AN ANALYSIS OF THE STRUCTURE AND FUNCTION OF PROTEIN TYROSINE PHOSPHATASE α

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ABSTRACT

The molecular basis for the control of cell growth, proliferation, and differentiation involves the coordinated activities of a diverse collection of molecules. Many, if not all, developmental processes are influenced by growth factors, extracellular matrix components, and/or adhesion molecules, which elicit changes in cytoskeletal organization, metabolism, and gene transcription. Signal transduction often involves the activation of tyrosine kinase activity associated with specific receptors. Receptor, or receptor-linked tyrosine kinases, then orchestrate the assembly of multimeric signaling complexes and regulate the activities of various downstream enzymes. Thus, tyrosine kinases form a critical link between the extracellular environment and the signal transduction machinery of the cytoplasm. The protein tyrosine phosphatases (PTPs), by dephosphorylating phosphotyrosine residues, play a critical, albeit less understood role in the regulation of tyrosine phosphorylation. A search for additional members of the PTP family, which now includes over 40 enzymes, led to the identification of PTPα, a widely-expressed receptor-like PTP. In studies aimed at characterizing this PTP, we expressed PTPα in both eukaryotic and bacterial cells. To assess the potential substrate specificity of these enzymes, select bacterially expressed PTPs were assayed with a group of synthetic phosphopeptide substrates using a modified colorimetric assay. These studies suggested that the substrate specificity of PTPs such as PTPβ may be influenced by the context of amino acids surrounding a given phosphotyrosine residue. Studies of the *in vivo* activity of PTPα, investigated by its overexpression in human epidermoid cells, revealed a role for PTPα in the activation and/or dephosphorylation of specific Src family kinases. Moreover, PTPα overexpression dramatically increased the cell-substratum adhesion of these cells and altered the tyrosine phosphorylation and associations of specific focal adhesion molecules, suggesting a role for this enzyme in cell-adhesion. Furthermore, an SH3 domain-binding motif was identified in the membrane-proximal

region of PTPα, which like similar proline-rich regions in various retroviral oncoproteins and cytokine receptors, was able to bind specific SH3 domains *in vitro*.

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ABBREVIATIONS

AMLV	Abelson murine leukemia virus
ASAP	actin-filament-associated protein
ATP	adenosine triphosphate
BPV	bovine papilloma virus
CD	cluster of differentiation
CEF	chicken embryo fibroblasts
CMV	cytomegalovirus
CSF-1-R	colony-stimulating factor receptor
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EGF-R	epidermal growth factor receptor
FACS	fluorescence activated cell sorter
FAK	focal adhesion kinase
FAT	focal adhesion targeting
FSV	feline sarcoma virus
GAP	GTPase activating protein
Grb2	growth factor receptor-bound protein 2
GST	Glutathione-S-Transferase
HA	hemagglutinin
HEK	human embryonal kidney
ICAM-1	intracellular adhesion molecule 1
Ig	immunoglobulin
IL-2	interleukin 2
IPTG	isopropyl-1-thio-β-D-galactopyranoside

LAR	leukocyte common antigen related protein
LFA-1	leukocyte function-associated antigen 1
LFA-3	leukocyte function-associated antigen 3
mAb	monoclonal antibody
MAP kinase	mitogen activated protein kinase
MGMP	malachite green microtiter-plate assay
MHC	major histocompatibility complex
MMTV	mouse mammary tumor virus
p-NPP	*para*-nitrophenylphosphate
PAO	phenylarsine oxide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF-R	platelet-derived growth factor receptor
PH	pleckstrin homology
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PLCγ1	phospholipase C gamma 1
PMA	phorbol myristic acid
PTB	phosphotyrosine binding
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
SYP	SH2-containing tyrosine phosphatase
TCR	T cell receptor

TGFα	transforming growth factor alpha
TNF-α	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen 4

PREFACE

Thesis Format

This thesis includes seven chapters. Chapters 2 through 4 focus on results contained within manuscripts either published (chapter 2) or in preparation (chapters 3 and 4). Each of these chapters is formatted as an expanded manuscript and contains an introductory paragraph and a discussion of the chapter's results. The first chapter is a general introduction to signal transduction involving protein tyrosine kinases and phosphatases and includes a general discussion of growth factor signaling, cell adhesion, and the function and regulation of Src family kinases. Chapter 5 contains a general summary of results presented in chapters 2 through 4. Chapter 6 summarizes the materials and methods used in each of preceding chapters. Finally, chapter 7 contains the references.

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CHAPTER 1

Introduction

1.1. General Overview and Thesis Objectives.

The growth, differentiation, and death of eukaryotic cells is governed by a highly complex number of interactions between molecules (Cohen et al., 1995). The identification of the participants in these interactions, and the elucidation of their functions, has led to many fundamental insights into the molecular mechanisms critical to the pathogenesis of various diseases. Importantly, the discovery of retrovirally-encoded oncogenes (*v-oncs*) suggested the involvement of genetic damage and altered gene expression in tumorigenesis (Bishop, 1991; Cantley et al., 1991). The identification of *v-src* , the transforming agent of Rous sarcoma virus (Rous, 1911), and its relationship to *c-src*, was instrumental in establishing this correlation (Bishop, 1987). Subsequent studies led to the identification of other oncogenes, including those encoding growth factors (e.g. *v-sis)*, growth factor receptors (e.g. *v-erbB* and *v-fms)*, small GTP-binding proteins (e.g. *v-ras*), cytoplasmic serine/threonine and tyrosine kinases (e.g. *v-raf*, *v-src*, *v-yes*, and *v-fgr)* and a variety of transcription factors (e.g. *v-myc*, *v-fos* and *v-jun*) (Cantley et al., 1991). Consistent with their role in oncogenesis, the cellular counterparts of these molecules are involved in normal cell growth, proliferation, and/or differentiation. Thus, the study of retroviral oncogenes heralded an era of inquiry into the molecules involved in the control of cell proliferation and differentiation. The investigation of DNA tumor

viruses and the role of DNA damage in carcinogenesis led to the concept of anti-oncogenes (or tumor suppressor genes), genes in which recessive, loss of function mutations, predispose cells to transformation (Bishop, 1987). Together such studies demonstrated the involvement of both proto- and anti-oncogenes in the signal transduction pathways that link signals from the extracellular environment to specific cellular responses in the cytoplasm and nucleus.

Receptors for growth factors, cytokines, cell surface associated-ligands, and extracellular matrix components provide a critical link between the extracellular environment and the cytoplasm. Receptor-stimulated signal transduction often results in either the activation of the intrinsic protein tyrosine kinase (PTK) activity of the receptor itself, or activation of receptor-associated PTKs (Ullrich and Schlessinger, 1990; Cantley et al., 1991). Studies aimed at uncovering the mechanisms of signal transduction revealed that tyrosine phosphorylation leads to the assembly of signaling complexes through the association of specific protein modules (Pawson, 1995; Cohen et al., 1995).

This thesis focuses on the identification and analysis of a member of the protein tyrosine phosphatase (PTP) family of enzymes. The PTPs comprise a growing family of proteins, demonstrating a complexity of structure comparable to that of the PTK family (Charbonneau and Tonks, 1992; Hunter, 1989; Pot and Dixon, 1992). Both groups of proteins are important elements within eukaryotic cell signal transduction pathways (Cantley et al., 1991; Ullrich and Schlessinger, 1990). The diversity of structure and the differential patterns of tissue specific expression suggest the possibility of multiple interactions taking place between these two groups of proteins during cell proliferation, differentiation, and metabolism. In reversing tyrosine phosphorylation, PTPs potentially regulate such important processes as the ligand-induced phosphorylation of PTK growth factor receptors and their substrates (Ullrich and Schlessinger, 1990), the phosphotyrosine-mediated binding of Src homology 2 (SH2) domain-containing proteins to tyrosine-phosphorylated substrates (Koch et al., 1991), and the activation state of Src

family PTKs (Hunter, 1987; Cantley et al., 1991). The amino acid sequence of PTP1B, the first member of this enzyme class to be identified, was determined by Charbonneau et al. in 1989. Following publication of the amino acid sequence of PTP1B and the demonstration of its relationship to CD45 (Charbonneau et al., 1988; Tonks et al., 1988), we set out to identify additional PTP family members by 'low-stringency' cDNA library-screening. This method resulted in the isolation and sequencing of a cDNA encoding LRP (leukocyte common antigen-related protein phosphatase) (Jirik et al., 1990), a receptor-like PTP subsequently renamed PTPα.

Although I contributed to the molecular cloning and DNA sequencing of this enzyme, this thesis focuses on an analysis of PTPα both in terms of the development of methods to express and characterize enzyme activity, and in the determination of the role of PTPα in cell physiology. These studies required the use of bacterial expression systems to obtain sufficient quantities of protein to facilitate analysis of enzyme activity and necessitated the development of assays to measure PTP activity. Experiments with recombinant enzymes were initiated to address questions concerning the substrate specificity of PTPs and to study PTPα structure-function.

To investigate PTPα function, both normal and mutant forms of PTPα were overexpressed in mammalian cells. These studies, in conjunction with an analysis of recombinant enzyme substrate specificity *in vitro*, held the promise of identifying the physiological substrates of PTPα. Interestingly, we found that PTPα dephosphorylated and activated specific Src family kinases. The activation of these kinases resulted in an increase in their association with and phosphorylation of specific Src kinase substrates. Furthermore, cells overexpressing PTPα displayed an increase in cell-substratum adhesion, suggesting a role for PTPα and Src kinases in the regulation of cell adhesion.

Finally, studies aimed at elucidating the nature of the interaction between PTPα and Src family kinases led to the identification of a proline-rich Src homology 3 (SH3) domain-binding site in the juxtamembrane region of PTPα. Characterization of the

binding specificity of this sequence, and the identification of similar sequences in various retroviral oncogenes and cytokine receptors, suggested a function for these sequences in the recruitment of substrates and/or the assembly of signal transduction complexes mediated by SH3 domain associations.

1.2. General Signal Transduction.

The conversion of information arriving at the cell-surface into specific messages in the cytoplasm involves a diverse array of membrane-linked receptors. Cell-surface receptors specific for myriad growth factors, cytokines and adhesion molecules, have evolved to regulate the growth, proliferation, differentiation, and death of cells during the complex course of development (Bishop, 1991; Ullrich and Schlessinger, 1990; Cantley et al., 1991). In the immune system, T and B cells generate receptors able to recognize the vast number of potential antigens encountered during host immune responses to potential pathogens (Gold and Matsuuchi, 1995).

The signal transduction pathways emanating from these receptors frequently involves a common group of molecules, the PTKs. The evolution of PTKs is thought to have coincided with the development of multicellularity in that all of the identified PTKs, excluding PTKs identified in *Dictyostelium,* are derived from metazoans (Hunter, 1987). Thus, it is likely that this class of enzyme evolved to mediate communication between cells of multicellular organisms. This concept is supported by the absence of PTKs in yeast and the apparent dichotomy between the membrane localization of PTKs and the largely cytoplasmic protein serine/threonine kinase family.

The level of tyrosine phosphorylation of specific proteins represents the balanced activities of both PTKs and PTPs. The existence of PTPs was inferred over 10 years ago by the observed slow time-dependent release of phosphate from epidermal growth factor (EGF) stimulated A431 cell-membrane preparations (Ushiro et al., 1980). Additionally, the finding that inhibitors of PTP activity were able to induce cell transformation pointed to a crucial role for PTPs in the regulation of cell proliferation (Karlund et al., 1985).

As this thesis focuses on the characterization of a member of the PTP family, this discussion will emphasize signal transduction pathways involving tyrosine kinases, with the realization of the necessity for PTPs in the regulation of PTK signal transduction.

This will include a brief discussion of growth factor, antigen, and cytokine receptor signaling, as these signal transduction pathways pertain to potential PTPα/EGF receptor (EGF-R) interactions discussed in chapter 3. Moreover, both antigen and cytokine receptor signaling are of relevance to models of Src kinase regulation and putative SH3 domain/cytokine receptor associations suggested in chapter 4. Additionally, due to the effects of PTPα expression on Src kinase activity and cell-substratum adhesion, a brief overview of Src family kinases and integrin-dependent cell adhesion will follow.

1.3. Growth Factor, Cytokine, and Antigen Receptor Signal Transduction.

*1.3.1. **Receptor tyrosine kinases.*** The tyrosine kinase family includes at least two broad structural classes, the receptor and cytoplasmic PTKs. Receptor tyrosine kinases possess an extracellular region which binds polypeptide ligands, a hydrophobic membrane-spanning segment, and a cytoplasmic kinase domain, which phosphorylates specific targets and is itself a target for 'autophosphorylation' (Ullrich and Schlessinger, 1990; Cooper, 1990). Cytoplasmic PTKs lack transmembrane domains, but may instead be tethered to cellular membranes via lipid anchors such as myristic acid or associated with other proteins via specific protein interaction motifs (Pawson, 1995; Cantley et al., 1991). Binding of polypeptide ligands to receptor tyrosine kinases induces receptor dimerization or oligomerization and subsequent kinase activation (Ullrich and Schlessinger, 1990). In the case of the EGF-R, dimerization results from monomeric ligand-induced conformational changes. Other receptors, such as those for platelet-derived growth factor (PDGF), colony-stimulating factor-1 (CSF-1), or steel factor (SF), dimerize presumably as a consequence of the bridging of two or more receptors by bivalent ligands (Ullrich and Schlessinger, 1990). Growth factor-induced activation results in the tyrosine phosphorylation of specific sites within the kinase cytoplasmic domains as well as

phosphorylation of exogenous substrates. Phosphorylation sites within the receptors are found predominately in non-catalytic regions of the molecules, i.e. the C-terminal tail of the EGF-R, or the kinase-insert regions of the PDGF- and CSF-1-like receptors. These tyrosine-phosphorylated residues then act as docking sites for various signal transduction molecules which associate with the activated receptors (Pawson and Gish, 1992; Pawson, 1995).

The discovery that SH2 domains found in many signal transduction molecules bind specific phosphotyrosine-containing sequences in the cytoplasmic domains of autophosphorylated receptors has dramatically changed the way in which we view signal transduction. The SH2 domain is a small protein module that facilitates the formation of protein complexes with tyrosine phosphorylated proteins (Pawson, 1995). However, SH2 domains do not randomly bind phosphotyrosine containing proteins. Instead, the binding specificity of individual SH2 domains is determined by the peptide sequence surrounding a given phosphotyrosine residue. Studies conducted by Songyang et al. (1993) have shown that residues at the +1 to +3 C-terminal to the phosphotyrosine residue, in the case of Src-like SH2s, or at the +1 to +5 positions for the phospholipase C-gamma 1 (PLCγ1) and SH2-containing protein tyrosine phosphatase-2 (SHP-2) SH2s, may be critical for SH2 domain binding specificity. Similarly, other modules such as SH3, pleckstrin homology (PH), phosphotyrosine binding (PTB) and WW domains may associate with other domain-specific ligands leading to the assembly of signal transduction protein complexes (Cohen et al., 1995; Pawson, 1995).

The PDGF receptor has served as a prototype for studies of signaling by tyrosine kinase receptors. Ligand-induced dimerization of the PDGF receptor results in the tyrosine phosphorylation of a variety of sites in the cytoplasmic domain (Escobedo et al., 1991; Kashisshian et al., 1992; Ullrich and Schlessinger, 1990; Cantley et al., 1991). This phosphorylation leads to the recruitment of signaling proteins including Src family kinases, the GTPase activating protein (p120 ras-GAP), PLC-γ1, the regulatory subunit of

phosphatidylinositol-3-kinase (PI3K), and SHP-2 (Syp) (Fig. 1.1) (Pawson, 1995). The activation or mobilization of these molecules to the membrane elicits a variety of responses within the cell including changes in ion exchange or transport, phosphatidylinositol metabolism, glucose metabolism or transport, guanine nucleotide levels associated with small G-proteins, and both tyrosine and serine/threonine phosphorylation.

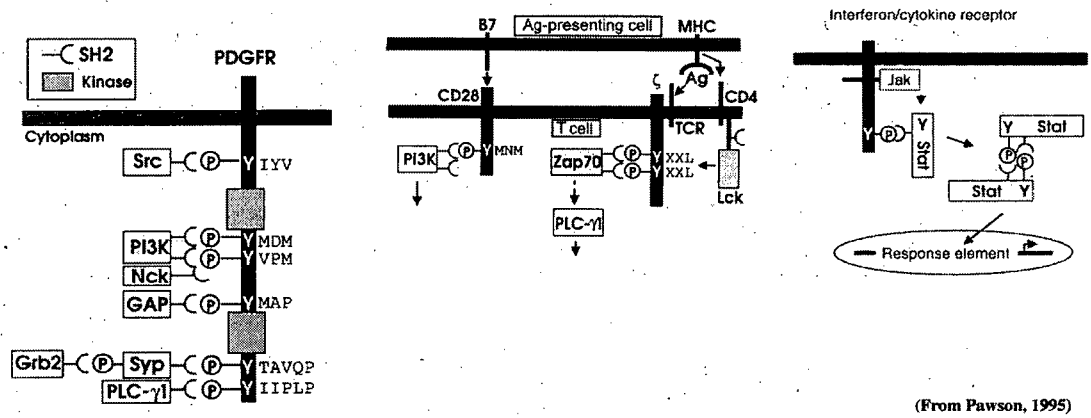


FIG. 1.1. Depiction of various receptor signal transduction complexes. The PDGF receptor and specific SH2 domain-containing molecules that associate with the autophosphorylated receptor are shown. Receptors for antigen or cytokines, which are indirectly coupled to cytoplasmic tyrosine kinases, are also illustrated. These receptors also induce both tyrosine phosphorylation and SH2-containing protein complex assembly in response to ligand-binding.

1.3.2. Antigen and cytokine receptors. In contrast to growth factor receptors which possess intrinsic tyrosine kinase activity, receptors for antigen (DeFranco, 1987; Bolen, 1991) and cytokines (Bazan, 1990; Taniguchi, 1995) are indirectly coupled to cytoplasmic tyrosine kinases. These receptors are also usually composed of multiple subunits. For example, the recognition of antigen by B cell receptors, or major

histocompatibility complex-associated antigen by T cells, involves receptor components which are devoid of enzymatic activity. Instead, these receptors are linked to specific molecules which recruit tyrosine kinases and contain sequences which when tyrosine phosphorylated may associate with signaling molecules containing SH2 domains (Gold and Matsuuchi, 1995). Thus, such receptors also initiate a ligand-dependent tyrosine phosphorylation cascade that results in both the regulation of specific enzymes and localization of signal transduction molecules to the membrane. Like the activation of classical tyrosine kinase receptors, cytokine or antigen receptor ligation results in receptor dimerization or oligomerization and activation of downstream enzymes such as PLC-γ, PI3K, tyrosine and serine/threonine kinases, and changes in ion-channel conductivity, metabolism, and gene transcription.

The PTKs activated by cytokine binding have until recently remained a mystery. Although the activation of Src family kinases is a common theme in cytokine signal transduction (Bolen, 1991; Taniguchi, 1995; Maher, 1993), the importance of the Jak family kinases was highlighted by complementation studies in mutant cell-lines defective in interferon (IFN) signaling (Velazquez et al., 1992). These and other studies pointed to Jak kinases as the receptor-associated kinases activated by cytokine binding (Ihle, 1995; Ihle and Kerr, 1995}. Cytokine binding is thought to lead to receptor dimerization or oligomerization resulting in Jak kinase cross-phosphorylation and activation. Cytokine receptors, composed of multiple receptor chains, may associate with more than one member of the Jak family, leading to ligand-dependent heterotypic aggregation of specific Jak kinases. A similar mechanism of signal transduction is employed by T and B cell antigen receptors. However, in these cases, receptor binding activates receptor-associated Src family kinases such as Fyn, Lck, Lyn, and/or Blk (Gold and Matsuuchi, 1995). Src family kinases are regulated differently than either receptor-tyrosine kinases or Jak family kinases, which both appear to be activated solely by aggregation induced autophosphorylation. The Src kinases are held in an inactive conformation by the

association of N-terminal domains with the tyrosine phosphorylated C-terminus (Roussel et al., 1991; Lui et al., 1993; Okada et al., 1993; Murphy et al., 1993; Superti-Furga et al., 1993; Erpel et al., 1995; Cobb and Parsons, 1993). Thus, the activation of Src kinases relies on either displacement of the N-terminal region from the C-terminus by allosteric effectors, or dephosphorylation of the C-terminus. This implies a requirement for conformational changes induced by receptor-ligand interactions and/or a requirement for PTP activity in the regulation of Src kinases. The role of the protein tyrosine phosphatase CD45 (to be discussed in greater detail below) in the activation of the Src kinases Lck and Fyn in hemopoeitic cells may represent a model by which Src kinases are activated by PTPs in other circumstances (Koretzky et al., 1990; Mustelin and Altman, 1990; Justement et al., 1991).

1.4. Src Family Kinases. First identified as the transforming agent of Rous sarcoma virus (Rous, 1911), *v-src* and the cellular product of the *c-src* gene, form the basis of our understanding of this class of tyrosine kinase. With the exception of ~60-80 residues at the N-terminus, Src kinases typically show significant sequence homology over the rest of their 505-543 residue sequence (Cooper, 1990). This similarity includes the presence of SH2 and SH3 domains, two modular domains first identified as regions of homology in Src family kinases, and a C-terminal tyrosine kinase domain (SH1) (Sadowski et al., 1986). These kinases are also typically modified by myristoylation which facilitates membrane localization (Shulz et al., 1985; Marchildon et al., 1984). To date, nine Src family kinases have been identified, including cellular homologues for the virally-encoded oncogenes detected in the genomes of avian sarcoma virus (*v-yes*) (Kitamura et al., 1982) and Gardner-Rasheed feline sarcoma virus (*v-fgr*) (discussed in chapter 4) (Naharro et al., 1984). Src family members such as Lck, Lyn, Blk, Hck, and Fgr display restricted tissue distribution, while others such as Src, Fyn and Yes are widely expressed (Cooper, 1990). Although many if not all of these kinases likely possess

the capacity to transform fibroblast cell lines when mutated and overexpressed (Bishop, 1987; Marth et al., 1988; Amrein and Sefton, 1988), determining the normal cellular function of these enzymes has been elusive.

1.4.1. Regulation of Src kinases. Clues to the nature of Src kinase regulation were apparent from examination of the differences between the sequences of virally-encoded, transforming versions of Src kinases, and their normal cellular counterparts. Indeed, overexpression of Src kinases does not result in cell transformation (Iba et al., 1984; Shalloway et al., 1984). Instead, transformation by these kinases requires the presence of mutations leading to kinase activation (Sefton et al., 1980; Tanaka and Fujita, 1986; Shalloway et al., 1984; Iba et al., 1984). For example, transforming versions of Fyn isolated by passaging Fyn-expressing retroviruses through tissue culture cells all contained C-terminal mutations (Kawakami et al., 1988). These mutants, and others isolated from various naturally occurring retroviruses, all contained mutations in residues corresponding to tyrosine-527 (Y527). Indeed, Src Y527F mutants and the corresponding mutation introduced into Lck and Hck allow these kinases to transform cells (Amrein and Sefton, 1988; Ziegler et al., 1989).

These and other studies, showing Src activation by PTPs present in cell lysates (Courtneidge, 1985; Cooper and King, 1986), suggest that Src kinases are regulated by the phosphorylation status of Y527 (Cooper et al., 1986) or its equivalent residue in other Src family members. Moreover, phosphopeptides based on the C-terminal Y527 sequence of Src are able to bind the Src SH2 domain, suggesting that the SH2 domain of Src contributes to the repression of activity through its association with the C-terminus (discussed in chapter 3) (Roussel et al., 1991; Sieh et al., 1993). This conclusion is

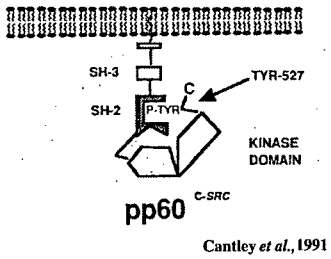


FIG. 1.2 Model of Src family kinase structure. Src kinases are thought to be held in an enzymatically inactive conformation by the association of N-terminal SH2 and SH3 domains with the tyrosine phosphorylated C-terminus.

supported by studies showing that a Src-specific mAb that inhibits enzyme activity recognizes an epitope composed of residues derived from both the N- and C-terminus (McCarley et al., 1987). Additionally, studies of Src regulation in yeast have demonstrated a requirement for both the SH2 and SH3 domains of Src for repression of kinase activity (Fig. 1.1) (Murphy et al., 1993; Superti-Furga et al., 1993; Erpel et al., 1995).

The maintenance of Src in the tyrosine phosphorylated, enzymatically-repressed state, requires the activity of the tyrosine kinase Csk (Okada et al., 1991; Nada et al., 1993; Murphy et al., 1993; Superti-Furga et al., 1993). This kinase shows relatively strict substrate specificity for Src kinases and its overexpression in cells transformed by a combination of *v-crk* and Src overexpression results in reversion of transformation (Sabe et al., 1992). Studies of Csk-deficient cells derived from Csk knockout mice have also clearly established a role of Csk in negative regulation of Src kinase activity (Imamoto and Soriano, 1993; Nada et al., 1993; Thomas et al., 1995). Deregulation of Src kinases can have serious consequences as homozygous mutant embryos derived from Csk -/- mice die at ~ day 9.5 and display gross developmental defects and inefficient blood circulation. Cell lines derived from these embryos contain constitutively activated Src

kinases and elevated levels of overall cellular tyrosine-phosphorylation (Imamoto and Soriano, 1993; Nada et al., 1993). One striking consequence of Src kinase activation due to the loss of Csk is the disruption of focal adhesions and the increased tyrosine phosphorylation of proteins such as paxillin, FAK, tensin, cortactin, and the cadherin adhesion protein, p120 (Thomas et al., 1995). These cell lines were also used to identify the regions of Csk required for localization to focal adhesions (Howell and Cooper, 1994). Intriguingly, mobilization of Csk to focal adhesions required both the SH2 and SH3 domains of Csk and the kinase activity of Src. Furthermore, Kaplan et al. (1994) demonstrated that mobilization of Src kinase to focal adhesions was dependent on the phosphorylation state of Y527. Together, these results suggest a model of Src kinase activation where dephosphorylation of Src kinases by specific PTPs leads to Src kinase-focal adhesion localization. Src may then recruit Csk to these sites by way of a kinase-dependent mechanism depending on both the SH2 and SH3 domains of Csk.

1.4.2. Src family kinase function. Support for the role of Src kinases in the regulation of integrin-dependent cell adhesion has also come from studies of *v-src* substrates (Schwartz et al., 1995). These include focal adhesion proteins such as paxillin, FAK, tensin, cortactin, the actin-filament-associated protein (ASAP), and the cadherin adhesion proteins p120 and β-catenin (Clark and Brugge, 1995; Thomas et al., 1995). As discussed above, a subset of these proteins are heavily tyrosine phosphorylated in Csk-/- cells (Thomas et al., 1995). Furthermore, studies in which Csk -/- mice were crossed with Src-/- or Fyn-/- mice have shown that substrates such as tensin and cortactin may be Src-specific substrates, while others such as FAK and paxillin may be substrates for both Src and Fyn (Thomas et al., 1995). However, the role of these substrates and the consequence of their tyrosine phosphorylation by Src family kinases is not understood.

Studies of receptor tyrosine kinase signal transduction have also pointed to a role for Src, Yes and Fyn in PDGF, CSF-1 and EGF dependent signaling. Src kinases have

been shown to associate with the PDGF and CSF-1 receptors by means of an SH2-phosphotyrosine-dependent interaction with receptor juxtamembrane regions (Kypta et al., 1990; Mori et al., 1993; Courtneidge et al., 1993). Although mutational studies of the receptor have been uninformative, other studies using microinjected inhibitory antibodies, or dominant negative versions of Src, have suggested a critical role for Src kinases in both PDGF- and EGF-induced DNA synthesis (Roche et al., 1995; Barone and Courtneidge, 1995). These studies demonstrated the necessity of Src kinases for a full 18 hours of the cell-cycle, until cells are committed to S-phase entry. Furthermore, these kinases appear to be responsible for the PDGF- and EGF-dependent *myc* gene induction. Indeed, the constitutive expression of Myc in cells in which Src kinases have been inactivated allows these cells to complete PDGF-dependent cell division (Barone and Courtneidge, 1995).

Studies of mice with Src kinases inactivated by homologous recombination in embryonic stem cells have identified specific tissues in which Src kinases have an indispensable function. For example, although Src is widely-expressed and likely plays an important role in growth factor-dependent gene induction and cell adhesion (discussed below), deletion of the Src gene by gene targeting was not lethal. Instead, these mice displayed specific defects in bone resorption, resulting in osteopetrosis (Soriano et al., 1991; Lowe et al., 1993). This phenotype is similar to that of *op* mouse in which CSF-1 expression is lost (Yoshida et al., 1990). Both src-/- and *op* mice contain a defect in osteoclast function, a subclass of cells of hemopoeitic origin that resorb bone. Thus, it is likely that Src has a specific substrate or function in osteoclasts which cannot be complemented by other Src family members. The phenotypic similarity between *op* and Src-/- mice may suggest an involvement of Src in CSF-1 receptor signaling. Other studies suggest a role for Src in mouse mammary tumor virus (MMTV) polyoma middle-T-induced mammary tumors in transgenic mice (Guy et al., 1995). MMTV-middle-T-

induced tumor frequency and severity was reduced in Src-/- mice. The requirement of Src for tumor induction was relatively specific as tumors developed normally in Yes-/- mice.

The role of Src kinases in the hematopoeitic system has been clearly demonstrated by gene ablation studies resulting in Lck-/-, Fyn-/-, and Hck-/-, Fgr-/- mutant mice. Loss of Lck, a T and B cell expressed Src kinase, was associated with a dramatic reduction in the number of thymocytes (5% of normal) (Molina et al., 1992). Remaining T-cells were blocked in development at the double positive stage, suggesting the requirement for Lck in thymocyte maturation. In contrast to the loss of Lck, gene ablation of the TCR-associated Fyn kinase did not appear to impact thymocyte development. Instead, mature thymocyte populations were impaired in their calcium mobilization response and proliferation induced by either anti-CD3 or anti-Thy-1 crosslinking (Stein et al., 1992; Appleby et al., 1992). However, peripheral T-cells obtained from these mice were relatively normal in terms of the above measured parameters . These results are consistent with those in which overexpression of Fyn in thymocytes led to enhanced calcium mobilization, tyrosine phosphorylation, and IL-2 production in response to TCR stimulation (Cook et al., 1991).

While the loss of either Src, Hck, or Fgr did not significantly impair immune function, the loss of both Src and Hck or Hck and Fgr resulted in specific immunological defects. Lowell et al. (1996) found that mice containing homozygous deletions of both Src and Hck contained defects in the bone marrow environment and two thirds of these mice died at birth. In contrast, mice lacking both Hck and Fgr were more susceptible to *Listeria monocytogenes* infection (Lowell et al., 1994). Additional studies of Hck-/-, Fgr-/- mice have demonstrated a profound defect in tumor necrosis factor (TNF)-induced β2- and β3-integrin-dependent neutrophil cell adhesion and associated respiratory burst in mutant animals (Lowell et al., 1996). These results suggest a role for Src kinases in integrin-dependent adhesion and provide evidence of the potential redundancy of Src kinases initially inferred by studies of Src, Yes, and Fyn pertaining to growth factor

receptor signaling. Experiments with mice deficient in both Src and Fyn or Src and Yes support the concept of Src kinase compensatory interactions (Stein et al., 1994). Mice lacking Src, Fyn, or Yes possess minor phenotypic defects (as previously discussed), however, mice containing deficiencies in both Src and Fyn, or Src and Yes, die perinatally. In contrast, most mice lacking both Fyn and Yes are viable but experience degenerative renal glomerulosclerosis.

Thus Src kinases, originally identified due to their role in tumor induction by various murine and avian oncogenic retroviruses, have proven to be a functionally diverse family of PTKs involved in the regulation of cell growth and differentiation. The requirement for Src kinases in the immune system and during development likely relates to their involvement in cell adhesion and growth factor, antigen, and cytokine receptor signal transduction.

1.5. Cell Adhesion. The organization of cells into the three dimensional tissues of organisms requires the coordinated interaction of neighboring cells and involves the actions of specific growth factors, cytokines, and adhesion molecules (Gumbiner, 1996). The regulated adhesion of cells for example, is required for immune cell function during leukocyte homing and activation and for the growth and metastasis of tumor cells (Springer, 1994). The adhesion molecules involved in cell-cell and cell-extracellular matrix (ECM) interactions provide the context in which cells respond to growth factors, cytokines, or antigens (Hynes, 1992). Adhesion molecules do not simply serve to link the actin cytoskeleton with specific extracellular ligands to form a static site of attachment, but instead are highly dynamic, having the ability to transduce signals (Clark and Brugge, 1995; Miyamoto et al., 1995). The classes of adhesion molecules responsible for cell-cell and cell-ECM interactions include members of the integrin, cadherin, immunoglobulin, selectin, and proteoglycan superfamilies (Gumbiner, 1996).

1.5.1. Integrin receptors and integrin-dependent signal transduction. Integrin receptors are heterodimeric receptors composed of α and β subunits (Hynes, 1992; Clark and Brugge, 1995; Schwartz et al., 1995). Multiple α and β chains combine to produce more than 20 different receptors specific for ECM components such as fibronectin, laminin, collagen and vitronectin, soluble ligands such as fibrinogen, or other cell surface receptors. The attachment of cells to the ECM is largely the responsibility of integrins and is critical for tissue integrity. Cells may attach either to the interstitial matrix, which is composed largely of collagen, or to the basement membrane of epithelia which is made up of collagen, laminin, fibronectin, proteoglycans and other molecules (Gumbiner, 1996). In tissue culture, integrins mediate the attachment of cells to the dish and are localized to the points at which the cells contact the substratum (Burridge et al., 1988). Within the cell, focal contacts are rich in phosphotyrosine-containing proteins and represent the sites at which microfilament bundles terminate at the cell membrane. Like receptors for growth factors, cytokines, and antigen, ligand binding to integrins induce receptor dimerization and clustering, which elicits a variety of cellular responses. Although integrins contain cytoplasmic regions devoid of enzymatic activity, integrin engagement by ligand results in tyrosine kinase activation (Ferrell and Martin, 1989; Golden et al., 1990; Guan et al., 1991; Guan and Shalloway, 1992; Schaller et al., 1992). The cytoplasmic region of the integrin receptor β chain contains sequences required for localization to focal adhesions and ligand-independent signal transduction (O'Toole et al., 1991). The β chain also contains sequences required to connect integrins with actin cytoskeletal proteins such as α-actinin (Otey et al., 1993). Clustering of integrin receptors results in the formation of a multi-protein complex including such proteins as α-actinin, vinculin, talin, paxillin, tensin, Src, and FAK (Fig. 1.3) (Clark and Brugge, 1995).

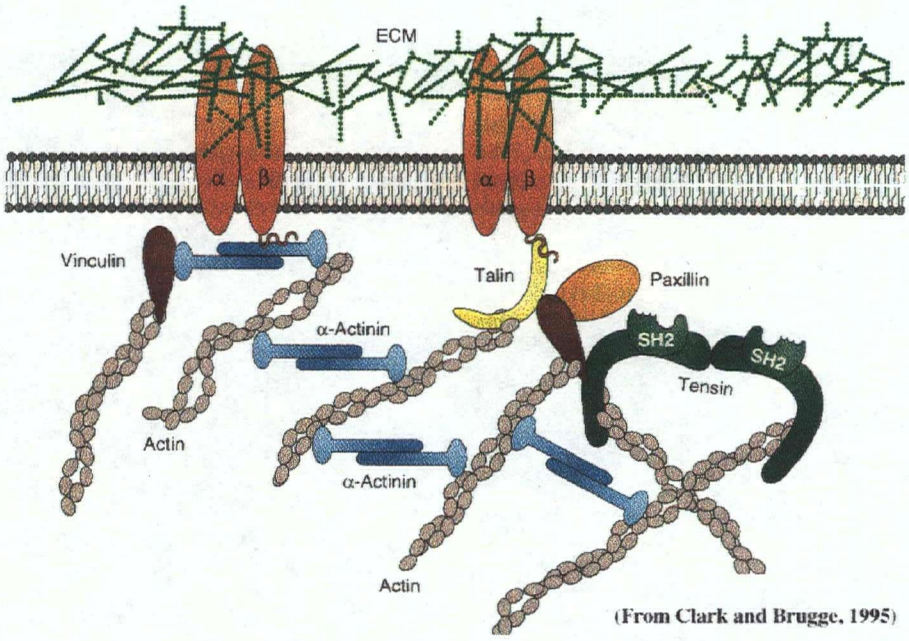


FIG. 1.3. Model of an integrin-ECM adhesion complex. The integrin family of adhesion receptors are composed of a heterodimeric combination of specific α and β chains. These chains form a link between extracellular matrix (ECM) ligands such as fibronectin and the cytoskeleton, leading to the formation of focal adhesions at sites of cell-ECM contact. The integrin receptor β chain is associated with cytosolic proteins such as α-actinin and talin which are linked to the actin cytoskeleton.. Other focal adhesion molecules include paxillin and FAK (not shown).

The formation of these complexes is believed to be important for stabilizing cell adhesion and regulating cell-shape, morphology, migration, and providing the context in which cells may respond to other signals (Gumbiner, 1996).

Tyrosine phosphorylation appears to be critical to integrin-dependent focal adhesion formation, as inhibitors of tyrosine kinases block focal adhesion formation (Burridge et al., 1992). Serine/threonine kinases such as PKC and Map kinases are also activated by cell-substratum adhesion (Vuori and Ruoslahti, 1993; Nakamura and Nishizuka, 1994; Chen et al., 1994; Schlaepfer et al., 1994). Indeed, many responses

observed after growth factor stimulation, such as calcium mobilization, phospholipid metabolism, changes in pH, and gene induction, are also observed following integrin crosslinking (Clark and Brugge, 1995; Miyamoto et al., 1995). The tyrosine kinase cascade initiated by integrin-ligand association involves multiple protein tyrosine kinases (Guan and Shalloway, 1992; Schaller et al., 1992; Clark and Brugge, 1993). However, a central role for the tyrosine kinase FAK has been suggested by studies showing FAK activation following integrin engagement (Guan and Shalloway, 1992; Schaller et al., 1992; Shattil et al., 1994; Kornberg et al., 1992). This kinase contains a C-terminal focal adhesion targeting (FAT) sequence that is necessary and sufficient for targeting FAK to focal adhesions as well as sequences involved in the association of FAK with paxillin (Fig. 1.4) (Hildebrand et al., 1993; Hildebrand et al., 1995). In addition, this kinase contains N-terminal sequences which bind to the cytoplasmic region of the β1-integrin receptor (Otey et al., 1994). FAK appears to be a Src kinase substrate and is directly associated with Src via Src SH2 domain-binding to tyrosine phosphorylated Y397 within the N-terminal domain of FAK (Guan and Shalloway, 1992; Schaller et al., 1994; Cobb et al., 1994). The binding of the Src kinase SH2 domain to FAK correlates with both FAK activation and tyrosine phosphorylation at a site which binds the Grb2 SH2 domain (Schlaepfer et al. 1994). Thus, activation of Src at focal adhesions may result in FAK activation and tyrosine phosphorylation, with corresponding Grb2-SOS dependent Ras activation. FAK is also able to associate with paxillin, a FAK and v-src substrate localized to focal adhesions (Van de Vijver et al., 1991; Turner et al., 1990; Schaller and Parsons, 1995b; Schaller and Parsons, 1995a). Tyrosine-phosphorylated paxillin has been shown to associate with the SH2 domains of both Csk and Crk (Schaller and Parsons, 1995a; Sabe et al., 1994; Birge et al., 1993). The association of these molecules with paxillin may act to either potentiate or inhibit the activity of Src by controlling the accessibility of Csk to Src (discussed in chapter 3) (Sabe et al., 1994; Sabe et al., 1992; Sabe et al., 1995).

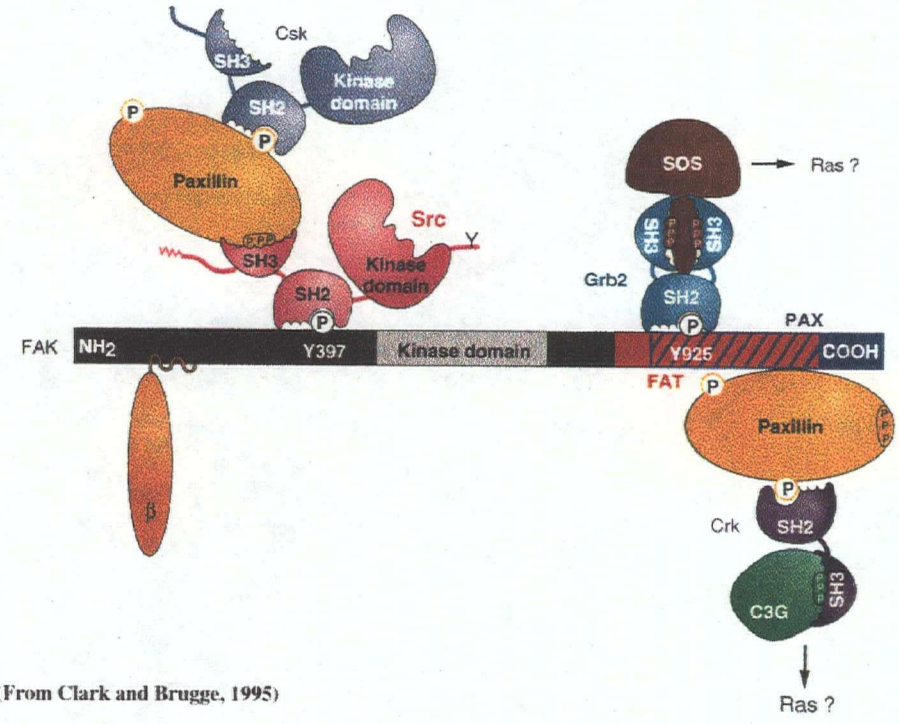


FIG. 1.4. The focal adhesion localized protein tyrosine kinase FAK. Shown are some of the proteins known to associate with FAK. The N-terminal region of FAK is thought to associate with the cytoplasmic tail of the integrin β chain. Sites of tyrosine phosphorylation at Y397, which binds Src kinase SH2 domains, and Y925, which associates with the adapter protein Grb2 are illustrated. FAK association with paxillin is also depicted.

Both FAK and paxillin are heavily tyrosine phosphorylated in *v-src* transformed cells (Guan and Shalloway, 1992; Glenney and Zokas, 1989). The fact that these cells grow in an anchorage-independent fashion has led to investigations of the role of FAK in this process. Chick embryo fibroblasts (CEF) which contain a temperature sensitive *src* allele (ts *LA29*) are transformed when grown at 35°C, but revert to a non-transformed phenotype when grown at 41°C (Fincham and Frame, 1995). Examination of FAK

regulation in these cells demonstrated that although FAK became heavily tyrosine phosphorylated at the permissive temperature, the protein was subsequently degraded. Thus, the degradation of FAK may reconcile the apparent tyrosine phosphorylation of FAK with *v-src* induced anchorage-independent growth. Other studies have shown that FAK may be involved more in the turnover of focal adhesions than in their ECM-dependent formation. This conclusion is supported by studies of cell lines derived from FAK-/- mice. Cells from these animals showed an impaired ability to migrate but contained an increased rather than decreased number of focal adhesions (Ilic et al., 1995). Studies of FRNKs (FAK related non-kinase), alternatively spliced versions of FAK in which only the C-terminal non-kinase and FAT region of the molecule are expressed (Schaller et al., 1993), have pointed to a role for the N-terminal region of FAK in cell-spreading on ECM (Richardson and Parsons, 1996).

1.5.2. Regulation of integrin binding. Affinity modulation is a process whereby the affinity of a specific integrin for ligand is controlled by signals received within the cell and transmitted to the receptor cytoplasmic regions (Diamond and Springer, 1994; Springer, 1995). Integrin receptors do not simply bind ligand when it is presented to them, but rather require signals making them competent to bind. Thus, while receptors for growth factors such as EGF may be desensitized by phosphorylation by serine/threonine kinases such as PKC, targeted for degradation, or internalized, their regulation does not seem to be as strict as that of integrin receptors. Affinity modulation requires conformational changes in the integrin extracellular domain in response to activation (Springer, 1995). Recent evidence suggests that the cytoplasmic domain of the α chain of the integrin receptor prevents the β chain from localizing to focal adhesions (O'Toole et al., 1991). Indeed, removal of the α chain cytoplasmic domain leads to constitutive focal adhesion localization and ligand binding competency (Briesewitz et al., 1993; Ylanne et al., 1993). This has led to the hypothesis that specific cytoplasmic tail-binding proteins

may regulate affinity. However, it is unclear whether known integrin-binding proteins such as talin, paxillin, α-actinin, or FAK are the tail-binding proteins responsible. Signals such as those from TNF, thrombin, lysophosphatidic acid, neuropeptides such as bombesin, vasopressin and endothelin, and various chemokines are known to confer integrin receptors with the ability to bind ligand (Clark and Brugge, 1995; Springer, 1995). Perhaps the best example of regulated integrin binding is observed with leukocyte adhesion to endothelium. In the three step model of leukocyte adhesion, selectin family members bind to specific leukocyte mucins, allowing cell-rolling on inflamed endothelium (Fig. 1.5) (Springer, 1994).

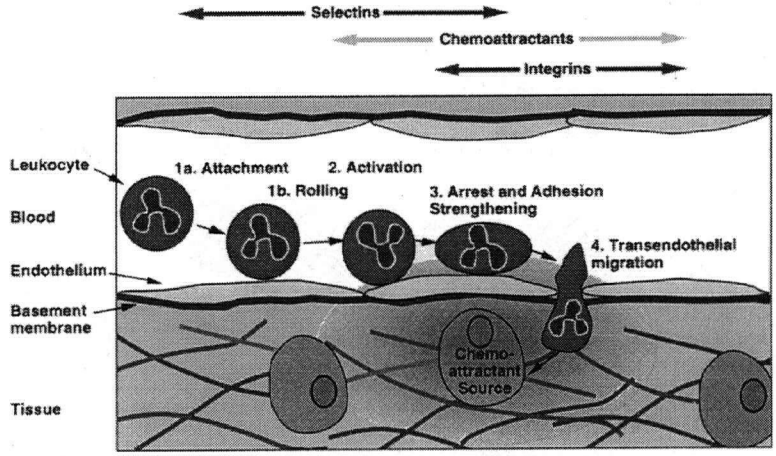


FIG. 1.5. Model of adhesion receptor interactions involved in the stable association of leukocytes with the endothelium. Leukocytes are thought to tether and roll on inflamed endothelium by means of selectin family receptor interactions with specific endothelial cell molecules. Signals from various chemokines to the leukocyte then initiate firm adhesion to the endothelium. Specific integrin-ligand interactions are then thought to mediate cell-migration into inflammatory sites (from Springer, 1994).

At this stage, although integrins such as LFA-1, Mac-1, or VLA-4 may be present on the leukocyte cell surface, their ability to mediate strong binding to endothelium proteins such as ICAM-1, -2, or -3, is impaired (Springer, 1995). Signals received by rolling leukocytes in the form of specific chemokines induce strong integrin-dependent cell adhesion. However, the underlying mechanism of action of these factors on integrin affinity is unclear. A role for Src kinases (as mentioned earlier) in affinity modulation was implied by results demonstrating that neutrophils derived from Fgr-/-/Hck-/- mice are defective in both β-2 and β-3 dependent cell-spreading on ECM (Lowell et al., 1996). Additionally, in mice that lack the β-2 integrin receptor, normal activation of Fgr is lost in response to TNF-dependent neutrophil cell-adhesion (Berton et al., 1994). Furthermore, thrombin activation of platelets is followed by the mobilization of Src from a detergent-soluble fraction to a detergent-insoluble fraction rich in cytoskeletal proteins (Clark and Brugge, 1993). This mobilization is dependent on the integrin αIIbβ3, as platelets obtained from Glanzmann's thrombasthenia patients in which this integrin is defective fail to mobilize Src kinases to this fraction. Thus, thrombin dependent activation of Src, which occurs normally in patient platelets, precedes integrin-dependent aggregation, and may play a role in normal platelet integrin-cytoskeletal localization and affinity modulation.

1.6. Protein Tyrosine Phosphatases.

The amino acid sequence of the first PTP was obtained by the purification and partial microsequencing of PTP1B in 1989 (Charbonneau et al., 1989). Surprisingly, although serine/threonine phosphatases such as PP2A and PP2B possess weak activity towards phosphotyrosine, the sequence of PTP1B bore no similarity to these enzymes. Thus, whereas serine/threonine and tyrosine kinases are related ancestrally, the PTPs show no relationship with serine/threonine phosphatases (Charbonneau and Tonks, 1992).

Another surprise arising from the sequence of PTP1B, was its similarity to tandemly repeated ~250 residue regions within the cytoplasmic domain of CD45 (Charbonneau et al., 1988). The demonstration that CD45 was in fact a PTP followed rapidly (Tonks et al., 1988). This finding, together with the isolation of LAR, a receptor-like PTP isolated by low-stringency screening of a cDNA library with a probe corresponding to CD45 (Streuli et al., 1988), suggested that there might be a large family of PTPs. Furthermore, the extracellular region of LAR was composed of both fibronectin type-III repeats and Ig domains reminiscent of the molecules NCAM and fasciclin II which are capable of homophilic interactions (Edelman and Crossin, 1991). This suggested that the extracellular domains of PTPs such as LAR might participate in contact inhibition of cell growth following homophilic engagement of extracellular domains of LAR on opposing cells. The identification of receptor-like PTPs such as CD45 and LAR suggested the possibility of highly regulated PTP involvement in signal transduction regulation and was the impetus for the isolation of additional family members by both PCR and low stringency library-screening techniques.

1.6.1. Structural diversity of PTP family members. To date more than 40 PTPs have been identified from organisms such as yeast, insects, nematodes, the proto-chordate *Styela plicata*, bacteria of the genus *Yersinia*, vaccinia virus, and mammals (Charbonneau and Tonks, 1992). Interestingly, the existence of PTPs in yeast may suggest that this family predates the PTKs, which have not been identified in yeast. This observation may relate to the requirement of dual specificity PTPs, which dephosphorylate serine, threonine and tyrosine residues, in the regulation of dual specificity kinase substrates such as cdc2. The similarity among PTPs is often restricted to an approximately 240 residue sequence containing the protein phosphatase catalytic domain within which lies the catalytic centre reactive cysteine residue (discussed below). Otherwise, members of this family exhibit highly divergent structures.

The receptor-like PTPs contain dissimilar extracellular domains and tandem cytoplasmic PTP domains (discussed in chapter 3). The extracellular region of CD45 is alternatively spliced, leading to the expression of at least six separate isoforms (Powrie and Mason, 1988; Sanders et al., 1988). Adding to this complexity, CD45 is heavily glycosylated at sites which vary according to extracellular domain exon usage (Thomas and Lefrancois, 1988). In contrast, PTPs such as LAR, PTPβ, PTPδ, PTPκ, and PTPμ have extracellular domain containing Ig domains and/or fibronectin type III motifs suggesting a role in adhesion (Mourey and Dixon, 1994; Brady-Kalnay and Tonks, 1995). The homophilic adhesion of these extracellular domains, initially anticipated from the structure of LAR, has recently been observed for both PTPκ and PTPμ (Brady-Kalnay et al., 1993; Gebbink et al., 1993; Sap et al., 1994). However, the regulation of cell-signaling by a homophilic interaction of PTP extracellular domains alone may be too simplistic. There is now evidence that PTPμ is able to associate with cadherins (adhesion receptors involved in cell-cell adhesion) which themselves are capable of homophilic adhesion (Brady-Kalnay et al., 1995).

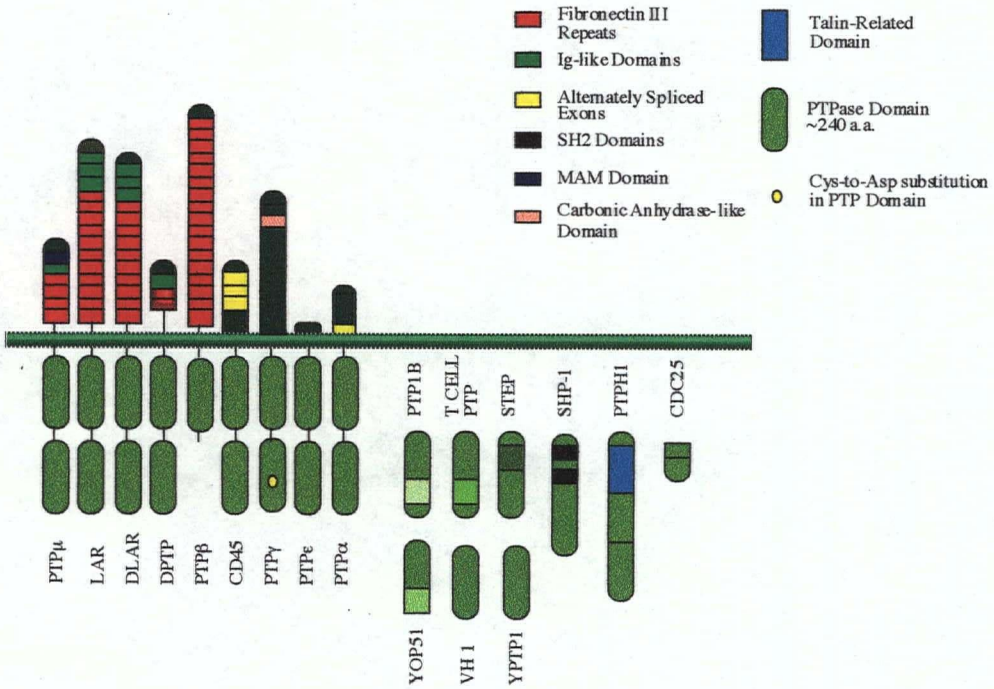


FIG. 1.6. Protein tyrosine phosphatase family diversity. PTPs may be either transmembrane receptor-like enzymes such as CD45 and PTPα, or cytosolic, like the SH2 domain-containing SHP-1. Receptor-like enzymes may contain large extracellular domains composed of fibronectin type III and Ig domains reminiscent of adhesion molecules. The extracellular domains of other PTPs, such as CD45 and PTPα, may be modified by alternative splicing. All receptor-like PTPs, with the exception of PTPβ-like enzymes, possess two cytoplasmic PTP domains. The PTPs YOP51, VH1, and YPTP1 correspond to enzymes isolated from *Yersinia*, vaccinia virus, and yeast respectively.

The homophilic adhesion of specific cadherins is known to be disrupted by tyrosine phosphorylation, thus PTPs such as PTPμ may regulate cell-cell adhesion by determining the phosphorylation status of cadherin-cytoskeletal complexes (Brady-Kalnay et al., 1995). Other examples of PTPs involved in cell adhesion include PTPζ, which is highly expressed on glial cells in both the peripheral and central nervous system and associates with NCAM, contactin, and NgCAM (Peles et al., 1995; Brady-Kalnay and Tonks, 1995). Moreover, expression of the *Drosophila* PTPs DLAR, DPTP10D, and DPTP99A

predominates in the central nervous systems at a time corresponding to neural outgrowth and pathfinding, suggesting a role for these PTPs in neuronal development (Tian et al., 1991).

PTPs such as PTPα and PTPε differ from the aforementioned receptor-like PTPs in that they possess relatively short extracellular domains lacking recognizable structural motifs (Jirik et al., 1990; Matthews et al., 1990; Krueger et al., 1990; Sap et al., 1990). The extracellular region of PTPα is modified by both alternative splicing and N- and O-linked glycosylation (Daum et al., 1994). However, unlike PTPs such as LAR and PTPμ, the extracellular regions of PTPα and PTPε do not suggest a specific function.

Other members of the PTP family are cytosolic and contain structural motifs that may lead to their localization to specific cellular compartments and/or regulate PTP activity. These motifs include SH2 domains in the PTPs SHP-1 (also designated PTP1C, HCP, and SHP) and SHP-2 (also referred to as PTP2C, Syp, PTP1D), regions similar to ezrin and band 4.1 in PTPs PTPH1 and GEG-01, and hydrophobic segments in the C-termini of T-cell PTP and PTP1B. PEST sequences, associated with rapidly degraded molecules, are found in the PTPs PEP and PTP-PEST (reviewed in Charbonneau and Tonks, 1992).

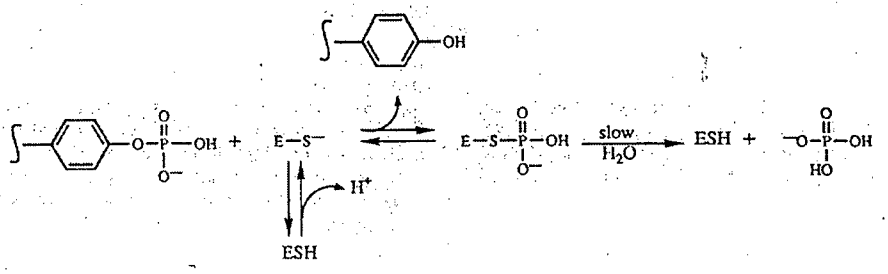
More distantly related to the receptor-like and the above mentioned cytosolic PTPs are the dual specificity PTPs such as cdc25, VH1, and CL100, which can dephosphorylate serine, threonine and tyrosine residues. This class of PTP may be involved in the regulation of kinases such as cdc2 and MAP-like kinases which are phosphorylated by dual specificity kinases at neighboring serine/threonine and tyrosine residues. Interestingly, overexpression of specific cdc25 isoforms in mouse fibroblast induces cell transformation, pointing to a growth-promoting activity for these enzymes (Galaktionov et al., 1995). In contrast, immediate early response genes such as the PTP CL100 may be involved in the inactivation of MAP-like kinases (Alessi et al., 1993).

Thus, within this sub-family of dual specificity PTPs there exists both growth promoting and growth inhibitory enzymes.

1.6.2. PTPs as anti-oncogenes. It was predicted that PTPs would represent a class of proteins able to counteract the actions of tyrosine kinases. It was hypothesized that specific PTPs might represent tumor-suppressor genes, which when disrupted by chromosomal aberrations or other mutations, would be associated with diseases such as cancer. Thus, the sequence determination of additional PTPs allowed the identification of the chromosomal localization of PTP genes. To date the only correlation supporting this hypothesis is the apparent loss of one allele of the PTPγ gene in 5 of 10 lung carcinomas, and 3 of 5 renal carcinomas (LaForgia et al., 1991). However, subsequent experiments have found that although one allele of the PTPγ gene was lost in the majority of renal carcinomas, no mutations were detected in the other PTPγ allele (Druck et al., 1995). Other observations, such as the suppression of Neu-induced 3T3 cell transformation by PTP1B (Brown-Shimer et al., 1992), or transformation of NRK-1 cells by PTP inhibitors (Klarlund, 1985) support the possibility of select PTPs being able to antagonize PTK-induced transformation. We have determined the chromosomal localization of PTPα (Jirik et al., 1992a), PTPβ (Harder et al., 1992) and LAR (Jirik et al., 1992b). Myc gene amplifications on chromosome 1p32 are a common feature of small-cell lung carcinoma (SCLC) cells. Interestingly, the LAR gene is also located on chromosome 1p32 and appeared to be coamplified with the *L-myc* gene in a SCLC cell line (Harder et al., 1995). However, although LAR mRNA and protein was detected in this cell line, its role in the growth of such tumor cell lines remains to be explored.

1.6.3. Structure/Function. Within the approximately 240 residue PTP catalytic domain lies the HCSAGVGR(S/T)G motif that defines members of the PTP family. Early studies reported the requirement of thiol-reducing agents for preserving enzymatic activity

(Tonks et al., 1988). This suggested the involvement of a cysteine residue in catalytic function. The requirement for the conserved cysteine residue was confirmed by studies demonstrating the radioactive labeling of this residue using C^{14}-iodoacetate, an irreversible sulfhydryl-directed inhibitor of PTP activity (Pot and Dixon, 1992). Other studies showing that site-directed mutagenesis of this cysteine residue to serine or alanine abolished enzymatic activity have confirmed the importance of this residue in catalytic activity (Streuli et al., 1990). Intriguingly, a PTP-like sequence identified as STYX (for phosphoserine/threonine/tyrosine interaction domain) that was recently identified in the EST-database is highly similar in sequence to the PTP catalytic domain but contains a glycine rather than cysteine residue in the catalytic centre. A glycine-to-cysteine substitution within the catalytic domain of STYX conferred catalytic activity to this otherwise inactive protein (Wishart et al. 1995). This finding suggests that specific PTP-like sequences may have a PTP-independent function. Indeed, the STYX protein may protect phosphorylated residues from dephosphorylation.



From Pot and Dixon, 1992

FIG. 1.7. Reaction mechanism of phospho-substrate dephosphorylation. ESH represents the reduced cysteine residue in the PTP active site. The thiol anion form of this residue is thought to act as a nucleophile in the attack of the phosphorylated substrate residue. Water attack of the thiol-phosphate intermediate then releases the enzyme and P_i.

Analysis of the mechanism of PTP activity by Guan and Dixon (1991) and Pot and Dixon (1992) have suggested that the nucleophilic cysteine within the catalytic core attacks the phosphate of phosphotyrosine containing substrates, releasing the dephosphorylated substrate, and creating a thiol-phosphate enzyme intermediate. The reaction is completed by water attack, regenerating the active enzyme and releasing inorganic phosphate (Fig. 1.7).

1.6.4. CD45. Perhaps the best example of the function of a receptor-like PTP comes from studies of the role of CD45 in lymphocyte activation. T and B cell receptor recognition of antigen results in the proliferation and/or differentiation of T and B cells into cells capable of carrying out specific immune functions. Alternatively, these cells may undergo apoptosis or become anergic depending on the context within which antigen is encountered (Gold and Matsuuchi, 1995). Signal transduction initiated by antigen recognition involves specific accessory chains associated with the cell-surface receptor chains and tyrosine kinase activation (Sefton and Campbell, 1991). As previously mentioned, Src kinases such as Lck and Fyn are critical for signal transduction from the T cell receptor. The necessity of CD45 for productive signaling from antigen receptors has been demonstrated by studies of mutant T and B cells deficient in CD45 (Pingel and Thomas, 1989; Koretzky et al., 1990; Koretszky et al., 1991; Weaver et al., 1991; Kishihara et al., 1993). T cells lacking CD45 possessed a drastically diminished ability to proliferate in response to TCR stimulation. Moreover, the ability of TCR crosslinking to induce tyrosine phosphorylation and Ca^{2+} mobilization was severely impaired in cells lacking CD45. However, cell proliferation in response to IL-2 was unaffected. As previously discussed, the activation of Src kinases likely involves dephosphorylation of their C-terminal tyrosine phosphorylation sites by specific PTPs. Experiments demonstrating CD45 dephosphorylation of Lck Y505 leading to kinase activation *in vitro*, together with the increased tyrosine phosphorylation of Lck observed in CD45

deficient T cells, point to a role for CD45 in Lck activation following TCR stimulation. A similar requirement for CD45 in membrane Ig-induced cell signaling was observed in CD45 negative B cells (Justement et al., 1991), suggesting that both T and B cells may require CD45 for receptor-specific signal transduction. Other studies have illustrated CD45-dependent activation of CD4-associated Lck and TCR-associated Fyn in T cells (Biffen et al., 1994). The activity of CD45 towards Lck and Fyn but not Src (Hurley et al., 1993; Burns et al., 1994) appears to be relatively specific, as expression of PTPα in a CD45 negative T cell line (BW5147) was unable to fully restore either TCR-dependent tyrosine phosphorylation or Lck and Fyn dephosphorylation/activation (Jabali et al. manuscript in preparation).

CD45-deficient mice have been produced to assess the importance of CD45 in the immune system *in vivo* (Kishihara et al., 1993). These mice lacked membrane expression of CD45 on B cells and on the majority of T cell populations. However, due to the design of the targeting construct, low levels of CD45 were expressed in mature thymocytes and peripheral T cell populations. Regardless, these mice displayed a block in thymocyte development at the $CD4^+$ $CD8^+$ double positive stage and contained dramatically reduced numbers of peripheral T cells . While B cell development appeared normal in these animals, B cell proliferation in response to surface IgM crosslinking was significantly impaired (Kishihara et al., 1993). Moreover, while tyrosine phosphorylation of both Ig-alpha and PLC-γ2 was normal in CD45-/- B cells, anti-Ig induced Ca^{2+} mobilization was strongly inhibited (Benatar et al., 1996).

CD45 may also have a role in integrin affinity modulation as antibodies to specific isoforms of CD45 induce LFA-1/ICAM-3-dependent homotypic thymocyte adhesion (Bernard et al., 1994). Although this study demonstrated that thymocyte adhesion was disrupted by inhibitors of oxidative phosphorylation, no evidence was provided that antibody-CD45 engagement resulted in Src kinase regulation. Intriguingly, Arroyo et al. (1994) demonstrated that anti-CD45 antibodies inhibited ICAM-3

dependent aggregation of T-cells. Moreover, anti-ICAM-3 and anti-LFA-1 induced tyrosine phosphorylation was blocked by preincubating the cells with anti-CD45 antibodies. These studies suggesting a role for CD45 in integrin-dependent cell adhesion may help to explain the increased A431 cell-substratum adhesion observed in cells overexpressing PTPα (discussed in chapter 3).

Studies of chimeras of CD45 containing the extracellular domain of the EGF receptor and the cytoplasmic region of CD45, demonstrated that CD45 may be functionally active as a monomer. Transfection of the EGF-R-CD45 chimeric receptor into CD45 negative T cells restored signaling. However, dimerization of the cytoplasmic domains of CD45 by EGF eliminated CD45-dependent TCR signaling (Desai et al., 1993). These results are intriguing as they suggest that PTPs, in contrast to PTKs, may be inactivated by dimerization. They may also explain certain results obtained with anti-CD45 antibodies discussed above.

1.7. The Receptor-Like Protein Tyrosine Phosphatase, PTPα.

In contrast to PTPs such as CD45, little information exists as to the function of PTPα in cell physiology. However, the wide tissue distribution of this PTP suggests that it may function in the regulation of a signal transduction pathway common to many cell types.

1.7.1. PTPα Structure. PTPα was isolated by screening a human HepG2 cell-line cDNA library with a probe consisting of CD45 catalytic domain sequences (Jirik et al., 1990). The isolation and sequencing of a full-length cDNA revealed a receptor-like PTP containing a relatively short extracellular domain (123 residues), followed by a putative transmembrane domain and tandem cytoplasmic regions homologous to PTP1B, CD45,

and LAR, the three phosphatases for which sequence information existed at the time (Fig. 1.8).

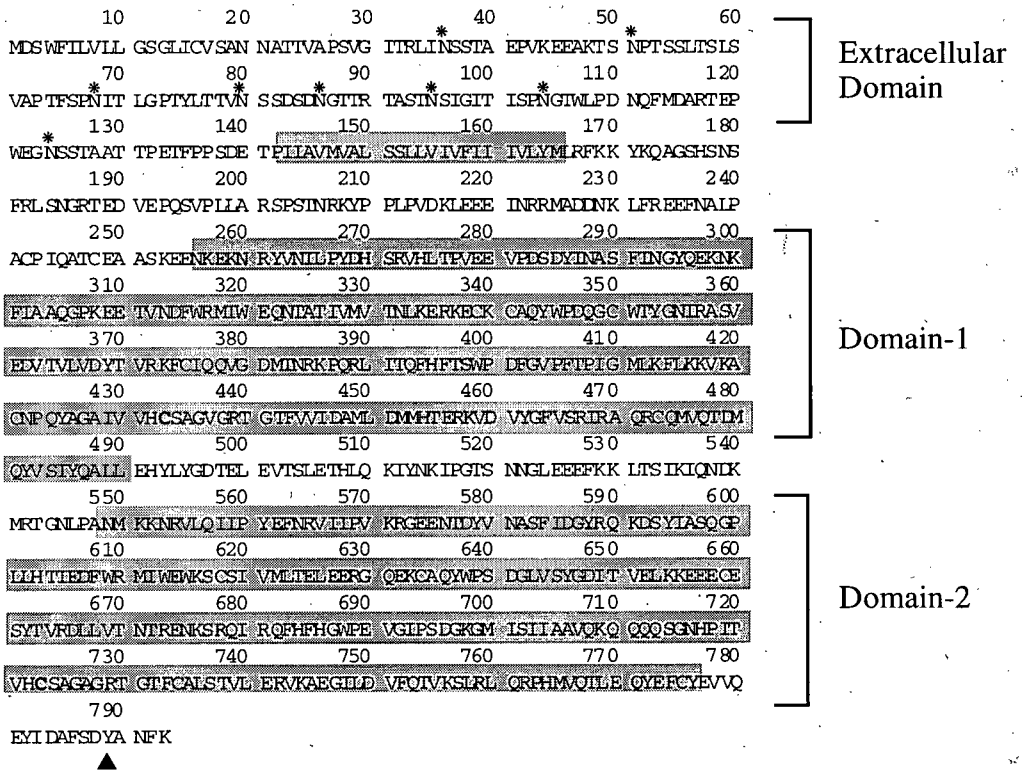


FIG. 1.8. The 793 residue sequence of human PTPα is shown. The extracellular domain, N-terminal PTP domain (Domain-1) and C-terminal PTP domain (Domain-2) are indicated. Potential sites of N-linked glycosylation are designated by a ✱ symbol. Cysteine residues in each PTP domain are in bold. Shading also illustrates the putative transmembrane region and two cytosolic PTP domains. The ▲ symbol designates the PTPα tyrosine phosphorylation site that associates with the Grb2 SH2 domain.

Although PTPα has a putative transmembrane domain and extracellular region, neither its structure or its expression pattern gave clues as to its physiological function. PTPα is expressed in a wide variety of tissues and cell lines (Jirik et al., 1990). Figure 1.9

shows PTPα immunoreactive protein in lysates derived from avian, murine and primate cells.

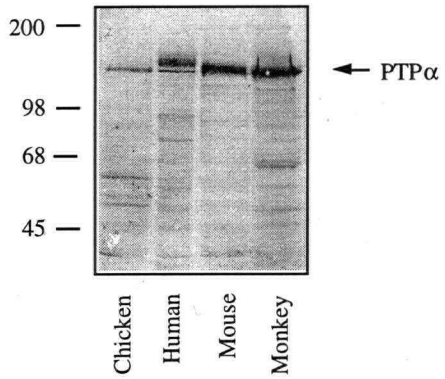


FIG. 1.9. Immunoblot of PTPα detected in lysates derived from chicken (fibroblasts), human (HepG2), mouse (NIH3T3), and monkey (COS) cells. The position of the 130-150 kDa PTPα protein is indicated. Immunoblotting was performed with anti-PTPα-1 antisera (produced as outlined in chapter 6).

*1.7.2. **Function.*** Clues to the function of PTPα came from studies of Zheng et al. (1992) who demonstrated that overexpression of PTPα in rat embryo fibroblasts resulted in cell transformation and tumorigenesis. PTPα overexpressing cells were not contact-inhibited, formed colonies in soft agar, and were highly tumorigenic when injected into nude mice. Investigations into the mechanism of cell transformation revealed that Src kinase isolated from transformed cells was dephosphorylated at both Y416 and Y527 correlating with a 3-6 fold activation of Src. The authors also demonstrated that recombinant PTPα was able to dephosphorylate ^{32}P labeled Src at Y527 *in vitro*. Moreover, MAP kinase was constitutively activated and localized to the nucleus in PTPα-transformed cells and the DNA-binding and transactivating ability of the transcription factor c-Jun was enhanced (Zheng and Pallen, 1994). However, it remains to be determined whether these changes were dependent on PTPα as opposed to being a secondary consequence of cell

transformation. It should be noted that we (Peacock, J., Harder, K., and Jirik, F.) and others (Schlessinger, J. personal communication) have tried to repeat these REF (or 3T3 for J.S.) transformation experiments without success. This may suggest that PTPα transformation requires additional genetic alterations to induce transformation.

PTPα may be involved in neuronal differentiation as den Hertog et al. (1993) found that expression of PTPα in P19 embryonal carcinoma cells biased their retinoic acid induced differentiation towards cells resembling neurons. Moreover, in support of a role of PTPα in the regulation of Src kinase, these cells contained elevated levels of Src kinase activity. PTPα mRNA expression was also increased in both serum deprived C1003 and dimethylsulfoxide (DMSO) treated N1E-115 cells preceding neuronal differentiation (den Hertog et al., 1993).

1.7.3. PTPα isoforms and covalent modification. Both alternative splicing and post-translational modification may result in multiple PTPα isoforms. Splicing variants include a form of PTPα with an exon removed from the N-terminal catalytic domain (Matthews et al., 1990) and a more commonly observed isoform containing an additional 9 residues in the extracellular domain (Daum et al., 1994). This latter isoform seems to be coordinately expressed with the shorter form of PTPα (Daum et al., 1994; Harder, K. and Jirik, F. unpublished observations). Intriguingly, the 9 residue insert is rich in basic residues and resembles furin-like protease recognition sites detected in other proteins (Hosaka et al., 1991). Proteolysis of the extracellular domains at sequences rich in arginine and lysine residues has been observed in other members of the PTP family (Streuli et al., 1992; Jiang et al., 1993; Pulido et al., 1995). We have evidence that the extra 9-residue containing isoform of PTPα (isolated from a human fetal brain cDNA library) may be subject to proteolysis (Fig. 1.10). The functional relevance of this and other PTP proteolysis is unclear, although in the case of PTPs such as LAR, proteolysis may allow receptor shreading in a cell density-dependent manner (Streuli et al., 1992).

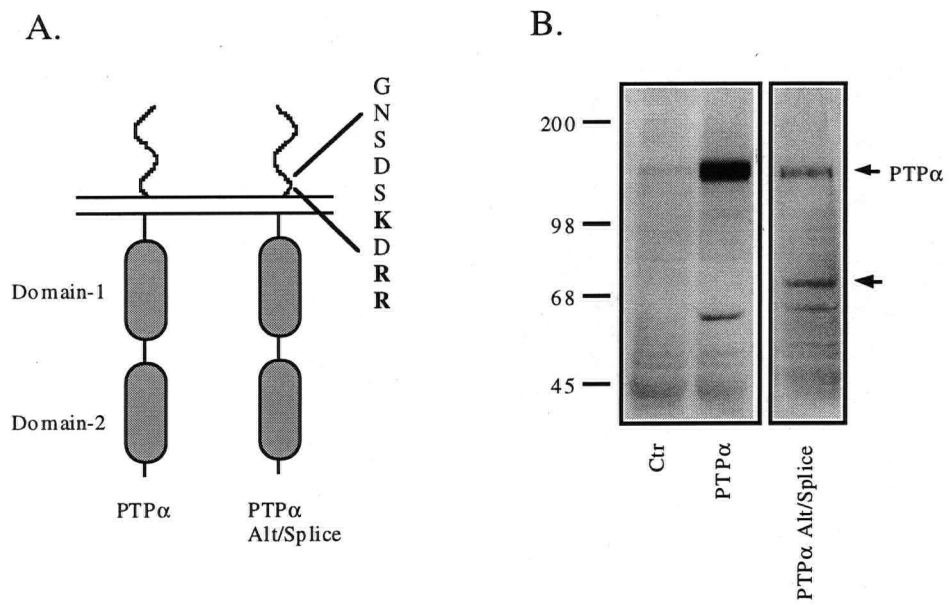


FIG. 1.10. PTPα is alternately spliced in the extracellular domain. (**A**) An alternately spliced form of PTPα contains a 9-residue encoding exon in the extracellular juxtamembrane-region. This sequence is rich in basic residues suggesting a site of proteolytic cleavage. (**B**) Lysate from control (Ctr) or PTPα transfected cells was separated by SDS-PAGE, transferred to membrane and blotted with anti-PTPα antisera (discussed in chapter 3). This antibody detects a 130-150 kDa immunoreactive species in transfected cells. Note that in cells transfected with the alternatively spliced form of PTPα (PTPα Alt./Splice) an additional immunoreactive species at ~70 kDa is apparent. A protein of this molecular weight would be expected if PTPα was proteolytically cleaved within the 9-residue sequence in the extracellular region.

PTPα is also modified by both serine and tyrosine phosphorylation. However, the functional relevance of serine phosphorylation of PTPα is unclear. While den Hertog et al. (1995) found that the activity of PTPα derived from TPA stimulated 293 or P19 cells was increased 2-3 fold, this result could not be confirmed by Tracey et al. (1995). The sites of serine phosphorylation have been mapped to serines 180, 204, and likely serine

202, in the membrane proximal region of PTPα (discussed in more detail in chapter 4). Tyrosine phosphorylation of PTPα appears to lead to the association of PTPα with the small adapter protein Grb2. Binding of Grb2 to PTPα is mediated primarily by the association of the Grb2 SH2 domain with tyrosine phosphorylated PTPα at Y789 (den Hertog et al., 1994; Su et al., 1994). There is also evidence that the C-terminal SH3 domain of Grb2 contributes to the PTPα binding (den Hertog and Hunter, 1996). The functional consequence of Grb2-PTPα association is enigmatic, as PTPα-associated Grb2 is not associated with the guanine nucleotide exchange factor SOS, and thus is unlikely to be involved in Ras activation. The possibility that Grb2 association with PTPα may attenuate phosphatase activity was suggested by the finding that the C-terminal SH3 domain of Grb2 bound to sequences near the active site within the N-terminal PTPα phosphatase domain (den Hertog and Hunter, 1996).

1.8. Thesis Objectives.

- To develop the methods and reagents required to investigate the function of PTPα.
- To assess the substrate specificity of PTPs by expressing and purifying select enzymes in bacteria. This required the development of methods to express, purify, and assay these enzymes.
- To test the hypothesis that PTPs such as PTPα may be able to antagonize the actions of growth factor receptor tyrosine kinases. These experiments required the expression of PTPα in A431 cells, a TGFα/EGF-R-dependent human epidermoid carcinoma cell line.
- To identify the *in vivo* substrates of PTPα in A431 cells.
- To investigate the role of PTPα in cell-substratum adhesion.
- To investigate the structural requirements for the association of PTPα with Src kinases.

CHAPTER 2

Bacterial expression and enzymatic characterization of protein tyrosine phosphatases α and β using synthetic phosphopeptides.

2.1. Summary

To explore PTP substrate specificity the intracellular domains of PTPα and PTPβ were expressed in bacteria using a modification of the pET bacterial expression system, or as Glutathione-S-Transferase (GST) fusion proteins, to facilitate analysis of enzyme activity. Bacterially expressed PTPβ was purified using epitope 'tagging' immunoaffinity chromatography, and characterized with respect to kinetic profile, substrate specificity, and potential modulators of enzyme activity. A chromogenic assay based on the malachite green method was employed for the detection of inorganic phosphate released from phosphopeptides by PTPα and PTPβ. This assay, modified so as to improve its sensitivity, was adapted to a 96-well microtiter-plate format, which provided linear detection of inorganic phosphate between 50 and 1000 pmol. To explore the substrate preferences of PTPβ, we generated four 13-residue synthetic phosphotyrosine-containing peptides that corresponded to sites of physiological tyrosine phosphorylation. The substrate preference of PTPβ was in the order Src Y527 > PDGFr Y740 > ERK1 Y204 >> CSF-1-R Y708 with K_m values ranging from 140 μM for Src Y527 to greater than 10 mM for CSF-1-R Y708 with corresponding K_{cat} values ranging between 76 and 258 s^{-1}. This variation in affinity was likely due to heterogeneity within the phosphopeptide

sequences, such as the presence of charged amino acids neighboring the phosphotyrosine residues. PTPβ appeared to favor phosphotyrosine residues residing in the context of uncharged amino acids. Enzymatic analysis of the intracellular domains of PTPα revealed PTP activity in both cytoplasmic domains. In contrast to PTPβ, PTPα displayed a K_m in the μM range towards the CSF-1-R Y708 peptide, suggesting that although PTPs such as PTPβ may possess some degree of substrate specificity determined by the context of amino acids surrounding a phosphotyrosine residue, other PTPs may not exhibit such selectivity.

2.2. Introduction

While PTPs show considerable heterogeneity within their non-catalytic domains (Charbonneau & Tonks, 1992; Pot & Dixon, 1992), their catalytic domains show striking evolutionary conservation. With few exceptions (Guan et al., 1991a; Ishibashi et al., 1992; Wo et al., 1992), PTPs isolated from yeast (Ottilie et al., 1991; Guan et al., 1991b; Ota & Varshavsky, 1992), *Styela plicata* (Matthews et al., 1991), *Drosophila melanogaster* (Streuli et al., 1989; Hariharan et al., 1991; Tian et al., 1991; Yang et al., 1991), *Caenorhabditis elegans* (Matthews et al., 1991) and mammals (Fischer et al., 1991), share highly characteristic stretches of amino acids. Both intracellular and receptor-like PTPs have been identified. Most receptor-like PTPs, such as the leukocyte common antigen (LCA or CD45), PTPα, and the leukocyte common antigen related protein (LAR), for example, possess two tandemly arranged catalytic domains (Charbonneau & Tonks, 1992; Pot & Dixon, 1992). With the exception of PTPα (Wang & Pallen, 1991), the C-terminal domain of these PTPs appears to be catalytically inactive, and may function primarily to regulate the specificity or activity of the N-terminal PTP domain (Strueli et al., 1990; Wang & Pallen, 1991). Alternatively, the C-terminal PTP domains may be catalytically active under as yet undefined conditions, or, like the STYX protein discussed in chapter 1, function in a PTP-independent manner (Wishart et al. 1995).

Although individual PTPs will likely be found to demonstrate selectivity towards particular phosphoprotein substrates *in vivo*, the potential role of the intracellular domain in such selectivity is unknown. To address this problem, we investigated the potential of the intracellular PTP domains to show substrate specificity *in vitro*. We selected the intracellular domains of PTPβ and PTPα to study the catalytic activities of PTPs *in vitro*. However, detailed analysis of phosphatase activity in terms of kinetic parameters and modifiers of enzyme activity was restricted to PTPβ. Although PTPβ belongs to the

group of receptor-like PTPs, PTPβ (Kreuger et al., 1990), a PTP specifically expressed in mouse brain (Hendriks et al., 1995), and the putative *Drosophila* PTPβ homologue, DPTP10D, are unique in that they possess only a single catalytic domain. DPTP10D is expressed in the CNS of the developing *Drosophila* embryo where it has a potential role in axonal outgrowth and guidance (Yang et al., 1991; Tian et al., 1991). The human PTPβ cDNA is predicted to encode a protein having a 1599 residue extracellular domain, a 21 residue transmembrane domain, and a 355 residue cytoplasmic domain (Krueger et al., 1990). The extracellular region of PTPβ contains a tandem array of 17 ~90 residue segments each containing a fibronectin type III repeat, while the extracellular domain of DPTP10D consists of 12 such units (Tian et al., 1991). Similar repeats have been found in membrane proteins involved in cell-cell interactions, such as the neuronal cell adhesion molecule N-CAM (Edelman, 1987). The single catalytic domain structure of PTPβ makes it ideal for addressing questions regarding the specificity and kinetics of the intact cytoplasmic domain of a receptor-like PTP. Moreover, the use of PTPβ avoided potential complications inherent to an analysis of double-catalytic domain PTPs due either to the presence of the C-terminal PTP domain, or to mutations introduced to remove this domain.

Human PTPα and PTPβ were selected for enzymatic characterization as part of a comparative study to examine whether PTPs are capable of showing substrate-specific differences in activity *in vitro*. To facilitate this analysis we employed a set of synthetic phosphopeptide substrates based on *in vivo* sites of tyrosine phosphorylation.

2.3. Results

2.3.1. Expression of PTPα and PTPβ in bacteria. To express PTPβ in bacteria, the cDNA sequence encoding the entire cytoplasmic domain of PTPβ was amplified from a human placental cDNA library using Vent DNA polymerase. This polymerase was selected for PCR amplification in view of its reported proof-reading ability (New England Biolabs). This cDNA and that of PTPα, isolated as previously described (Jirik et al., 1990), were then introduced into the pET-11a bacterial expression vector (Studier et al., 1990). This vector was modified by addition of an epitope 'tag' decapeptide sequence derived from the influenza hemagglutinin (HA) protein (Field et al., 1988). PTP cDNAs cloned into this plasmid were expressed as fusion proteins consisting of a 12 residue amino terminus S-10 leader peptide from the pET-11a vector, the decapeptide HA-tag flanked by two glycine spacer residues, followed by the PTP cytoplasmic domains (Fig. 2.1). This plasmid was developed with the aim of facilitating the isolation of proteins expressed in this system by epitope 'tagging' immunoaffinity purification. In addition the 12CA5 (HA) epitope allows immunodetection of proteins expressed in bacteria in the absence of protein-specific antisera. The pET system developed by Studier et al. possesses a variety of unique features. Specifically, expression of recombinant proteins is under the control of the T7 RNA polymerase, the production of which is tightly controlled. T7 RNA polymerase is supplied by a copy of the T7 RNA polymerase gene contained within the lysogenic phage DE3 in the bacterial strain *BL21 (DE3)*. Both expression of T7 RNA polymerase and specific cDNA sequences are under the control of the lac operon. Addition of IPTG to bacterial cultures induces the expression of T7 RNA polymerase which leads to the expression of introduced cDNA sequences placed downstream of the T7 RNA polymerase promoter. An inducible expression system such as this is desirable for the efficient expression of proteins which may be toxic to bacteria.

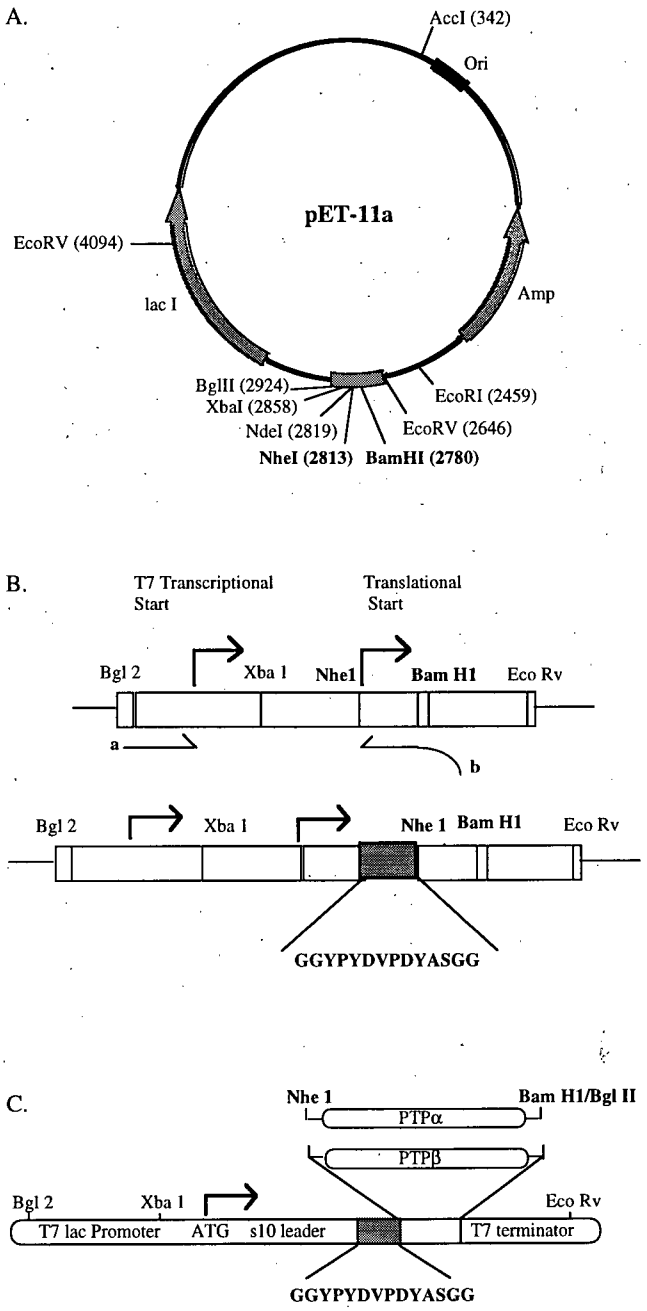


FIG. 2.1. (**A**) Outline of the pET-11a plasmid. (**B**) Construction of the modified pET-11a expression vector. Cloning sites and positions of both the T7 transcriptional and translational starts sites are illustrated. The relative positions of PCR primers a and b used to incorporate the epitope tag are shown. Nucleotides encoding the peptide sequence GGYPYDVPDYASGG were introduced into the pET-11a plasmid: This sequence is recognized by the monoclonal antibody 12CA5. (**C**) The cDNAs encoding PTPα and PTPβ were introduced into the *Nhe 1* and *Bam H1* sites as shown.

Bacterially expressed PTPα and PTPβ were detected by immunoblotting total cell lysates with the mAb 12CA5 (Fig. 2.2). Protein of the predicted molecular weight was detected in the presence of IPTG. In contrast, no detectable expression was observed in the absence of IPTG.

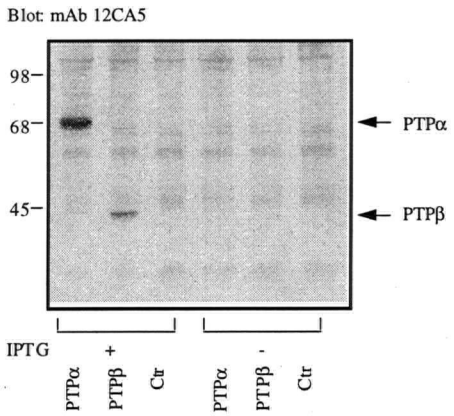


FIG. 2.2. Bacterial cell lysate was separated by SDS-PAGE, transferred to membrane, and immunoblotted with the mAb 12CA5. Lanes correspond to bacterial lysates derived from pET-11a-tag-PTPα (PTPα), pET-11a-tag-PTPβ (PTPβ), and control pET-11a-tag (Ctr) expressing cells. Lysates were obtained from either IPTG induced (+) or uninduced (-) cell cultures as indicated. The positions of PTPα and PTPβ are shown.

To obtain sufficient quantities of recombinant PTPs for a detailed kinetic analysis, large scale protein purification was required. To determine whether the 12CA5 mAb was able to immunoprecipitate the N-terminally 'tagged' PTPs, we immobilized the mAb 12CA5 to protein A Sepharose using the cross-linking agent dimethylpimidilate. This matrix was then incubated with ^{35}S-labeled bacterial lysates. ^{35}S-labeled proteins of molecular weights corresponding to PTPα and PTPβ were specifically immunoprecipitated with 12CA5 mAb-protein A Sepharose from IPTG-induced bacterial cell lysates (Fig. 2.3). The use of ^{35}S-labeled cells and immobilized monoclonal antibody

(mAb) obviated the problem of masking of PTPβ by co-migrating immunoglobulin heavy chain on SDS-PAGE gels. These results demonstrated that the immobilized antibody was able to bind to and immunoprecipitate the 'tagged' PTPs from bacterial lysates. However, low yields of immunoprecipitated protein were observed and ^{35}S-labeled bands corresponding to PTPα and PTPβ were not detected in lanes containing total cell lysate (Fig. 2.3).

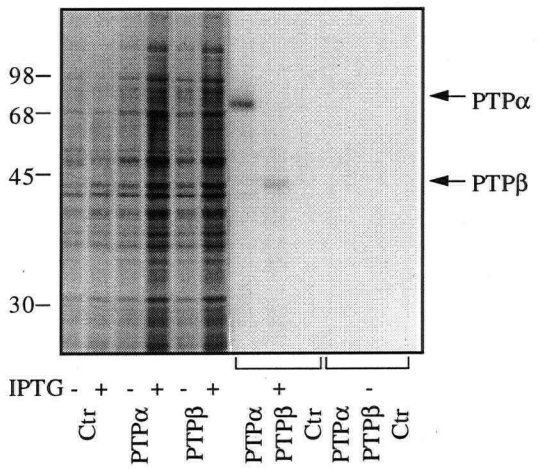


Fig. 2.3. Autoradiograph of ^{35}S-labeled bacterial proteins. Lysates were derived from bacteria containing pET-11a-tag vector (Ctr), pET-11a-tag-PTPα (PTPα), or bacteria containing pET-11a-tag-PTPβ plasmid (PTPβ). Lanes 1-6 correspond to total cell lysates from cells either induced with IPTG (+) or uninduced (-) as indicated. Lanes 7-12 contain 12CA5 immunoprecipitated ^{35}S-labeled protein from IPTG induced (+) or uninduced cells (-). The positions of PTPα and PTPβ are indicated.

The 12CA5 mAb-Sepharose matrix was then used to immunopurify larger quantities of each PTP. PTPα and PTPβ were immunopurified with the 12CA5 matrix and eluted with HA peptide at 30^{0}C. Protein purification and elution was assessed by western blot analysis with the mAb 12CA5 (Fig. 2.4A). To determine whether the recombinant PTPs were catalytically active, we assayed PTP activity with ^{32}P–labeled

tyrosine phosphorylated T-cell receptor ζ-chain peptide. ^{32}P release was measured by scintillation counting as outlined in materials and methods. Both PTPα and PTPβ were catalytically active as demonstrated by dephosphorylation of tyrosine phosphorylated ζ-chain (Fig. 2.4B)

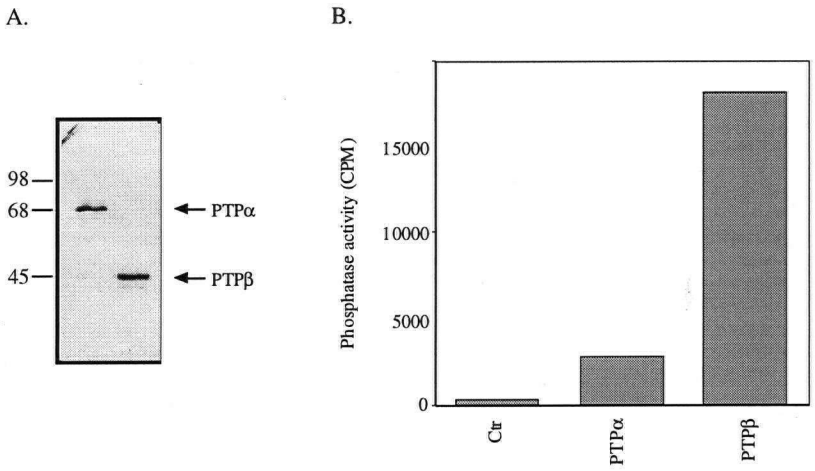


Fig. 2.4. Bacterially-expressed PTPα and PTPβ are enzymatically active. (**A**) Both PTPα and PTPβ were immunoprecipitated from IPTG induced bacterial cell lysates with 12CA5-protein A Sepharose. Recombinant PTPs were eluted with synthetic peptide corresponding to the HA epitope as described in materials and methods. A fraction of each purification was fractionated by SDS-PAGE, transferred to membrane, and immunoblotted with the 12CA5 mAb. (**B**) A sample of each enzyme, or eluate from a control bacterial lysate 12CA5-protein A Sepharose purification, was incubated with ^{32}P-labeled tyrosine phosphorylated ζ-chain peptide. Phosphatase specific ^{32}P release was then determined by scintillation counting.

2.3.2. Bacterial expression and purification of PTPβ. To further characterize PTP activity we focused on the large scale purification and kinetic analysis of PTPβ. PTPβ was chosen in light of its consistently higher expression levels, its higher catalytic activity, and because its single catalytic domain structure was more amenable to

Michaelis-Menten kinetics. PTPβ was purified to apparent homogeneity in quantities sufficient for kinetic studies by immunoaffinity purification on the 12CA5 mAb affinity column followed by MonoQ-Sepharose chromatography, as outlined in the experimental procedures section (Fig. 2.5).

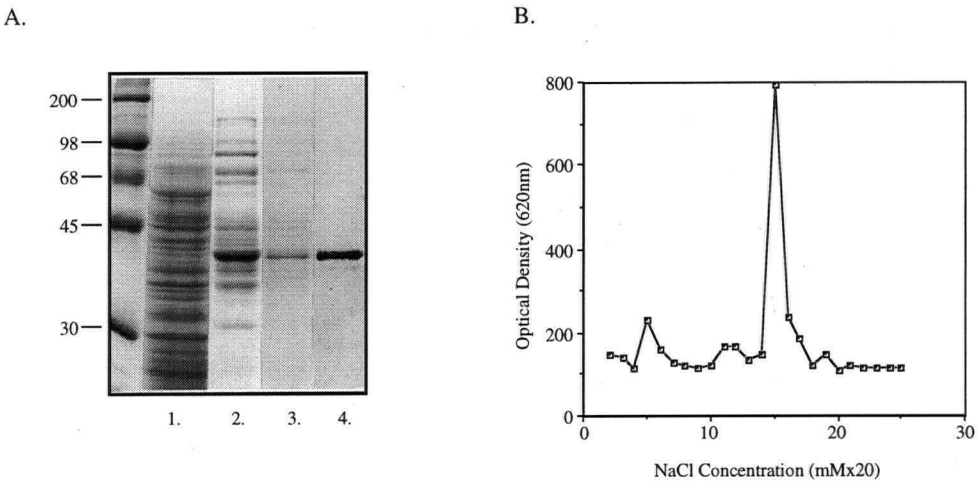


FIG. 2.5. PTPβ purification by immunoaffinity and monoQ Sepharose chromatography. (**A**) Proteins were separated by SDS-PAGE and stained with Coomassie Blue (lanes 1, 2, and 3), or transferred to membrane and immunoblotted with the 12CA5 mAb (lane 4). Lane 1, bacterial total cell lysate. Lane 2, protein eluate from the mAb 12CA5 immunoaffinity column. Lane 3, PTPβ following MonoQ chromatography. Lane 4, 12CA5 immunoblot of the partially purified PTPβ intracellular domain. (**B**) MonoQ fractionation of PTPβ. PTPβ was eluted from the MonoQ column with a 100-500 mM NaCl gradient. Analysis of PTP activity in each fraction was determined as outlined in materials and methods. Fractions containing PTPβ were pooled and saved.

Typically, between 10-50 μg PTPβ/litre culture was recovered from the 12CA5 column upon peptide elution. Increased recovery of protein was achieved by re-application of the bacterial lysate to regenerated affinity columns. PTPβ was quantified by comparison to serially-diluted bovine serum albumin and ovalbumin standards. The

enzyme was stable for over 4 months when stored in 20% glycerol at -80°C, or for 3-4 weeks when stored at 4°C. The enzyme, however, became inactive when subjected to repeated freeze-thawing.

2.3.3. Malachite green microtiter-plate assay. The analysis of PTP activity has previously relied on the measurement of ^{32}P release from various tyrosine phosphorylated substrates (i.e. ζ-chain assay, as above). However, such assays require the use of freshly prepared radioactive substrates, a supply of tyrosine kinase activity, and demand laborious substrate purification procedures. PTP substrates prepared in this way are also usually phosphorylated to a low stoichiometry leading to potential problems of product inhibition during enzyme kinetic analysis. To facilitate the analysis of PTPs, we modified the malachite green colorimetric assay, an assay based on the detection of phosphate in acidic solutions of malachite green. In the presence of inorganic phosphate, the color of malachite green changes from orange to dark green, allowing colorimetric detection of phosphate release. Malachite green has been used previously for the detection of inorganic phosphate in the analysis of calcineurin (Lanzetta et al., 1979) and more recently in the study of the activities of the PTPs LAR and CD45 on synthetic phosphopeptides (Cho et al., 1991, 1992). However, the assays used in these studies were relatively insensitive. For example, previous assays provided linear detection between 1 nmol and 10 nmol of P_i (Lanzetta et al., 1979; Cho et al., 1991, 1992). Additionally, these assays were not favorable for kinetic analysis or large scale screening for modifiers of enzyme activity due to cumbersome spectrophotometer color detection. We modified the malachite green assay by adapting it to a microtiter-plate format and optimized the assay with respect to a variety of conditions. We found that the addition of non-ionic detergents was crucial for linear detection of P_i. Specifically, the addition of 0.01 % Tween 20 was required for linear P_i detection above 300 pmol (Fig. 2.6A). Color development times were optimized to between 15 and 30 min. This incubation time was particularly

important when Pi levels in the 1-2 nmole range were present (Fig. 2.6B). We also found that a wavelength of 620 nM was optimal for color detection (Fig. 2.6C).

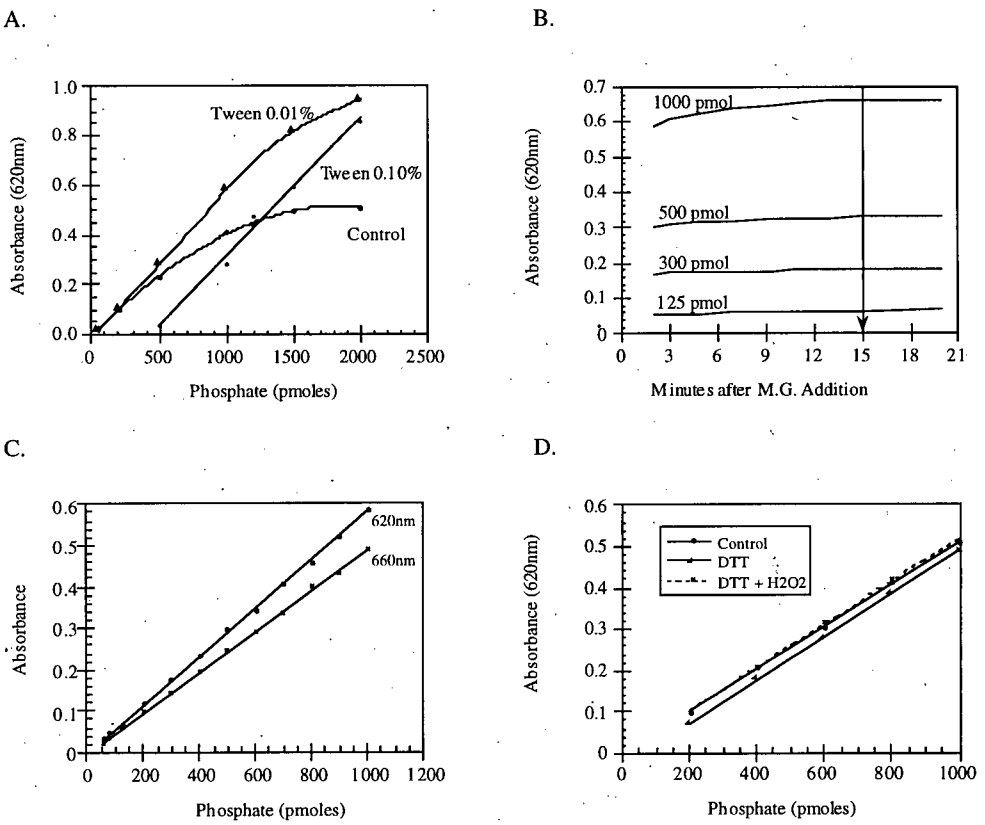


FIG. 2.6. The effect of non-ionic detergent concentrations (**A**), color development time (**B**), wavelength (**C**), and reducing agents (**D**) on P_i detection was determined. The absorbance (620 or 660 nM) of malachite green solution in each well was determined in the presence of the outlined quantities of P_i.

The activity of PTPs requires the presence of reducing agents to maintain the reduced state of a critical cysteine residue in the catalytic site centre. However, the presence of dithiothreitol (DTT) used in the PTP assays interfered with malachite green color development. Standard PTP assays contain between 1-5 mM DTT. We found that addition of equimolar concentrations of the oxidizing agent H_2O_2 abolished the

inhibitory effects of reducing agents on the malachite green microtiter-plate (MGMP) assay (Fig. 2.6D). Subsequent studies have determined that reducing agents such as 2-mercaptoethanol (2-ME) at concentrations between 1-5 mM are not inhibitory to the MGMP assay. Either the addition of H_2O_2, or the use of 2-ME circumvented problems with reducing agent on color development. Fig. 2.7 illustrates a standard curve for P_i detection with the modified malachite green microtiter-plate assay; P_i detection is linear between 50 pmoles and 1000 pmoles.

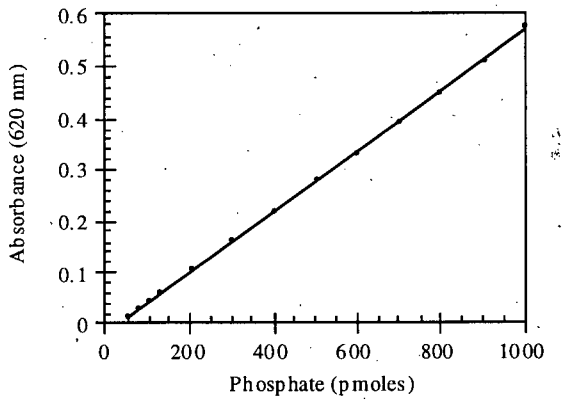


FIG. 2.7. Malachite green microtiter-plate assay standard curve. The absorbance (620 nM) of appropriately diluted P_i standards was determined 15 min after the addition of 50 µl malachite green solution to each well.

While 1 nmol P_i yielded an absorbance of 0.1 unit in previous assays, the microtiter-plate assay reported here achieved an absorbance of 0.6 for the same quantity of P_i. Using the microtiter-plate assay, enzyme reactions and released P_i detection are carried out in microtiter-plate wells, eliminating the need for sample transfer and chamber cleaning associated with the use of cuvettes when a spectrophotometer is used for P_i detection. The microtiter-plate reader also has the advantage of allowing optical density determination of multiple reaction samples simultaneously.

The MGMP assay has also been successfully adapted for the detection of serine/threonine phosphatase activity (Roberge et al., 1994).

2.3.4. Preparation of phosphotyrosine substrate peptides. To study both the reaction kinetics of PTPβ, and the substrate preferences of this PTP, four 13-residue peptides were synthesized containing phosphotyrosine residues corresponding to sites of *in vivo* phosphorylation. PTPβ catalyzed release of P_i from the various synthetic phosphopeptides was then determined using the MGMP assay. As little information exists regarding the three-dimensional structure of tyrosine phosphorylation sites within intact proteins, the assumption was made that such sites might be located at the centre of an exposed segment of primary sequence. Thus, the phosphotyrosine residue was placed centrally within the synthetic peptides. Peptides of 13 residues were selected: (i) to maintain as much of the sequences normally surrounding the phosphotyrosine residues as possible, while yet being of a length that minimized the potential for peptide-specific secondary structures; and (ii) for their heterogeneity with regard to the composition of the residues flanking the tyrosine residue. The following phosphopeptides were synthesized (Table I): (1) A peptide containing the C-terminal phosphorylation site of Src. This sequence contains Tyr 527 which has been linked to the negative regulation of Src kinase activity (discussed in chapter 1 and reviewed in Hunter, 1987). (2) A peptide corresponding to sequence containing Tyr 740 of the human PDGF-R β-chain. Autophosphorylation on tyrosine residues triggered by ligand binding to growth factor receptors such as the PDGF-R or CSF-1-R leads to an increase in the tyrosine kinase activity of these receptors, and also to the binding of second messenger molecules such as PI3K (Koch et al., 1991; Cantley et al., 1991). Phosphorylation of the β-chain of the PDGF-R on Tyr 740 and Tyr 751 has been shown to mediate PI3K association with the activated receptor both *in vitro* and *in vivo* (Escobedo et al., 1991; Kashishian et al., 1992). (3) A peptide corresponding to the sequence containing Tyr 204 of the human

extracellular signal-regulated kinase (ERK) 1 kinase. The ERK or mitogen-activated protein (MAP) kinases are a family of phosphorylation-regulated kinases thought to form a link between membrane PTK activation and serine/threonine phosphorylation cascades in the cytoplasm (Posada & Cooper, 1992; Thomas, 1992). Phosphorylation of ERK1 on Tyr 204 and Thr 202 , is associated with increased activity of this serine/threonine kinase (Anderson et al. 1990; Payne et al., 1991). (4) A peptide corresponding to the sequences flanking tyrosine 708 of the human CSF-1-R. Although the CSF-1-R Tyr 708, and its murine counterpart Tyr 706, are known to be phosphorylated following stimulation with CSF-1 (van der Geer and Hunter, 1990; Reedijk et al., 1992), the function of this phosphorylation is unknown. As the sequence surrounding this Tyr is identical in the CSF-1-R of both species, a conserved function is suggested for this region.

2.3.5. Kinetic analysis of PTPβ with phosphotyrosyl containing peptides. The specificity of PTPβ for phosphotyrosine was tested using the free amino acids phosphotyrosine, phosphothreonine, and phosphoserine as substrates. While PTPβ catalyzed the hydrolysis of phosphotyrosine, it showed no detectable activity toward the other phospho-amino acids tested (data not shown).

Table I: Kinetic Parameters of PTPβ

Substrate		K_m *mM*	V_{max} *umol/min/mg*	K_{cat} s^{-1}
TSTEPQY*QPGENL	*src*^{Tyr527}	0.142± 0.009	350 ± 10	258
GESDGGY*MDMSKD	PDGF-R^{Tyr740}	0.175± 0.007	272 ± 4	200
TGFLTEY*VATRWY	ERK1^{Tyr204}	0.330± 0.03	240 ± 13	177
IHLEKKY*VRRDSG	CSF-1R^{Tyr708}	>10.0	163	120
	p-NPP	2.5 ±0.5	103 ± 9	76

[a] kinetic data were generated under conditions in which less than 20% of substrate was converted to product, and were analysed using the enzyme kinetics program Enzfitter (Biosoft U.K.). All assays were performed in duplicate with similar results obtained in two or more separate experiments

Y*- phosphotyrosine

Previous studies of the first catalytic domain of LAR demonstrated that the affinity of this PTP for different members of a panel of synthetic phosphopeptides varied between 27 μM (a 12-mer) and 4.1 mM (a 6-mer), a 150-fold difference (Cho et al., 1991). However, the peptides selected for this analysis of LAR were not uniform in length, varying between 6 and 12 residues in length. Variation, with a range of 2 to 8 residues, was also present with respect to the number of residues flanking a given phosphotyrosine. Thus, some of the observed differences in the affinity of LAR for these substrates was likely due to the overall design of the synthetic peptides. In this kinetic study of PTPβ, the phosphopeptides selected were homogeneous with respect to length as well as to placement of the phosphotyrosine residue within the sequence (Table I). The results of the kinetic analysis of PTPβ are presented in Table I.

K_{cat} values ranged between 76 and 258 s^{-1}, based on a PTPβ molecular weight of 44,000. A K_{cat} of 258 s^{-1} for the phosphotyrosyl peptide Src Y527 is approximately 3.7 times that observed for LAR-D1 on a similar Src Y527 phosphopeptide (Cho et al., 1991). Comparison of the catalytic activity of PTPβ on Src Y527 with the activities of CD45 and LAR previously reported, revealed that PTPβ had a V_{max} approximately twice that of CD45, and six times greater than that of LAR-D1 on Src Y527 phosphopeptide (Cho et al., 1992). In addition, the V_{max} obtained with the Src Y527 substrate is approximately 30-times greater than that reported for PTPβ using phosphorylated RR-Src peptide as a substrate (Wang & Pallen, 1992). Although this difference in activity may have been due to differences intrinsic to the peptide substrates employed, it likely resulted from differences in the pH of the enzyme assays. Wang and Pallen (1992) assayed PTPβ activity at pH 6.0. We found PTPβ favoured more alkaline conditions, with a narrow optimum at pH 8.0 (data not shown). This was similar to the previously reported pH optimum for this PTP (Cho et al., 1992).

A K_m of 140 μM for the Src Y527 phosphotyrosyl peptide was similar to that of 170 μM obtained for the PDGFr Y740 peptide. The K_m for the ERK1 Y204 peptide at

330 μM was approximately twice that of either the Src Y527 or the PDGFr Y740 peptide. Peptide CSF-1-R Y708 was a relatively poor substrate for PTPβ. The K_m for this peptide was greater than 10 mM, approximately 90-times higher than that of the Src Y527 peptide. The sequences surrounding the tyrosine residue of the CSF-1-R peptide are unusual as compared to the majority of sites of tyrosine phosphorylation that have been identified (Pearson & Kemp, 1991), in that the phosphotyrosine residue is flanked on the amino terminal side by two basic (Lys-Lys) residues and by two additional basic (Arg-Arg) residues on the C-terminal side of the phosphotyrosine residue. Peptide ERK1 Y204 contains an acidic (Glu) residue neighboring tyrosine 204. In contrast, peptides Src Y527 and PDGFr Y740, the best substrates of PTPβ in this study, contained uncharged amino acids surrounding the phosphotyrosine residue. PTPβ catalyzed the removal of phosphate from *p*-NPP with a Km of 2.5 mM, a K_m superior to that for peptide CSF-1-R Y708. This finding suggests that the peptide sequence surrounding the phosphotyrosine residue of the CSF-1-R Y708 may inhibit PTPβ recognition of this site.

2.3.6. PTP activity modifiers. The activity of PTPβ was tested in the presence of a variety of potential modifiers. PTPβ was inhibited by the cations Ca^{2+}, Mg^{2+} and Mn^{2+} (Fig. 2.8A). Mn^{2+} inhibition of PTPβ was the strongest of the three cations tested with 26 % of control activity at 10 mM Mn^{2+}. The inhibition observed however, required high concentrations of the three cations tested. Itoh et al. (1992) found that both Ca^{2+} and Mg^{2+} stimulated PTPβ activity at concentrations of 10 mM. Interestingly, we found that when the substrate concentration was increased 10-fold, from 200 μM to 2 mM Src Y527 phosphopeptide, the inhibitory effects of these cations was eliminated, and a stimulation of 107 % and 110 % was observed for 10 mM Ca^{2+} and Mg^{2+} (data not shown). The PTP inhibitors vanadate and molybdate also inhibited PTPβ activity with IC_{50}s of 130 nM and 160 μM for vanadate and molybdate respectively (Fig. 2.8B and C).

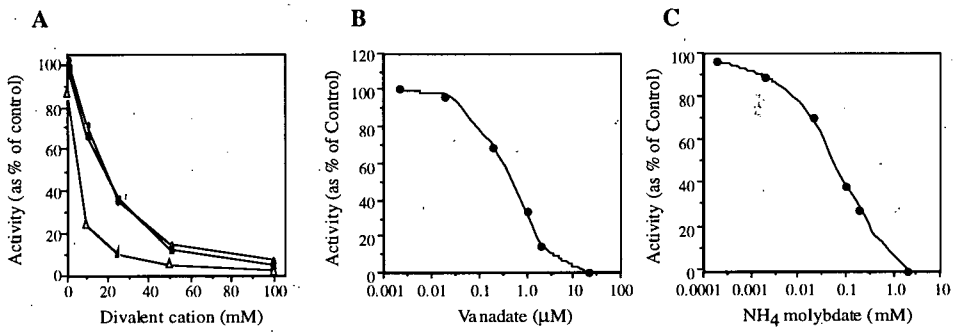


FIG. 2.8. The effects of various potential modifiers of PTPβ activity. (**A**) Effects of the cations (○) Ca^{2+}, (■) Mg^{2+} and (Δ) Mn^{2+} (**B**), sodium vanadate and (**C**), NH_4 molybdate. Values are expressed as % of control activity of PTPβ using 200 μM Src Y527 substrate peptide.

The effects of other potential modifiers of enzyme activity are summarized in table II. The serine/threonine protein phosphatase inhibitor NaF marginally stimulated the activity of PTPβ at a concentration of 10 μM , whereas 72 % of control activity was observed at 10 mM NaF. In contrast, Zn^{2+} was a potent inhibitor of PTPβ activity at low μM concentrations. Phenylarsine oxide (PAO) has been reported to be a potent inhibitor of the PTP CD45 with an IC_{50} between 5 μM and 10 μM (Garcia-Morales et al., 1990). In this study PAO stimulated the activity of PTPβ at concentrations reported to strongly inhibit CD45. PAO has been reported to have no effect on rat LAR at concentrations 10-fold higher than those used to inhibit CD45 (Pot et al., 1991). Together these results and those of Pot et al. suggest that PAO may be specific for CD45 or perhaps for a subgroup of PTPs. The polycation spermine has been reported to stimulate the catalytic activity of PTPβ by 244% and 265% at 10μM and 100 μM respectively, while inhibiting at 10 mM when tested on the artificial substrate [^{32}P] Raytide (Itoh et al., 1992). The effects of various concentrations of spermine on the activity of PTPβ on the substrates Src Y527 (Table II) and *p*-NPP (data not shown) were tested. Spermine had little or no effect at 20

μM and 200 μM, whereas inhibition by spermine was observed with concentrations of 2.0 mM and 20 mM. The nature of the differences between these results and those of Itoh et al. (1992) with respect to the effects of spermine, $Ca^{2+}$and Mg^{2+} on the activity of PTPβ are unclear. They may reflect the nature of the substrates used, or the conditions used in the enzyme assays. Alternatively, the different fusion sequences at the N-termini of the proteins might affect their susceptibility to specific modulators. The polyanion heparin, and the polymer Glu/Tyr (4:1), both strongly inhibited the activity of PTPβ at 1.0 μM. Inhibition was observed with both compounds at concentrations as low as 10 nM. Kinetic analysis of placental PTPs (Tonks et al., 1988) indicated that poly-Glu/Tyr (4:1) inhibition of PTP 1B was noncompetitive, and suggested a binding site for polyanionic molecules distinct from the active site. Studies of the effects of various polyanionic and polycationic amino acid polymers such as poly-Glu/Tyr (4:1) on the enzymatic activities of PTPβ, LAR and CD45 have revealed a positive correlation between the tyrosine content of such polymers and their potency as inhibitors (Itoh et al., 1992).

Table II: modulators of PTPβ activity with 200 μM *src*Tyr527 peptide

Effector	Concentration	PTPase Activity (% of Control)
$ZnCl_2$	0.1 μM	106
	1.0 μM	70
	5.0 μM	58
	25.0 μM	40
	75.0 μM	24
NaF	10.0 μM	114
	100.0 μM	110
	1.0 mM	105
	10.0 mM	72
EDTA	1.0 mM	98
	10.0 mM	81
Spermine	20.0 μM	102
	200.0 μM	102
	2.0 mM	85
	20.0 mM	19
Heparin	10.0 nM	93
	0.1 μM	68
	1.0 μM	32
	10.0 μM	17
Poly E/Y (4:1)	10.0 nM	80
	0.1 μM	46
	1.0 μM	33
PAO	10.0 μM	112
	100.0 μM	110
	1.0 mM	104

a All assay were performed in duplicate, in two or more separate experiments

*2.3.7. **Bacterial expression of PTPβ using the pGex system.*** Although the modified pET-11a-tag vector allowed the purification of PTPβ by immunoaffinity techniques in quantities sufficient for enzyme analysis, the yields of purified protein where consistently lower than those reported for other expression systems. Moreover, high level expression and purification of PTPα was not possible using this system.

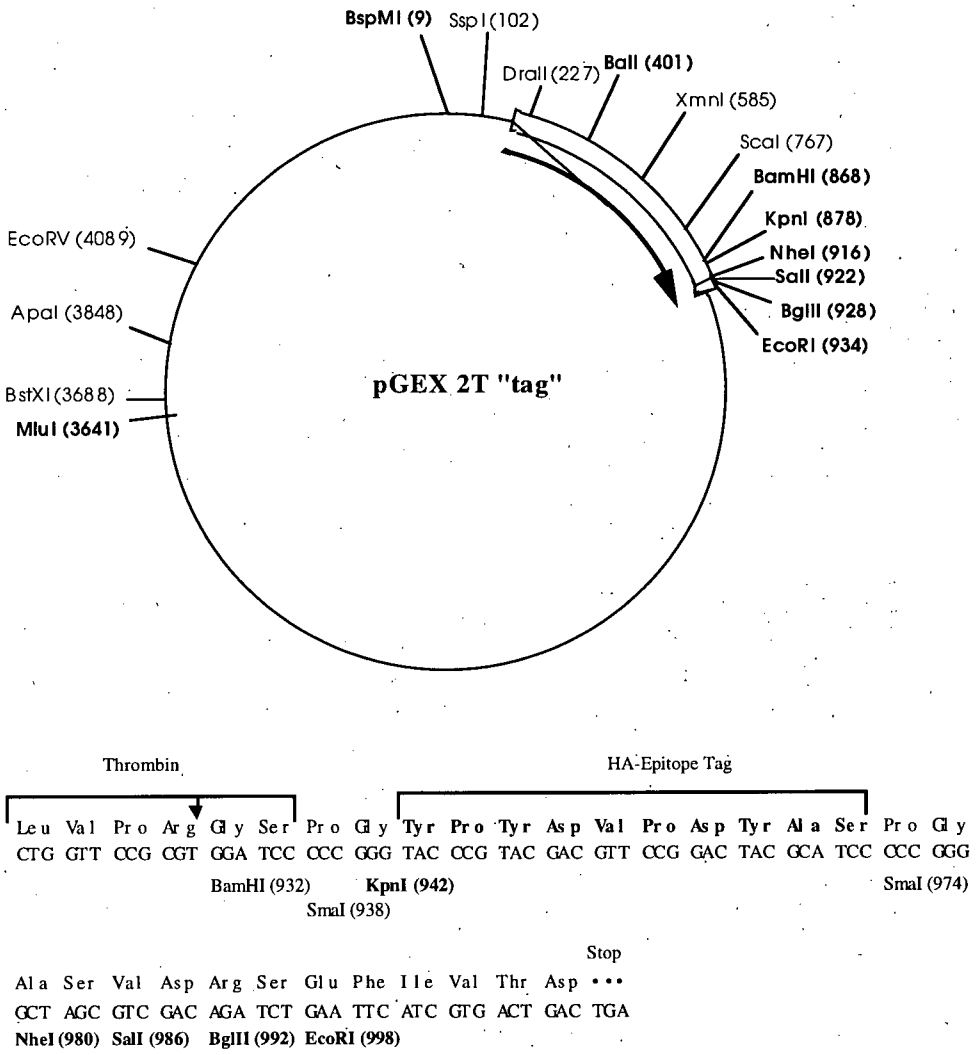


FIG. 2.9. Outline of the pGex-2T-tag plasmid. The multiple cloning site of the modified pGex-2T-tag vector is shown. The thrombin cleavage site and sequence encoding the 12CA5 epitope tag are indicated. Regions encoding the cytoplasmic PTP domains of PTPα and PTPβ were subcloned into the *NheI* and *BglII* sites of pGex-2T-tag.

To address these concerns we modified the existing pGex bacterial expression plamid pGex-2T to accommodate cDNAs designed for the pET system. This involved the addition of the HA-epitope encoding sequence to pGex 2T in the context of an expanded polylinker (Fig. 2.9). These modifications provided the advantages of the pET system, such as the ability to detect proteins with the 12CA5 mAb, with high level protein production and glutathione Sepharose/Glutathione-S-Transferase (GST) purification procedures provided by the pGex system. The cDNA encoding PTPβ was subcloned into pGex-2T-tag and protein expression and purification was assessed. Proteins expressed with the pGex system exist as fusion proteins with GST. However, thrombin cleavage allows the removal of GST from the C-terminal fusion partner. PTPβ was purified with glutathione Sepharose and cleaved with increasing concentrations of thrombin (Fig. 2.10).

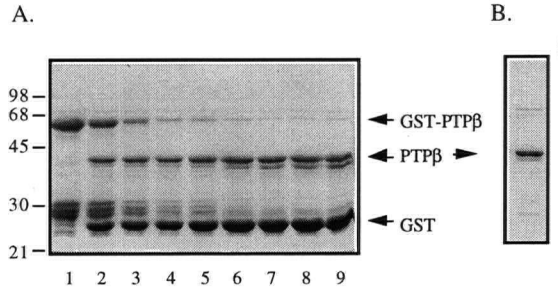


FIG.2.10. Bacterial expression and thrombin cleavage of PTPβ. (A) PTPβ was expressed in bacteria using the pGex system. Recombinant protein was purified with glutathione Sepharose and either left uncleaved (lane 1) or cleaved with increasing concentrations of thrombin (lanes 2-9). Reactions were then separated by SDS-PAGE and the gel stained with Coomassie brilliant blue. Positions of uncleaved GST-PTPβ, thrombin-cleaved PTPβ, and free GST are indicated. (**B**) Glutathione purified PTPβ was thrombin cleaved and a sample of supernatant was separated by SDS-PAGE and stained with Coomassie blue.

The addition of thrombin resulted in the appearance of an approximately 40 kDa protein and an intensely staining protein at 28 kDa corresponding to GST. However, at

high thrombin concentrations another protein product was observed that corresponded to a proteolytic fragment of PTPβ (Fig. 2.10). Thus, although use of the pGex system resulted in dramatically higher protein production and facilitated protein purification, care was required for the production of GST-free recombinant protein.

2.3.8. Bacterial expression and characterization of PTPα enzymatic activity. Various forms of PTPα were expressed and purified using the pGex-2T-tag expression system. The entire cytoplasmic region of PTPα (PTPα-D1+D2), the membrane-proximal catalytic

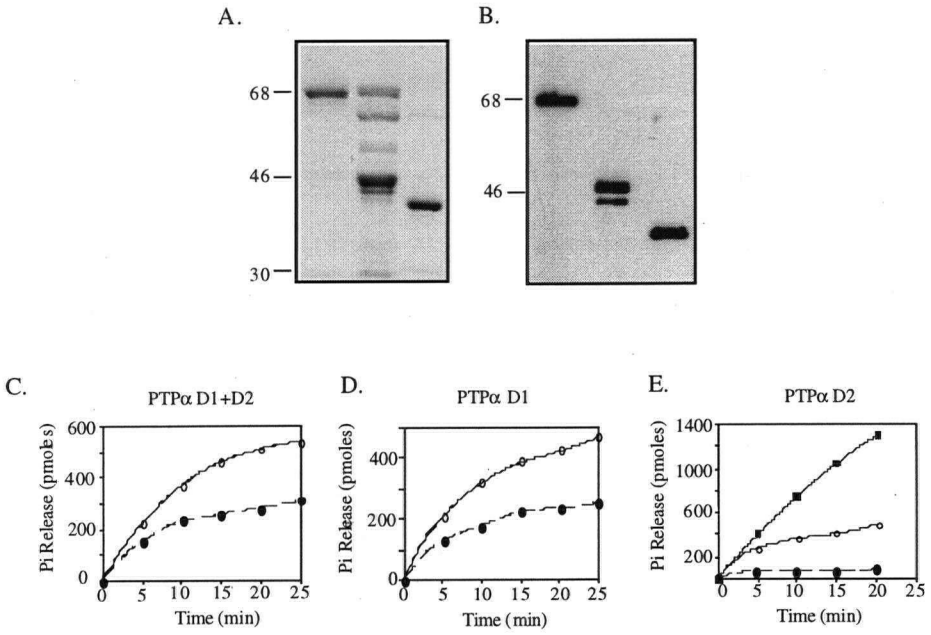


FIG. 2.11. Bacterial expression and purification of various regions of PTPα. The entire cytoplasmic region of PTPα (PTPα-D1+D2) lane 1, the membrane-proximal catalytic domain (PTPα-D1) lane 2, and the second catalytic domain alone (PTPα-D2) lane 3 were partially purified with glutathione Sepharose and thrombin cleaved before being fractionated by SDS-PAGE and either (**A**) stained with Coomassie blue or (**B**) immunoblotted with the 12CA5 mAb. Partially purified enzymes corresponding to (**C**) PTPα-D1+D2, (**D**) PTPα-D1, and (**E**) PTPα-D2, were then assayed for activity against *p*-NPP (■) (for PTPα-D2) or the Src Y527 (○), and CSF-1 Y708 (●) phosphopeptides.

domain (PTPα-D1) and the second catalytic domain alone (PTPα-D2) were expressed as GST-fusion proteins. Glutathione Sepharose-purified, thrombin-cleaved recombinant enzymes were fractionated by SDS-PAGE and stained with Coomassie blue (Fig. 2.11A) or transferred to membrane and immunoblotted with the mAb 12CA5, specific for the N-terminal epitope tag in each fusion protein (Fig. 2.11B). Purified enzymes were incubated with phosphopeptides corresponding to the C-terminal sequence of Src Y527 or with CSF-1-R Y708 and phosphate release was determined using the MGMP assay. PTPα-D1+D2 and PTPα-D1 catalyzed the dephosphorylation of each phosphopeptide, both showing a preference for the src Y527 peptide substrate over peptide CSF-1-R Y708 (Fig. 2.11C and D). Enzyme activity of the second catalytic domain of PTPα was demonstrated by its ability to hydrolyze *p*-NPP and dephosphorylate the Src Y527 phosphopeptide (Fig. 2.11E). PTPα-D2, like PTPα-D1+D2 and PTPα-D1, preferred the Src Y527 phosphopeptide substrate to peptide CSF-1-R Y708. However, detection of PTPα-D2 activity required greater than 10 fold more enzyme than those fusion proteins containing PTPα-D1. Preliminary analysis of the kinetics of PTPα dependent dephosphorylation of these peptides revealed surprisingly similar K_ms (Table III). These results were in sharp contrast to the K_ms observed for these peptides with PTPβ.

Table III:Kinetic Parameters of PTPα dom1+2 and dom1 alone

Substrate		PTPα dom1+2 K_m	PTPα dom1 K_m
		mM	mM
TSTEPQY*QPGENL	*src*Tyr527	0.070	0.046
IHLEKKY*VRRDSG	CSF-1RTyr708	0.197	0.184

[a] kinetic data were generated under conditions in which less than 20% of substrate was converted to product, and were analysed using the enzyme kinetics program Enzfitter (Biosoft U.K.). All assays were performed in duplicate with similar results obtained in two or more separate experiments.

Y*- phosphotyrosine

2.4. Discussion

This work was initiated to develop an efficient method for the production and characterization of recombinant PTPs. To facilitate the study of PTP enzyme activity, enzymes were expressed and partially purified using a variety of bacterial expression systems. Although PTPα and PTPβ were initially expressed with the pET bacterial expression system, subsequent PTP expression and purification was facilitated by using the pGex system. Recombinant enzymes were used to assess the substrate specificity and the structure function of these enzymes by assaying their activity against 13 residue synthetic phosphopeptides corresponding to physiological sites of tyrosine phosphorylation.

Bacterial expression of the cytoplasmic domains of PTPα either alone or in combination was also useful in assessing PTPα catalytic activity in terms of its double domain structure. Both the N- and C-terminal domains of PTPα dephosphorylated Src Y527 phosphopeptide. Thus, unlike the C-terminal PTP domains of LAR and CD45, the second catalytic domain of PTPα is catalytically active (Strueli et al., 1990). This is in agreement with results of Wang and Pallen (1991) who demonstrated activity of PTPα domain-2 against *p*-NPP and RR-src. We also found that PTPα-D2 preferred p-NPP to phosphopeptide based substrates.

Much is known about the location and function of tyrosine phosphorylation sites within intracellular proteins. The insulin receptor β-chain, the epidermal growth factor receptor, and the kinase-insert regions of several other receptor PTKs, such as the PDGF and CSF-1 receptors, contain physiologically important phosphotyrosine residues that are found in close proximity to one another. For example, the kinase-insert region of the PDGF-R contains three tyrosine phosphorylation sites within a stretch of 32 amino acid residues (Kashishian et al., 1992; Kazlaukas et al., 1992; Reedijk et al., 1992). The human insulin receptor β-chain (Ullrich et al., 1985) has three autophosphorylation sites

at residues 1146, 1150 and 1151 (White et al., 1988; Flores-Riveros et al., 1989). These observations suggest that PTKs and PTPs, likely recognize substrate tyrosine and phosphotyrosine residues, respectively, within the context of a short segment of primary sequence *in vivo*.

As the PTPs demonstrate great heterogeneity in their non-catalytic domains, it might be argued that the specificity of a given PTP for its phosphoprotein substrate might be determined by protein-protein associations that rely on the non-catalytic regions of the PTPs. If correct, isolated PTP catalytic domains, tested *in vitro* on phosphopeptide substrates, would be predicted to show a relative lack of specificity. As demonstrated by the substrate specificity of PTPβ, this may not be the case. In addition, there is other evidence of PTPs exhibiting substrate-specific differences in activity (Cho et al., 1991; Wang and Pallen, 1991; Cho et al., 1992; Ramachandran et al., 1992). In particular, a triphosphorylated synthetic peptide, derived from the insulin receptor β-chain sequence, has been used in a study using the PTPs CD45, LAR, T-cell PTP, and PTP 1B (Ramachandran et al., 1992). In these experiments, the PTPs differed in the order with which they dephosphorylated the triphosphorylated peptide, illustrating PTP substrate preferences within this 12 residue sequence.

This analysis of PTPα and PTPβ has extended the observation that specific PTPs are able to show substrate-specific differences in activity *in vitro*. PTPβ in particular demonstrated variations in affinity for the synthetic phosphopeptides selected. These variations appeared to be determined by the composition of the sequences flanking the phosphotyrosine residue. Specifically, the CSF-1 Y708 peptide was a very poor substrate for PTPβ. Subsequent studies conducted in this lab with an extended panel of phosphopeptides have demonstrated that SHP-2, like PTPβ, discriminated against phosphopeptides rich in basic residues (Dechert et al., 1994). Whether the observed differences in specificity are of relevance to the *in vivo* functions of these PTPs remains to be determined, however one may speculate that it is unlikely that PTPs such as PTPβ

and SHP-2 will be found to select substrates sequences rich in basic residues. If other PTPs exhibit similar differences in specificity when tested on panels of synthetic phosphopeptide substrates, this information may prove helpful in predicting the *in vivo* substrate(s) of a given PTP.

CHAPTER 3

Increased cell-substratum adhesion and activation of Src family kinases by overexpression of PTPα

3.1 Summary

The role of protein tyrosine phosphatases (PTPs) in the regulation of cell growth, differentiation and cell adhesion is poorly understood. To explore the possibility that specific PTPs may alter cell signaling pathways initiated by growth factor receptor stimulation, we overexpressed the receptor-like PTP, PTPα, in the human epidermoid carcinoma cell line A431. These cells overexpress the epidermal growth factor (EGF) receptor tyrosine kinase and proliferate in response to autocrine production of transforming growth factor-alpha (TGFα). Although PTPα was unable to alter the growth kinetics of A431 cells in either the presence or absence of EGF, PTPα expression increased A431 cell-substratum adhesion and rescued these cells from EGF induced cell-rounding. Moreover, PTPα overexpression was associated with the activation and/or dephosphorylation of specific members of the Src tyrosine kinase family. In support of a role for PTPα in the regulation of cell adhesion, there was an increase in association of Src kinases with the integrin-associated molecule, focal adhesion kinase (FAK) in PTPα expressing cells. Additionally, paxillin, a v-Src substrate localized at focal adhesions, displayed increased tyrosine phosphorylation in PTPα overexpressing cells. There was also increased association of Csk with paxillin, suggesting a possible feedback

mechanism for the control of Src kinase activity in PTPα overexpressing cells. Together these results suggest that PTPα may have a role in the regulation of cell-substratum adhesion through the regulation of Src kinase activity.

3.2 Introduction

Reversible protein phosphorylation is a widely employed mechanism for regulating enzyme activity, the assembly and location of protein complexes, and for controlling transcription within eukaryotic cells (Pawson, 1995; Cohen et al., 1995). Protein phosphorylation also regulates changes in the organization of the cytoskeleton in response to the adhesion of cells to the extracellular matrix (ECM) or to other cells during such processes as embryo morphogenesis, cell migration, tumor cell metastases, and cellular differentiation (Springer, 1994; Swartz et al., 1995). Indeed, like growth factor receptor signal transduction, engagement of cell-adhesion molecules is rapidly followed by the activation of specific protein tyrosine kinases and the subsequent assembly of multimeric protein complexes at the sites of cell adhesion (Swartz et al., 1995). Moreover, many of the signal transduction molecules activated or phosphorylated in response to ligand-binding to growth factor receptors are also regulated by cell adhesion (Clark and Brugge, 1995; Miyamoto et al., 1995).

The human epidermoid carcinoma cell line A431 has proven to be invaluable to studies of cell growth, differentiation, and cell adhesion regulated by receptor tyrosine kinase signaling. These cells express high levels of the EGF receptor on their surface ($0.5\text{-}3.0 \times 10^6$/cell) (Gill et al., 1981) and proliferate in response to autocrine production of transforming growth factor-alpha (TGFα) (Van de Vijver et al., 1991). Interestingly, while EGF enhances the growth of A431 cell-derived tumors in nude mice, when grown as a monolayer in tissue culture these cells are growth inhibited and terminally differentiate in response to high concentrations of EGF (Gill et al., 1981; Barnes et al., 1982; Ginsberg et al., 1985; MacLeod et al., 1986; Santon et al., 1986; King and Sartorelli, 1986). EGF stimulation of A431 cells also causes dramatic changes in cell morphology, including extensive membrane ruffling, filopodia extension, and changes in cytoskeletal organization and cell adhesion which culminate in the rounding-up and

retraction of these cells from the substratum (Chinkers et al., 1979; Schlessinger et al., 1981; Bretscher et al., 1989).

The involvement of the protein tyrosine phosphatase (PTP) family of enzymes in the regulation of growth factor receptor signal transduction and cell adhesion is still poorly understood. However, as enzymes that regulate the levels of cellular phosphotyrosine, they represent a potentially significant point of control in processes such as cell proliferation, differentiation, and cell adhesion (Charbonneau and Tonks, 1992; Mauro and Dixon, 1994; Fashena and Zinn, 1995). We overexpressed the receptor-like protein tyrosine phosphatase, PTPα, in A431 cells to assess the ability of this phosphatase to regulate EGF receptor-dependent signal transduction.

PTPα expression in A431 cells led to an apparent increase in cell-substratum adhesion and inhibited cell rounding and lifting-off induced by EGF stimulation. However, overexpression of PTPα failed to antagonize EGF-dependent growth inhibition of A431 cells, and did not alter the growth characteristics of unstimulated cells. PTPα expression resulted in the dephosphorylation and activation of specific Src family kinases. Moreover, Src kinases immunoprecipitated from PTPα over-expressing cells were associated with elevated levels of focal adhesion kinase $pp125^{FAK}$(FAK), a molecule activated by integrin-dependent cell adhesion to the substratum, v-Src transformation, and neuropeptide stimulation (reviewed in Zachary and Rozengurt, 1992). PTPα overexpression was also associated with increased tyrosine phosphorylation of paxillin, a protein associated with members of the integrin receptor family and a putative substrate of FAK and/or Src kinases (Glenney and Zokas, 1989; Turner et al., 1990; Schaller et al., 1992). Paxillin immunoprecipitated from PTPα overexpressing cells was complexed with increased levels of Csk, suggesting a possible feedback loop in the regulation of Src activity in these cells and supporting previous observations linking the activation of Src kinases with changes in the intracellular localization of Csk (Sabe et al., 1992; Howell and Cooper, 1994; Sabe et al., 1994; Thomas et al., 1995; Sabe et al.,

1995). The activation of Src kinases together with the observed changes in cell adhesion, tyrosine phosphorylation and the association of FAK and paxillin with Src and Csk kinases respectively, suggests that PTPα may have a role in the regulation of cell-substratum adhesion.

3.3. Results.

3.3.1. Expression of PTPα in A431 cells. To investigate whether the expression of a protein tyrosine phosphatase could interfere with epidermal growth factor receptor signal transduction, we overexpressed the 123 residue extracellular domain-containing isoform of PTPα in A431 cells. PTPα, is composed of a heavily N- and O-glycosylated 123- or 132-residue alternatively spliced extracellular domain, a transmembrane domain, and a cytoplasmic tail bearing two tandemly duplicated phosphatase domains (Jirik et al., 1990; Daum et al., 1994).

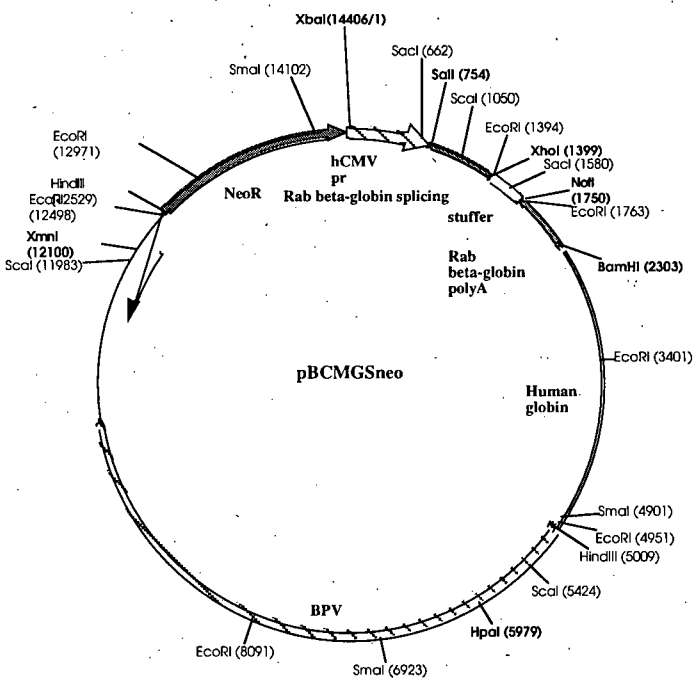


FIG. 3.1. The plasmid pBCMGsNeo was used to overexpress PTPα in A431 cells. This plasmid contains the cytomegalovirus immediate early promoter, rabbit and human β-globin splicing sites, the neomycin resistance gene, and approximately 69% of the genome from bovine papilloma virus (BPV). The BPV sequences allow episomal plasmid replication in a wide variety of cell types.

A431 cells normally express low levels of PTPα (Fig. 3.2A). Both wild type PTPα and PTPα (D1 C433A), a catalytically inactive mutant form of PTPα containing a Cys433Ala substitution within the membrane-proximal catalytic domain (D1), were overexpressed in A431 cells using the CMV promoter based episomal plasmid expression vector pBCMGsNeo (Fig. 3.1) (Karasuyama and Melchers, 1988). PTPα expression was determined by western blot analysis of A431 total cell lysates with anti-peptide antibodies (anti-PTPα-1) (Fig. 3.2A) or anti-recombinant PTPα specific antibodies (anti-PTPα-2) (data not shown). These antibodies recognized proteins of approximately 130-150, 100, 85 and 68 kDa. It has been previously shown that both N- and O-glycosylation of the predicted 85 kDa PTPα polypeptide chain produces a molecule of between 130-150 kDa (Daum et al., 1994). A 100 kDa form of PTPα observed in immunoblots of PTPα lysates (Fig. 3.2A) and immunoprecipitated from PTPα and PTPα (D1 C433A) A431 cell lysates with antibodies specific for the extracellular region of PTPα (Fig. 3.2B) likely represents an N-glycosylated precursor of the larger form. This is in agreement with the findings of Daum et al. (1994) who reported that antibodies against baculovirus-expressed PTPα recognized a glycosylation-dependent epitope in the extracellular domain of PTPα. The anti-PTPα-ext antibodies used in this study were generated against amino acids 20-60 within the extracellular domain of PTPα, a region containing multiple potential N- and O-glycosylated residues. Thus, it is likely that the PTP-ext antisera recognized an incompletely glycosylated PTPα species. However, this antibody stained the surface of PTPα overexpressing cells (Fig. 3.2C panels 2 and 3) but not control vector transfected cells (Fig. 3.2C panel 1), suggesting that both the N- and N- plus O-glycosylated forms of PTPα are present at the cell surface. Regardless of this possibility, cell surface staining of PTPα transfected cells, demonstrated the presence of PTPα at the cell surface of transfected A431 cells.

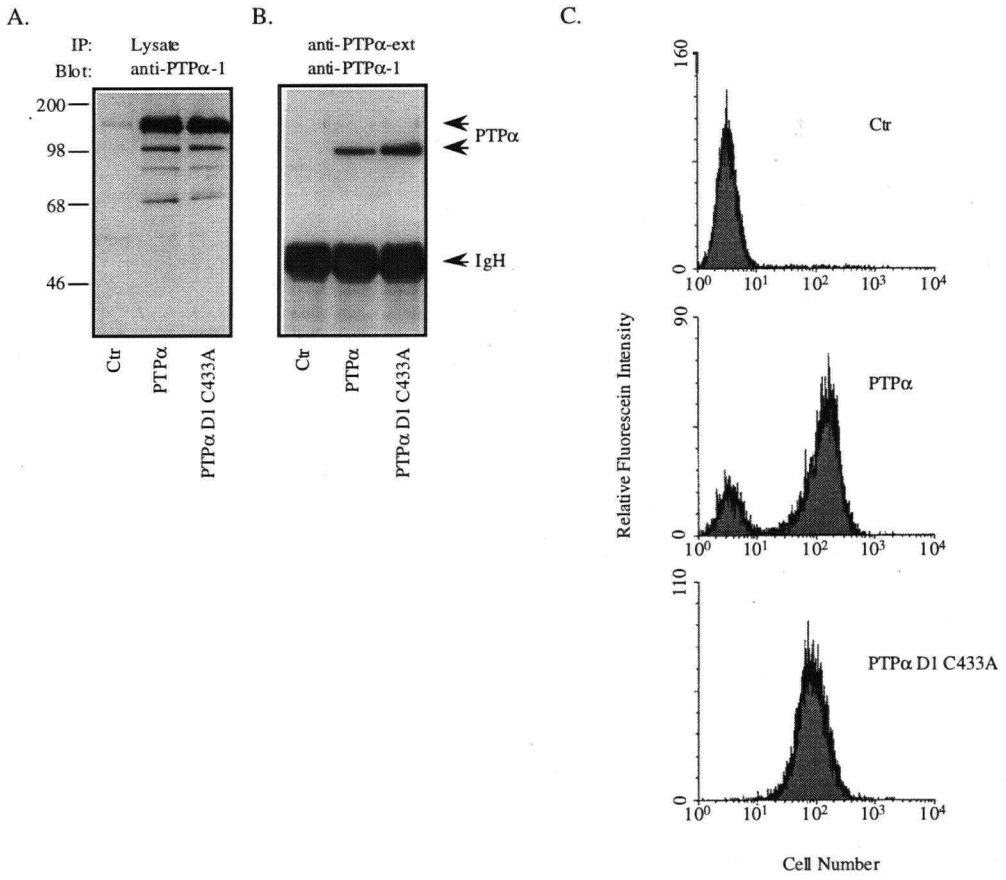


FIG. 3.2. (**A**) Cell lysates from control vector alone (Ctr), PTPα, and PTPα (D1-C433A) transfected cells were separated by SDS-PAGE, transferred to membrane, and immunoblotted with anti-PTPα-1 antibodies. (**B**) Alternatively, PTPα was immunoprecipitated from A431 lysates with anti-PTPα-ext antisera and immunoblotted with anti-PTPα-1 antibodies. (**C**) Antibodies specific for the extracellular domain of PTPα were used to determine cell-surface expression levels of PTPα in the transfected A431 cells.

3.3.2. PTPα phosphatase activity from A431 cells. We investigated the activity of PTPα isolated from lysates derived from the A431 cell lines. Due to the preference of the various recombinant PTPα proteins for the Src Y527 phosphopeptide (chapter 2), this peptide was used as a substrate with which to assay PTPα activity from A431 cell lysates. Anti-PTPα immunoprecipitates from PTPα overexpressing A431 cells possessed high

levels of phosphatase activity against the Src Y527 phosphopeptide (Fig. 3.3). Control vector alone transfected A431 cells showed low but detectable PTPα activity, while cells expressing PTPα (D1 C433A), containing a cysteine-to-alanine substitution in domain-1, showed no detectable PTP activity (Fig. 3.3). Thus, although the second catalytic domain was active when expressed and purified from bacteria, no domain-2 specific activity was detected in the context of an inactive domain-1. This may have been a consequence of the lower protein concentrations used in the immunoprecipitation assay as compared to the recombinant assay, or, alternatively, mutation of domain-1 may inhibit the activity of domain-2. This latter possibility is supported by the results of Wang et al. (1991) who demonstrated that mutation of bacterially expressed PTPα domain-1 abolished the *in vitro* catalytic activity of associated domain-2. Due to the lack of detectable activity of PTPα (D1 C433A) against Src Y527 phosphopeptide we considered PTPα (D1 C433A) expressing cells, in combination with vector alone transfected cells, to be controls in the study of PTPα activity in A431 cells.

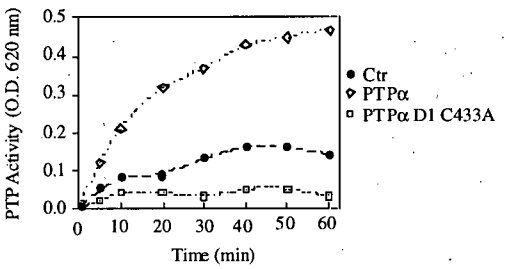


FIG. 3.3. PTPα activity derived from A431 cell lysates. PTPα was immunoprecipitated from vector alone control (Ctr), PTPα and PTPα D1C433A transfected A431 cells and PTP activity was determined by assaying the dephosphorylation of Src 527 phosphopeptide substrate. Released phosphate was determined by the addition of malachite green followed by spectrophotometer measurement at 620 nM.

3.3.3. A431 cells expressing PTPα are resistant to the cell rounding and adhesion disrupting effects of EGF. To assess whether PTPα overexpression in A431 cells could alter the growth response of A431 cells exposed to EGF, we treated control and PTPα transfected A431 cells with various concentrations of EGF and measured the degree of cell growth. Maximal inhibition of A431 cell growth was observed at between 1-5 ng/ml EGF (Fig. 3.4A inset).

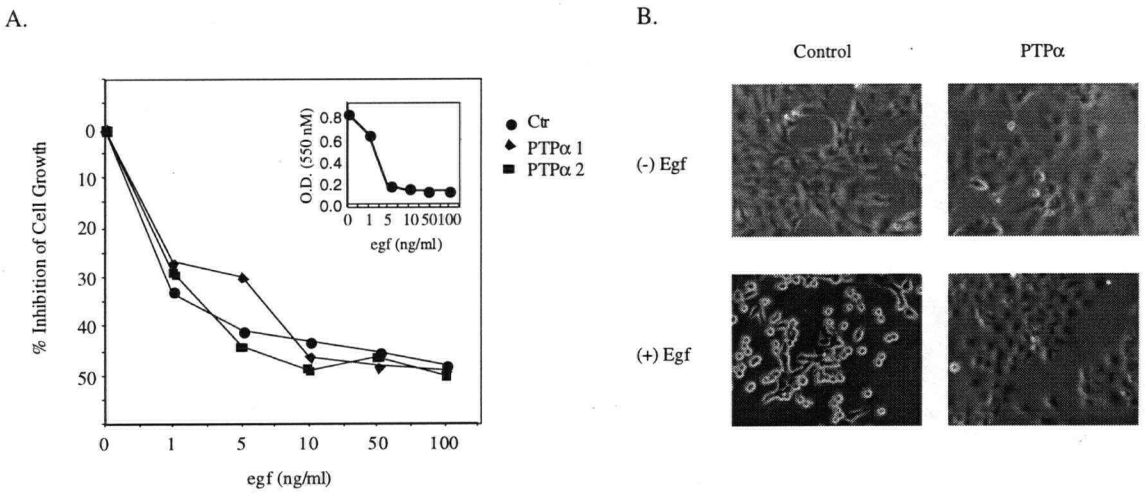


FIG. 3.4. (**A**) Inhibition of A431 cell growth by EGF (inset). The growth of Ctr, PTPα-1 and PTPα-2 transfected A431 cells in the presence of the outlined concentrations of EGF was assessed. Curves represent the % growth inhibition of each A431 cell line after 4 days of growth in the specified concentration of EGF. Cell numbers were assessed by MTT staining of cells as described in the materials and methods section. (**B**) PTPα overexpressing A431 cells are resistant to the cell rounding effects of EGF. Control vector alone or PTPα transfected A431 cells were either stimulated with 100 ng/ml EGF for 10 min (+) or left untreated (-).

The susceptibility of PTPα transfected A431 cells to EGF growth inhibition was then evaluated (Fig. 3.4A). The expression of PTPα in A431 cells was unable to rescue

these cells from the growth inhibitory effects of EGF. However, we did detect a dramatic change in the phenotype of PTPα expressing cells after exposure to EGF. Whereas treatment of control A431 cells with EGF resulted in the rounding and lift off of these cells from the substratum, cells expressing PTPα appeared to be resistant to these effects of EGF (Fig. 3.4B).

3.3.4. Increased cell-substratum adhesion of PTPα expressing cells. Additionally, A431 cells expressing PTPα that were grown in the absence of EGF were more adherent to the substratum than control A431 cells. This characteristic was quantified using an adhesion assay based on measuring the resistance of PTPα expressing cells to cell rounding and removal from the substratum in the presence of PBS (described in chapter 6). Whereas over 80% of control A431 cells were removed from the substratum by 4 washes in PBS, only 30% of A431 cells expressing PTPα were removed (Fig. 3.5A). To determine whether this characteristic was dependent on the catalytic activity of PTPα, or mediated by the extracellular domain, and independent of PTP activity, we assessed whether PTPα (D1 C433A) expressing cells were also more adherent. To differentiate the lower adhesive properties of the control and PTPα (D1 C433A) cell lines the adhesion assay was modified by reducing the number and duration of PBS washes. Although PTPα (D1 C433A) protein levels were slightly higher than wild type PTPα levels (Fig. 3.2A, B), these cells were removed from the substratum more readily than control A431 cells (Fig. 3.5B). These results argue that the catalytic activity of PTPα is required for the observed change in cell-substratum adhesion. In support of this correlation, the addition of the PTP inhibitor pervanadate to the PBS washes reduced the adhesion of the PTPα expressing cells to levels similar to control A431 cells (Fig 3.5A). Pervanadate treatment was also effective at reducing the adhesion of control and PTPα (D1 C433A) expressing cells, suggesting a general role for PTPs in maintaining A431 cell adhesion to the substratum (Fig 3.5A, B).

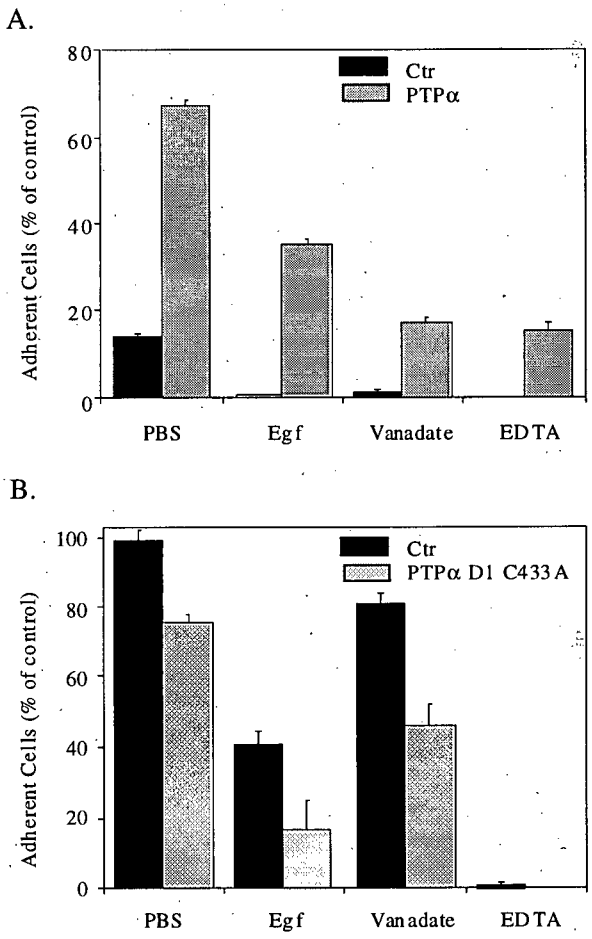


FIG. 3.5. (**A**) The adhesion of control and PTPα overexpressing cells was assessed by measurement of cell-substratum adhesion as outlined in chapter 6. The percentage of cells remaining bound to the substratum after 4 washes (10 min each) in PBS, PBS+EGF, PBS+pervanadate, or PBS+EDTA was compared to untreated cells and plotted as adherent cells as a % of control. (**B**) The adhesion of control transfected A431 cells was compared to cells expressing PTPα (D1 C433A) as in (A) except that cells were washed 3 times (5 min each) to distinguish the lower adhesive properties of control and PTPα (D1 C433A) expressing A431s. Error bars correspond to the standard error of the mean for 4 replicate wells for each point.

The effect of PTPα on EGF-induced release of A431 cells from the substratum was also assessed. Whereas almost 100% of control A431 cells were removed from the substratum by exposure to EGF, almost 40% of PTPα transfected cells remained firmly adherent and spread (Fig. 3.4B, 3.5A). In contrast, PTPα (D1 C433A) expressing cells appeared to be more sensitive to the adhesive disrupting actions of EGF than control transfected cells (Fig. 3.5B). The addition of EDTA to the PBS washes was effective at accelerating the removal of the three cell lines from the substratum (Fig. 3.5A, B). Thus, the expression of PTPα in A431 cells increased cell adhesion in the absence of EGF and inhibited the EGF-induced release of these cells from the substratum.

The increased adhesion of PTPα expressing cells may have been a consequence of augmented or altered expression of ECM proteins by PTPα overexpressing cells. However, the transfer of control A431 cells onto plates on which PTPα expressing cells had been previously grown did not alter the adherence characteristics of the transferred cells (data not shown). This suggested that the adhesion of PTPα expressing cells was intrinsic to these cells and not a result of altered ECM production. We have plated these cells on fibronectin, laminin, collagen, and vitronectin to determine whether a specific ECM component was mediating the increased cell-substratum adhesion. However, no significant difference in the adhesion of control and PTPα expressing cells to any of these isolated ECM components was detected (data not shown).

3.3.5. Potential substrates of PTPα. The expression of PTPα in A431 cells led to specific changes in the phosphotyrosine content of surprisingly few proteins. Anti-phosphotyrosine immunoblots of total cell lysates from control, PTPα, or PTPα (D1 C433A) transfected cells revealed reduced phosphotyrosine levels in proteins with molecular masses of 50-65 kDa in PTPα expressing cells (Fig. 3.6A). It has been previously reported that overexpression of PTPα in rat embryo fibroblasts (Zheng et al., 1992) and P19 cells (den Hertog et al., 1993) led to the activation of Src kinase.

However, these studies did not address whether PTPα expression resulted in a general decrease in cellular phosphotyrosine content, or whether PTPα activity was specific for Src. To determine whether the A431 50-65 kDa tyrosine phosphorylated proteins corresponded to Src kinases(s) we immunoprecipitated Src, Yes, Fyn, and Lyn and assessed their phosphotyrosine content. Reduced phosphotyrosine levels were detected for Src, Yes and Fyn, while Lyn phosphotyrosine levels appeared unchanged (Fig. 3.6B). Interestingly, dephosphorylation of Src and Yes was observed only in cells expressing active PTPα while Fyn phosphotyrosine content was also apparently reduced in PTPα (D1 C433A) expressing cells. However, in contrast to the other Src family kinases, Fyn protein levels were reduced by overexpression of either PTPα or PTPα (D1 C433A) (Fig. 3.6C).

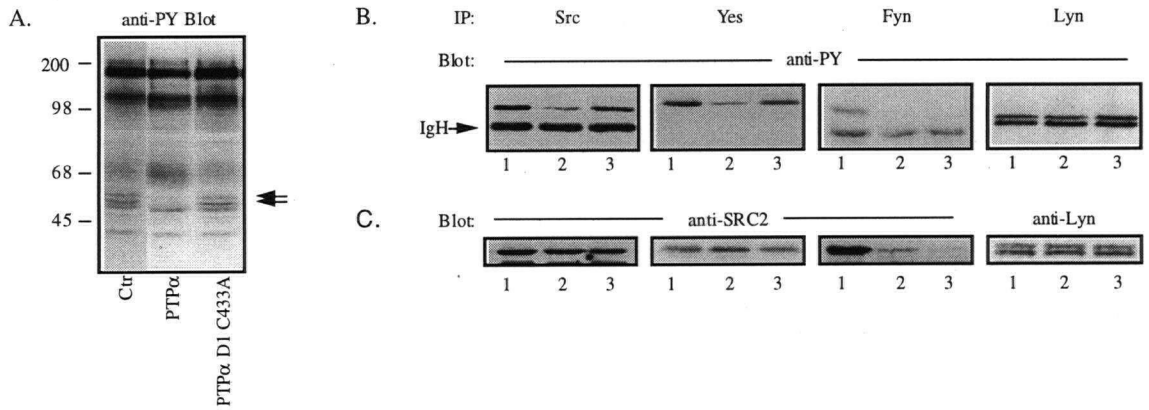


FIG. 3.6. (**A**) Total cell lysate from control vector transfected (Ctr), PTPα, and PTPα (D1 C433A) A431 cells was separated by SDS-PAGE, transferred to membrane, and immunoblotted with anti-phosphotyrosine antibodies. Arrows indicate proteins containing diminished anti-phosphotyrosine immunoreactivity. (**B**) Src family members were immunoprecipitated with antibodies specific for Src, Yes, Fyn or Lyn from Ctr (1), PTPα (2), and PTPα (D1 C433A) (3) A431 lysates and immunoblotted with anti-phosphotyrosine or (**C**) kinase specific antiserum.

We have determined that the change in Fyn protein levels was not a consequence of changes in the protein solubility (data not shown). Further studies will be required to determine how PTPα expression resulted in reduced Fyn kinase protein levels in A431 cells.

3.3.6. PTPα expression results in Src kinase activation. Src family kinases are both positively and negatively regulated by tyrosine phosphorylation (Cooper and Howell, 1993; Cooper, 1990). C-terminal phosphorylation leads to the inhibition of enzyme activity through a proposed intramolecular association between the N-terminal SH2 and SH3 domains and the C-terminus (Roussel et al., 1991; Lui et al., 1993; Okada et al., 1993; Murphy et al., 1993; Superti-Furga et al., 1993; Erpel et al., 1995; Cobb and Parsons, 1993). In contrast, tyrosine phosphorylation within the catalytic domain leads to enhanced enzyme activity (Jove and Hanafusa, 1987). To determine the effect of PTPα expression on Src kinase activity, we immunoprecipitated Src from two PTPα overexpressing A431 cell lines.These two lines expressed approximately equal levels of PTPα protein (Fig. 3.7A) and contained similar levels of PTPα enzyme activity against the Src Y527 phosphopeptide (Fig. 3.7B). Src kinase immunoprecipitated from these cells contained lower phosphotyrosine levels than Src immunoprecipitated from control A431 cells (Fig. 3.8A,B). The relative activity of Src was determined by autokinase and enolase assays (Fig. 3.8C,D). Src kinase isolated from the PTPα overexpressing cell lines was approximately 3-fold more active than control A431 derived Src kinase (Fig. 3.8E) suggesting that PTPα induces the activation of Src kinase by regulating the phosphorylation status of Src Y527.

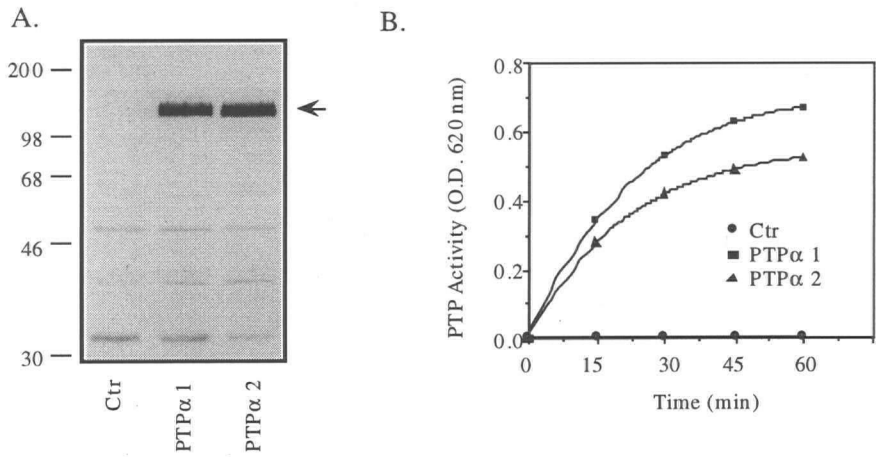


FIG. 3.7. (**A**) Western blot analysis of lysates derived from control (Ctr) and two A431 clones expressing similar levels of PTPα. The arrow indicates the position of PTPα. (**B**) PTPα was immunoprecipitated from A431 cell lysates obtained from cells as in (A) and PTP activity was assessed by measuring the dephosphorylation of Src Y-527 phosphopeptide using the MGMP assay. P_i release was determined by optical density measurement at 620 nM at the indicated times.

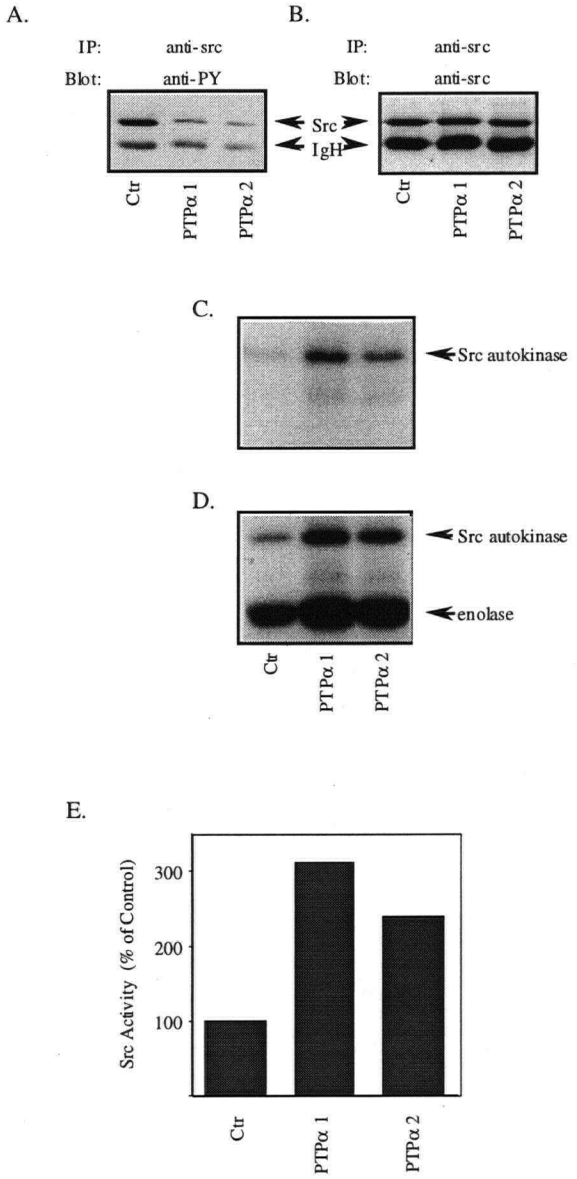


FIG. 3.8. (**A**) Src kinase was immunoprecipitated from Ctr and PTPα overexpressing A431 cells (PTPα 1, PTPα 2) and immunoblotted with anti-phosphotyrosine or (**B**) anti-Src specific antisera. (**C**) The relative activity of Src kinase obtained from either Ctr or PTPα overexpressing cell lysate was determined by autokinase (**C**) or enolase assays (**D**). Relative Src activity was determined by scintillation counting of ^{32}P-labeled enolase (**E**).

Dephosphorylation and activation of Src is thought to correlate with changes in its cellular localization and protein association by freeing both the SH2 and SH3 domains to bind specific targets. This model predicts that a Src kinase activated by dephosphorylation of Y527 should exhibit enhanced SH2 domain mediated binding to exogenous phosphopeptides (Roussel et al., 1991; Sieh et al., 1993). We detected a marked increase in the amount of Src kinase retained on Src Y527 phosphopeptide beads incubated with lysates derived from cells expressing PTPα as compared to control or PTPα (D1 C433A) cell lysates (Fig. 3.9 panel 2). Control non-phosphorylated Src Y527 beads did not retain Src kinase from any of the cell lysates (Fig. 3.9 panel 3).

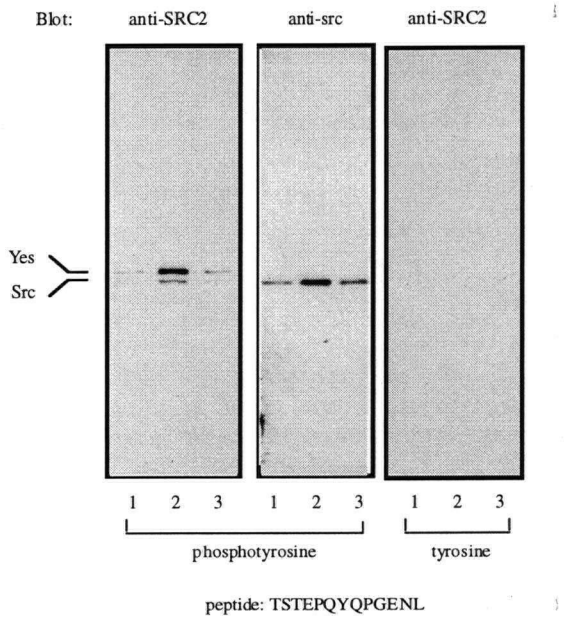


FIG. 3.9. Src Y527 phosphopeptide binding assay. Lysates from control (1), PTPα (2), and PTPα (D1 C433A) expressing A431 cells were incubated with Src Y527 phosphopeptide conjugated CnBr Sepharose. Bead retained enzyme was then detected with anti-Src antisera (panel-anti-src) or antibodies specific for Src, Fyn and Yes tyrosine kinases (panel-anti-SRC2). Beads conjugated to non-phosphorylated Src Y527 peptide were used as a control in this assay.

Interestingly, SRC2 immunoblots of Src Y527 phosphopeptide Sepharose immunoprecipitates revealed an additional band of approximately 62 kDa (Fig. 3.9 panel A). Although we did not have an antibody able to immunoblot Yes specifically, the SRC2 immunoblot and the previously observed reduction in phosphotyrosine content of Yes in anti-Yes immunoprecipitates (Fig. 3.6B) suggests that PTPα expression also results in the activation of Yes kinase.

3.3.7. PTPα is inducibly tyrosine phosphorylated in response to EGF stimulation of A431 cells. It has been previously reported that PTPα is a substrate of Src kinase *in vitro* and likely *in vivo* (den Hertog et al., 1994). Moreover, we have observed that PTPα is tyrosine phosphorylated in NIH 3T3, HEK 293, and A431 cells in the absence of growth factor stimulation (Fig. 3.10 and data not shown).

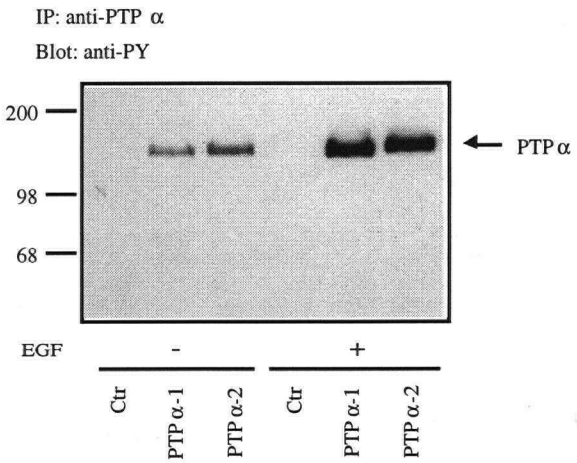


FIG. 3.10. PTPα is inducibly tyrosine phosphorylated following EGF stimulation of A431 cells. PTPα was immunoprecipitated from cell-lysates derived from control (Ctr), and two PTPα transfected A431 cell lines (PTPα-1, PTPα-2) and immunoblotted with antiphosphotyrosine antisera. Cells were either untreated (-) or stimulated for 10 min with EGF (+) before lysis. Blots were reprobed with anti-PTPα antisera to ensure equal quantities of PTPα were immunoprecipitated in each condition (data not shown).

To determine whether PTPα is a substrate for growth factor PTKs such as the EGF-R, we assessed whether PTPα was tyrosine phosphorylated in response to EGF stimulation of A431 cells. Intriguingly, EGF stimulation of A431 cells led to a dramatic increase in the tyrosine phosphorylation of PTPα (Fig. 3.10). Additionally, we found that PTPα was tyrosine phosphorylated by the PTKs Lck (data not shown) and Csk *in vitro* (Fig. 3.11). *In vitro* tyrosine phosphorylation was restricted predominantly to the second catalytic domain of PTPα. However, a PTPα Y772F mutant (Fig. 3.11 PTPα-QFE) was still tyrosine phosphorylated by Csk. Thus, in contrast to CD45, which is phosphorylated by Csk at tyrosine residue Y1193 (Autero et al., 1994), the equivalent residue in PTPα (Y772) was not a major Csk phosphorylation site. The receptor-like PTP, LAR, was not a substrate of Csk in these *in vitro* kinase assays.

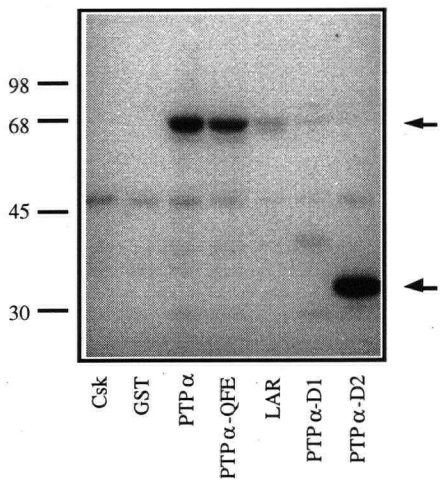


FIG. 3.11. PTPα is phosphorylated by Csk *in vitro*. Recombinant proteins corresponding to GST, thrombin cleaved forms of PTPα, PTPα-QFE, LAR, PTPα-D1, and PTPα-D2 were incubated with Csk in the presence of ^{32}P γATP for 30 min. Tyrosine phosphorylated proteins were visualized by autoradiography.

Two dimensional phosphopeptide analysis of tryptic peptides derived from Csk phosphorylated PTPα revealed at least two major sites of *in vitro* tyrosine phosphorylation. These sites were present in both GST-PTPα (containing D1+D2) and GST-PTPα-D2 but not GST-PTPα-D1 fusion proteins (data not shown). Mass spectrometry analysis of these trypsinized tyrosine phosphorylated PTPα-D2 derived peptides demonstrated that tyrosine residues 579 and 789 were phosphorylated by Csk *in vitro* (Amankwa, L., Harder, K., and Jirik, F., unpublished observations). PTPα Y789 has been shown previously to be phosphorylated *in vivo* in 293, 3T3, and L6 myoblast cells (den Hertog et al., 1994; Su et al., 1994). This site conforms to a Grb2 SH2 domain binding site and binds Grb2 *in vivo* (den Hertog et al., 1994; Su et al., 1994). The role of Grb2 bound to PTPα is unclear however, as Grb2 bound to PTPα was not associated with the guanine nucleotide exchange factor SOS. Thus, it is unlikely that Grb2 association with PTPα is involved in Ras activation. We have not determined whether the *in vivo,* EGF-stimulated tyrosine phosphorylation of PTPα occurs at Y789, however, that PTPα is a target of EGF-R stimulated phosphorylation suggests that this enzyme may be involved in some aspect of growth factor signaling.

Interestingly, incubation of GST-fusions containing the cytoplasmic domains of PTPα with A431 lysates, also revealed a tyrosine kinase activity which was retained by PTPα and LAR, but not GST-beads (Fig. 3.12). The association of this kinase was relatively stable, withstanding thorough washing in lysis buffer containing 1 % NP40, 0.1 % SDS and 0.5 % deoxycholate. However, immunoblotting of these precipitations failed to detect either Csk or Src family kinases. Thus we have been unable to determine the identity of this kinase.

3.3.8. PTPα substrate specificity. An interesting consequence of PTPα overexpression in A431 cells was the selective reduction of phosphotyrosine levels in Src family kinases. Indeed, other phosphotyrosine containing proteins exhibited increased anti-

phosphotyrosine immunoreactivity (Fig. 3.13A). Specifically, we observed increased phosphotyrosine staining of 70 kDa and 110-130 kDa proteins in PTPα over-expressing cells. Increased levels of phosphotyrosine were not observed in cells expressing PTPα (D1 C433A).

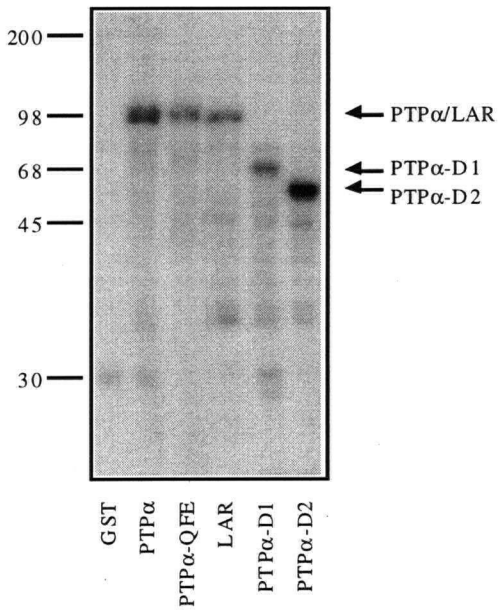


FIG. 3.12. PTPα associates with tyrosine kinase activity from A431 cells lysate preparations. GST-fusion proteins of PTPα, PTPα-QFE, LAR, PTPα-D1, and PTPα-D2 were incubated with A431 cell-lysates, bead bound fusion proteins were then washed extensively in lysis buffer (containing 1.0 % NP40, 0.1 % SDS, 0.5 % deoxycholate) and subjected to kinase assays in the presence of P^{32} γATP. Gels were then soaked in 1M KOH (55°C for 1.5 hours), dried, and exposed to autoradiography film. The positions of tyrosine phosphorylated fusion proteins are indicated.

C-terminal regulatory phosphorylation site Y527F mutants of c-Src, v-Src, and activate Src family kinases in Csk-/- mouse embryo fibroblasts have been shown to induce the tyrosine phosphorylation of specific proteins (Thomas et al., 1995; Cooper and Howell, 1993; Cooper, 1990). An interesting feature of many Src family kinase substrates is their localization and/or involvement in the organization of the cytoskeleton. This

correlation and the observed changes in cell adhesion of PTPα expressing A431 cells suggested that PTPα may be involved in the regulation of Src kinase(s) localized at focal adhesions.

3.3.9. Increased Src kinase(s) association with FAK in PTPα expressing cells. To investigate whether the proteins with increased phosphotyrosine levels in PTPα expressing A431 cells were associated with Src kinase(s), we immunoprecipitated Src, Yes and Fyn from cells with SRC2 antisera. Immunoblotting of these immunoprecipitates with anti-phosphotyrosine antibodies revealed proteins of 110-130 and approximately 220 kDa (Fig. 3.13B). The activation of Src has been shown to enhance the phosphorylation and activation of focal adhesion kinase (FAK), a 125 kDa kinase localized at focal adhesions and activated in response to integrin-dependent cell adhesion (Thomas et al., 1995; Guan and Shalloway, 1992; Schaller et al., 1992; Shattil et al., 1994; Kornberg et al., 1992). Others have reported the SH2 domain-dependent association of Src family kinases with FAK (Schaller et al., 1994; Cobb et al., 1994). To determine if the 125 kDa phosphoprotein which co-immunoprecipitated with Src kinase(s) was FAK, we immunoblotted SRC2 immunoprecipitates with anti-FAK antibodies. Increased quantities of FAK were observed in Src kinase immunoprecipitates from PTPα overexpressing cells (Fig. 3.13C). In keeping with enhanced association of Src kinase(s) with FAK, we observed that the level of FAK tyrosine phosphorylation was elevated in cells expressing PTPα (Fig. 3.13D). In contrast, in cells expressing PTPα (D1 C433A), FAK phosphotyrosine levels were approximately 50% lower than FAK isolated from control A431 cells (Fig 3.15A). FAK protein levels were similar in the three cell lines examined (Fig. 3.13E).

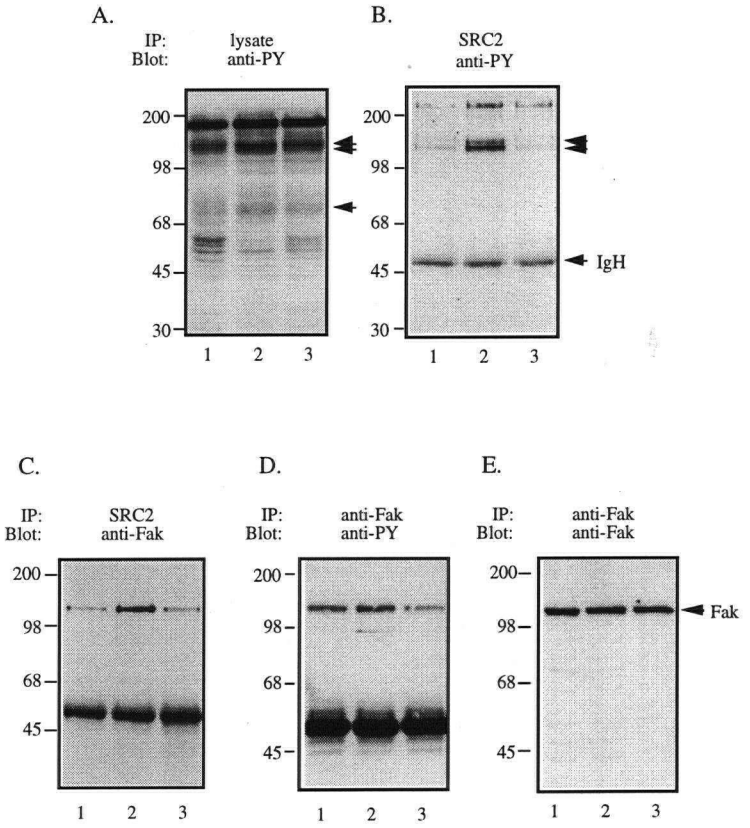


FIG. 3.13. Lysate from control (1), PTPα (2), and PTPα (D1 C433A) (3) expressing A431 cells was separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies (**A**), or subjected to immunoprecipitation with SRC2 antisera and immunoblotted with anti-phosphotyrosine (**B**), anti-FAK (**C**), or used to immunoprecipitate FAK. Anti-FAK immunoprecipitates were then immunoblotted with anti-phosphotyrosine (**D**), or anti-FAK (**E**). Arrows in (A) indicate proteins with increased tyrosine phosphorylation levels.

We have not determined the identity of the other 110-130 kDa or 220 kDa phosphoproteins co-immunoprecipitating with Src kinase(s) from A431 cells (Fig. 3.13B). However, likely candidates include p110-p130 (Reynolds et al., 1992; Kanner et al., 1992; Petch et al., 1994) and p220 tensin (Bockholt et al., 1992), which are potential substrates of Src kinase(s) and regulated by cell-substratum adhesion.

3.3.10. Enhanced paxillin tyrosine phosphorylation in PTPα overexpressing cells. The protein exhibiting the most dramatic increase in phosphotyrosine content had a molecular mass of approximately 70 kDa and was not co-immunoprecipitated with Src kinase(s) (data not shown). Paxillin is ~70 kDa and has been previously shown to be a substrate of Src kinases in *v-src* transformed cells (Glenney and Zokas, 1989; Turner et al., 1990). We immunoprecipitated paxillin from control, PTPα, and PTPα (D1 C433A) expressing cells and immunoblotted these immunoprecipitates with anti-phosphotyrosine antibodies. Dramatically enhanced anti-phosphotyrosine immunoreactivity was detected in paxillin obtained from PTPα overexpressing cells (Fig. 3.14A), while immunoprecipitated paxillin protein levels were similar among the three cell lines (Fig. 3.14B). Densitometric analysis of anti-phosphotyrosine immunoblots of paxillin immunoprecipitates revealed approximately 5-fold greater anti-phosphotyrosine immunoreactivity in paxillin obtained from PTPα expressing cells (Fig. 3.15B).

3.3.11. Increased Csk/Paxillin association in PTPα expressing cells. Src kinase-dependent tyrosine phosphorylation of paxillin is thought to result in the recruitment of Csk to phosphorylated paxillin through the interaction of the Csk SH2 domain with phosphorylated tyrosine residues in paxillin (Sabe et al., 1992; Sabe et al., 1995). Sabe et al. (1992 and 1995) have shown that v-Crk expression led to a 3-4 fold activation of Src kinase, while overexpression of Csk blocked Crk-dependent transformation of 3Y1 cells. The authors suggest that v-Crk expression inhibits the ability of Csk to associate with paxillin at sites of Src kinase activity (Sabe et al., 1995). This may prevent Csk from negatively regulating the catalytic activity of Src resulting in enhanced Src kinase activity. Thus, Src kinase regulation can be thought of as a balance between the activity and localization of Csk and the activities of specific PTPs. The activation of Src kinase(s) by PTPs may result from the dephosphorylation of paxillin directly, preventing

the mobilization of Csk to sites of Src activity, or, by the direct dephosphorylation of Src kinase(s) C-termini or both. PTPα expression enhanced the tyrosine phosphorylation of paxillin, suggesting that Src kinase(s) rather than paxillin are substrates of PTPα. A prediction based on this model is that PTPα-dependent Src kinase(s) phosphorylation of paxillin would result in the recruitment of Csk to sites of Src activity. Indeed, we observed that Csk immunoprecipitated from PTPα overexpressing cells was complexed with a heavily tyrosine phosphorylated group of proteins of approximately 70 kDa (Fig. 3.14C) which immunoblotted with anti-paxillin specific antibodies (Fig. 3.14D). Thus, Src kinase

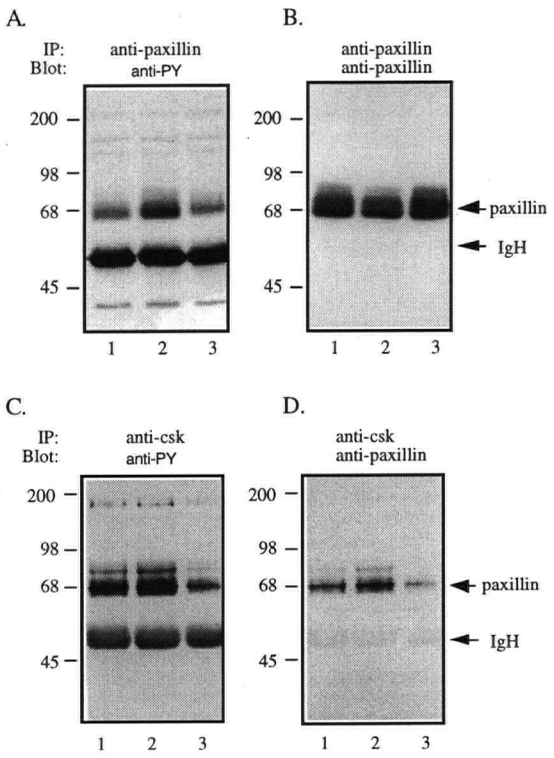


FIG. 3.14. Paxillin was immunoprecipitated from control (1), PTPα (2), and PTPα (D1 C433A) expressing A431 cell lysates, separated by SDS-PAGE, and immunoblotted with anti-phosphotyrosine (**A**), and anti-paxillin antibodies (**B**). Csk was immunoprecipitated as above and immunoblotted with anti-phosphotyrosine (**C**), and anti-paxillin antibodies (**D**).

activation by PTPα may result in Csk mobilization to tyrosine phosphorylated paxillin with subsequent inhibition of Src activity.

Densitometric analysis of Csk immunoprecipitates immunoblotted with anti-paxillin antibodies detected approximately 2 fold more paxillin co-immunoprecipitating with Csk in PTPα expressing cells. In contrast, within cells expressing PTPα (D1 C433A), the levels of paxillin co-immunoprecipitating with Csk was reduced by approximately 50% as compared to control cells (Fig. 3.15C).

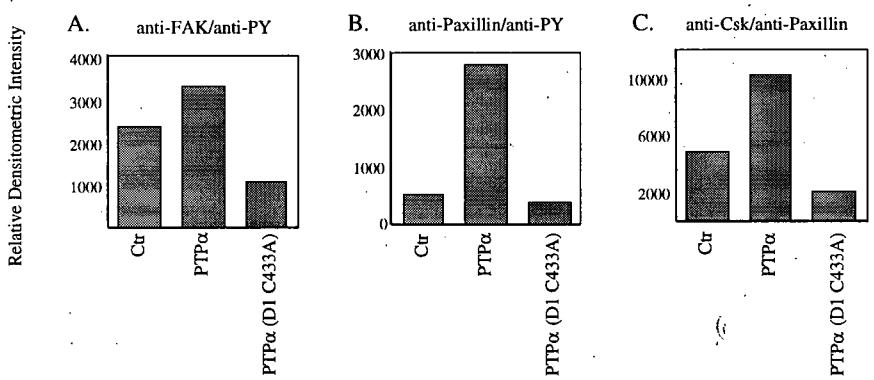


FIG. 3.15. Densitometric analysis of immunoreactive proteins in Figs 3.13. and 3.14. (**A**) FAK was immunoprecipitated from Ctr, PTPα, and PTPα (D1 C433A) expressing A431 cell lysates and the anti-phosphotyrosine immunoreactivity intensity plotted in arbitrary units. (**B**) Anti-phosphotyrosine immunoreactivity intensity of paxillin immunoprecipitated from lysates as above (**C**) Relative scan-intensity of anti-paxillin immunoreactive protein in an anti-CSK immunoprecipitate from the outlined cell lysates.

3.4. Discussion.

These studies were initiated to establish whether expression of PTPα in A431 cells would alter EGF receptor signal transduction. Although PTPα was efficiently expressed in A431 cells, its expression did not alter the rate of cell proliferation in either the presence or absence of exogenous EGF. Consistent with this finding, the EGF receptor isolated from control, PTPα or PTPα (D1 C433A) transfected cells revealed similar levels of phosphotyrosine (data not shown). Although we have not excluded the possibility that PTPα dephosphorylated specific sites within the EGF receptor, the inability of PTPα to alter autocrine TGFα-dependent cell growth suggests that this phosphatase does not dephosphorylate the EGF receptor.

Although PTPα expression did not alter EGF inhibition of cell proliferation, an increase in PTPα overexpressing cell cell-substratum adhesion was observed. This characteristic was dependent on the catalytic activity of PTPα, as the adhesion of PTPα (D1 C433A) expressing cells was similar to, or lower than, control A431 cells. This difference between control and PTPα-transfected cells, however, was most evident upon EGF treatment. While control and PTPα (D1 C433A) cells rounded-up and lifted-off the substratum in the presence of EGF, a large fraction of PTPα expressing cells remained spread and adherent. Thus, although PTPα appeared to be unable to dephosphorylate the EGF receptor directly, it was able to interrupt the cell-substratum adhesion-disrupting effect of EGF. These finding are similar to those of Moller et al. (1995) who reported that PTPα- and PTPε-transfected baby hamster kidney cells overexpressing the insulin receptor remained adherent in the presence of insulin while control cells lifted-off the substratum. As the authors noted, their assay would likely detect any tyrosine phosphatase capable of interfering with signal transduction pathways leading to cell-rounding and lift-off from the substratum. Although reduced phosphotyrosine content of the insulin receptor was observed, it is also possible that PTPα and PTPε regulate a common signaling pathway through which diverse growth factor receptors effect changes in cell adhesion. Importantly,

insulin receptor stimulation has been previously shown to lead to the dephosphorylation of FAK (Pillay et al., 1995). Thus, rather than inhibiting the effect of insulin by direct dephosphorylation of the insulin receptor, PTPα and PTPε may regulate a tyrosine kinase pathway involved in the regulation of cell-substratum adhesion. In support of this possibility, we have shown that PTPα expression in A431 cells leads to enhanced tyrosine phosphorylation of FAK and an increase in FAK/Src kinase association. The protection of PTPα expressing A431 cells from EGF-induced cell-rounding and lift off suggests that PTPα may modulate the ability of multiple receptors to regulate cell adhesion.

Precisely how PTPα interferes with the cell adhesion-disrupting effects of EGF is unclear. Other studies have demonstrated the involvement of phospholipid-derived second messengers such as arachidonic acid in the regulation of EGF induced A431 cell-rounding (Peppelenbosch et al., 1993). Although our results do not exclude the possibility that PTPα expression alters the production of cytosolic phospholipase-A2 (cPLA2) metabolites, our observations suggest a direct role for PTPα in cell-substratum adhesion via regulation of Src family kinase activity. We found that PTPα overexpression resulted in the dephosphorylation of Src and Yes kinases, and resulted in an approximately 3-fold increase in Src kinase activity. Indeed, arguing for the specificity of PTPα for Src family kinases, other cellular proteins showed enhanced tyrosine phosphorylation in PTPα expressing cells. Furthermore, in support of a role for PTPα activation of Src family kinase(s) localized at focal adhesions, we observed an increase in the association of Src kinase(s) with FAK and an increase in the tyrosine phosphorylation and association of paxillin with Csk. Superficially, these results appear to be in conflict with the anchorage-independence observed following v-Src transformation or activated Src overexpression (discussed in chapter 1). However, studies demonstrating enhanced tyrosine phosphorylation of Src kinase substrates during the adhesion of cells to the substratum, the integrin-dependent activation of Src in thrombin-stimulated platelets (Clark and Brugge, 1993), defects in Src-/- fibroblast spreading on fibronectin, the activation of Src kinase

during normal fibroblast cell spreading on fibronectin (Kaplan et al., 1995), and studies showing the requirement of Hck and Fgr for integrin-dependent neutrophil cell adhesion (Lowell et al., 1996), suggest that Src kinase(s) play a critical role in integrin-dependent cell adhesion. Moreover, studies of the role of Csk in the regulation of Src kinases also support a role for Src kinases at focal adhesions (Bergman et al., 1995). Howell and Cooper (1994) demonstrated that the SH2 and SH3 domains of Csk are required for the mobilization of Csk to sites of Src activity in structures resembling podosomes. They also found that Csk localization to focal adhesions required Src kinase activity. Thus, the localization of Src and Csk to focal adhesions may be regulated positively by dephosphorylation of Y527 or negatively by Csk. Indeed, Kaplan et al. (1994) found that mutation of Src Y527 resulted in a change in Src localization from endosomes to focal adhesions. They also demonstrated that the first 251 residues of Src were sufficient for the targeting of this kinase to focal adhesions. Intriguingly, this region of Src was also sufficient to enhance FAK tyrosine phosphorylation and to increase anti-phosphotyrosine staining of focal adhesions (Kaplan et al., 1995). Other studies have demonstrated Src kinase involvement in neural cell adhesion and/or migration mediated by adhesion molecules such as NCAM and L1 (Beggs et al., 1994; Ignelzi et al., 1994).

While it is tempting to speculate that PTPα may be the molecule responsible for Src Y527 dephosphorylation and mobilization to focal adhesions, it remains to be determined which class of adhesion molecule is responsible for the PTPα-dependent change in adhesion observed in A431 cells. It also remains to be determined how Src kinase activation might lead to increased cell adhesion. Although the affinity of integrin family receptors for ECM components can be regulated in an 'inside-out' manner (discussed in chapter 1 and Springer, 1994) we have no evidence that PTPα activity regulates the affinity of integrin receptors specifically. However, our observations that PTPα overexpression activates Src kinase(s), enhances cell-substratum adhesion, and alters the interactions and phosphorylation states of known focal adhesion-associated molecules, suggest a role for

PTPα in the regulation of cell adhesion.

CHAPTER 4

PTPα, retrovirally-encoded oncoproteins, and members of the cytokine receptor superfamily contain a novel SH3 domain-binding motif

4.1. Summary

PTPα expression has been associated with Src kinase activation in various cell systems (chapter 3). We found that PTPα co-immunoprecipitated with Src kinase(s) in the human epidermoid cell line A431. In studies aimed at elucidating the nature of the interaction between these molecules, we identified a proline-rich membrane-proximal region within PTPα able to bind SH3 domains *in vitro*. Using filter, solution, and BIAcore binding assays, a PXXP motif within this region was identified that exhibited a preference for the Lyn and Fyn kinase SH3 domains. Moreover, binding of Lyn SH3 domain to the membrane proximal region of PTPα required basic amino acids N-terminal to the PXXP motif, consistent with a class I SH3 domain binding orientation. Furthermore, specific members of the cytokine receptor superfamily and proteins encoded by viral oncogenes contain a proline-rich motif which is similar in sequence to the PTPα proline-rich sequence. We show that peptides derived from these sequences are able to bind SH3 domains *in vitro*. These findings suggest that specific retrovirally-encoded oncoproteins as well as members of the PTP and cytokine receptor superfamilies contain a membrane proximal motif that may mediate association with SH3 domain-containing proteins within cells.

4.2. Introduction

Information arriving at the cell surface in the form of soluble factors, extracellular matrix components or cell surface-associated ligands is converted into a vast array of second messenger systems within the cell. Activation of cell surface receptor or receptor-associated tyrosine kinases, for example, leads to the recruitment of a variety of molecules to the plasma membrane (Pawson and Gish, 1992). Recent studies have highlighted the modular nature of such molecules, many of which contain various combinations of Src homology (SH) 2, SH3, pleckstrin homology (PH), and/or phosphotyrosine binding (PTB) domains. These modules are required for the assembly of signaling complexes which are responsible for the transmission of phosphorylation signals at the membrane to the cytoplasm and ultimately the nucleus (Cohen et al., 1995; Pawson, 1995).

The SH3 domain is a 50-70 amino acid module thought to mediate interactions between proteins (Cohen et al., 1995). The precise function of the SH3 domain within specific signaling pathways is still incompletely understood. However, the identification of 3BP1 and 3BP2 as ligands for the Abl SH3 domain by expression library screening has aided in determining SH3 domain binding specificity (Cicchetti et al., 1992). The regions of 3BP1 and 3BP2 found to mediate association with the Abl SH3 domain were localized to an approximately 10 amino acid sequence in both proteins, rich in proline residues (Ren et al., 1993). The SH3 binding specificity has been further defined using phage-displayed peptides and biased peptide libraries (Chen et al., 1993; Yu et al., 1994; Sparks et al., 1994; Cheadle et al., 1994; Rickles et al., 1994). The structure of the SH3 domain module complexed to proline-rich peptides has also been determined. The SH3 domain itself is formed by two perpendicular β sheets composed of 5 antiparallel β strands with binding centered around a core XPpXP motif within the ligand. Peptide binding on the exposed hydrophobic face of the SH3 domain is mediated largely by hydrophobic

interactions of the peptide with a cluster of aromatic residues conserved among SH3 domains. However, specific acidic residues in the RT loop of the SH3 domain also make important contacts with basic amino acids within the polyproline ligand (Cohen et al., 1995).

Both the SH2 and SH3 domains of Src kinases contribute to the repression of kinase activity through the proposed intramolecular association of these domains with the tyrosine phosphorylated C-terminus (Okada et al., 1993; Howell and Cooper, 1994; Kaplan et al., 1994; Erpel et al., 1995). However, constitutively active forms of Src kinase which contain SH3 domain mutations are unable to transform NIH 3T3 cells, suggesting that SH3 domain interactions potentiate the transforming ability of this kinase (Erpel et al., 1995). Conversely, the SH3 domain of Abl has been shown to inhibit the ability of this kinase to transform cells (Jackson et al., 1993). In other systems, such as in the regulation of the dynamin GTPase, or the PI3K p85 subunit, SH3 domain binding is associated with enzyme activation (Pleiman et al., 1994; Gout et al., 1993). In yeast, osmotic shock results in initiation of a MAP kinase cascade that is in part regulated by the association and activation of a polyproline-containing MAP kinase kinase (Pbs2p) with the SH3 domain of the transmembrane osmosensor Sho1p (Maeda et al., 1995). Moreover, the production of reactive oxygen intermediates within phagocytic cells is governed by the NADPH oxidase complex, the assembly of which involves SH3-polyproline interactions (McPhail, 1994). The SH3 domain also has a role in determining the compartmentalization of specific signaling molecules. For example, the SH3 domains of PLC γ and Grb2 are required for localization to the cytoskeleton, and to membrane ruffles, respectively (Bar-Sagi et al., 1993), while a polyproline peptide within the amiloride-sensitive sodium channel leads to the localization of this protein to the apical surface of epithelial cells (Rotin et al., 1994). In addition, the prevalence of SH3 domains in molecules required for the assembly and function of the cortical cytoskeleton such as non-muscle myosin 1b and the *Saccharomyces cerevisiae* proteins Abp1, Bem1, and

Sla1, also suggest an important role for the SH3 domain in the regulation of cellular architecture (Drubin et al., 1990; Drubin, 1991; Holtzman et al., 1993).

We have shown that overexpression of PTPα in A431 cells results in the activation and/or dephosphorylation of Src and Yes kinases (chapter 3). Futhermore, ectopic expression of PTPα in embryonal carcinoma P19 cells resulted in neuronal differentiation in response to retinoic acid (den Hertog et al., 1993), while PTPα over-expression in rat embryo fibroblasts was reported to lead to cell transformation and tumorigenesis (Zheng et al., 1992). In these studies an increase in Src kinase activity was observed as a result of PTPα expression. In support of this correlation, PTPα co-immunoprecipitated with Src kinase(s) from the human epidermoid carcinoma cell line A431. Furthermore, we demonstrate that PTPα contains a membrane proximal polyproline SH3-binding motif that exhibits specificity for the SH3 domains of Lyn and Fyn protein tyrosine kinases *in vitro*. In addition, the Lyn SH3 domain is able to bind to similar polyproline sequences in proteins encoded by oncogenic retroviruses such as Abelson's murine leukemia virus (AMLV) and Gardner-Rasheed feline sarcoma virus (FSV). The Lyn SH3 domain also bound proline-rich sequences present in members of the cytokine receptor superfamily, including the growth hormone (GH) receptor and the alpha chains of the receptors for interleukin-3 (IL-3), interleukin 5 (IL-5), and granulocyte-macrophage colony stimulating factor (GM-CSF). The identification of SH3 domain-binding motifs in the membrane-proximal regions of these molecules supports the hypothesis that SH3 domain containing proteins may interact at this site within these diverse signal transduction molecules.

4.3. Results

4.3.1. Co-immunoprecipitation of Src family kinase(s) with PTPα.

We (chapter 3) and others (Zheng et al., 1992; den Hertog et al., 1993) have observed that overexpression of PTPα in cells can lead to Src kinase activation. However, the nature of the interaction between these molecules is not known. The human epidermoid carcinoma cell line A431 expresses Src, Fyn, Yes (Fig. 4.1A) and Lyn (chapter 3) tyrosine kinases. To assess whether PTPα is physically associated with Src family kinases in these cells, we overexpressed PTPα and a catalytically inactive mutant form of PTPα (D1 C433A) (discussed in chapter 3) in A431 cells (Fig. 4.1B). PTPα association with Src kinases was investigated by western blot analysis of SRC2 immunoprecipitates with anti-PTPα specific antibodies. The antibody SRC2 recognizes Src, Fyn and Yes kinases (Fig. 4.1A). We detected PTPα in SRC2 immunoprecipitations from cells expressing PTPα (Fig. 4.1C), but not in rabbit antisera-matched control anti-Csk kinase immunoprecipitations (Fig. 4.1D). Interestingly, in cells expressing the catalytically inactive mutant, PTPα (D1 C433A), less PTPα was co-immunoprecipitated with the Src kinases despite higher expression levels of this mutant phosphatase (Fig. 4.1C, lane 3). This may reflect a difference in the ability of the SRC2 antibody to immunoprecipitate active versus inactive Src kinases in PTPα expressing cells. Alternatively, it is possible that PTPα phosphatase activity modifies Src kinases in a way that allows them to associate either directly or indirectly with PTPα.

4.3.2. PTPα contains a Src family kinase SH3 domain-binding motif. PTPα likely activates Src by directly dephosphorylating the C-terminal regulatory tyrosine residue (Y527) responsible for repression of kinase activity. This phosphotyrosine residue associates with the SH2 domain of Src through a proposed intramolecular mechanism which also involves the SH3 domain (Cooper and Howell, 1993).

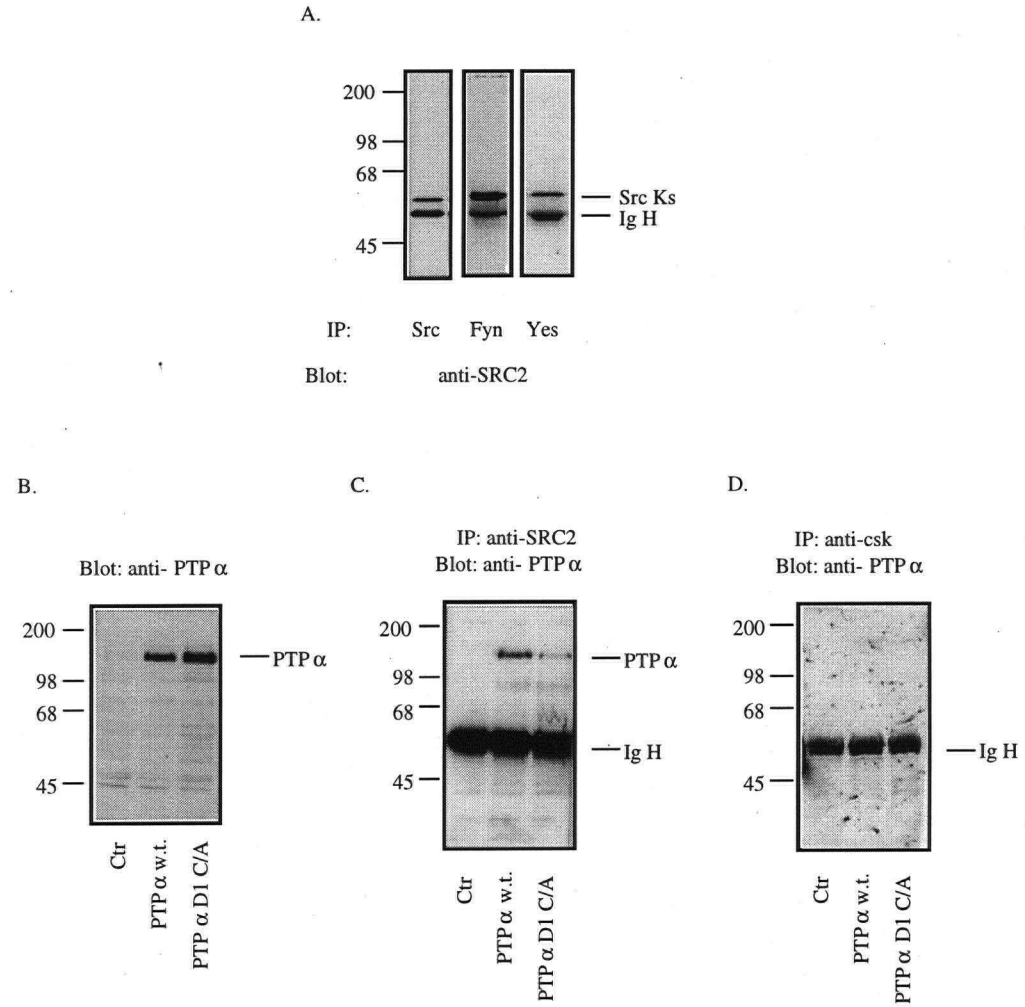


FIG. 4.1. PTPα co-immunoprecipitates with Src family kinases. (**A**) Src (lane 1), Fyn (lane 2), and Yes (lane 3) were immunoprecipitated from A431 cell lysates with kinase specific antibodies and immunoblotted with SRC2 antisera. (**B**) Total cell lysate from untransfected (lane 1), w.t. PTPα transfected (lane 2), or PTPα (D1 C433A) mutant transfected A431 cells (lane 3) was separated by SDS-PAGE and blotted with anti-PTPα-specific antibodies. (**C**) The Src family kinases Src, Fyn, and Yes were immunoprecipitated with the SRC2 antibody from lysates prepared from control A431 (lane 1), w.t. PTPα-transfected A431 (lane 2), and PTPα (D1 C433A) mutant transfected A431 cells (lane 3). Associated PTPα was then detected by immunoblotting with anti-PTPα-specific antibodies. (**D**) Csk kinase was immunoprecipitated from the above A431 lysates and blotted with anti-PTPα specific antibodies as a control.

Thus, the phosphorylation state of Src tyrosine 527 appears to control the protein interactions and sub-cellular localization of Src by regulating both the SH2 and SH3 domain's availability to bind other proteins (Howell and Cooper, 1994; Kaplan et al., 1994). We hypothesized that dephosphorylation of the Src kinase(s) regulatory C-terminal tyrosine phosphorylation site by PTPα might allow their SH2 and/or SH3 domains to associate directly with PTPα. As the *in vitro* binding specificity of Src family kinase SH3 domains has been determined to be RPLPPXP and RPLPXXP by phage display techniques (Sparks et al., 1994; Rickles et al., 1994; Cheadle et al., 1994) or RXLPPLP by combinatorial peptide library analysis (Yu et al., 1994), we examined the primary sequence of human PTPα for similar polyproline motifs. This search revealed the sequence RKYPPLPVDK, in the membrane-proximal region between the transmembrane domain and first PTP domain (Fig. 4.2). Although this motif lies in a region whose sequence is not conserved amongst the receptor-like PTPs, similar polyproline motifs are present in the membrane-proximal regions of the receptor-like PTPs LAR, PTPδ, PTPσ, and PTPε (Fig. 4.13).

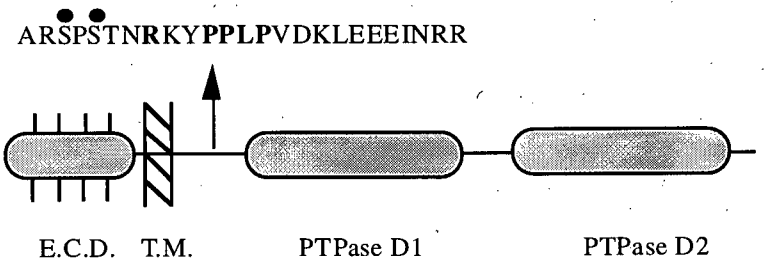


FIG. 4.2. Structure of PTPα. The extracellular domain (E.C.D.), transmembrane (T.M.) and two phosphatase domains (D1 and D2) of PTPα are shown. A stretch of the membrane proximal region sequence is shown. Key residues thought to mediate SH3 domain binding are highlighted by bold lettering. Serine phosphorylation sites, Ser 202 and Ser 204 within the proline-rich sequence, are indicated by closed circles.

4.3.3. Interaction of PTPα with Src family kinase SH3 domains by filter binding assay. To assess whether the membrane-proximal region of PTPα is capable of binding SH3 domains, we performed a filter binding assay analysis of this region with a variety of SH3 domains. The 70 amino acid region (containing the putative polyproline SH3-binding motif) between the transmembrane domain of PTPα and the first phosphatase domain, was expressed as a GST fusion protein. As positive controls to monitor SH3 binding, the Src family kinase SH3 consensus peptide RPLPPLP, and the 11 amino acid peptide 3BP1 APTMPPPLPPV (corresponding to the *in vitro* Abl SH3-binding site), were also expressed as GST fusion proteins. Lysate from bacteria expressing GST alone served as a negative control. Total cell lysates from bacteria expressing the various GST-polyproline peptide fusions were separated by SDS-PAGE and blotted with biotinylated GST, or biotinylated GST-fusion proteins containing the SH3 domains of Src, Yes, Fyn, Lyn, Abl, PI3K, and GAP (Fig. 4.3).

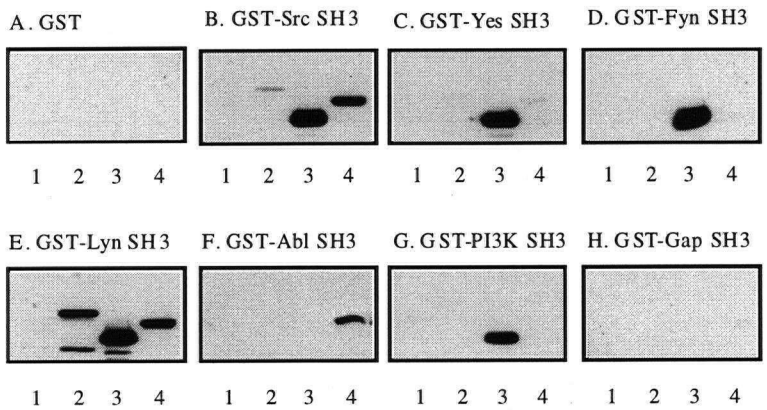


FIG. 4.3. Filter Binding Assay of the PTPα membrane proximal region with SH3 domains. Total cell lysates from bacteria expressing GST (lane 1), GST-PTPα 70 a.a. (lane 2), GST-RPLPPLP (Src family SH3-binding consensus) (lane 3), and GST-APTMPPPLPPV (Abl SH3-binding sequence from 3BP1) (Lane 4), were fractionated by 15% SDS-PAGE, transferred to membrane and blotted with biotinylated

SH3 domains as indicated. Bound SH3 domains were then detected with streptavidin horseradish peroxidase conjugate.

The PTPα-derived 70-residue peptide (GST-PTPα 70 a.a.) bound well to the Lyn SH3 domain (Fig. 4.3 panel E, lane 2). There was also low but detectable binding of this peptide to the Src SH3 domain (Fig. 4.3 panel B, lane 2). In contrast, no binding could be detected to the SH3 domains of Yes, Fyn, Abl, PI3K, or GAP. Strong binding to the Src kinase consensus 7-residue peptide was observed for all of the SH3 domains tested, with the exception of the Abl and GAP SH3 domains, which showed no reactivity (Fig. 4.3 lane 3, panels B, C, D, E, G). The 3BP1 peptide was bound by Abl, Src and Lyn SH3 domains (Fig. 4.3 lane 4, panels B, E, F). These results suggest that the region between the transmembrane domain and the first catalytic domain of PTPα is capable of binding Src family kinase SH3 domains.

4.3.4. Binding of the Lyn SH3 domain to PTPα requires an intact PXXP motif. To further define the region of PTPα mediating the observed association with the Lyn SH3 domain, we expressed a smaller 18 residue PTPα-derived peptide (PSTNRKY**P**PL**P**VDKLEEE) containing the putative PXXP SH3-binding motif. The Lyn SH3 bound strongly to this peptide (Fig. 4.4A lane 3). Furthermore, binding was abolished by a proline-to-alanine substitution within the PXXP motif (PSTNRKY**P**PLAVDKLEEE) (Fig. 4.4A lane 4), indicating that an intact PXXP sequence is required for Lyn SH3 domain association with this region of PTPα.

4.3.5. The Lyn SH3 domain binds PTPα in a class I orientation. Structural studies have determined that in the case of class I ligands, SH3 domains bind to a core PXXP motif in conjunction with critical contacts with basic residues N-terminal to the PXXP motif, or in the case of class II associations, with basic residues C-terminal to the PXXP

motif (Feng et al., 1994; Lim et al., 1994). The PTPα sequence we have defined contains an arginine at position -3, a lysine at position -2, and a lysine at the +6 position relative to the PXXP motif ($R^{-3}K^{-2}XP^{0}XXP^{+3}XXK^{+6}$). To determine whether the Lyn SH3 domain bound to the PTPα peptide in a class I or class II orientation, we expressed two smaller peptides: PTPα class I (RKY**PPLP**) and PTPα class II (Y**PPLP**VDK).

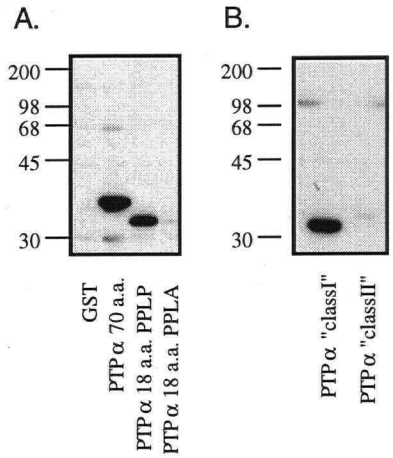


FIG. 4.4. The PXXP motif of PTPα is required for association of the Lyn SH3 domain. (**A**) Total cell lysate from bacteria expressing GST (lane 1), GST-PTPα 70 a.a. (lane 2), GST-PTPα 18 a.a. PPLP (lane 3), and GST-PTPα 18 a.a. PPLA (lane 4) were separated by SDS-PAGE and blotted with the biotinylated Lyn SH3 domain. (**B**) PTPα binds the Lyn SH3 domain in a class I orientation. Bacterial lysates from cells expressing GST-PTPα "class I" RKYPPLP (lane 1), and GST-PTPα "class II" YPPLPVDK (lane 2) were separated as above and blotted with the biotinylated Lyn SH3 domain.

While the Lyn SH3 domain bound to the PTPα class I peptide, it failed to show detectable binding to the PTPα class II peptide (Fig. 4.4B). These results suggest that the Lyn SH3 domain binds to the PTPα sequence in a class I orientation, and also demonstrate the importance of residues outside the core PXXP motif in SH3 binding. These findings support the hypothesis that this proline-rich segment of PTPα may be an SH3-binding site and suggest that this region contains the elements required for class I

SH3-binding. The possibility remains however, that another SH3 domain that requires a lysine at the +6 position of the PXXP motif may bind to this sequence in a class II orientation.

4.3.6. Solution binding of SH3 domains to PTPα. The binding of the Lyn SH3 domain to PTPα in the filter binding assay revealed that the PTPα PXXP motif could bind SH3 domains. However, we wanted to determine if this region could interact with SH3 domains in solution. We expressed specific proline-rich peptides in bacteria to determine conditions favorable for SH3 domain binding. Peptide sequences were introduced into the bacterial expression vector PinPoint-Xa (Promega). Recombinant proteins expressed using the PinPoint system are fusion proteins containing approximately 13 kDa derived from the enzyme transcarboxylase and are biotinylated at a single site at the N-terminus. This system was used to express the phage display derived peptide sequence RPLPPLP (called Sparks in Fig. 4.5) (Sparks et al., 1994), the Abl SH3 domain ligand 3BP1-APTMPPPLPPV (Ren et al., 1993), and peptides corresponding to the proline rich region of PTPα-PPLP PSTNRKYPPLP(A)VDKLEEE or PTPα-PPLP PSTNRKYPPLA VDKLEEE which contains a proline-to-alanine substitution in the PXXP motif (Fig. 4.5). Bacterial lysates containing these biotinylated fusion proteins were then incubated with GST-Lyn SH3 domain. Lyn SH3 domain associated protein was recovered with glutathione Sepharose, separated by SDS-PAGE, transferred to membrane, and immunoblotted with horseradish peroxidase-linked streptavidin. Only the proline rich peptide corresponding to the phage display sequence was immunoprecipitated with the Lyn SH3 domain (Fig. 4.5 lane 5).

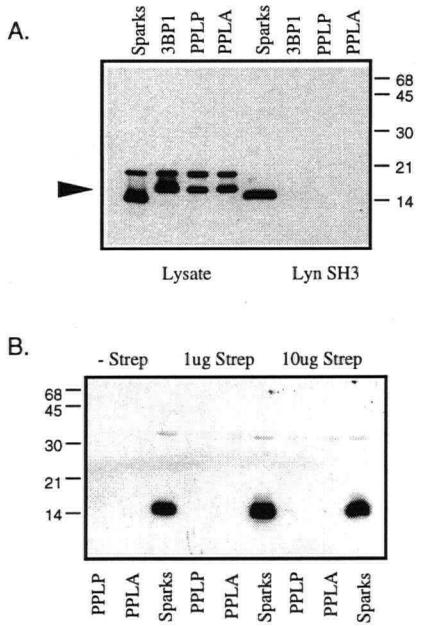


FIG. 4.5. (**A**) Lysates of bacteria expressing PinPoint peptides corresponding to the phage display peptide RPLPPLP (Sparks), 3BP-1, PTPα-PPLP, and PTPα-PPLA were separated by SDS-PAGE, transferred to nitrocellulose and blotted with horseradish peroxidase-linked streptavidin. The biotinylated fusion proteins are depicted by a ▲ symbol. The bands above the fusion proteins, visible in total cell lysate lanes, correspond to the endogenous biotinylated bacterial transcarboxylase protein. The Lyn SH3 domain was then used to immunoprecipitate the various proline-rich fusion proteins from the above lysates. (**B**) To determine if oligomerization is required for SH3 domain binding, streptavidin was added to the various bacterial lysates desrcribed above and the Lyn SH3 domain was again used in immunoprecipitations.

Binding of this fusion protein to the Lyn SH3 domain allowed us to analyze various SH3 domain binding conditions. We investigated whether clustering of the biotinylated polyproline peptides aided peptide association with the Lyn SH3 domain by increasing the avidity of the interaction. As fusion proteins expressed in this system are biotinylated, we assessed whether the addition of tetravalent streptavidin facilitated the association of the Lyn SH3 domain with the other polyproline peptides . However, the addition of streptavidin did not lead to stable association of the Lyn SH3 domain with the

PTPα derived peptides (Fig. 4.5B).

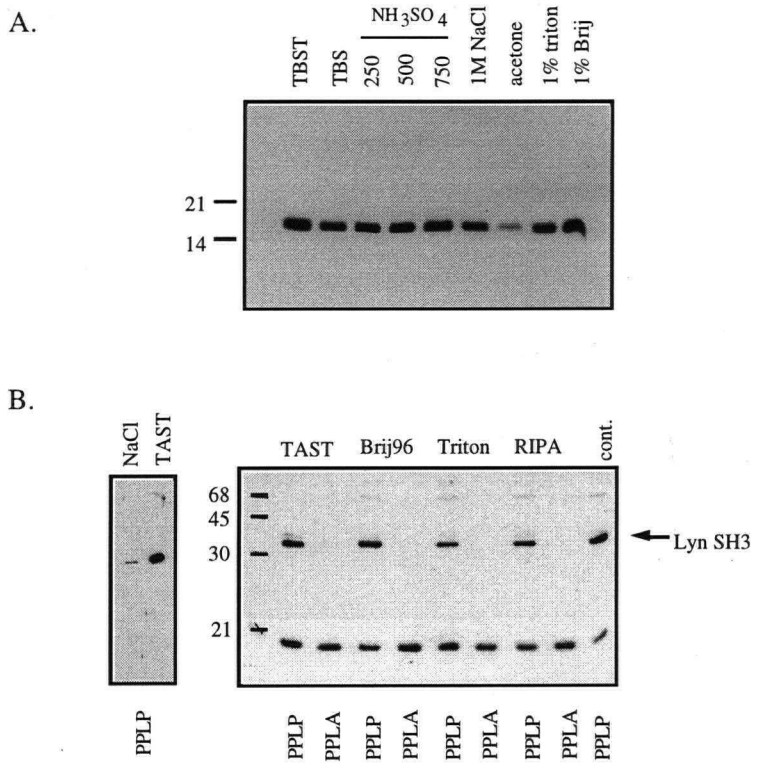


FIG. 4.6. (**A**) PinPoint-RPLPPLP bacterially expressed peptide (sparks) was used to assess SH3 domain binding conditions. Bacterial lysates in TBST (Tris pH 7.5, 150 mM NaCl, Tween 20), TBS (Tris pH 7.5, 150 mM NaCl), TBS containing increasing concentrations of ammonium sulfate (250 mM-750 mM), 1M NaCl, acetone (recombinant protein precipitated in acetone and resuspended in dH_20), 1 % triton X-100 (in TBS) or 1 % Brij 96 (in TBS) were incubated with 1 μg of Lyn SH3 domain. The Lyn SH3 domain was recovered with glutathione Sepharose and precipitated material was detected with horseradish peroxidase-linked streptavidin. (**B**) Biotinylated synthetic peptides corresponding to the proline-rich region of PTPα (PPLP), or (PPLA) were used to immunoprecipitate the Lyn SH3 domain from buffers containing NaCl (TBST containing 150 mM NaCl), TAST (TBST, 750 mM ammonium sulfate) or TAST buffer containing various detergents (RIPA: 1 % triton X-100, 0.1 % SDS, 0.5 % deoxycholate)

The lack of association of the Lyn SH3 domain with cell derived PTPα led us to suspect that the cell lysis buffer was not compatible with stable SH3-PTPα binding. To assess this possibility we made use of the PinPoint expressed phage display peptide to characterize Lyn SH3 binding in various buffer conditions. We found that the addition of high concentrations of ammonium sulfate favored SH3 domain binding (Fig. 4.6A). This was particularly apparent when a biotinylated synthetic peptide corresponding to PTPα was employed. Binding of the Lyn SH3 domain to the PTPα peptide was dramatically enhanced in the presence of 750 mM ammonium sulfate (TAST) (Fig. 4.6B, panel 1). The effect of different detergents on SH3 binding was also assessed. The presence of Tween 20, Brij 96, Triton X-100, or RIPA buffer (containing SDS, deoxycholate, and NP-40) did not significantly affect SH3 binding.

A biotinylated 25-residue synthetic PTPα-derived polyproline peptide (PPLP) was effective at binding the Lyn SH3 domain in the presence of TAST buffer. We then assessed whether the other SH3 domains were able to bind to the PTPα-PPLP peptide or to a 25 residue biotinylated control peptide derived from the sequence of 3BP1. Biotinylated peptides were recovered from solutions containing the outlined SH3 domains with streptavidin beads and associated SH3 domains were detected with anti-GST antibodies. Binding of the Abl SH3 domain by the 3BP1 peptide beads served as a positive control in this assay (Fig. 4.7A, B). Using this strategy we detected strong binding of the PTPα-derived peptide to the Lyn and Fyn SH3 domains. The PI3K, Yes, Abl and Src SH3 domains and the Grb2 protein also bound to the PTPα peptide at levels greater than GST alone (Fig. 4.7A). These results were unexpected due to the filter binding assay results, demonstrating only Lyn and Src SH3 domain binding to the PTPα sequence. We then repeated this assay using a biotinylated 25 amino acid peptide (PPLA) containing an alanine in the +3 position of the PXXP motif of PTPα. Binding of the SH3 domains was dramatically diminished by this single amino acid substitution (Fig. 4.7B), demonstrating the requirement for an intact PXXP motif for the binding of individual

SH3 domains.

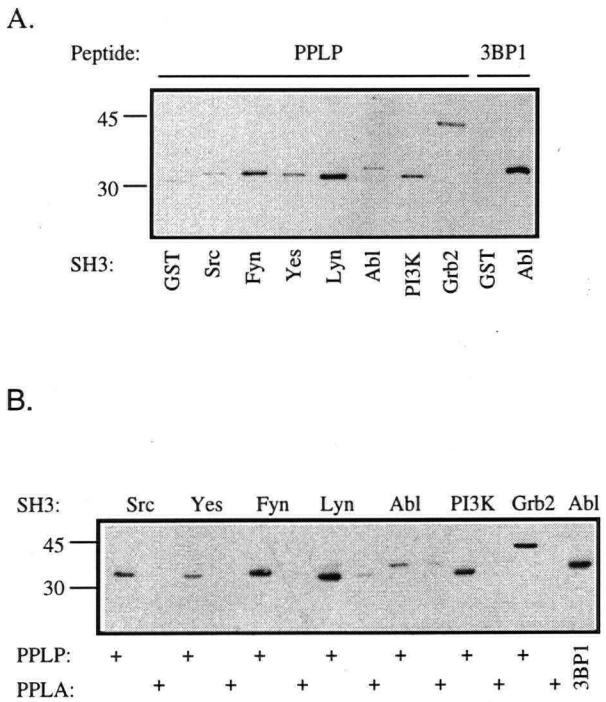


FIG. 4.7. PTPα binds SH3 domains in solution. (**A**) GST-SH3 domain fusion proteins were incubated with a biotinylated 25 a.a PTPα peptide (PPLP lanes 1-7) or with a biotinylated 25 a.a. 3BP1 derived peptide (3BP1 lanes 8 and 9) in TAST buffer as described in materials and methods. SH3 domains bound to the individual peptides were eluted in Laemmli buffer and separated by SDS-PAGE. The SH3 domains were then detected by blotting with anti-GST antibodies. (**B**) An intact PXXP motif is required for SH3 domain association. SH3 domains were incubated as above with biotinylated PTPα peptide (PPLP) or alternatively, with a peptide containing a proline to alanine mutation in the PXXP motif (PPLA). Abl SH3 domain binding to 3BP1 is shown as a control. Peptide associated SH3 domains were detected as above.

4.3.7. BIAcore analysis of SH3 domain binding. SH3 domain binding was evaluated further by BIAcore to assess the relative affinities of the interactions between each SH3 domain and the PTPα-peptide. The optical biosensor has been used previously to

examine interactions between SH3 domains and polyproline peptide ligands (Rickles et al., 1994; Lee et al., 1995; Sastry et al., 1995). The biotinylated PTPα 25-mer was immobilized onto a streptavidin-coated sensor chip and the binding of SH3 domains was monitored. Again, binding of the Abl SH3 domain to the 3BP1 peptide served as a positive control (Fig. 4.8B). Consistent with the results of both the filter binding and solution binding assays, the Lyn SH3 bound to the immobilized PTPα peptide with the highest relative affinity (Fig. 4.8A). This result, and the overall ranking of SH3 domain binding was comparable to the results obtained from the solution binding assay.

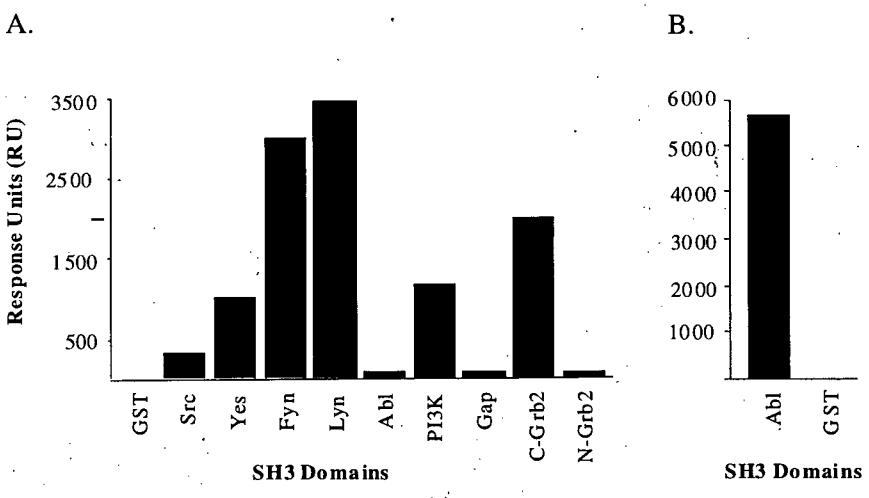


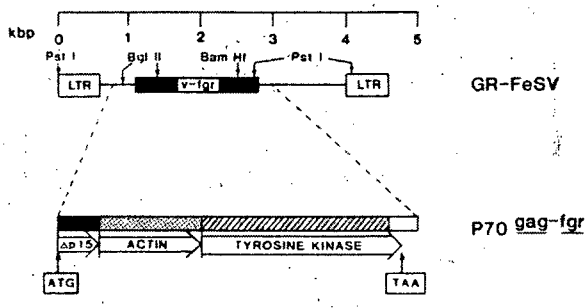
FIG. 4.8. Measurement of SH3 domain binding to PTPα using BIAcore. The association of free SH3 domains with an immobilized 25 a.a. PTPα-derived peptide was recorded by measuring the change in refractive index at the surface of the BIAcore sensor chip. Relative binding of the individual SH3 domains is reflected by the change in response units (RU). (**A**) The binding of free GST or GST-SH3 domain fusion proteins to immobilized PTPα peptide. (**B**) Binding of GST-Abl SH3 domain or GST to immobilized 3BP1 peptide. All binding assays were performed in PBS buffer.

Interestingly, the Fyn SH3 domain bound to the immobilized peptide almost as

well as the Lyn SH3. This result was similar to the findings of (Lee et al., 1995) who found that the Fyn and Hck SH3 domains bound a 12 residue HIV-1 Nef peptide with similar affinity in solution. However, binding of the Fyn SH3 domain to the Nef peptide could not be detected by filter binding assay (Saksela et al., 1995). We have determined that the absence of Fyn binding to the PTPα derived peptide by filter binding assay was not a result of SH3 domain biotinylation, as both biotinylated and non-biotinylated Fyn SH3 domains associated equally well with the PTPα peptide using the BIAcore method (data not shown). It is possible that a structural determinant required for the binding of the Fyn, but not the Lyn SH3 domain was destroyed by SDS-PAGE.

4.3.8. The feline sarcoma virus (FSV) protein p70 gag-fgr contains a polyproline sequence which binds SH3 domains in vitro and enhances viral transformation efficiency. During our analysis of the putative SH3 domain binding sequence of PTPα, we found that specific murine retroviruses contained DNA sequences encoding polyproline sequences similar to that of PTPα and other SH3 domain peptide ligands. The FSV retrovirus contains at least two regions of gene sequences corresponding to host murine gene products. Specifically, viral *gag* sequences are fused to both a *γ-actin* gene segment and *v-fgr* tyrosine kinase encoding DNA (Fig. 4.9A, B) (Naharro *et al.*, 1984). The FSV gene product, p70 gag-fgr, contains both the SH2 and tyrosine kinase domains of c-Fgr fused to N-terminal sequences derived from viral gag and γ-actin proteins. Interestingly, the polyproline sequence contained within the FSV genome is identical to SH3 domain selected peptides obtained *in vitro* by phage display techniques (Fig. 4.9B). Sugita *et al.* (1989) demonstrated that the FSV gene product, p70 gag-fgr, encoded an active tyrosine kinase which was able to transform murine fibroblasts. The authors went on to show that while the actin domain was dispensable for transforming activity, removal of the proline-rich region dramatically impaired transforming ability. However, the authors had no explanation for the requirement of this region for transformation.

A.



B.

SH3 domain binding site?

```
                 110        120        130        140        150        160        170
(fgr)      LPPPKPPTSLPQPHSPQPARALCRPAVCRPRPLPPLPPTAMEEEVAALVIDNGSGMCKAGFAGDDAPRAVFPSIV
                                                   :::: ::::::::: ::::::::::::::::::::
(γ-Actin)                                          MEEEIAALVIDNGSAMCKAGFAGDDAPRAVFPSIV
                                                        10         20         30

                  180        190        200        210        220        230        240        250
(fgr)      GRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLN
           ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
(γ-Actin)  GRPRHQGVMVGMGQKDSYVGDEAQSKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLN
              40         50         60         70         80         90         100        110

                  260        270        280        290
(fgr)      PKANREKMTQIMFETFNIPSNYVAPVDSIQAEEWYFGKIGRKDAERQLL
           ::::::::::::::::: :  :::    :::
(γ-Actin)  PKANREKMTQIMFETFNTPAMYVA----IQAVLSLYASGRTTGIVMDSG
                 120        130            140        150
```

C.

Transfection analysis of GR-FeSV deletion mutants[a]

	DNA clone	Focus formation (FFU/pmol viral DNA)[b]	Colony formation (CFU/pmol viral DNA)
	pGR-FeSV	3,777	1,890
polyproline (-) →	pd119	372	1,890
actin (-) →	pD141	7,533	1,965
kinase (-) →	pd269	<1	1,920

[a] Transfection of NIH 3T3 cells was performed by using the calcium phosphate technique. Focus formation or G418-resistant colony formation was scored at 14 to 21 days. Results represent the average of three independent experiments.

[b] FFU, Focus-forming units.

From Naharro *et al.*, 1984 and Sugita *et al.*, 1989

FIG. 4.9. Taken from references noted at figure bottom (**A**) Structure of the Feline sarcoma virus. The size of the retroviral genome is shown above the illustration depicting viral LTRs and *v-fgr* sequences. The relative orientation of p15-gag, γ-actin and tyrosine kinase domains are shown. (**B**) Protein sequence showing the fusion between viral gag sequences, the region of γ-actin homology and the N-terminal region of the *v-fgr* tyrosine kinase domain. The putative SH3 domain binding sequence is indicated. (**C**) Summary of data reported by Sugita *et al.*, 1989. Shown are transformation efficiency data for wild type FSV *v-fgr* virus (pGR-FeSV), and mutants lacking the polyproline-rich sequence (pd119), γ-actin sequences (pD141), or tyrosine kinase domain (pd269).

The abelson-murine leukemia virus (AMLV) transforming protein v-Abl also contains a proline-rich sequence similar to that of FSV, lying N-terminal to the v-Abl tyrosine kinase domain (Reddy et al., 1983). Alignment of this sequence with that of PTPα, FSV, 3BP-1 and the phage display derived peptide reveals considerable similarity (Fig. 4.10A). In particular, the AMLV sequence is similar to the Abl SH3 domain binding peptide 3BP-1, while the FSV p70 gag-fgr sequence resembles that of the Src family kinase binding consensus peptides obtained by phage display and biased peptide library studies (Chen et al., 1993; Yu et al., 1994; Sparks et al., 1994; Cheadle et al., 1994; Rickles et al., 1994). These similarities suggested that the proline-rich sequences of each viral protein might recruit either Abl or endogenous Src family kinases to the gag fusion kinase domains. Such a model is attractive in view of experiments demonstrating tyrosine kinase activation by kinase dimerization (Fig. 4.10C) (Ullrich and Schlessinger, 1990). To determine if these viral sequences are ligands for SH3 domains of specific cellular kinases, we expressed the FSV and AMLV polyproline rich sequences as GST fusion proteins and assessed SH3 domain binding to these sequences by filter binding assays (Fig. 4.10B). The Lyn SH3 domain bound well to the FSV peptide, producing the strongest signal observed in these studies. The Lyn SH3 domain also bound to the AMLV-derived peptide. However, although the AMLV and 3BP-1 sequences are similar,

poor binding was detected between the Abl SH3 domain and the AMLV sequence. GST and GST fusions of PTPα and 3BP-1 served as controls in these assays.

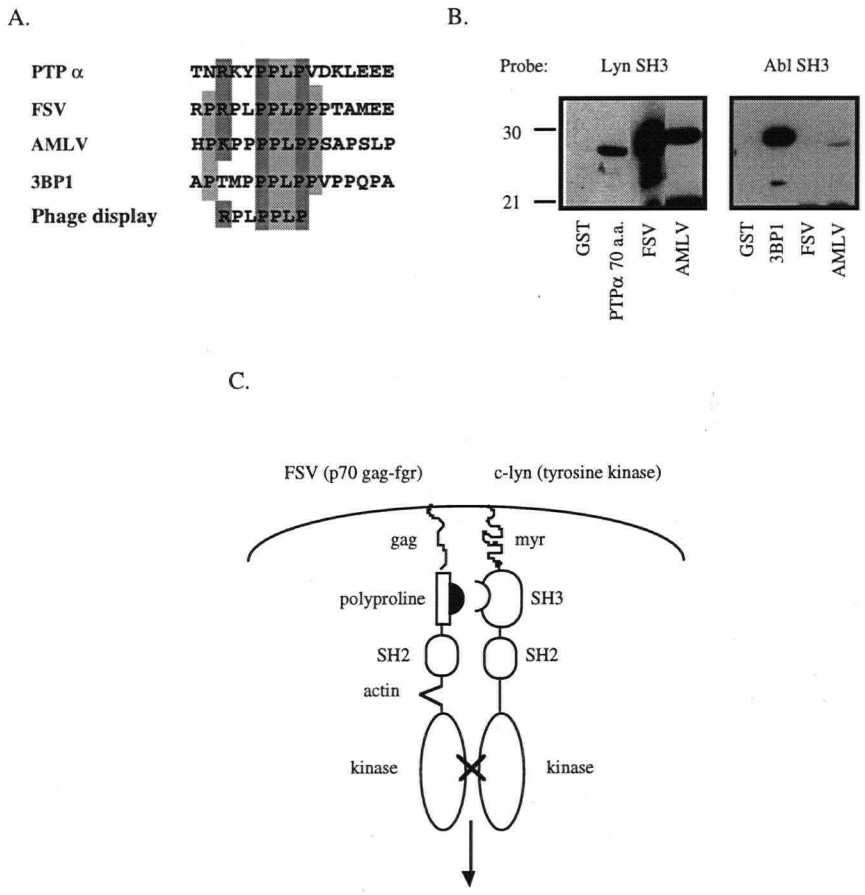


FIG. 4.10. (**A**) Protein alignment of the proline rich regions of PTPα, FSV, AMLV, 3BP1, and Src family kinase SH3 domain binding consensus peptide obtained by phage display. (**B**) Filter binding assay showing the binding of the Lyn and Abl SH3 domains to the proline-rich sequences of PTPα, FSV, AMLV, and 3BP1. All peptides were expressed as GST-fusion proteins in bacteria. Total cell lysates was separated by SDS-PAGE, transferred to membrane and blotted with the biotinylated Lyn SH3 domain. Lysate from bacteria expressing GST alone was used as a negative control. (**C**) Model of an interaction between an SH3 domain containing molecule (such as Lyn) and the proline rich region of FSV p70 gag-fgr. The arrow indicates kinase activation through protein dimerization.

4.3.9. Generation of a model for the detection of in vivo association of SH3 domains with polyproline ligands. The inability to show stable association of SH3 domains with PTPα derived from eukaryotic cells prompted us to speculate that certain SH3 domain associations are not stable enough to be detected by standard co-immunoprecipitation techniques. To address this possibility, we developed a system which we predicted would facilitate the detection of such interactions within eukaryotic cells.

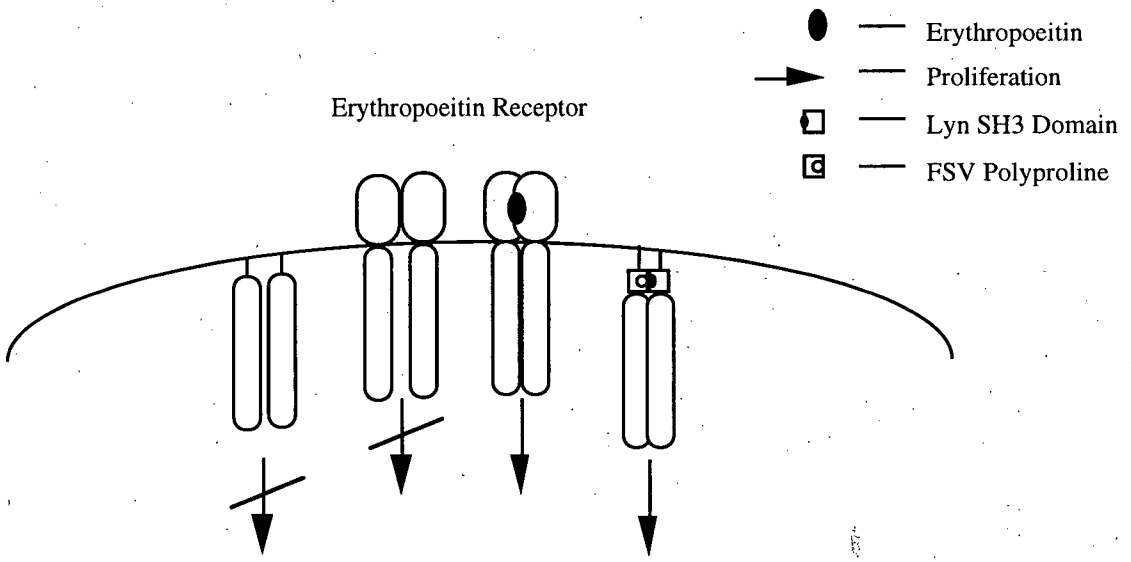


FIG. 4.11. This figure depicts a model for the detection of interacting protein domain modules in vivo. In the presence of erythropoeitin, the EPO receptor dimerizes inducing signal transduction leading to proliferation (in responsive cells such as BAF3s). Fusion proteins were created which contained the Src myristylation sequence allowing membrane localization, followed by either the Lyn SH3 domain, or the FSV polyproline sequence. These sequences were fused to the entire cytoplasmic region of the EPO receptor and cotransfected into Baf3 cells. It was hypothesized that this system might allow the detection of interacting domains which although able to interact *in vivo*, are not stable enough to study by co-precipitation.

A variety of studies have demonstrated that ligand specific binding to cell surface receptors leads to dimerization of their respective cytoplasmic domains. In the case of growth factor receptors, this dimerization results in signal transduction leading to cell growth, division or cell survival (Ullrich and Schlessinger, 1990). The receptor dimerization model has been studied extensively using the epidermal growth factor receptor (EGF-R), growth hormone (GH) and erythropoietin receptors (EPO-R) among others. In the appropriate cell type, erythropoietin induced receptor dimerization leads to cell proliferation in the absence of other factors (Ohashi et al., 1994). Alternatively, receptors may be dimerized by incorporation of cysteine residues in the extracellular segment creating disulfide bonds between neighboring receptors (Yoshimura et al., 1990; Watowich et al., 1992), or artificially with chimeric receptors with extracellular domains specific for other growth factors (Ohashi et al., 1994).

Due to the apparently strong affinity of the Lyn SH3 domain for the FSV polyproline sequence, we reasoned that the binding of these two elements, grafted into the EPO-R, might lead to receptor dimerization with corresponding EPO-independent growth of previously EPO-dependent cells. Thus, the FSV proline-rich peptide sequence was used to assess the feasibility of this approach before testing of the PTPα polyproline sequence in this system. The Lyn SH3 domain and the FSV polyproline sequence were each subcloned into the eukaryotic expression vector pEFBOS. This expression plasmid contains the EF1α ribosomal protein promoter and the neomycin resistance gene (Mizushima & Nagata, 1990) . Constructs included the Src myristylation sequence (Niklinska et al., 1994) to provide membrane localization of the cytoplasmic domain of the EPO-R and two copies of the myc epitope tag to allow immunodetection of expressed protein. The final EPO-R fusion proteins contained 15 residues corresponding to the Src myristylation sequence, followed by two copies of the myc epitope, the Lyn SH3 domain, FSV polyproline peptide, or no insert, followed by the entire cytoplasmic region of the

EPO-R at the C-terminus. The ability of these constructs to be expressed was assessed by transient transfection of human embryonal kidney 293 cells. Lysates from transfected cells were prepared and fusion protein expression was detected by western blot analysis with anti-myc tag antibodies. The three fusion proteins were all efficiently expressed in 293 cells (Fig. 4.12).

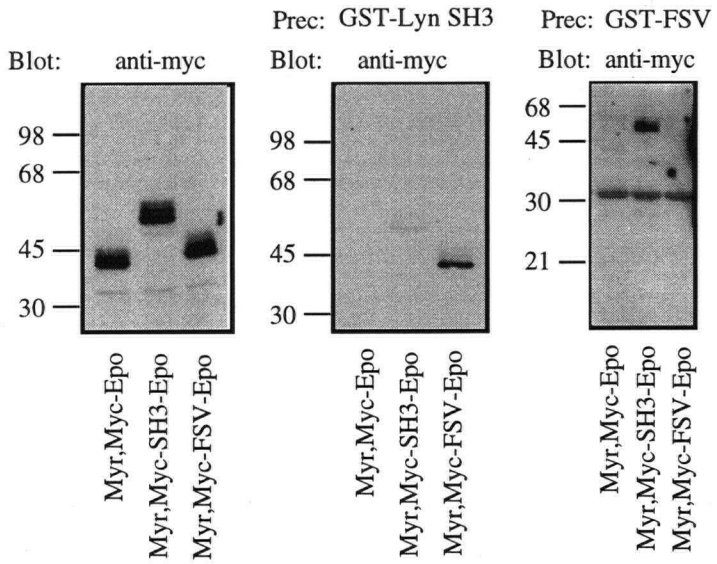


FIG. 4.12. Chimeric receptors containing the Src myristylation sequence, two copies of the myc epitope, and either no insert, the Lyn SH3 domain, or the FSV proline-rich region, followed by the cytoplasmic domain of the EPO receptor, were transiently transfected into human embryonal kidney 293 cells by lipofection. Lysate was prepared from cells 2-days post transfection and protein expression was assessed by immunoblotting with mAb 9E10 which recognizes the myc epitope in each fusion protein. These lysates were also used to assess the ability of either the Lyn SH3 domain to bind the FSV-chimeric receptor, or the FSV peptide to precipitate the Lyn SH3 domain containing fusion protein.

We then assessed whether the Lyn SH3 domain and the FSV peptide were able to precipitate the FSV-EPO-R and Lyn-SH3-EPO-R fusion proteins from 293 cell lysates.

The Lyn SH3 domain and recombinant GST-FSV protein were each able to co-immunoprecipitate their corresponding partners from 293 total cell lysate (Fig. 4.12 panels 2 and 3). However, we were unable to obtain EPO-independent cells when both of these EPO-R-fusions were co-transfected into EPO-dependent Baf3 cells (derived by Levings M., and Schrader, J.). Thus, although we could demonstrate *in vitro* association of the FSV peptide with the Lyn SH3 domain, and could co-immunoprecipitate each EPO-R fusion protein with either the Lyn SH3 domain or FSV peptide, these molecules did not associate in a way that was able to induce EPO-independent cell growth.

***4.3.10. A PXXP motif within cytokine receptors can bind SH3* domains.** We have demonstrated that the PXXP motif in the membrane-proximal region of PTPα is able to bind various SH3 domains *in vitro*. We identified similar polyproline motifs within specific retroviruses (as discussed above), members of the cytokine receptor superfamily, other receptor-like PTPs, and the Tec family of protein tyrosine kinases (Fig. 4.13). Specific binding of SH3 domains to the proline-rich region of the Tec family kinase Btk has been reported (Cheng et al., 1994; Yang et al., 1995). In the case of the cytokine receptors and other PTPs, the PXXP motif is located just inside the plasma membrane in a location similar to that of the PTPα-PXXP motif. The similarities of sequence and location suggested that these regions represent homologous motifs that interact with SH3 domain-containing molecules. To assess whether the polyproline-region of these proteins could bind SH3 domains *in vitro*, we expressed 10 amino acid peptides containing these motifs in bacteria.

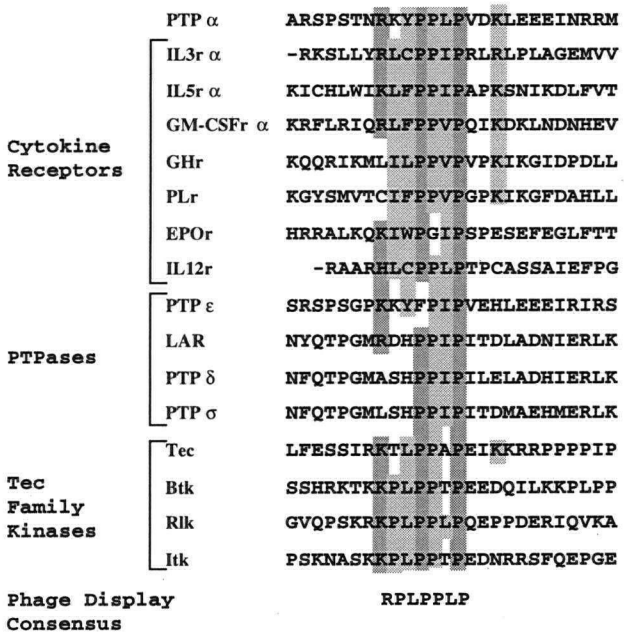


FIG. 4.13. Alignment of various polyproline containing protein sequences with the membrane-proximal proline-rich sequence of PTPα. Shading illustrates the common PXXP motifs in each sequence and emphasizes the potential structural features important for SH3 binding. A Src family kinase SH3 domain binding consensus peptide is shown at bottom. Abbreviations: PTPα, (human, protein tyrosine phosphatase alpha); IL-3rα, (murine, interleukin-3 receptor alpha chain); IL-5rα, (human, interleukin-5 receptor alpha chain); GM-CSFrα, (human, granulocyte-macrophage colony stimulating factor receptor alpha chain); GH-R, (human, growth hormone receptor); PL-R, (human, prolactin receptor); EPO-R, (human, erythropoietin receptor); PTPε, (human, protein tyrosine phosphatase epsilon); LAR, (human, leukocyte common antigen related phosphatase); PTPδ, (human, protein tyrosine phosphatase delta); PTPσ, (Rat, protein tyrosine phosphatase sigma); Tec, (mouse, tyrosine kinase expressed in hepatocellular carcinoma); Btk, (mouse, Bruton's tyrosine kinase); Rlk, (mouse, resting lymphocyte kinase); Itk, (mouse, IL-2 inducible T-cell kinase).

The ability of these GST-polyproline fusion peptides to bind the Lyn SH3 domain was then assessed by filter binding assay. Interestingly, the polyproline membrane-proximal peptides derived from the alpha chains of the receptors for IL-3 (RLCPPIPRLR), IL-5

(KLFPPIPAPK), and GM-CSF (RLFPPVPQIK) were all able to bind the Lyn SH3 domain, as was the polyproline sequence from the growth hormone receptor (LILPPVPVPK) (Fig. 4.14A). The binding observed did not appear to be due to non-specific binding of the Lyn SH3 domain to random polyproline sequences, however, as sequences derived from the erythropoietin receptor (KIWPGIPSPE) and LAR PTP (RDHPPIPITD) did not bind the Lyn SH3 domain (Fig. 4.14A). To assess whether binding of the Lyn SH3 domain to the cytokine receptor peptides required an intact PXXP motif, as was observed for the PTPα-derived peptides, a proline-to-alanine substitution was introduced into the sequence of the IL-3 receptor alpha chain peptide (RLFPPIAHMK). This mutation abolished detectable binding of the Lyn SH3 domain to the IL-3 receptor alpha chain peptide (Fig. 4.14B).

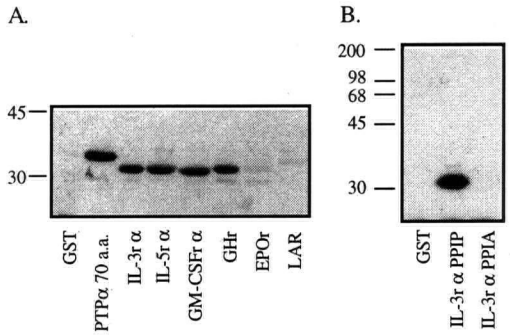


FIG. 4.14. Cytokine receptors contain an SH3 domain binding motif. (**A**) Total cell lysates from bacteria expressing GST (lane 1), GST-PTPα 70 a.a (lane 2), GST-IL-3r α (lane 3), GST-IL-5r α (lane 4), GST-GM-CSFr α (lane 5), GST-GHr (lane 6), GST-EPO-R (lane 7), and GST-LAR (lane 8) were separated by SDS-PAGE, transferred to membrane and blotted with the biotinylated Lyn SH3 domain. (**B**) An intact PXXP motif is required for Lyn association with the IL-3r α chain. Bacterial cell lysates expressing GST (lane 1), GST-IL-3r α PPIP (lane 2), and GST-IL-3r α PPIA (lane 3) were blotted as above with the Lyn SH3 domain.

4.4. Discussion

We have employed filter, solution, and BIAcore binding assays to identify an SH3 domain-binding motif in the membrane-proximal region of PTPα. This sequence showed a preference for the SH3 domains of the Lyn and Fyn protein-tyrosine kinases, although variable binding was observed to other SH3 domains. We also demonstrated that similar polyproline sequences are present in specific murine retroviral proteins and in the membrane-proximal regions of certain members of the cytokine receptor family. Moreover, peptides bearing these sequences are able to bind the Lyn SH3 domain *in vitro*. In addition, binding of the Lyn SH3 domain to the polyproline regions of both PTPα and the IL-3 receptor alpha chain required an intact PXXP motif. Furthermore, Lyn SH3 domain binding to the PXXP motif of PTPα required basic amino acids N-terminal to the proline-rich region, consistent with this being a class I binding site. Previous studies have shown that the presence of such basic amino acids determine the orientation of the peptide ligand on the face of the SH3 domain (Feng et al., 1994; Lim et al., 1994). Class I SH3 ligands, with the notable exception of the 3BP1-Abl SH3 interaction, usually contain an arginine or lysine residue at the -3 position of the PXXP motif. This residue forms a salt bridge with a conserved acidic residue within the SH3 domain (D99 in the Src SH3). The binding of the PTPα class I peptide, but not the class II peptide to the Lyn SH3 domain, suggests that the arginine and/or lysine residue at position -3 and/or -2 of the PTPα-PXXP motif may interact with the equivalent of Src aspartate 99 in the Lyn SH3 domain.

These studies were initiated to elucidate the nature of the association of PTPα with Src kinase(s) within the human epidermoid cell line A431. We have been able to co-immunoprecipitate PTPα with Src kinase(s) from A431 cells over-expressing PTPα using the antibody SRC2 which recognizes Src, Yes, and Fyn. Thus far, however, we have been unable to show that binding of the PTPα-PXXP motif to Src kinase SH3 domains *in vivo*

is responsible for this association. Although the SH3 domain of Lyn formed the strongest association with PTPα *in vitro,* these two molecules did not stably associate when co-expressed in 293 cells (data not shown). We also investigated the possibility that SH3 domain binding to this region may regulate the enzymatic activity of PTPα. However, no change in the *in vitro* catalytic activity of PTPα was observed in the presence of SH3 domains (data not shown). It is possible that the PTPα proline-rich region cooperates with another domain within PTPα, or an associated molecule, to enhance the association of Src kinase(s) with PTPα. Alternatively, the proline-rich region of PTPα may associate with higher affinity with an as yet untested or unidentified SH3 domain-containing molecule. It is also possible that this region of PTPα serves to position specific molecules for dephosphorylation by PTPα, a process which may not require the formation of a stable association. Further studies will be required to address these interesting possibilities.

Others have had similar difficulty correlating the *in vitro* binding specificities of SH3 domains with binding to specific intracellular proteins. The Nef protein of HIV-1, for example, has been shown to bind the SH3 domains of Hck and Lyn tyrosine kinases *in vitro*, however, stable association of cell-derived Nef protein with these kinases could not be demonstrated *in vivo* (Saksela et al., 1995). Similarly, the proline-rich region of BTK bound the SH3 domains of Lyn, Fyn, and Hck *in vitro*, but was not stably associated with these kinases in lysates obtained from cells expressing these molecules (Cheng et al., 1994; Yang et al., 1995). The herpesvirus Saimiri TIP protein associates with Lck in virus-infected T-lymphocytes by means of the binding of two disparate regions of the TIP protein with Lck. Although a proline-rich region within TIP bound to the Lck SH3 domain, this region alone was insufficient for strong binding to Lck (Jung et al., 1995). This suggests that some SH3-polyproline interactions are of insufficient affinity to allow detection by standard co-immunoprecipitation techniques. Other interactions, such as SH2-phosphotyrosine binding, may be required in combination with SH3-polyproline

binding, to create a stable association between specific proteins. Indeed, Cowburn et al. (1995) have shown that the binding affinity of a GST fusion protein containing the Abl SH3 and SH2 domains was significantly enhanced when both SH3 and SH2 domain ligands were present within the same synthetic peptide target. Intriguingly, the C-terminal SH3 domain of Grb2 bound the proline-rich region of PTPα *in vitro*. This finding, and the observation that the SH2 domain of Grb2 binds tyrosine phosphorylated PTPα, may suggest that Grb2 associates with PTPα in both an SH2 and SH3 domain-dependent manner. Although, den Hertog *et al.* (1996) found a binding site for the Grb2 C-terminal SH3 domain in PTPα-D1 it is possible that the proline-rich region of PTPα contributes to this association.

The difficulty in detecting what might be low affinity, yet physiologically relevant SH3-polyproline interactions, led to the design of an *in vivo* system which we thought would allow detection of such associations. However, co-expression of membrane-linked chimeric molecules containing the Lyn SH3 domain and the FSV polyproline sequence fused to the EPO receptor cytoplasmic tail failed to provide factor-independent Baf cell proliferation. Several possibilities exist to explain this result. It is possible that the affinity of SH3 domains for polyproline ligands was insufficient to maintain EPO-R cytoplasmic domain dimers long enough to induce cell proliferation. This interpretation is supported by the results of Spencer *et al.* (1993) who showed that a single FK506 immunophilin-binding domain incorporated into the T-cell receptor ζ-chain was insufficient for reporter gene induction in Jurkat T-cells. However, the use of three such domains in tandem resulted in strong gene induction in the presence of FK1012. These results suggest that the use of two or more SH3 domains and polyproline ligands in tandem may be required to provide Baf cell factor-independent growth. It is also possible that placement of the SH3 and FSV sequences in the juxtamembrane region of the EPO-R interfered with the assembly of signal transduction molecules required for proliferation or that these motifs were unable to interact due to steric hindrance. To address these possibilities use of

multiple copies of the SH3 and FSV regions or placement of these domains at the C-terminus of the EPO receptor would be required.

An important question raised by studies of SH3 domain polyproline-ligand interactions is how specificity of SH3 domain binding is achieved. Studies using phage-displayed or combinatorial library proline-rich peptides have defined an optimal peptide sequence for the binding of Src, Yes, Fyn, Lyn, PI3K and Abl kinase SH3 domains (Rickles et al., 1994; Rickles et al., 1995). We found that the GST-RPLPPLP Src family kinase SH3 domain consensus peptide used in this study was bound by all but two of the SH3 domains tested. In contrast, the 3BP1 and PTPα sequences were more discriminating in terms of specific SH3 domain binding. Rickles *et al.* (1995) have shown that residues outside a preferred core RPLPPLP sequence can enhance the specificity of SH3 domain association. However, the possibility remains that residues other than those selected by phage display and combinatorial library analysis, within a shorter sub-optimal core sequence, may be required for discrimination of individual SH3 domains *in vivo*. In the case of Crk association with the guanine exchange factor C3G, both the Crk SH3 domain binding specificity and binding affinity were found to be contained within a 9 residue peptide derived from C3G (Knudsen et al., 1995). In addition, the 10 amino acid polyproline-rich region of Btk bound to the SH3 domains of Fyn, Lyn and Hck, but not to other structurally similar Src family kinase SH3 domains (Cheng et al., 1994). Similarly, the Hck SH3 domain, but not other SH3 domains, bound with high affinity and specificity to the HIV-1 Nef protein. Interestingly, high affinity binding was dependent on a single residue within the RT loop of the Hck SH3 domain which when introduced into the Fyn SH3 domain, supplanted high affinity Nef binding to the Fyn SH3 domain (Lee et al., 1995). We have observed a preference of the PTPα polyproline sequence for the SH3 domains of Lyn, Fyn and to a lesser extent Grb2 (C-term). These studies suggest that PTPα-, HIV-1 Nef-, and Btk-like sequences which diverge from the optimal Src family core PXXP motifs, may be able to provide the specificity required to distinguish

specific SH3 domains *in vivo*.

The membrane proximal region of PTPα contains at least three sites of serine phosphorylation raising the intriguing possibility that serine phosphorylation is required for, or, disrupts, specific SH3 domain association with PTPα. Tracey *et al.* (1995) have shown that serine 180 and serine 204 of PTPα are phosphorylated by PKC *in vitro* and within phorbol-ester stimulated 293 cells over-expressing PTPα. The authors also suggested that serine 202 of PTPα may be phosphorylated by a proline-directed kinase such as a MAP kinase. Serine 202 and 204 reside immediately N-terminal to the PTPα-PXXP motif (ARS*PS*TNRKY**PPLP**). The presence of these phosphorylation sites supports the notion that this PTPα-sequence lies within an exposed region of the molecule and a potential site of protein-protein interaction. In support of the possibility that phosphorylation of PTPα may regulate SH3 domain binding, serine 202 and 204 lie at positions equivalent to valine 1 and leucine 3 within the synthetic Src SH3 domain *in vitro* ligands VSL12 (**V**S**L**ARRPLPPLP) and VPL12 (**V**P**L**ARRPLPPLP). NMR studies of these peptides complexed with the Src SH3 domain revealed that valine 1 and leucine 3 formed specific contacts with the n-src and RT loops of the Src SH3 domain. In particular, leucine 3 was found to interact with threonine 98 of the Src SH3 domain. The authors suggested that the preference of the Lyn and PI3K SH3 domains for arginine rather than leucine in the corresponding position of their ligands might be accounted for by the presence of acidic residues, rather than threonine in their respective SH3 domains (Feng et al., 1995). Based on these predictions, phosphorylation of the PTPα sequence might be expected to enhance binding of SH3 domains containing a basic residue at this position (such as the spectrin and Blk SH3 domains), or, to inhibit the binding of SH3 domains such as Lyn and Fyn, which contain a corresponding acidic residue. We are currently investigating the effects of serine phosphorylation on the PTPα-SH3 domain interaction.

Finally, we have found that members of the cytokine receptor superfamily contain

a proline-rich region that is similar in sequence and location to the proline-rich region in PTPα. The PTPα sequence is similar to the IL-3, IL-5, and GM-CSF receptor alpha chain sequences in that each sequence contains an arginine or lysine residue at the -3 position of the PXXP motif, an aromatic residue flanking the first proline residue, and a lysine or arginine residue at the +6 position in addition to the core PXXP sequence (Fig. 4.13). The similarity of these PXXP motifs suggests that these polyproline-containing peptides may have a similar overall topology. Proline-rich SH3 domain ligands form a left-handed type II helix that completes a helical turn every three residues (Williamson, 1994). Thus, both the amino- and carboxy-terminal basic residues present in the PTPα and cytokine receptor PXXP motifs may be on the same face of the polyproline helix and be able to make specific contact with their respective SH3 domain partners. However, binding of the Lyn SH3 domain to the growth hormone receptor polyproline sequence was observed despite the fact that this sequence lacked a basic residue at the -3 position of the PXXP motif. Similarly, the SH3 domains of Src and Lyn bound the 3BP1 peptide used in this study even though this peptide lacked an appropriate basic residue at the -3 position of the PXXP motif. In contrast, the polyproline sequences of the erythropoietin receptor and LAR PTP, both of which contain PXXP motifs and basic residues at the -3 position, showed no detectable binding to the Lyn SH3. This observation illustrates the difficulty in predicting SH3-binding motifs based on simple examination of primary amino acid sequences, and suggests that a complex process of discrimination occurs between a prospective SH3 domain and potential polyproline partner. This selectivity likely includes both the requirement for specific structural elements essential for binding and the absence of amino acids which may hinder binding of specific SH3 domains.

SH3 domain polyproline interactions may also be important in signal transduction from cytokine receptors. Dimerization of the growth hormone receptor, or the IL-3, IL-5, or GM-CSF cytokine-specific receptor alpha chains with a common beta chain, initiates tyrosine phosphorylation and the recruitment and activation of various signal transduction

molecules (Miyajima et al., 1993; Ihle, 1995; Ihle and Kerr, 1995). The cytoplasmic domains of the IL-3, IL-5 and GM-CSF receptor alpha chains have little similarity with each other except for a short proline-rich juxtamembrane sequence. Despite this limited homology, the membrane-proximal regions of various cytokine receptors are required for initiation of tyrosine phosphorylation and mitogenic signaling in response to cytokine binding (Ihle, 1995). Indeed, disruption of these polyproline sequences by mutagenesis ablates the ability of specific cytokine receptors to transduce a mitogenic signal (Murakami et al., 1991; Polotskaya et al., 1993; Takaki et al., 1994; Narazaki et al., 1994; Cornelis et al., 1995; Wang and Wood, 1995; Avalos et al., 1995). We have now shown that these proline-rich regions of the IL-3, IL-5 and GM-CSF receptor alpha chains and the membrane-proximal region of the growth hormone receptor can bind to SH3 domains *in vitro*. Although the Jak family kinases have been shown to be both associated with and crucial for cytokine receptor-initiated signal transduction (Ihle and Kerr, 1995), other tyrosine kinases such as Hck, Lck, Lyn, Fyn, Yes, Btk and Tec have also been identified as being activated and/or associated with specific cytokine receptors (Hatakeyama et al., 1991; Schieven et al., 1992; Corey et al., 1993; Kobayashi et al., 1993; Ernst et al., 1994; Seckinger and Fougereau, 1994; Machide et al., 1995; Sato et al., 1994; Mano et al., 1995; Pazdrak et al., 1995). An intriguing possibility is that one or more of these molecules may associate with the membrane-proximal region of cytokine receptors in an SH3 domain-dependent manner. Alternatively, the SH3 domain-binding regions of these receptors may be required for their association with specific cytoskeletal structures that are required for the localization and/or activation of Jak family kinases. The conservation of amino acids required for SH3 domain association and the similarity of the cytokine receptor motifs to the PTPα proline rich region raises the possibility that these regions represent functionally homologous units within these distinct molecules. The regulation and specificity of the protein interactions mediated by these proline-rich regions represents a future challenge in the study of both PTPα and cytokine receptor signaling.

CHAPTER 5

General Discussion

The growth, proliferation, metabolism, and differentiation of cells involves a wide variety of molecules integrated into complex signal transduction pathways acting to organize specific cellular responses to diverse stimuli. Many cellular activities involve the co-ordinated activities of both protein kinases and phosphatases. Tyrosine phosphorylation may act to regulate the activity of specific enzymes and/or the assembly of various signal transduction complexes. In this role, reversible phosphorylation may induce changes in gene transcription, cellular metabolism, and alter cellular architecture during cell migration, cell-substratum adhesion, and cytokinesis.

This thesis has focused on the analysis of PTPα, a member of the PTP family isolated in this lab in 1990. Although this thesis was aimed at determining the role of PTPα in cell physiology, in more general terms the aim was also to investigate the activity of this class of enzyme in terms of both catalytic activity and substrate specificity. This involved the production of recombinant enzymes and required the development of methods to characterize these molecules. PTPα analysis also necessitated expression of this enzyme within mammalian cells and required the development of reagents such as antibodies and mutant forms of the enzyme to assess *in vivo* function.

5.1. Bacterial expression, purification, and characterization of PTPα and PTPβ. At the onset of these enzyme/substrate specificity studies it was unclear as to whether PTPs would display substrate specificity. In fact, PTPs were generally regarded as house-

keeping enzymes, unlikely to discriminate between phosphotyrosine-containing substrates. However, the rapid identification of additional members of the family with diverse structural characteristics and expression patterns, suggested that like PTKs, PTPs might be highly regulated and exhibit substrate preferences. In studies aimed at assessing this possibility, PTPα and PTPβ were expressed in bacteria using either the pGex system or a modification of the pET bacterial expression system developed by Studier et al. (1990). PTPs were purified by epitope tagging immuno-affinity chromatography and characterized using a variety of phosphotyrosine-containing synthetic peptides and a modified malachite green assay. This assay provided sensitive, colormetric detection of PTP activity. Peptide substrates were chosen to maximize the variability of residues surrounding a centrally located phosphotyrosine residue and represented *in vivo* sites of tyrosine phosphorylation. For example, a peptide based on the autophosphorylation site of the CSF-1 receptor, CSF-1-R Y708, was selected because of its high basic residue content surrounding the phosphotyrosine residue (CSF-1-R Y708, IHLEKKpYVRRDSG). These studies demonstrated the substrate specificity of PTPβ, which favored substrates lacking basic residues neighboring the phosphotyrosine residue. This preference was reflected by a Km of ~140 μM for Src Y527, a peptide comprised of largely neutral and acidic residues (Src Y527, TSTEPQpYQPGENL), while the Km of PTPβ for peptide CSF-1-R Y708 was greater than 10 mM. Subsequent studies have suggested that other PTPs such as SHP-2 may share this selectivity (Dechert et al., 1994). However, this may not be a general characteristic of PTPs as the K_m of PTPα with the CSF-1-R Y708 peptide was similar to that observed with peptide Src Y527. Thus, although these studies demonstrated that certain PTPs do display specific peptide substrate preferences, the contribution of substrate or PTP cellular compartmentalization and protein substrate secondary structure need to be investigated to fully define the selectivity of this enzyme family.

Bacterial expression of the cytoplasmic domains of PTPα either alone or in

combination was also useful in demonstrating the activity intrinsic to the second catalytic domain of PTPα. Intriguingly, this domain was the most active on *p*-NPP as opposed to phosphopeptide based substrates. Although dephosphorylation of the Src Y527 phosphopeptide was observed, these assays required large quantities of recombinant domain-2. To date, PTPα domain-2 is the only C-terminal PTP domain which has displayed catalytic activity *in vitro*. Thus, the role of these domains in catalytic function is as yet unclear. It has been suggested that these domains may act to regulate the activity of the N-terminal PTP domain (Strueli et al., 1990) or become active *in vivo* in response to specific stimuli or covalent modification.

PTPα-transfected A431 cells were useful in demonstrating that mutants of the enzyme, containing an inactive N-terminal catalytic domain but retaining the C-terminal domain, were inactive in immunoprecipitation PTP assays. This result supported those of the recombinant enzyme assays, which demonstrated that the majority of PTP activity resided in the N-terminal domain of PTPα. Thus, it is unlikely that the low activity of recombinant C-terminal phosphatase domains reflects problems associated with bacterial expression of these enzymes. However, we found that A431 cells transfected with a mutant form of PTPα, containing a cysteine-to-alanine mutation in the C-terminal catalytic domain, either did not express the enzyme or quickly lost expression (data not shown) suggesting a critical function for this domain. Further studies with these mutants in combination with either transient or inducible expression systems in mammalian cells may aid in the determination of PTPα domain-2 function.

Interestingly, the C-terminal domain of PTPα was tyrosine phosphorylated by kinases such as Csk and Lck *in vitro* and by an unidentified tyrosine kinase *in vivo*. We have mapped the *in vitro* sites of phosphorylation to Y579 and Y789 within PTPα domain-2 by phosphopeptide mapping and mass spectrophotometry analysis (Amankwa, L., Harder, K., and Jirik, F., unpublished observations). Other studies have determined that tyrosine phosphorylation at Y789 results in Grb2 association with PTPα (den Hertog

et al., 1994; Su et al., 1994), however the consequence of this association in terms of regulation of domain-2 activity or signal transduction complex assembly are unclear. PTPα was also inducibly tyrosine phosphorylated in EGF-stimulated A431 cells. However, due to auto-dephosphorylation of PTPα, we were unable to determine the effects of phosphorylation on PTP activity. Regardless, these results suggest that PTPα may be involved in some aspect of growth factor receptor PTK signaling.

*5.2. **Expression of PTPα in mammalian cells.*** We overexpressed PTPα in A431 cells to assess the potential of PTPα to antagonize PTK-dependent cell growth. A431 cells overexpress the EGF receptor and are dependent on autocrine production of TGFα (Van de Vijer et al., 1991). Paradoxically, these cells are growth inhibited by high concentrations of EGF. Thus, these cells provided a model system in which the consequences of PTPα expression on both autocrine TGFα-EGF-R-dependent cell growth, and EGF-induced growth inhibition could be examined. High level expression of PTPα in A431 cells was achieved using the CMV promoter based episomal plasmid pBCMGsNeo (Karasuyama and Melchers, 1988). Antibodies produced against either synthetic peptides based on PTPα sequences, or bacterially expressed PTPα, detected a dominant immunoreactive protein of 130-150 kDa in transfected cells. Moreover, antibodies directed against the extracellular region of PTPα stained the surface of transfected cells, confirming the expected plasma-membrane localization of this PTP. However, the expression of PTPα in A431 cells was unable to protect these cells from EGF-induced growth inhibition. Moreover, control and transfected cells grew at similar rates, suggesting that PTPα was unable to regulate EGF-R-dependent growth or differentiation. Consistent with this observation, EGF-R obtained from PTPα expressing cells contained similar levels of phosphotyrosine as control A431 cell-derived receptor. Furthermore, nude mice injected with either control-transfected or PTPα overexpressing A431 cells developed tumors of similar size and with a similar latency period (data not

shown).

5.3. PTPα expression results in Src kinase regulation and alters cell-substratum adhesion. Although PTPα was unable to disrupt EGF-R growth or differentiation signals, we did observe a dramatic increase in cell-substratum adhesion of PTPα overexpressing cells. This characteristic appeared to be dependent on the catalytic activity of PTPα, as cells containing an inactive mutant form of PTPα, with a cysteine-to-alanine mutation in domain-1, appeared less adherent than control cells. Furthermore, treatment of A431 cells with EGF typically results in the rounding and lifting-off of these cells from the substratum. However, we found that cells expressing PTPα remained adherent and spread in the presence of EGF. Thus, cells expressing PTPα displayed increased cell-substratum adhesion in the absence of EGF, and were resistant to the morphological changes and adhesion disrupting effects that normally follow EGF stimulation of A431 cells.

To investigate the underlying mechanism for this effect on adhesion, we examined potential substrates of PTPα catalytic activity. PTPα overexpression led to the activation and/or dephosphorylation of the tyrosine kinases Src and Yes. Intriguingly, these molecules appeared to be the only proteins which displayed reduced levels of phosphotyrosine detectable in total cell lysates. Indeed, other proteins such as Fak and paxillin contained increased phosphotyrosine levels. These results and those suggesting that the EGF-R was unaffected by PTPα expression, suggest that certain PTPs may possess restricted substrate specific activities. Thus, in contrast to the results of the recombinant enzyme/phosphopeptide assays which suggested that PTPα possessed little substrate specificity, these results demonstrate that PTPα may select a narrow subset of substrates within cells. Results demonstrating elevated levels of phosphotyrosine in the Src kinase substrates Fak and paxillin are particularly intriguing. PTPα may either dephosphorylate and activate Src kinases within a cellular compartment which lacks other potential substrates, or, PTPα may be complexed with Src kinases in a way which

precludes its accessibility to kinase substrates such as Fak and paxillin which are substrates of Src kinases. Alternatively, dephosphorylation of Src kinases may be separated temporally from the tyrosine phosphorylation of specific Src kinase substrates, with PTP activity being inhibited following Src activation. Specific SH2 domain association with phosphotyrosine residues may also protect these sites from the activity of PTPα. The activation of Src kinases by PTPα is reminiscent of the role of CD45 in the activation of Lck and Fyn in lymphocytes. These PTPs appear to be specific for Src family kinases which they regulate by dephosphorylation of negative regulatory tyrosine residues.

The relationship between Src kinase activation and cell-substratum adhesion is unclear. Although we observed an increase in the association of Src kinases with Fak and an increase in paxillin tyrosine phosphorylation it is not known how such changes act to alter cell-substratum adhesion. It is clear, however, that paxillin and Fak are involved in focal adhesion structure or assembly (Fig. 5.1). Furthermore, data demonstrating localization of Src kinases to focal adhesions and results correlating Src kinase activation with cell-adhesion point to these kinases being involved in either the formation of adhesion structures or signal transduction in response to adhesion. Our results demonstrating increased cell-substratum adhesion of A431 cells, suggest that PTPα may act to regulate Src kinases involved in focal adhesions (Fig. 5.1). In addition, an increase in Csk association with paxillin may provide a mechanism for the negative regulation of Src kinases in PTPα expressing cells. However, it will be important to assess the effects of PTPα overexpression on the adhesion of other cell types. The possibility that the cell-substratum adhesion effects of PTPα are a consequences of unphysiological PTPα overexpression must also be considered.

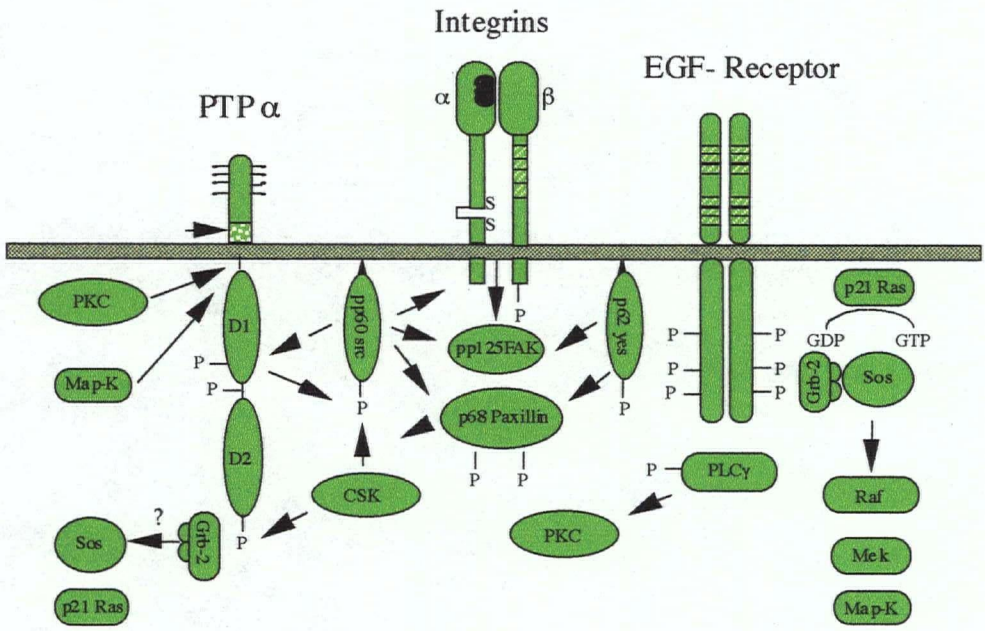


FIG. 5.1. Illustration of potential PTPα/integrin/EGF receptor interactions. The binding of integrin receptor extracellular domains to ECM-ligands such as fibronectin leads to the activation of FAK and the tyrosine phosphorylation of paxillin. We have shown that PTPα expression in A431 cells results in Src kinase activation, enhances Src/FAK association, and increases cell-substratum adhesion. Moreover, the v-Src and FAK substrate paxillin is hyperphosphorylated in PTPα expressing cells. Arrows indicate potential interactions between molecules in terms of either association, phosphorylation, or activation. The EGF-R is included in this figure as PTPα was a target of EGF-induced tyrosine phosphorylation in A431 cells. In addition PTPα expression appeared to modulate cell-substratum adhesion and cell-shape changes induced by EGF.

5.4 PTPα, various retroviral oncoproteins, and specific cytokine receptors contain an SH3 domain-binding motif. Investigations into the nature of the interaction between PTPα and Src family kinases led to studies of a proline-rich region in the membrane-proximal region of PTPα. However, although PTPα and Src kinases co-immunoprecipitated from A431 cells, we were unable to demonstrate involvement of Src kinase SH3 domains with the proline-rich region of PTPα in the association of these

molecules. A variety of *in vitro* studies demonstrated the ability of this region to bind SH3 domains. Thus, it is likely that this region is at least partially responsible for either the association of an SH3 domain-containing substrate with PTPα or for SH3 domain-dependent PTPα subcellular localization. It is also possible that this region functions within a specific cell type which expresses a specific SH3 domain-containing molecule. Alternatively, this region may be involved in the regulation of PTPα activity by association with a protein such as Grb2 (den Hertog and Hunter, 1996). A mutant form of PTPα containing proline-to-alanine substitutions within this putative SH3 domain binding site have been constructed. This mutant has been transfected into A431 cells and analysis of both Src kinase association and activation by PTPα are being explored.

Studies of this proline-rich SH3 domain binding region in PTPα led to the identification of other similar proline-rich putative SH3 domain ligands in other proteins. For example, proline-rich sequences within oncogenic retroviral proteins derived from FSV and AMLV bound SH3 domains *in vitro*. One of these regions within p70-gag-fgr has been shown previously to facilitate transformation of 3T3 cells (Sugita et al., 1989). We also identified proline-rich sequences in various cytokine receptors which were able to bind SH3 domains *in vitro*. However, like those PTPα and retroviral sequences, additional studies will be required to confirm SH3 domain association with these regions *in vivo*. It's likely that these proline-rich sequences may provide critical sites for association for SH3 domain containing molecules involved in signaling from these molecules.

CHAPTER 6

Materials and Methods

6.1. For Chapter 2

6.1.1. General. A human placenta λ Zap cDNA library was obtained from Stratagene (catalogue #936203). Vent DNA Polymerase was purchased from New England Biolabs. Restriction enzymes and protease inhibitors were purchased from Boehringer Mannheim. The pET-11a bacterial expression vector and the *E. coli* strain BL21 (DE3) were from Novagen (Studier et al., 1990). Mouse ascites fluid containing the monoclonal antibody 12CA5 was obtained from BAbCO (Field et al., 1988). Prestained molecular weight markers were from Amersham. The DNA sequencing kit, MonoQ column, protein A and cyanogen bromide Sepharose were from Pharmacia LKB Biotechnology Inc. Radioisotopes were from ICN and Amersham. A Biotec model 309 microtiter plate reader was used. Half-volume microtiter plates were from Canlab (Costar catalogue #3690). Malachite green (1% w/v solution) was from BDH. Ammonium molybdate was from Sigma and Tween 20, EIA grade, was purchased from Bio-Rad. *p*-nitrophenylphosphate (*p*-NPP) was obtained from Sigma. *t*-Butyl oxy carbonyl tyrosine (o) phosphate dibenzyl was obtained from Peninsula Laboratories. All other reagents and chemicals were from BDH or Sigma.

6.1.2. Cloning of PTPα and PTPβ intracellular domains.. The sequences of the PTPβ 5' and 3' primers:, CTCTCGCTAGCAGACAGAAAGTGAGCCA TGGTCGAG and CTCAGATCTCTCAATGCCTTGAATAGACTGGATC, respectively, were derived from the published sequence of human PTPβ (Krueger et al., 1990), and were synthesized on an Applied Biosystems DNA Synthesizer (model 391). The 5' and 3' primers correspond to nucleotides 1594 through 1619 and 1900 through 1926, respectively, of human PTPβ. The 5' primer contains additional nucleotides encoding an *Nhe* 1 restriction endonuclease site, while the 3' oligonucleotide contains a *Bgl* II site. 15 µl of the human placental λ-Zap cDNA library was combined with an equal volume of dH_2O and heated at 70°C for 10 min followed by chilling on ice. To this reaction 200 µM dNTPs, 5 µl 10x Vent buffer, 5 µl 10x bovine serum albumin (10 mg/ml), and 3 Units of Vent DNA polymerase were added to a final volume of 50 µl. Polymerase chain reaction (PCR) amplification (30 cycles of 94°C for 1.5 min, 50°C for 1.75 min, and 72°C extension for 2 min) produced a 1.07 kilobase fragment. This fragment was gel-purified and ligated into *EcoR* V restriction endonuclease digested Bluescript plasmid (Stratagene) to give the plasmid pPTPβ. Similarly, oligonucleotides corresponding to specific sequences of PTPα were used to PCR the entire cytoplasmic domain of PTPα (PTPα-D1+D2, a.a. 167-793), the first catalytic domain (PTPα-D1, a.a. 167-555), and the C-terminal phosphatase domain (PTPα-D2, a.a. 510-793). Each PCR fragment was verified by DNA sequencing using the chain termination method (Sanger et al., 1977), digested with *Nhe* 1 and *Bgl* II, and subcloned into *Nhe* 1 and *BamH* 1/ *Bgl II* digested pET-11a-tag and/or pGex-2T-tag plasmids.

6.1.3. Modification of pET-11a and pGex-2T bacterial expression vectors. To facilitate both the detection and purification of recombinant proteins expressed in bacteria, the pET-11a vector, and more recently the pGex-2T vector, were modified by the addition of a nucleic acid sequence encoding a 10-residue segment located at the N-terminus of the

expressed protein. This sequence corresponds to a peptide derived from the influenza virus hemagglutinin protein that is recognized by the monoclonal antibody 12CA5 (Field et al., 1988). PCR was used to incorporate this decapeptide 'HA tag' sequence along with two flanking glycine spacers into the pET-11a plasmid (Fig. 1). 50 pmols each of primer-A (GGATCGAGATCTCGATCCCGCGAAATT) and primer-B (CTCGCTAG CACCGCCAGATGCGTAGTCCGGAACGTCGTACGGGTAACCGCCGCGGCCCAT TTGCTGTCCACCAGTCATGCTAGTCATATG), containing the fusion sequence, were added to 100 ng pET-11a template DNA, 10x PCR buffer, 200 µM dNTPs and 2.5 Units of *Taq* DNA polymerase in a volume of 100 µl. The fragment resulting from PCR amplification (30 cycles of 96^{o} C for 45 sec, 50^{o} C for 90 sec, 72^{o} C for 120 sec) was subcloned into Bluescript plasmid, the DNA sequence determined, and then directionally subcloned into *Bgl* II and *Nhe* 1 digested pET-11a.

The pGex-2T plasmid was also modified by incorporation of sequences encoding the 'HA tag'. Two overlapping oligonucleotides were synthesized: **A**-5'-GATCCCCCGGGTACCCGTACGACGTTCCGGACTACGCATCCCCCGGGGCTAG CGTCGACAGATCTG-3' and **B**-5'-AATTCAGATCTGTCGACGCTAGCCCCGGGG ATGCGTAGTCCGGAACGTCGTACGGGTACCCGGGG-3'. Each oligo was diluted to 25 pmoles/µl in dH_2O and heated to 95^{o}C for 5 min, the oligos were combined and shifted to 70^{o}C for 5 min, 45^{o}C for 5 min, and then to 37^{o}C for 20 min. The annealed oligos were diluted 1/50 in dH_2O (250 fmoles/µl) and ligated into *BamH1*, *EcoR1* digested pGex-2T. Thrombin cleavage of Glutathione-S-Transferase (GST) fusion proteins expressed with this plasmid result in proteins containing the HA epitope at the N-terminus.

6.1.4. Immunological methods. Monoclonal antibody 12CA5 was purified from ascites fluid by passage over a sheep anti-mouse IgG cyanogen bromide-linked Sepharose column. 12CA5 was used for immunological detection of bacterially-expressed protein in

conjunction with alkaline phosphatase-linked goat anti-mouse IgG heavy and light chain specific antisera (Calbiochem). A 12CA5 affinity column was prepared by cross-linking 4 mg of the mAb (IgG2b isotype) to 2 ml protein A Sepharose (50% slurry) using the cross-linking agent dimethylpimelimidate (Harlow and Lane, 1988).

6.1.5. Buffers. Buffer A: 25 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM 2-mercaptoethanol. Buffer B: same as buffer A except that soybean trypsin inhibitor (0.6 μg/ml), leupeptin (0.5 μg/ml), phenylmethylsulfonylfluoride (1 mg/ml), and pepstatin (0.7 μg/ml) were added. Buffer C: 25 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM 2-mercaptoethanol, 0.5% (v/v) Tween-20. Buffer D: 25 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM dithiothreitol (DTT), 1 mg/ml decapeptide (YPYDVPDYAS). Buffer E: 100 mM Tris-HCl pH 8.0, 5 mM DTT.

6.1.6. Small-scale expression of ^{35}S labeled PTPs. 15 ml BL21(DE3) bacterial cultures containing plasmids pET-11a-tag PTPα or PTPβ were grown in minimal-9 (M9) media containing $MgSO_4$ and 25 ug/ml ampicillin (amp) overnight to an optical density at 600 nM of ~0.9. Cultures were divided in half and (1 mM) isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to half of the cultures. The cells were induced for 1 hour before pelleting and resuspending in M9 media (without $MgSO_4$) containing 25μl ^{35}S labeled H_2SO_4 (ICN, 1383 mCi/ml). Cells were grown for an additional 20 min before lysis and analysis.

6.1.7. Bacterial expression of pET-11a-tag PTPα and PTPβ. The pET-11a-tag-PTPβ and pET-11a-tag-PTPα plasmids was transformed into competent *BL21 (DE3)* bacteria. This strain contains an integrated copy of the T7 RNA polymerase gene carried by the bacteriophage *DE3*, and is under the control of the IPTG inducible *Lac* UV5 promoter (Studier et al., 1990). 20 ml overnight cultures of *BL21 (DE3)* bacteria grown in 100

µg/ml ampicillin/Luria broth were added to 2x1 litre of this same medium and grown with shaking to an O.D. 600 of ~0.7 at 37° C in the absence of IPTG. Recombinant PTP production was stimulated by the addition of IPTG (1 mM). After 4-5 hours of induced growth the bacterial cells were pelleted by centrifugation at 3,500 rpm for 15 min at 4°C (Beckman J-6B). The cells were washed in buffer A, sedimented and then resuspended in 5 ml buffer B. 550 µl of 10 mg/ml lysozyme was added and the mixture was incubated on ice for 15 min. To ensure bacterial lysis, 3 cycles of alternating freeze-thaws on dry ice/ethanol were performed, followed by addition of 55 µl of 1M $MgCl_2$ and 25 µl DNase 1 (10 mg/ml) to the lysate. This mixture was incubated at 25°C for 20 min, 100 µl of 500 mM EDTA and 600 µl of 10% Triton X-100 were then added, and the lysate was incubated for a further 30 min at 25°C. The extract was subjected to ultra-centrifugation (Sorvall RC 80, SW55Ti rotor) at 35,000 rpm at 4°C for 45 min and the pellet discarded.

6.1.8. PTP purification. The bacterial extract supernatants were added to protein A-12CA5 affinity columns (prepared as previously described) and rotated at 4°C overnight. Each protein-bead matrix was washed with 50 ml of buffer C at 4°C. Elution of PTPα and PTPβ bound to the columns was achieved by resuspending the beads in each column with 1.5 ml buffer D warmed to 30°C containing 1 mg/ml of the decapeptide (YPYDVPDYAS) (Field et al., 1988). The column mixtures were incubated for 15 min at 30°C before collection of the eluate. This elution procedure was repeated and the two elutions were combined and concentrated to a volume of ~500 µl with a Centricon 30 microconcentrator (Amicon). The concentrated eluate was then loaded onto a MonoQ anion exchange column (for PTPβ) (1 ml bed volume) equilibrated with 25 mM Tris-HCl pH 7.5, 5 mM DTT. The column was eluted with a 0.1-0.5 M NaCl gradient using a Pharmacia Fast Protein Liquid Chromatography system. PTP activity in 250 µl column fractions was assessed as described below. Peak fractions were pooled, glycerol was added to 20% (v/v), and then aliquots were frozen in liquid nitrogen before storage at

-80°C. The 12CA5 affinity column was regenerated for repeated use by eluting the decapeptide with 10 column volumes of 100 mM triethylamine pH 11.5, followed by equilibration in 50 mM Tris-HCl pH 7.5, 150 mM NaCl.

6.1.9. Bacterial expression and purification of pGex expressed PTPα and PTPβ. Luria broth cultures (500 ml) of UT5600 bacteria (New England Biolabs) containing the various pGex plasmids were grown to O.D. 600 ~0.6-0.9 at 37°C. Cells were then shifted to 26°C and induced overnight with 100 μM IPTG. Bacteria were sedimented and lysed by sonication in buffer composed of 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol and 1 mM PMSF. Triton X-100 was added to 1% final concentration and cellular debris was removed by ultra-centrifugation at 30,000 x *g*. The supernatant was removed and incubated with 1 ml of a 50% slurry of glutathione Sepharose (Pharmacia Biotech) for 1 hour. The beads were thoroughly washed before cleavage in 1 ml of buffer containing 50 mM Tris pH 8.0, 2.5 mM $CaCl_2$, 150 mM NaCl, 10 mM β-mercaptoethanol and 50 μl thrombin (400 μg/ml). Glycerol was added to 15% final concentration before storage at -80°C.

6.1.10. Lck tyrosine-phosphorylated ζ-chain PTP assay. Preparation of PTP substrate; 100 μg of ζ-chain peptide was tyrosine-phosphorylated with 10 μl (25 μg) of Lck (supplied by Julian Watts) in buffer (25 mM Tris pH 7.5, 10 mM MgCl2, 1.0 mM DTT) containing 50 μCi ^{32}P-γ-ATP, and 250 μM unlabeled ATP for 1.5 hours at 30°C. The reaction mixture was HPLC purified, dried down to remove the acetonitrile, resuspended in 500 μl dH_2O, and again dried down to remove remaining acetonitrile. The ζ-chain peptide was again resuspended in dH_2O for use as a PTP substrate. For the PTP assay 150,000 CPM of ^{32}P labeled ζ-chain was combined with the appropriate dilution of PTP in buffer containing 25 mM Tris pH 8.0 (PTPβ) or 25 mM MES (2-[N-Morpholino]ethanesulfonic acid) pH 6.0 (PTPα), in a final volume of 50 μl. Reactions

were carried out for 15-30 min at 30°C. 50 µl of 25 mg/ml bovine serum albumin (BSA) and 350 µl of 30% trichloroacetic acid were then added and reactions were chilled on ice for 15 min to precipitate the ζ-chain peptide. Precipitated protein was pelleted in a microfuge and 300 µl of supernate was scintillation counted to determine the amount of ^{32}P liberated from the ζ-chain peptide by phosphatase treatment.

6.1.11. Malachite green solution preparation. One volume of 4.2% (w/v) ammonium molybdate in 4 N HCl was added to 3 volumes of 0.045% (w/v) malachite green. This solution was stirred for a minimum of 30 min before filtering through a 0.22 µM filter (Millipore). The filtered solution was stored for up to 6 months at 4° C. Tween 20 (0.01% v/v) was added to an aliquot of filtered malachite green solution prior to use.

6.1.12. Generation of inorganic phosphate standard curve. Standards were prepared from KH_2PO_4 that had been desiccated at 80° C for 5 hours. Appropriately diluted inorganic phosphate (P_i) standards in a volume of 25 µl were delivered to half-volume wells of a 96-well microtiter plate, followed by the addition of 50 µl of the malachite green-Tween 20 solution. After 15 min of color development at room temperature the optical density of each well was measured in an ELISA plate reader fitted with a 620 nm filter.

6.1.13. Synthesis of phosphotyrosine containing peptides. Peptides were synthesized using solid-phase methods with *t*-butyloxy carbonyl (t-boc) and alpha-protected amino acids, with appropriate side chain protection, as described (Clark-Lewis et al., 1991). Phosphopeptides were synthesized with *t*-boc Tyr PO_4 $(Bzl)_2OH$. After chain assembly, the peptides were de-protected via trifluoromethane sulfonic acid (TFMSA). Each 100 mg of peptide resin was stripped with 100 µl thioanisole, 50 µl ethanedithiol and 1 ml of trifluoroacetic acid. 100 µl of TFMSA was added and allowed to react for 2 hours at

room temperature. The peptide was precipitated with diethyl ether, washed, and then dissolved in 6 M guanidine HCl, pH 8.5. For purification this mixture was loaded directly onto reverse phase HPLC (Clark-Lewis et al., 1991). The mass of each peptide was confirmed using ionspray mass spectrometry on a model AP1 III triple quadrapole mass spectrometer (SCIEX, Thornhill, Ont) with a liquid delivery interface. The amino acid composition was confirmed by amino acid analysis.

6.1.14. Quantitation of phosphotyrosine containing peptides. Phosphopeptides were weighed on an analytical balance and dissolved in the appropriate volume of dH_2O. Peptides with lower solubility were dissolved in dH_2O to saturation and centrifuged at 14,000 rpm and the supernatants saved. The concentration of each phosphopeptide solution was determined by measuring P_i release by acid hydrolysis (Hasegawa et al. 1982) using the malachite green microtiter plate assay. Briefly, the phosphopeptides were completely hydrolyzed by acid cleavage in 50% (v/v) perchloric acid and 50% (v/v) sulfuric acid at 150°C for 16 hr. Liberated P_i was then detected using the malachite green assay. Values for P_i release were then obtained by comparison with the malachite green standard curve. This allowed equimolar concentrations of each peptide to be determined, and was particularly useful with peptides that demonstrated incomplete solubility.

6.1.15. Measurement of PTP activity. Enzymatic activity of PTPα and PTPβ on various phosphotyrosine containing substrates were determined using the malachite green microtiter plate assay. Enzyme reactions were carried out on half-volume 96-well plates in a final volume of 25 µl. Appropriately diluted enzymes, substrate peptides, and potential modifiers of enzyme activity (for PTPβ), were incubated for 5-15 min at 25°C in buffer E at pH 8.0 (PTPβ) or buffer E containing 25 mM MES pH 6.0 (PTPα). Reactions were terminated by the addition of 50 µl of malachite green solution and then incubated for 15 min at room temperature before optical density measurement at 620 nm.

The detection of MonoQ Sepharose column fractions containing PTPβ was achieved by assaying for PTP activity essentially as above except that 5 mM *p*-NPP was used as substrate. Reactions were carried out for 15 min before addition of 50 µl of malachite green solution and optical density determination.

6.2. For Chapter 3

6.2.1. Cells and plasmids. NIH 3T3, 293 and A431 cells were obtained from the American Type Culture Collection and were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS), antibiotics and 50 µM β-mercaptoethanol. The PTPα cDNA was obtained from a human HepG2 cell line cDNA library (Stratagene) by low stringency screening with a cDNA probe encompassing a region of the first catalytic domain of CD45 as previously described (Jirik et al., 1990). The wild type and catalytically inactive form of PTPα (containing a cysteine-to-alanine mutation at residue 433 within the first catalytic domain, D1 C433A) were subcloned into the eukaryotic expression vector pBCMGsNeo. This vector contains the cytomegalovirus promoter and 79% of the bovine papilloma virus genome which allows episomal plasmid replication (Karasuyama and Melchers, 1988). Plasmids were electroporated into A431 cells and G418 resistant clones were selected.

6.2.2. Antibodies. PTPα specific antibodies were produced by repeated immunization of New Zealand white rabbits with recombinant PTPα cytoplasmic domain containing residues 167-793 (PTPα-2), or N-terminal cysteine-linked keyhole limpet hemocyanin conjugated synthetic peptides (Ziltner et al., 1987), corresponding to amino acids 20-60 within the extracellular domain of PTPα (PTPα-ext), or amino acids 512-558, corresponding to the region between the two PTP domains (PTPα-1). All antibodies were affinity purified on thiol Sepharose-peptide or CnBr Sepharose-recombinant PTPα

specific affinity columns. The anti-Src mAb 327 (provided by J. Brugge, ARIAD Pharmaceuticals), anti-Fyn mAb (provided by R. Perlmutter, University of Washington), anti-Yes antiserum (from J. Bolen, Bristol-Myers Squibb Pharmaceutical Research Institute), and anti-Csk antisera (from J. Cooper and B. Howel, Fred Hutchinson Cancer Research Centre) were used to specifically immunoprecipitate each kinase. The antiserum SRC2 (Santa Cruz Biotechnology), which is specific for C-terminal peptide sequence 509-533 of Src and the conserved sequences of Fyn and Yes, was also used to immunoprecipitate and immunoblot Src, Fyn, and Yes. Antibodies against phosphotyrosine (4G10), paxillin, FAK, and src (GD11) were obtained from Upstate Biotechnology Inc. and Transduction Laboratories.

6.2.3. Cell lysis, immunoblotting, and kinase assays. A431 cells were lysed on ice for 30 min in buffer containing 1% Nonidet P-40, 10% glycerol, 50 mM NaCl, 50 mM Tris pH 7.5, 2 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mM soybean trypsin inhibitor, and 100 μM leupeptin. Insoluble cellular debris was removed by ultracentrifugation at 30,000 x *g* for 30 min Protein concentration was estimated with the bicinchoninic acid (BCA) assay (Pierce). PTPα expressing clones were then selected based on western blot analysis of total cell lysates of G418 resistant A431 cells.

6.2.4. Kinase Assays. Src kinase activity from the various A431 clones was assessed by autokinase and enolase assay. Src kinase was immunoprecipitated from 500 μg of cell lysate with 1 μg of mAb 327. Immune-complexes were collected with 75 μl of a 30% slurry of rabbit anti-mouse IgG preabsorbed protein A Sepharose. Beads were washed in cell lysis buffer, RIPA buffer (lysis buffer containing 1% NP-40, 0.5% deoxycholic acid and 0.1% SDS) and then kinase buffer (100 mM PIPES [piperazine-*N*, *N*'-bis 2-ethanesulfonic acid] pH 7.0, 5 mM $MnCl_2$, and 10 μM vanadate) before resuspension in

kinase buffer containing 10 μCi [γ-^{32}P] ATP (3000 Ci/mmole) with or without 10 μg of acid denatured enolase. Kinase assays were performed at 25ºC for 15 min before termination by addition of 2 x Laemmli buffer. 50% of each immunoprecipitation was immunoblotted with anti-Src antisera to ensure equal quantities of Src were used for each assay.

6.2.5. Flow cytometry. A431 cells transfected with vector alone, vector containing PTPα, or catalytically inactive PTPα (D1 C433A) were lightly trypsinized in 0.05% trypsin containing 1 mM EDTA. Cells were then thoroughly washed in cell culture media and approximately 10^6 cells were incubated with anti-PTPα-ext antibody (5 μg/ml) in 1 ml of phosphate buffered saline (PBS) containing 2% FCS for 1 hour. Cells were then washed in PBS/FCS and incubated with a 1/200 dilution of FITC-conjugated goat-anti-rabbit FAb2 before analysis by FACScan Flow Cytometry with FACScan Research Software (Becton Dickinson, Mountain View, CA).

6.2.6. Malachite green phosphatase assay. Phosphatase activity was determined using the malachite green microtiter-plate phosphatase (MGMP) assay to detect the release of phosphate from phosphotyrosine-containing synthetic peptides as previously described (Harder et al., 1994; Ng et al., 1995). Briefly, PTPα immunoprecipitated directly from A431 cell lysate, was incubated with phosphopeptides in buffer containing 25 mM MES pH 6.0 and 0.1 mM β-mercaptoethanol. Enzyme reactions were carried out on half-volume microtiter plates in a final volume of 25 μl for the indicated times. Phosphatase reactions were terminated and free phosphate was detected by the addition of 100 μl of malachite green solution to each well. The change in absorbance at 620 nM of each well was measured in an ELISA plate reader and the amount of phosphate released was determined by comparison to an inorganic phosphate standard curve (Harder et al., 1994). Peptides used in this study corresponded to the C-terminal regulatory phosphorylation

site of Src (Y527-TSTEPQ**Y**QPGENL) or to an autophosphorylation site of the CSF-1 receptor (Y708-IHLEKK**Y**VRRDSG). The activity of PTPα domain-2 towards *para*-nitrophenylphosphate (*p*-NPP) was detected using the MGMP assay as above.

6.2.7. A431/EGF growth inhibition assay. Control transfected A431 and PTPα over-expressing A431 cells were plated at 10^4 cells/well in quadruplicate in 96 well plates. EGF was added at the specified concentrations and cell growth was determined 4 days later by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrozolium bromide) assay (Boehringer Mannheim Inc.).

6.2.8. Cell-substratum adhesion assay. During the routine passaging of A431 clones expressing PTPα we observed that these cells were resistant to the low concentrations of trypsin used to remove cells from the substratum. To quantitate this characteristic an adhesion assay was developed to assess cell adhesion to the substratum. Cells containing vector alone, or cells expressing PTPα or PTPα (D1 C433A) were plated in 96 well flat-bottom plates in quadruplicate at 10^4 cells/well. Cells were allowed to adhere and spread in serum containing media to 75-85% confluency (1-2 days). The medium was then carefully removed so as not to disturb the cell monolayer and the cells were gently washed 3-5 times with 100 µl of PBS/well/wash over a period of 20-30 min. During this treatment control cells rounded and lifted off the substratum in a manner dependent on the number and duration of washes in PBS. To distinguish the lower adhesive properties of control transfected A431 cells and those expressing PTPα (D1 C433A) the number and duration of PBS washes was reduced. The wash solutions were then discarded and 100 µl of culture medium was added to each well. One hour later 25 µl of MTT (5 mg/ml) was added and the cells remaining in each well were incubated for 2.5 hours. The medium was then carefully removed, 100 µl of DMSO was added to each well to dissolve the precipitated MTT crystals, and the optical density at 550 nM was determined in an

ELISA plate reader. The optical density of each well was compared to untreated wells to determine the percentage of cells removed by each treatment. To analyze changes in cell adhesion in the presence of agents known to alter cell-substratum adhesion, EGF (100 ng/ml), pervanadate (100 μM vanadate, 2 mM H_2O_2), or EDTA (10 mM) were added to the PBS and the cells were treated as above.

6.2.9. Peptide binding assay. Lysate from A431 cells containing vector alone or from PTPα, or PTPα (D1 C433A) expressing cells was incubated for 1 hour with Src Y527 phosphopeptide immobilized on CnBr activated Sepharose. Beads were thoroughly washed in cell lysis buffer before resuspension in Laemmli buffer. Precipitated Src family kinases were separated by 10% SDS PAGE, transferred to Durolose membrane (Stratagene) and immunoblotted with SRC2 antisera or the Src specific antibody (GD11). Immobilized non-phosphorylated Src Y527 peptide were used as a control. Blots were developed using horseradish peroxidase-linked goat-anti-rabbit antiserum and the enhanced chemiluminescence (ECL) system (Amersham).

6.3. For Chapter 4

6.3.1. Western blotting and immunoprecipitation. A431 cells were grown and lysed as outlined above. The Src family kinases Src, Yes, and Fyn were specifically immunoprecipitated from approximately 500 μg of A431 total cell lysate with the Src-specific mAb 327 (provided by Joan Brugge, ARIAD Pharmaceuticals), an anti-Fyn mAb (provided by Roger Perlmutter, University of Washington) or an anti-Yes antiserum (provided by Joseph Bolen, Bristol-Myers Squibb Pharmaceutical Research Institute). Immunoprecipitated Src family kinases were then immunoblotted with the antibody SRC2 (Santa Cruz Biotechnology). This polyclonal antibody was raised against the C-terminal residues 509-533 of Src but also recognizes conserved sequences in Fyn and Yes

kinases. SRC2 antibody was used for Src kinase-PTPα co-immunoprecipitations due to its superior immunoprecipitating ability. Src kinases were immunoprecipitated with approximately 2 μg of SRC2 antiserum from 1-2 mg of A431 lysate. PTPα was then detected by immunoblotting with anti-PTPα-specific antibodies and developed using a horseradish peroxidase-linked goat anti-rabbit antiserum and the enhanced chemiluminescence (ECL) system (Amersham).

6.3.2. Bacterial expression. Proteins were expressed and purified from bacteria with the pGex system (Pharmacia Biotech) as outlined above. The membrane-proximal 70 amino acid region, between the transmembrane domain and the first PTP domain (amino acids 167-237), was subcloned into pGex-2T-tag. pGex expression plasmids for the SH3 domains of p21 Ras GTPase activating protein (GAP), PI3K, and Src were kindly provided by Megan Brown (Fred Hutchinson Cancer Research Centre). The pGex Lyn SH3 domain construct was provided by Zhigang Weng (ARIAD Pharmaceuticals). The Fyn, Yes and Abl SH3 domains were PCR amplified from cDNA templates and subcloned into pGex 2T. Luria broth cultures (500 ml) of UT5600 bacteria (New England Biolabs) containing the various pGex plasmids were grown to O.D. 0.6-0.9 at 37°C. Cells were then shifted to 26°C and induced overnight with 100 μM IPTG. Bacteria were sedimented and lysed by sonication in phosphate buffered saline (PBS) containing 1 mM PMSF. Triton X-100 was then added to 1% final concentration. Cellular debris was removed by ultra-centrifugation at 30,000 x g. The supernatant was removed and incubated with 1 ml of a 50% slurry of glutathione Sepharose (Pharmacia Biotech) for 1 hour. The beads were then extensively washed in PBS containing 1% Triton X-100. The GST-SH3 domain fusions were eluted from the beads with 5x1 ml aliquots of dH_2O. The various short polyproline-containing peptide fragments were produced by synthesizing complementary oligonucleotides with appropriate restriction enzyme site overhangs. These fragments were then subcloned into pGex 2T. All GST-peptide fusions contain the

amino acids ASPGAS amino terminal to the introduced peptide sequence and the amino acids EFIVSD at the carboxy terminus. Small-scale cultures containing these peptide fusions were produced and lysates were prepared as above.

6.3.3. Protein Biotinylation. Biotinylation of GST-SH3 domain fusion proteins was carried out as previously described (Mayer et al., 1991). Briefly, between 1-2 mg of GST-SH3 domain was incubated with 50 µg of N-hydroxysuccinimide biotin/mg SH3 domain in 100 mM borate buffer pH 8.8. Fusion proteins were biotinylated for ~3.5 hours before addition of NH_4Cl_2 to 10 mM final concentration. Biotinylation reactions were loaded onto PBS washed PD10 columns (Pharmacia) and eluted in 3.5 mls PBS.

6.3.4. Filter binding assay of polyproline peptides using biotinylated SH3 domains. Whole bacterial cell lysates containing the various GST-peptide fusions were fractionated by SDS polyacrylamide gel (15%) electrophoresis (SDS-PAGE). Proteins were transferred to Duralose membrane (Stratagene) and reversibly stained with Ponceau-S to confirm equal loading of fusion protein between lanes. Filters were blocked in 5% bovine serum albumin (BSA) in Tris pH 7.5, 150 mM NaCl, 0.05 % Tween 20 (TBST). Blocked membranes were incubated with biotinylated SH3 domains at a concentration of 3 µg/ml in TBST containing 0.5% BSA for 2 hours at 4°C. Blots were thoroughly washed in TBST, incubated with streptavidin horseradish peroxidase conjugate (1:3000), again washed in TBST and then developed with the ECL system.

6.3.5. Solution binding assay. (1). Pinpoint polyproline peptides: To assess conditions favorable to SH3-polyproline interactions we expressed various polyproline sequences in the bacterial expression vector Pinpoint (Promega). Bacterial expression using this vector results in the biotinylation of bacterially expressed fusion proteins at a single site at the N-terminus . Recombinant expression and cell lysis was carried out as above. Briefly ~3

ml cultures of IPTG induced UT5600 bacteria expressing the polyproline sequences were lysed in 1 ml of lysis buffer. Approximately 900 µl of TBST containing 2 µg of GST-Lyn SH3 domain was added to 100 µl of lysate and incubated for 2 hours at 4°C. 20 µl of washed glutathione Sepharose was then added, lysates were incubated for 1 hour, and then beads were washed 4 times in TBST before elution in Laemmli buffer. SH3 domain-associated biotinylated fusion proteins were detected with horseradish peroxidase-linked streptavidin. (2) Biotinylated synthetic proline-rich peptides: SH3 domains (1 µg) were incubated for 2 hours with 1-5 µg of the biotinylated peptides: 3BP1, GTSLRAPTMPPPLPPVPPQPARRQS; PTPα (PPLP), ARSPSTNRKYPPLPVD KLEEEINRR; or PTPα (PPLA), ARSPSTNRKYPPLAVDKLEEEINRR in TAST buffer (Tris pH 8.0, 750 mM ammonium sulfate, 0.05% Tween 20). Peptides were recovered by the addition of 30 µl of a 50% slurry of Ultra-Link streptavidin beads (Pierce). Beads were incubated for 1-2 hours and then washed 4 times in TAST buffer and then once in TBST before resuspension in Laemmli buffer. Peptide-bound GST-SH3 fusion protein was fractionated by SDS-PAGE and transferred to Durolose membrane. SH3 domains were detected by immunoblotting with anti-GST antibodies (Molecular Probes, Inc.) at a dilution of 1:30 000.

6.3.6. Surface plasmon resonance. Binding assays were conducted using the BIAcore Biosensor (Pharmacia Biosensor). Biotinylated polyproline containing-peptides were coupled to SA 5 biosensor chips containing approximately 4000 Response Units (RU) of pre-immobilized streptavidin. Immobilization was carried out in phosphate buffered saline (10 mM $NaPO_4$, 150 mM NaCl, 0.1 mM EDTA, pH 7.4, containing 0.005% P20) at 25°C with a flow rate of 5 µl/min. Approximately 715 RU of peptide was bound to each chip. GST-SH3 domains were diluted in PBS (50-100 µg/ml) and applied to the biosensor chip (30 µl) at a flow rate of 5 µl/min. The biosensor chip peptide surface was regenerated with a 10 µl pulse of 60 mM NaOH.

6.3.7. Transient transfection of HEK 293 cells. 293 cells were transiently transfected by lipofection (Gibco, BRL). Cells were plated at 1.5 x 10^6 cells/well in 6-well plates the day before transfection. The following day 2.5 µg of DNA in 100 µl serum free media (SFM) (Optimem, Gibco BRL) was combined with 12 µl of lipofectamine in 100 µl of SFM. This mixture (200 µl) was incubated for 45 min. During this incubation the 293 cells were gently washed (2x1 ml) in SFM media to remove any serum. The above mixture (200 µl) was then layer onto the cells and 800 ml of SFM was added to each well. Transfection proceeded for 5-7 hours before termination by the addition of 1ml of 20% serum containing media to each well. The cells were grown for 1-2 days before lysis and analysis.

CHAPTER 7

References

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