein tyrosine phosphatase activity by ionomycin in T cells." *Science*, 253(5026), 1423-5.

Ostman, A., Yang, Q., and Tonks, N. K. (1994). "Expression of DEP-1, a receptor-like protein-tyrosine-phosphatase, is enhanced with increasing cell density." *Proceedings of the National Academy of Sciences of the United States of America*, 91(21), 9680-4.

Pawson, T., Guyden, J., Kung, T. H., Radke, K., Gilmore, T., and Martin, G. S. (1980). "A strain of Fujinami sarcoma virus which is temperature-sensitive in protein phosphorylation and cellular transformation." *Cell*, 22(3), 767-75.

Pelech, S. L., and Sanghera, J. S. (1992). "MAP kinases: charting the regulatory pathways [comment]." *Science*, 257(5075), 1355-6.

Peles, E., Nativ, M., Campbell, P. L., Sakurai, T., Martinez, R., Lev, S., Clary, D. O., Schilling, J., Barnea, G., Plowman, G. D., and et al. (1995). "The carbonic anhydrase domain of receptor tyrosine phosphatase beta is a functional ligand for the axonal cell recognition molecule contactin." *Cell*, 82(2), 251-60.

Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T., and Pelicci, P. G. (1992). "A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction." *Cell*, 70(1), 93-104.

Perkins, L. A., Larsen, I., and Perrimon, N. (1992). "corkscrew encodes a putative protein tyrosine phosphatase that functions to transduce the terminal signal from the receptor tyrosine kinase torso." *Cell*, 70(2), 225-36.

Perlmutter, R. M., Levin, S. D., Appleby, M. W., Anderson, S. J., and Alberola-Ila, J. (1993). "Regulation of lymphocyte function by protein phosphorylation." *Annual Review of Immunology*, 11, 451-99.

Pingel, J. T., and Thomas, M. L. (1989). "Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation." *Cell*, 58(6), 1055-65.

Piwnica-Worms, H., Saunders, K. B., Roberts, T. M., Smith, A. E., and Cheng, S. H. (1987). "Tyrosine phosphorylation regulates the biochemical and biological properties of pp60c-src." *Cell*, 49(1), 75-82.

Pleiman, C. M., Hertz, W. M., and Cambier, J. C. (1994). "Activation of phosphatidylinositol-3' kinase by Src-family kinase SH3 binding to the p85 subunit." *Science*, 263(5153), 1609-12.

Plutzky, J., Neel, B. G., and Rosenberg, R. D. (1992). "Isolation of a src homology 2-containing tyrosine phosphatase." *Proceedings of the National Academy of Sciences of the United States of America*, 89(3), 1123-7.

Porfiri, E., and McCormick, F. (1996). "Regulation of epidermal growth factor receptor signaling by phosphorylation of the ras exchange factor hSOS1." *Journal of Biological Chemistry*, 271(10), 5871-7.

Pot, D. A., and Dixon, J. E. (1992). "Active site labeling of a receptor-like protein tyrosine phosphatase." *Journal of Biological Chemistry*, 267(1), 140-3.

Pot, D. A., Woodford, T. A., Remboutsika, E., Haun, R. S., and Dixon, J. E. (1991). "Cloning, bacterial expression, purification, and characterization of the cytoplasmic domain of rat LAR, a receptor-like protein tyrosine phosphatase." *Journal of Biological Chemistry*, 266(29), 19688-96.

Potts, J. R., and Campbell, I. D. (1994). "Fibronectin structure and assembly." *Current Opinion in Cell Biology*, 6(5), 648-55.

Prigent, S. A., Pillay, T. S., Ravichandran, K. S., and Gullick, W. J. (1995). "Binding of Shc to the NPXY motif is mediated by its N-terminal domain." *Journal of Biological Chemistry*, 270(38), 22097-100.

Pronk, G. J., McGlade, J., Pelicci, G., Pawson, T., and Bos, J. L. (1993). "Insulin-induced phosphorylation of the 46- and 52-kDa Shc proteins." *Journal of Biological Chemistry*, 268(8), 5748-53.

Puil, L., Liu, J., Gish, G., Mbamalu, G., Bowtell, D., Pelicci, P. G., Arlinghaus, R., and Pawson, T. (1994). "Bcr-Abl oncoproteins bind directly to activators of the Ras signalling pathway." *EMBO Journal*, 13(4), 764-73.

Rao, Y., Wu, X. F., Gariepy, J., Rutishauser, U., and Siu, C. H. (1992). "Identification of a peptide sequence involved in homophilic binding in the neural cell adhesion molecule NCAM." *Journal of Cell Biology*, 118(4), 937-49.

Rechsteiner, M. (1987). "Ubiquitin-mediated pathways for intracellular proteolysis." *Annual Review of Cell Biology*, 3, 1-30.

Rechsteiner, M. (1991). "Natural substrates of the ubiquitin proteolytic pathway." *Cell*, 66(4), 615-8.

Reddy, R. S., and Swarup, G. (1995). "Alternative splicing generates four different forms of a non-transmembrane protein tyrosine phosphatase mRNA." *DNA & Cell Biology*, 14(12), 1007-15.

Ren, R., Ye, Z. S., and Baltimore, D. (1994). "Abl protein-tyrosine kinase selects the Crk adapter as a substrate using SH3-binding sites." *Genes & Development*, 8(7), 783-95.

Reynolds, F. H., Jr., Todaro, G. J., Fryling, C., and Stephenson, J. R. (1981). "Human transforming growth factors induce tyrosine phosphorylation of EGF receptors." *Nature*, 292(5820), 259-62.

Roach, P. J. (1991). "Multisite and hierarchal protein phosphorylation." *Journal of Biological Chemistry*, 266(22), 14139-42.

Roberts, D. L., Bennett, D. W., and Forst, S. A. (1994). "Identification of

the site of phosphorylation on the osmosensor, EnvZ, of Escherichia coli." *Journal of Biological Chemistry*, 269(12), 8728-33.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). "Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis." *Science*, 234(4774), 364-8.

Rohan, P. J., Davis, P., Moskaluk, C. A., Kearns, M., Krutzsch, H., Siebenlist, U., and Kelly, K. (1993a). "PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase." *Science*, 259(5102), 1763-6.

Rohan, P. J., Davis, P., Moskaluk, C. A., Kearns, M., Krutzsch, H., Siebenlist, U., and Kelly, K. (1993b). "PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase." *Science*, 259(5102), 1763-6.

Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., and Bowtell, D. (1993). "The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1 [see comments]." *Nature*, 363(6424), 83-5.

Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G., and et al. (1992). "Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases." *Nature*, 360(6405), 689-92.

Ruff-Jamison, S., McGlade, J., Pawson, T., Chen, K., and Cohen, S. (1993). "Epidermal growth factor stimulates the tyrosine phosphorylation of SHC in the mouse." *Journal of Biological Chemistry*, 268(11), 7610-2.

Sahin, M., Dowling, J. J., and Hockfield, S. (1995). "Seven protein tyrosine phosphatases are differentially expressed in the developing rat brain." *Journal of Comparative Neurology*, 351(4), 617-31.

Sahin, M., and Hockfield, S. (1993). "Protein tyrosine phosphatases expressed in the developing rat brain." *Journal of Neuroscience*, 13(11), 4968-78.

Salama, S. R., Hendricks, K. B., and Thorner, J. (1994). "G1 cyclin degradation: the PEST motif of yeast Cln2 is necessary, but not sufficient, for rapid protein turnover." *Molecular & Cellular Biology*, 14(12), 7953-66.

Salcini, A. E., McGlade, J., Pelicci, G., Nicoletti, I., Pawson, T., and Pelicci, P. G. (1994). "Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins." *Oncogene*, 9(10), 2827-36.

Sap, J., D'Eustachio, P., Givol, D., and Schlessinger, J. (1990). "Cloning and expression of a widely expressed receptor tyrosine phosphatase." *Proceedings of the National Academy of Sciences of the United States of America*, 87(16), 6112-6.

Sastry, S. K., and Horwitz, A. F. (1993). "Integrin cytoplasmic domains: mediators of cytoskeletal linkages and extra- and intracellular initiated transmembrane signaling." *Current Opinion in Cell Biology*, 5(5), 819-31.

Sawada, M., Ogata, M., Fujino, Y., and Hamaoka, T. (1994). "cDNA cloning of a novel protein tyrosine phosphatase with homology to cytoskeletal protein 4.1 and its expression in T-lineage cells." *Biochemical & Biophysical Research Communications*, 203(1), 479-84.

Schievella, A. R., Paige, L. A., Johnson, K. A., Hill, D. E., and Erikson, R. L. (1993). "Protein tyrosine phosphatase 1B undergoes mitosis-specific phosphorylation on serine." *Cell Growth & Differentiation*, 4(4), 239-46.

Schlessinger, J. (1993). "How receptor tyrosine kinases activate Ras." *Trends in Biochemical Sciences*, 18(8), 273-5.

Sefton, B. M., and Hunter, T. (1984). "Tyrosine protein kinases." *Advances in Cyclic Nucleotide & Protein Phosphorylation Research*, 18, 195-226.

Sefton, B. M., Hunter, T., Ball, E. H., and Singer, S. J. (1981). "Vinculin: a cytoskeletal target of the transforming protein of Rous sarcoma virus." *Cell*, 24(1), 165-74.

Sefton, B. M., Hunter, T., Beemon, K., and Eckhart, W. (1980). "Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus." *Cell*, 20(3), 807-16.

Segatto, O., Pelicci, G., Giuli, S., Digiesi, G., Di Fiore, P. P., McGlade, J., Pawson, T., and Pelicci, P. G. (1993). "Shc products are substrates of erbB-2 kinase." *Oncogene*, 8(8), 2105-12.

Seimiya, H., Sawabe, T., Inazawa, J., and Tsuruo, T. (1995). "Cloning,

expression and chromosomal localization of a novel gene for protein tyrosine phosphatase (PTP-U2) induced by various differentiation-inducing agents." *Oncogene,* 10(9), 1731-8.

Shen, S. H., Bastien, L., Posner, B. I., and Chretien, P. (1991). "A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases [published erratum appears in Nature 1991 Oct 31;353(6347):868]." *Nature,* 352(6337), 736-9.

Shultz, L. D. (1988). "Pleiotropic effects of deleterious alleles at the "motheaten" locus." *Current Topics in Microbiology & Immunology,* 137, 216-22.

Shultz, L. D. (1991). "Hematopoiesis and models of immunodeficiency." *Seminars in Immunology,* 3(6), 397-408.

Shultz, L. D., Coman, D. R., Bailey, C. L., Beamer, W. G., and Sidman, C. L. (1984). ""Viable motheaten," a new allele at the motheaten locus. I. Pathology." *American Journal of Pathology,* 116(2), 179-92.

Shultz, L. D., and Green, M. C. (1976). "Motheaten, an immunodeficient mutant of the mouse. II. Depressed immune competence and elevated serum immunoglobulins." *Journal of Immunology,* 116(4), 936-43.

Shultz, L. D., Schweitzer, P. A., Rajan, T. V., Yi, T., Ihle, J. N., Matthews, R. J., Thomas, M. L., and Beier, D. R. (1993). "Mutations at the murine motheaten locus are within the hematopoietic cell protein-tyrosine phosphatase (Hcph) gene." *Cell,* 73(7), 1445-54.

Sidman, C. L., Shultz, L. D., and Unanue, E. R. (1978). "The mouse mutant "motheaten." II. Functional studies of the immune system." *Journal of Immunology,* 121(6), 2399-404.

Skolnik, E. Y., Batzer, A., Li, N., Lee, C. H., Lowenstein, E., Mohammadi, M., Margolis, B., and Schlessinger, J. (1993). "The function of GRB2 in linking the insulin receptor to Ras signaling pathways." *Science,* 260(5116), 1953-5.

Smith, D. L., Bruegger, B. B., Halpern, R. M., and Smith, R. A. (1973). "New histone kinases in nuclei of rat tissues." *Nature,* 246(5428), 103-4.

Smith, D. L., Chen, C. C., Bruegger, B. B., Holtz, S. L., Halpern, R. M., and Smith, R. A. (1974). "Characterization of protein kinases forming acid-labile histone phosphates in Walker-256 carcinosarcoma cell

nuclei." *Biochemistry*, 13(18), 3780-5.

Smith, M. R., DeGudicibus, S. J., and Stacey, D. W. (1986). "Requirement for c-ras proteins during viral oncogene transformation." *Nature*, 320(6062), 540-3.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., and et al. (1993). "SH2 domains recognize specific phosphopeptide sequences." *Cell*, 72(5), 767-78.

Sparks, A. B., Quilliam, L. A., Thorn, J. M., Der, C. J., and Kay, B. K. (1994). "Identification and characterization of Src SH3 ligands from phage-displayed random peptide libraries." *Journal of Biological Chemistry*, 269(39), 23853-6.

Stock, J. B., Stock, A. M., and Mottonen, J. M. (1990). "Signal transduction in bacteria." *Nature*, 344(6265), 395-400.

Stoker, A. W. (1994). "Isoforms of a novel cell adhesion molecule-like protein tyrosine phosphatase are implicated in neural development." *Mechanisms of Development*, 46(3), 201-17.

Stone, R. L., and Dixon, J. E. (1994). "Protein-tyrosine phosphatases." *Journal of Biological Chemistry*, 269(50), 31323-6.

Strausfeld, U., Fernandez, A., Capony, J. P., Girard, F., Lautredou, N., Derancourt, J., Labbe, J. C., and Lamb, N. J. (1994). "Activation of p34cdc2 protein kinase by microinjection of human cdc25C into mammalian cells. Requirement for prior phosphorylation of cdc25C by p34cdc2 on sites phosphorylated at mitosis." *Journal of Biological Chemistry*, 269(8), 5989-6000.

Streuli, M., Krueger, N. X., Tsai, A. Y., and Saito, H. (1989). "A family of receptor-linked protein tyrosine phosphatases in humans and Drosophila." *Proceedings of the National Academy of Sciences of the United States of America*, 86(22), 8698-702.

Stuckey, J. A., Schubert, H. L., Fauman, E. B., Zhang, Z. Y., Dixon, J. E., and Saper, M. A. (1994). "Crystal structure of Yersinia protein tyrosine phosphatase at 2.5 A and the complex with tungstate [see comments]." *Nature*, 370(6490), 571-5.

Su, J., Batzer, A., and Sap, J. (1994). "Receptor tyrosine phosphatase R-

PTP-alpha is tyrosine-phosphorylated and associated with the adaptor protein Grb2." *Journal of Biological Chemistry*, 269(29), 18731-4.

Sugimoto, S., Lechleider, R. J., Shoelson, S. E., Neel, B. G., and Walsh, C. T. (1993). "Expression, purification, and characterization of SH2-containing protein tyrosine phosphatase, SH-PTP2." *Journal of Biological Chemistry*, 268(30), 22771-6.

Sun, H., Charles, C. H., Lau, L. F., and Tonks, N. K. (1993a). "MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo." *Cell*, 75(3), 487-93.

Sun, X. J., Crimmins, D. L., Myers, M. G., Jr., Miralpeix, M., and White, M. F. (1993b). "Pleiotropic insulin signals are engaged by multisite phosphorylation of IRS-1." *Molecular & Cellular Biology*, 13(12), 7418-28.

Sun, X. J., Wang, L. M., Zhang, Y., Yenush, L., Myers, M. G., Jr., Glasheen, E., Lane, W. S., Pierce, J. H., and White, M. F. (1995). "Role of IRS-2 in insulin and cytokine signalling." *Nature*, 377(6545), 173-7.

Swarup, G., Dasgupta, J. D., and Garbers, D. L. (1984). "Tyrosine-specific protein kinases of normal tissues." *Advances in Enzyme Regulation*, 22, 267-88.

Taborsky, G. (1974). "Phosphoproteins." *Advances in Protein Chemistry*, 28, 1-210.

Takahashi, T., and Shirasawa, T. (1994). "Molecular cloning of rat JAK3, a novel member of the JAK family of protein tyrosine kinases." *FEBS Letters*, 342(2), 124-8.

Takekawa, M., Itoh, F., Hinoda, Y., Adachi, M., Ariyama, T., Inazawa, J., Imai, K., and Yachi, A. (1994). "Chromosomal localization of the protein tyrosine phosphatase G1 gene and characterization of the aberrant transcripts in human colon cancer cells." *FEBS Letters*, 339(3), 222-8.

Takekawa, M., Itoh, F., Hinoda, Y., Arimura, Y., Toyota, M., Sekiya, M., Adachi, M., Imai, K., and Yachi, A. (1992). "Cloning and characterization of a human cDNA encoding a novel putative cytoplasmic protein-tyrosine-phosphatase." *Biochemical & Biophysical Research Communications*, 189(2), 1223-30.

Tan, X., Stover, D. R., and Walsh, K. A. (1993). "Demonstration of protein tyrosine phosphatase activity in the second of two homologous domains of CD45." *Journal of Biological Chemistry*, 268(10), 6835-8.

Tang, T. L., Freeman, R. M., Jr., O'Reilly, A. M., Neel, B. G., and Sokol, S. Y. (1995). "The SH2-containing protein-tyrosine phosphatase SH-PTP2 is required upstream of MAP kinase for early Xenopus development." *Cell*, 80(3), 473-83.

Thomas, M. L. (1989). "The leukocyte common antigen family." *Annual Review of Immunology*, 7, 339-69.

Thomas, P. E., Wharram, B. L., Goyal, M., Wiggins, J. E., Holzman, L. B., and Wiggins, R. C. (1994). "GLEPP1, a renal glomerular epithelial cell (podocyte) membrane protein-tyrosine phosphatase. Identification, molecular cloning, and characterization in rabbit." *Journal of Biological Chemistry*, 269(31), 19953-62.

Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S., and Brugge, J. S. (1992). "Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases." *Cell*, 68(6), 1031-40.

Tian, S. S., Tsoulfas, P., and Zinn, K. (1991). "Three receptor-linked protein-tyrosine phosphatases are selectively expressed on central nervous system axons in the Drosophila embryo." *Cell*, 67(4), 675-80.

Tillmann, U., Wagner, J., Boerboom, D., Westphal, H., and Tremblay, M. L. (1994). "Nuclear localization and cell cycle regulation of a murine protein tyrosine phosphatase." *Molecular & Cellular Biology*, 14(5), 3030-40.

Tonks, N. K., Charbonneau, H., Diltz, C. D., Fischer, E. H., and Walsh, K. A. (1988a). "Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase." *Biochemistry*, 27(24), 8695-701.

Tonks, N. K., Diltz, C. D., and Fischer, E. H. (1988b). "Characterization of the major protein-tyrosine-phosphatases of human placenta." *Journal of Biological Chemistry*, 263(14), 6731-7.

Tonks, N. K., Diltz, C. D., and Fischer, E. H. (1990). "CD45, an integral membrane protein tyrosine phosphatase. Characterization of enzyme activity." *Journal of Biological Chemistry*, 265(18), 10674-80.

Tracy, S., van der Geer, P., and Hunter, T. (1995). "The receptor-like

protein-tyrosine phosphatase, RPTP alpha, is phosphorylated by protein kinase C on two serines close to the inner face of the plasma membrane." *Journal of Biological Chemistry*, 270(18), 10587-94.

Trowbridge, I. S. (1991). "CD45. A prototype for transmembrane protein tyrosine phosphatases." *Journal of Biological Chemistry*, 266(35), 23517-20.

Trowbridge, I. S., Ostergaard, H. L., and Johnson, P. (1991). "CD45: a leukocyte-specific member of the protein tyrosine phosphatase family." *Biochimica et Biophysica Acta*, 1095(1), 46-56.

Trowbridge, I. S., and Thomas, M. L. (1994). "CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development." *Annual Review of Immunology*, 12, 85-116.

Trub, T., Choi, W. E., Wolf, G., Ottinger, E., Chen, Y., Weiss, M., and Shoelson, S. E. (1995). "Specificity of the PTB domain of Shc for beta turn-forming pentapeptide motifs amino-terminal to phosphotyrosine." *Journal of Biological Chemistry*, 270(31), 18205-8.

Tsui, H. W., Siminovitch, K. A., de Souza, L., and Tsui, F. W. (1993). "Motheaten and viable motheaten mice have mutations in the haematopoietic cell phosphatase gene." *Nature Genetics*, 4(2), 124-9.

Ushiro, H., and Cohen, S. (1980). "Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes." *Journal of Biological Chemistry*, 255(18), 8363-5.

Valentine, M. A., Widmer, M. B., Ledbetter, J. A., Pinault, F., Voice, R., Clark, E. A., Gallis, B., and Brautigan, D. L. (1991). "Interleukin 2 stimulates serine phosphorylation of CD45 in CTLL-2.4 cells." *European Journal of Immunology*, 21(4), 913-9.

van Biesen, T., Hawes, B. E., Luttrell, D. K., Krueger, K. M., Touhara, K., Porfiri, E., Sakaue, M., Luttrell, L. M., and Lefkowitz, R. J. (1995). "Receptor-tyrosine-kinase- and G beta gamma-mediated MAP kinase activation by a common signalling pathway [see comments]." *Nature*, 376(6543), 781-4.

van der Geer, P., and Pawson, T. (1995). "The PTB domain: a new protein module implicated in signal transduction." *Trends in Biochemical Sciences*, 20(7), 277-80.

van der Geer, P., Wiley, S., Lai, V. K., Olivier, J. P., Gish, G. D., Stephens, R., Kaplan, D., Shoelson, S., and Pawson, T. (1995). "A conserved amino-terminal Shc domain binds to phosphotyrosine motifs in activated receptors and phosphopeptides." *Current Biology*, 5(4), 404-12.

Van Etten, R. L. (1982). "Human prostatic acid phosphatase: a histidine phosphatase." *Annals of the New York Academy of Sciences*, 390, 27-51.

Vogel, W., Lammers, R., Huang, J., and Ullrich, A. (1993). "Activation of a phosphotyrosine phosphatase by tyrosine phosphorylation." *Science*, 259(5101), 1611-4.

Wagner, J., Boerboom, D., and Tremblay, M. L. (1994). "Molecular cloning and tissue-specific RNA processing of a murine receptor-type protein tyrosine phosphatase." *European Journal of Biochemistry*, 226(3), 773-82.

Wakim, B. T., Picken, M. M., and DeLange, R. J. (1990). "Identification and partial purification of a chromatin bound calmodulin activated histone 3 kinase from calf thymus." *Biochemical & Biophysical Research Communications*, 171(1), 84-90.

Walinder, O. (1968). "Identification of a phosphate-incorporating protein from bovine liver as nucleoside diphosphate kinase and isolation of 1-32P-phosphohistidine, 3-32P-phosphohistidine, and N-epsilon-32P-phospholysine from erythrocytic nucleoside diphosphate kinase, incubated with adenosine triphosphate-32P." *Journal of Biological Chemistry*, 243(14), 3947-52.

Walker, M. S., and DeMoss, J. A. (1993). "Phosphorylation and dephosphorylation catalyzed in vitro by purified components of the nitrate sensing system, NarX and NarL." *Journal of Biological Chemistry*, 268(12), 8391-3.

Walton, K. M., and Dixon, J. E. (1993). "Protein tyrosine phosphatases." *Annual Review of Biochemistry*, 62, 101-20.

Wang, Y., and Pallen, C. J. (1991). "The receptor-like protein tyrosine phosphatase HPTP alpha has two active catalytic domains with distinct substrate specificities." *EMBO Journal*, 10(11), 3231-7.

Waters, S. B., Holt, K. H., Ross, S. E., Syu, L. J., Guan, K. L., Saltiel, A. R., Koretzky, G. A., and Pessin, J. E. (1995b). "Desensitization of Ras activation by a feedback disassociation of the SOS-Grb2 complex."

Journal of Biological Chemistry, 270(36), 20883-6.

Waters, S. B., Yamauchi, K., and Pessin, J. E. (1995a). "Insulin-stimulated disassociation of the SOS-Grb2 complex." *Molecular & Cellular Biology*, 15(5), 2791-9.

Weaver, C. T., Pingel, J. T., Nelson, J. O., and Thomas, M. L. (1991). "CD8+ T-cell clones deficient in the expression of the CD45 protein tyrosine phosphatase have impaired responses to T-cell receptor stimuli." *Molecular & Cellular Biology*, 11(9), 4415-22.

Wei, Y. F., and Matthews, H. R. (1990). "A filter-based protein kinase assay selective for alkali-stable protein phosphorylation and suitable for acid-labile protein phosphorylation." *Analytical Biochemistry*, 190(2), 188-92.

Weiss, A., and Littman, D. R. (1994). "Signal transduction by lymphocyte antigen receptors." *Cell*, 76(2), 263-74.

White, M. F., and Kahn, C. R. (1994). "The insulin signaling system." *Journal of Biological Chemistry*, 269(1), 1-4.

White, M. F., Livingston, J. N., Backer, J. M., Lauris, V., Dull, T. J., Ullrich, A., and Kahn, C. R. (1988). "Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity." *Cell*, 54(5), 641-9.

Wieland, T., Nurnberg, B., Ulibarri, I., Kaldenberg-Stasch, S., Schultz, G., and Jakobs, K. H. (1993). "Guanine nucleotide-specific phosphate transfer by guanine nucleotide-binding regulatory protein beta-subunits. Characterization of the phosphorylated amino acid." *Journal of Biological Chemistry*, 268(24), 18111-8.

Wilks, A. F., Harpur, A. G., Kurban, R. R., Ralph, S. J., Zurcher, G., and Ziemiecki, A. (1991). "Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase." *Molecular & Cellular Biology*, 11(4), 2057-65.

Williamson, M. P. (1994). "The structure and function of proline-rich regions in proteins." *Biochemical Journal*, 297(Pt 2), 249-60.

Witt, D. P., and Gordon, J. A. (1980). "Specific dephosphorylation of membrane proteins in Rous sarcoma virus-transformed chick embryo fibroblasts." *Nature*, 287(5779), 241-4.

Witte, O. N., Goff, S., Rosenberg, N., and Baltimore, D. (1980). "A transformation-defective mutant of Abelson murine leukemia virus lacks protein kinase activity." *Proceedings of the National Academy of Sciences of the United States of America*, 77(8), 4993-7.

Wittekind, M., Mapelli, C., Farmer, B. T. n., Suen, K. L., Goldfarb, V., Tsao, J., Lavoie, T., Barbacid, M., Meyers, C. A., and Mueller, L. (1994). "Orientation of peptide fragments from Sos proteins bound to the N-terminal SH3 domain of Grb2 determined by NMR spectroscopy." *Biochemistry*, 33(46), 13531-9.

Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., and Ihle, J. N. (1993). "JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin." *Cell*, 74(2), 227-36.

Wolf, G., Trub, T., Ottinger, E., Groninga, L., Lynch, A., White, M. F., Miyazaki, M., Lee, J., and Shoelson, S. E. (1995). "PTB domains of IRS-1 and Shc have distinct but overlapping binding specificities." *Journal of Biological Chemistry*, 270(46), 27407-10.

Wong, C., Faiola, B., Wu, W., and Kennelly, P. J. (1993). "Phosphohistidine and phospholysine phosphatase activities in the rat: potential protein-lysine and protein-histidine phosphatases?" *Biochemical Journal*, 296(Pt 2), 293-6.

Wood, K. W., Sarnecki, C., Roberts, T. M., and Blenis, J. (1992). "ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK." *Cell*, 68(6), 1041-50.

Wu, X., Knudsen, B., Feller, S. M., Zheng, J., Sali, A., Cowburn, D., Hanafusa, H., and Kuriyan, J. (1995). "Structural basis for the specific interaction of lysine-containing proline-rich peptides with the N-terminal SH3 domain of c-Crk." *Structure*, 3(2), 215-26.

Xiao, S., Rose, D. W., Sasaoka, T., Maegawa, H., Burke, T. R., Jr., Roller, P. P., Shoelson, S. E., and Olefsky, J. M. (1994). "Syp (SH-PTP2) is a positive mediator of growth factor-stimulated mitogenic signal transduction." *Journal of Biological Chemistry*, 269(33), 21244-8.

Yajnik, V., Blaikie, P., Bork, P., and Margolis, B. (1996). "Identification of residues within the SHC phosphotyrosine binding/phosphotyrosine

interaction domain crucial for phosphopeptide interaction." *Journal of Biological Chemistry*, 271(4), 1813-6.

Yan, H., Grossman, A., Wang, H., D'Eustachio, P., Mossie, K., Musacchio, J. M., Silvennoinen, O., and Schlessinger, J. (1993). "A novel receptor tyrosine phosphatase-sigma that is highly expressed in the nervous system." *Journal of Biological Chemistry*, 268(33), 24880-6.

Yang, Q., Co, D., Sommercorn, J., and Tonks, N. K. (1993). "Cloning and expression of PTP-PEST. A novel, human, nontransmembrane protein tyrosine phosphatase [published erratum appears in J Biol Chem 1993 Aug 15;268(23):17650]." *Journal of Biological Chemistry*, 268(9), 6622-8.

Yang, Q., and 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." *Proceedings of the National Academy of Sciences of the United States of America*, 88(14), 5949-53.

Yang, X. H., Seow, K. T., Bahri, S. M., Oon, S. H., and Chia, W. (1991). "Two Drosophila receptor-like tyrosine phosphatase genes are expressed in a subset of developing axons and pioneer neurons in the embryonic CNS." *Cell*, 67(4), 661-73.

Yi, T., Cleveland, J. L., and Ihle, J. N. (1991). "Identification of novel protein tyrosine phosphatases of hematopoietic cells by polymerase chain reaction amplification." *Blood*, 78(9), 2222-8.

Yi, T., and Ihle, J. N. (1993). "Association of hematopoietic cell phosphatase with c-Kit after stimulation with c-Kit ligand." *Molecular & Cellular Biology*, 13(6), 3350-8.

Yi, T., Mui, A. L., Krystal, G., and Ihle, J. N. (1993). "Hematopoietic cell phosphatase associates with the interleukin-3 (IL-3) receptor beta chain and down-regulates IL-3-induced tyrosine phosphorylation and mitogenesis." *Molecular & Cellular Biology*, 13(12), 7577-86.

Yi, T. L., Cleveland, J. L., and Ihle, J. N. (1992). "Protein tyrosine phosphatase containing SH2 domains: characterization, preferential expression in hematopoietic cells, and localization to human chromosome 12p12-p13." *Molecular & Cellular Biology*, 12(2), 836-46.

Yokoyama, K., Ohmori, H., and Kumon, A. (1993). "Isolation of N omega-phosphoarginine hydrolase from rat liver and its physical

properties." *Journal of Biochemistry*, 113(2), 236-40.

Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, S. L. (1994). "Structural basis for the binding of proline-rich peptides to SH3 domains." *Cell*, 76(5), 933-45.

Zetterqvist, O. (1967a). "Further studies on acid-labile [32P]phosphate bound to high-molecular weight material from rat-liver cell sap after incubation with [32P]adenosine triphosphate." *Biochimica et Biophysica Acta*, 141(3), 533-9.

Zetterqvist, O. (1967b). "Studies on acid-labile [32P]phosphate in different chromatographic fractions of high-molecular weight material from rat-liver cell sap after incubation with [32P]adenosine triphosphate." *Biochimica et Biophysica Acta*, 141(3), 540-6.

Zhang, Z. Y., and Dixon, J. E. (1993). "Active site labeling of the Yersinia protein tyrosine phosphatase: the determination of the pKa of the active site cysteine and the function of the conserved histidine 402." *Biochemistry*, 32(36), 9340-5.

Zhang, Z. Y., and VanEtten, R. L. (1991). "Pre-steady-state and steady-state kinetic analysis of the low molecular weight phosphotyrosyl protein phosphatase from bovine heart." *Journal of Biological Chemistry*, 266(3), 1516-25.

Zheng, X. M., Wang, Y., and Pallen, C. J. (1992). "Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase." *Nature*, 359(6393), 336-9.

Zhou, M. M., Ravichandran, K. S., Olejniczak, E. F., Petros, A. M., Meadows, R. P., Sattler, M., Harlan, J. E., Wade, W. S., Burakoff, S. J., and Fesik, S. W. (1995a). "Structure and ligand recognition of the phosphotyrosine binding domain of Shc." *Nature*, 378(6557), 584-92.

Zhou, S., Margolis, B., Chaudhuri, M., Shoelson, S. E., and Cantley, L. C. (1995b). "The phosphotyrosine interaction domain of SHC recognizes tyrosine-phosphorylated NPXY motif." *Journal of Biological Chemistry*, 270(25), 14863-6.

Zhuo, S., Clemens, J. C., Stone, R. L., and Dixon, J. E. (1994). "Mutational analysis of a Ser/Thr phosphatase. Identification of residues important in phosphoesterase substrate binding and catalysis." *Journal of Biological Chemistry*, 269(42), 26234-8.

Ziemiecki, A., and Friis, R. R. (1980). "Phosphorylation of pp60src and the cycloheximide insensitive activation of the pp60src-associated kinase activity of transformation-defective temperature-sensitive mutants of Rous sarcoma virus." *Virology*, 106(2), 391-4.

Zinn, K. (1993). "Drosophila protein tyrosine phosphatases." *Seminars in Cell Biology*, 4(6), 397-401.

Zondag, G. C., Koningstein, G. M., Jiang, Y. P., Sap, J., Moolenaar, W. H., and Gebbink, M. F. (1995). "Homophilic interactions mediated by receptor tyrosine phosphatases mu and kappa. A critical role for the novel extracellular MAM domain [published erratum appears in J Biol Chem 1995 Oct 13;270(41):24621]." *Journal of Biological Chemistry*, 270(24), 14247-50.

IMAGE EVALUATION
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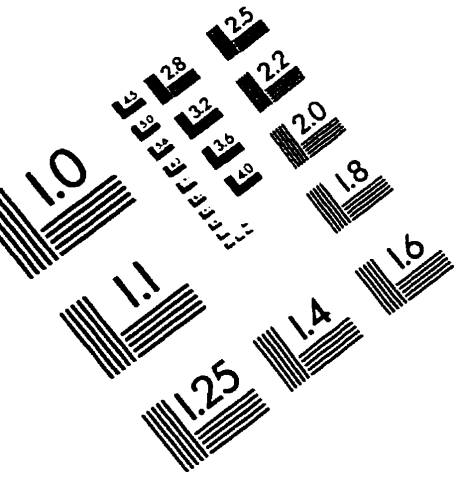

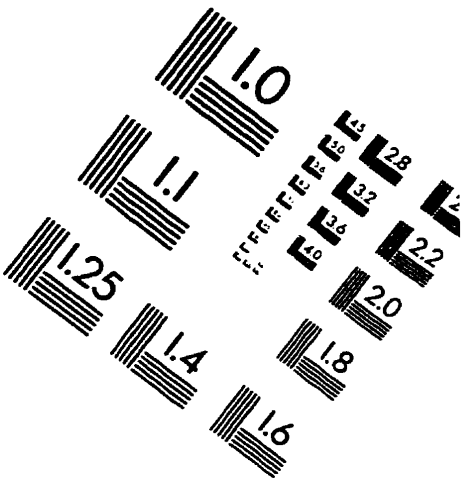

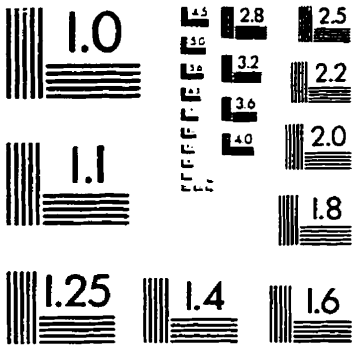

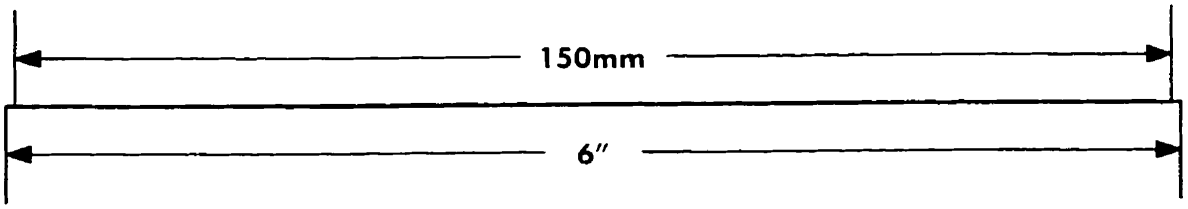

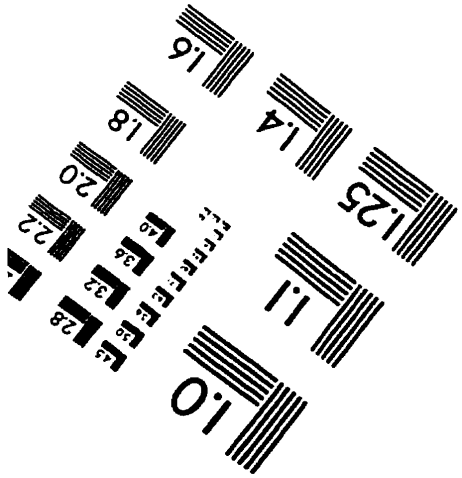

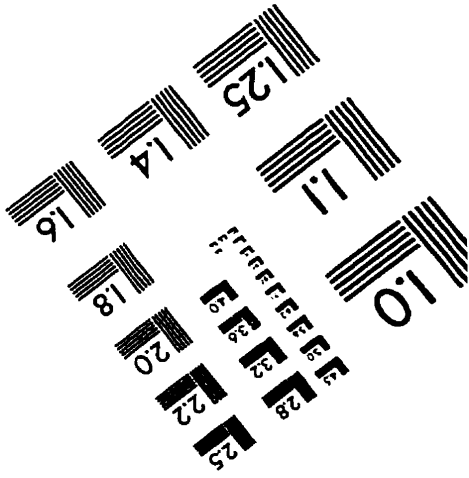

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