THE REGULATION AND FUNCTION OF PROTEIN TYROSINE PHOSPHATASE ALPHA (PTPα) TYROSINE PHOSPHORYLATION

by

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ABSTRACT

Protein tyrosine phosphatase alpha (PTPα) is a ubiquitously expressed receptor protein tyrosine phosphatase that functions as an early upstream regulator in the integrin signaling pathway. The integrins, by interacting with extracellular matrix components, regulate cell growth, migration, and survival, and are functionally linked to multiple aspects of cancer biology such as invasion and metastasis. PTPα plays a major role in the integrin signaling cascade by activating Src family kinases (SFKs), which are required for the full activation of the central signaling molecule focal adhesion kinase (FAK). The importance of PTPα in integrin signaling is demonstrated by defects observed in integrin-mediated cytoskeletal rearrangement and focal adhesion formation in PTPα-deficient fibroblasts. PTPα contains a tyrosine phosphorylation site, Tyr-789, located in the intracellular C-terminal tail. Tyr-789 phosphorylation is shown to not affect PTPα catalytic activity, but allows binding of Src and the adaptor protein Grb2. Little is known about the regulation and function of PTPα Tyr-789 phosphorylation. Recent work from our laboratory has discovered that PTPα Tyr-789 phosphorylation is positively regulated upon integrin engagement and is functionally required for integrin-induced cytoskeletal reorganization events, suggesting the importance of the phospho-Tyr-789 motif in PTPα-mediated signaling events. In a search for other regulators of PTPα Tyr-789 phosphorylation, IGF-1, and potentially aFGF, LPA, and PMA, were found to positively regulate PTPα Tyr-789 phosphorylation. These inductions occurred in a Src/Fyn/Yes-independent manner, indicating an involvement of likely non-SFK cellular kinases distinct from those involved in integrin-induced PTPα phosphorylation. Although PTPα Tyr-789 phosphorylation mediates integrin-induced cytoskeletal remodeling, this

phosphorylation event did not appear to act upstream of the Rho family of small GTPases that are key regulators of cellular actin structures. To further understand the precise action of PTPα Tyr-789 phosphorylation in integrin (and other) signaling pathway, the interaction between the PTPα phospho-Tyr-789 motif and other cellular proteins was investigated. Integrin-induced PTPα Tyr-789 phosphorylation was accompanied by increased Grb2 recruitment to phospho-PTPα, which provides for a mechanism by which Grb2-interacting signaling proteins are recruited to PTPα to mediate downstream integrin signaling events. In addition, several proteins representing potential PTPα phospho-Y789 interacting proteins were isolated by (phospho)peptide affinity purification. The identification of these proteins and validation of their interactions with PTPα may reveal the precise signaling role of PTPα Tyr-789 phosphorylation.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
C2	protein kinase C conserved region 2
CH2	Cdc25 homology region 2
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
DSP	dual specific protein phosphatase
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal related kinase
EyA	eyes absent
FAK	focal adhesion kinase
FAT	focal adhesion targeting domain
FBS	fetal bovine serum
FERM	protein 4.1, ezrin, radixin, moesin domain
FGF	fibroblast growth factor
FN	fibronectin
GAP	GTPase activating protein

GDI	GDP dissociation inhibitor
GEF	guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GST	glutathione S transferase
HRP	horse radish peroxidase
IGF-1	insulin-like growth factor 1
IGF-IR	insulin-like growth factor receptor 1
IPTG	iso-propyl-thio-β-D galactopyranside
KLH	keyhole limpet hemocyanin
LB	Luria broth
LCA	Leukocyte common antigen
LD	leucine-rich domain
LMPTP	low molecular weight PTP
LPA	lysophosphatidic acid
LRP	LCA-related protein
MAPK	mitogen-activated protein kinase
MKP	MAPK phosphatase
min	minute
MKP	MAPK phosphatase
NMDA	N-methyl-D-aspartate
NP-40	Nonidet P-40
OD	optical density
PAK	p21-activated kinase

PBD	p21-binding domain
PBM	PDZ binding motif
PBS	phosphate-buffered saline
PI3K	phosphoinositide 3 kinase
PIP3	phosphatidylinositol-3,4,5-triphosphate
Pix	PAK-interacting exchange factor
PKB	protein kinase B
PKC	protein kinase C
PKD	protein kinase D
PKL	paxillin kinase linker
PLL	poly-L-lysine
PMA	phorbol myristate acetate
PMSF	phenylmethylsulphonylfluoride
PP1	4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]-pyrimidine
PP2	4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine
PRL	protein in regenerating liver
PTB	phosphotyrosine binding
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PTPα	protein tyrosine phosphatase alpha
PVDF	poly vinylidene fluoride
RBD	Rho binding domain
RTK	receptor tyrosine kinase

SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
SFK	Src family kinase
SH2	Src homology 2
SH3	Src homology 3
Sos	son of sevenless
WT	wildtype

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CHAPTER 1

INTRODUCTION

1.1 Protein Tyrosine Phosphatase Superfamily

Protein tyrosine phosphorylation is a common post-translational modification that serves a fundamental role in intracellular signal transduction events. These events regulate important cell processes including proliferation, differentiation, migration, metabolism, survival/death, and others. The addition of a phosphate group to the tyrosyl residue is catalyzed by protein tyrosine kinases (PTKs) and the reverse dephosphorylation reaction by protein tyrosine phosphatases (PTPs). A protein may contain several tyrosine phosphorylation sites, and phosphorylation at different sites can regulate its function in various ways including enzymatic activity, stability, cellular localization, and interaction with other proteins. The coordinated and balanced actions of cellular PTKs and PTPs thus are critical for proper protein function and signaling. Dysregulation of PTKs and PTPs, resulting in excessive or diminished phosphorylation of target proteins, has been implicated in the pathogenesis of several human diseases including cancer.

The first PTP (PTP1B) was purified and characterized in 1988 (Tonks *et al.*, 1988), ten years after the first PTK. Since then, there has been emerging interest and recognition that PTPs play roles as significant as those of PTKs in modulating cell signal transduction. PTPs encompass a large family of enzymes with 107 genes identified in the human genome, comparable to the number of genes encoding PTKs (Alonso *et al.*, 2004). The PTPs show no sequence similarities with other types of phosphatases such as

serine/threonine phosphatases or alkaline phosphatases. The PTP superfamily (Figure 1.1) is divided into four subfamilies based on their structure and catalytic mechanism: Class I Cys-based PTPs, Class II Cys-based low molecular weight PTP (LMPTP), Class III Cys-based Cdc25 PTPs, and Asp-based EyA PTPs (Wang *et al.*, 2003; Alonso *et al.*, 2004; Tonks, 2006).

The Class I Cys-based PTPs contain 38 classical tyrosine-specific PTPs and 61 dual specific protein phosphatases (DSP). The classical, strictly tyrosine-specific PTPs can be further divided into transmembrane receptor-like RPTPs (such as PTPα, CD45, and LAR) and intracellular non-receptor NRPTPs (such as PTP1B, PTP-PEST, and SHP2). Unlike the classical tyrosine-specific PTPs, the DSPs are much more diverse in their substrate specificity. They include the mitogen-activated protein kinase (MAPK) phosphatases (MKPs), which have dual phosphothreonine and phosphotyrosine phosphatase activities towards MAPKs; the PTENs, which have additional lipid phosphatase activity; and other less well characterized DSPs such as PRLs.

The Class II Cys-based PTPs contain only one member in humans, the 18kD LMPTP. LMPTP is an evolutionarily well conserved, cytosolic phosphotyrosine-specific phosphatase with related enzymes that are widely distributed in prokaryotes and eukaryotes. Unlike other PTPs which have additional regulatory or targeting domains, LMPTP appears to be composed of a single catalytic domain. The physiological function of LMPTP is not well understood.

The Class III Cys-based PTPs include the three rhodanese-related Cdc25 cell cycle regulators, which function as dual phosphothreonine and phosphotyrosine phosphatases for the Cdks. Despite the diversity in amino acid sequences and substrate specificity, all PTPs with the exception of Asp-based EyA PTPs have a conserved signature motif (H/V)**C**(X_5)**R**(S/T) at their active site and utilize a similar catalytic mechanism. EyA is a newly identified PTP family that has phosphotyrosine and phosphoserine phosphatase activities (Rayapureddi *et al.*, 2003; Rebay *et al.*, 2005). The EyA family is unique as its members employ aspartic acid, instead of cysteine, at their active sites. More studies are required to structurally and functionally characterize this new PTP family.

1.2 Protein Tyrosine Phosphatase Alpha (PTPα)

PTPα was first discovered in 1990 through cDNA library screens using probes corresponding to phosphatase domain sequences of the RPTP CD45 (also known as leukocyte common antigen or LCA) (Kaplan *et al.*, 1990; Krueger *et al.*, 1990; Matthews *et al.*, 1990; Sap *et al.*, 1990). The gene was mapped to chromosome 2 in mouse (Sap *et al.*, 1990) and chromosome 20 (20p13) in human (Rao *et al.*, 1992), and was found to encode a widely expressed RPTP with particularly high expression in the brain. The protein has been named LCA-related protein (LRP), protein tyrosine phosphatase alpha (PTPα), or PTPRA. The name PTPα is used throughout this thesis.

1.2.1 Structure

Human PTPα has 802 amino acids and is structurally composed of an extracellular domain, a transmembrane domain, and a cytoplasmic region containing two tandem

catalytic domains (Figure 1.2). Two alternatively spliced isoforms have been identified, with one bearing an additional 9 amino acid insert located three residues N-terminal to the transmembrane domain. The two isoforms exhibited comparable enzymatic activity in an *in vitro* phosphatase assay (Daum *et al.*, 1994). Recent work by Kapp *et al.* (2007) has revealed a tissue-specific expression of the larger form in the brain and some skeletal muscles, while the shorter form is ubiquitously expressed. In addition, the two forms appear to exhibit differences in their potential to induce Src-dependent focus formation (Kapp *et al.*, 2007). Although the molecular basis for this requires further investigation, this finding indicates that the two isoforms may exhibit functional differences *in vivo*.

Compared to other RPTPs, the extracellular domain of PTPα is considerably shorter (around 135 amino acids) and lacks cell adhesion-like motifs such as immunoglobulin (Ig) and fibronectin (FN)-III domains. So far, the only molecule known to interact with this domain is the neural cell adhesion molecule contactin. PTPα associates with contactin *in cis*, and the complex is proposed to function as a signaling platform involved in neuronal differentiation (Zeng *et al.*, 1999). The extracellular domain of PTPα undergoes extensive N- and O-glycosylation to yield the mature 130 kD protein (Daum *et al.*, 1994).

Similar to other RPTPs, PTPα contains two tandem phosphatase domains, D1 and D2. While the membrane proximal D1 domain is responsible for the majority of phosphotyrosyl protein phosphatase activity, the membrane distal D2 domain exhibits some activity towards the low molecular weight substrate *p*-nitrophenyl phosphate, but not towards phosphotyrosyl peptides (Wang *et al.*, 1991). The preservation of the highly

conserved RPTP D2 domains through evolution suggests a functional involvement of D2 in the proper action and signaling of RPTPs. The D2 domain of PTPα has been shown to interact with the PDZ domain of the scaffold protein PSD-95 to regulate N-methyl-D-aspartate (NMDA) receptor signaling (Lei *et al.*, 2002) in learning and memory. In addition, the D2 domain is implicated in the formation of homo- and/or hetero-dimers with other RPTPs to regulate PTPα activity (see section 1.2.3.1). The same phenomenon has also been observed with other RPTPs, suggesting a common regulatory function of the D2 domain in initiating protein-protein interactions. However, it cannot be ruled out that the D2 domain may act on different cellular substrates that have yet to be identified.

1.2.2 Cellular Substrates

1.2.2.1 Src Family Kinases

Studies involving the ectopic expression of PTPα led to the identification of the protein tyrosine kinase Src as PTPα substrate. Src is the prototype member of the Src family kinases (SFKs), a family that encompasses nine structurally-related cellular protein tyrosine kinases: Src, Fyn, Yes, Lyn, Lck, Hck, Blk, Fgr, and Yrk. While Src, Fyn, and Yes are widely expressed, other SFKs have restricted tissue and/or cell-type expression (Thomas *et al.*, 1997). The SFKs share similar structural domains and arrangements, and their activities are regulated by a common mechanism involving a switch between the closed inactive and the open active conformations (Figure 1.3). Src, in its inactive state, is phosphorylated at Tyr-527 (Y527) at the C-terminal tail, which binds intramolecularly to its SH2 domain. This interaction, together with the interaction between the SH3 domain and the linker region between the SH2 and the kinase domains, disrupt the catalytic

active site and render the kinase inactive (Xu *et al.*, 1997). During activation, disruption of these intramolecular interactions and dephosphorylation of the inhibitory Y527 residue release Src from its inactive closed conformation. This is followed by autophosphorylation of src itself at Tyr-416 (Y416) to achieve full activation. PTPα is one of several phosphatases that can catalyze Src Y527 dephosphorylation to induce Src activation.

In both rat embryonic fibroblasts and P19 embryonal carcinoma cell lines, overexpression of PTPα resulted in reduced Src phosphorylation at Y527 (Zheng *et al.*, 1992; den Hertog *et al.*, 1993). In accord with this, brain and embryonic fibroblasts isolated from PTPα knockout mice showed enhanced Src and Fyn phosphorylation at Y527 and reduced kinase activity (Ponniah *et al.*, 1999; Su *et al.*, 1999). A broader action of PTPα on other SFKs, Yes and Lck, has also been demonstrated (Harder *et al.*, 1998; Le *et al.*, 2006). Interestingly, the activity of these SFKs is not completely abolished in the PTPα knockout system. Other phosphatases, such as PTP1B, SHP1, and SHP2, are also implicated in the dephosphorylation and activation of SFKs (Somani *et al.*, 1997; Arregui *et al.*, 1998; Oh *et al.*, 1999). SFKs are involved in a wide range of signaling pathways including those initiated by integrins, growth factor receptors, G protein-coupled receptors, and others (Erpel *et al.*, 1995). It is likely that different Src phosphatases are utilized depending on the signaling pathway and cell type.

1.2.2.2 p130Cas

p130Cas was identified as a potential PTPα substrate in a substrate trapping pull-down experiment utilizing a GST-fusion protein comprising the catalytically inactive PTPα-D1

domain, D1-C433S (Buist *et al.*, 2000). PTPα exhibited *in vitro* phosphatase activity towards p130Cas. *In vivo* activity was indicated by the reduced p130Cas tyrosine phosphorylation observed upon co-expression of PTPα. Thus far, the functional significance of this role of PTPα is not understood.

1.2.2.3 Kv1.2 Potassium Channel

The activities of ion channels are regulated by tyrosine phosphorylation. Stimulation of a neurotransmitter receptor, the m1 muscarinic acetylcholine receptor, by carbachol treatment can induce tyrosine phosphorylation of the voltage-gated Kv1.2 potassium channel and leads to the suppression of the channel activity (Huang *et al.*, 1993). The suppression of Kv1.2 involves multiple signaling events and can be induced by a protein kinase C activator, phorbol 12-myristate 13-acetate (PMA). PTPα is implicated in the dephosphorylation of Kv1.2 (Tsai *et al.*, 1999). Co-overexpression of PTPα with Kv1.2 partially reverses the PMA-induced tyrosine phosphorylation and activity suppression of the Kv1.2 ion channel following carbachol treatment, indicating that PTPα functions downstream of m1 muscarinic acetylcholine receptor signaling to mediate Kv1.2 ion channel dephosphorylation. It remains to be determined whether PTPα directly or indirectly dephosphorylates Kv1.2.

1.2.3 Regulation

1.2.3.1 Dimerization

The crystal structure of the PTPα D1 catalytic domain revealed an intermolecular dimer formation mediated by the N-terminal helix-loop-helix wedge-like structure of one monomer binding to the active site of the other (Bilwes *et al.*, 1996). This interaction was predicted to negatively regulate phosphatase activity due to the occlusion of the active sites. One study utilizing PTPα mutants with single cysteine mutations in the juxtamembrane region demonstrated that forced PTPα dimerization by disulfide-bond linkage led to inhibition of the PTPα phosphatase activity (Jiang *et al.*, 1999). However, the same study also pointed out that the inhibitory effect might only be exerted under specific orientation of the dimer counterparts. Ectopic expression of PTPα chimeric proteins fused to the green fluorescent protein derivatives YFP and CFP confirmed the formation of PTPα homodimers *in vivo* by fluorescence resonance energy transfer (FRET) assay (Tertoolen *et al.*, 2001). The formation and stabilization of the dimer interaction appeared to involve multiple domains including the extracellular, transmembrane, D1, and D2 domains (Jiang *et al.*, 2000; Tertoolen *et al.*, 2001). In addition to PTPα, CD45 also possesses dimerizing potential (Felberg *et al.*, 1998). It has thus been proposed that similar to receptor tyrosine kinases (RTKs), the activities of these RPTPs can be regulated by homo- and/or hetero- dimerization in response to certain signals (Blanchetot *et al.*, 2002b). Oxidative stress was identified as a potential trigger for inducing and/or stabilizing PTPα dimer formation (Blanchetot *et al.*, 2002a). Nevertheless, it is not known whether endogenous PTPα dimerizes at its physiological expression level. Further

investigations are required to elucidate the cellular cues for and functions of PTPα dimerization.

1.2.3.2 Tyrosine Phosphorylation

Tyrosine phosphorylation of PTPα was first reported in NIH 3T3 cells metabolically labeled with [^{32}P]orthophosphate (den Hertog *et al.*, 1994). It was estimated that about 20% of the endogenous PTPα is tyrosine phosphorylated, and the site was mapped to Tyr-789 (Y789) located five residues from the cytoplasmic C-terminus. Co-expression of Src with PTPα increased PTPα Y789 phosphorylation, which implicates Src to be one of the responsible kinases for phosphorylation at this site (den Hertog *et al.*, 1994). Mutation of the Y789 site to Phe resulted in phosphatase activity comparable to wildtype PTPα, indicating that this phosphorylation does not affect the intrinsic catalytic activity (Su *et al.*, 1996). A sequence comparison of the PTPα Y789 phosphorylation site and surrounding residues with SH2 domain binding motifs identified Grb2, a ubiquitously expressed adaptor protein, as a potential binding partner for Y789-phosphorylated PTPα (den Hertog *et al.*, 1994; Su *et al.*, 1994). Grb2 indeed associates with PTPα *in vivo* and the interaction is mediated by phospho-Y789 of PTPα and the SH2 domain of Grb2. In growth factor signaling, Grb2 is usually recruited to the activated receptor in conjunction with its binding partner and Ras activator Son of sevenless (Sos), leading to the activation of the Ras signaling pathway. Thus far however, Sos has not been found in the PTPα/Grb2 complex and it is suggested that PTPα may interfere with the Grb2 and Sos interaction (Su *et al.*, 1996). The functional implication of PTPα and Grb2 association in Ras or other signaling pathways still requires further investigation.

Besides Grb2, Src can directly associate with phospho-Y789 of PTPα (Zheng *et al.*, 2000). Zheng *et al.* (2000) proposed that phospho-Y789 of PTPα can competitively bind to and displace phospho-Y527 of Src from the Src SH2 domain. This interaction facilitates PTPα-mediated Src dephosphorylation and activation by positioning PTPα and Src in close proximity and by freeing the inhibitory Src phospho-Y527 site for dephosphorylation by PTPα. Consistent with this proposed model, phosphorylation of PTPα Y789 is required for PTPα-mediated Src dephosphorylation and activation in mitosis, although PTPα Y789 phosphorylation is not altered during mitosis (Zheng *et al.*, 2001). Grb2 was demonstrated to have a greater binding affinity for PTPα Y789 than Src, and thus may prevent PTPα-mediated Src recruitment and dephosphorylation (Zheng *et al.*, 2000). In mitosis, this is overcome by serine phosphorylation of PTPα in the intracellular juxtamembrane region (see Section 1.2.3.3). This decreases the affinity of Grb2 for PTPα as a result of PTPα conformational changes (Zheng *et al.*, 2001). It is not known if the same displacement mechanism involving the direct association of PTPα and Src is utilized in signaling pathways other than mitosis. However, since PTPα Y789 phosphorylation is not required for integrin-induced PTPα-mediated Src dephosphorylation and activation (see Section 1.4.2) (Chen *et al.*, 2006), other signaling events/molecules may be responsible for exposing the Src inhibitory Y527 residue and for indirectly linking PTPα and Src.

In addition to serving as a binding motif for Grb2 and Src, PTPα Y789 has been implicated in the proper localization of PTPα to focal adhesions, which are points of contacts between the cell and the substratum (Lammers *et al.*, 2000). Recent work from

our lab has revealed a new role of PTPα Y789 phosphorylation in mediating integrin-induced cytoskeletal reorganization (see Section 1.4.2) (Chen *et al.*, 2006). Furthermore, we showed that phosphorylation of PTPα Y789 is increased during integrin signaling. In a model of oxidative stress, Hao *et al.* (2006b) demonstrated that H_2O_2 negatively regulates PTPα Y789 phosphorylation in various cell lines including embryonic fibroblasts. The functional implication of this downregulation of PTPα Y789 phosphorylation in PTPα-dependent H_2O_2-induced protein kinase D (PKD) activation (Hao *et al.*, 2006a) is not known. Taken together, these evidence suggest that PTPα Y789 is functionally involved in various PTPα-mediated signaling events including Src dephosphorylation and activation, and cytoskeletal reorganization. These functions of PTPα Y789 are dependent on its phosphorylation status which can be regulated accordingly in different signaling pathways.

1.2.3.3 Serine Phosphorylation

The catalytic activity of PTPα can be positively regulated by protein kinase C (PKC)-mediated serine phosphorylation of PTPα at Ser-180 (S180) and Ser-204 (S204) located in the intracellular juxtamembrane region (den Hertog *et al.*, 1995; Tracy *et al.*, 1995). In addition, serine phosphorylation of PTPα decreases its affinity for Grb2 (Zheng *et al.*, 2001). These two effects of PTPα serine phosphorylation were proposed to be important mechanisms by which PTPα mediates Src dephosphorylation and activation in mitosis (Zheng *et al.*, 2001).

1.2.4 Cellular Functions

PTPα is a well characterized and ubiquitously expressed positive regulator of SFKs. PTPα knockout mice are viable and do not display gross phenotypic abnormalities (Ponniah *et al.*, 1999; Su *et al.*, 1999). However, closer examination of PTPα overexpression and knockout systems at the molecular level has revealed that PTPα functions in a variety of cell processes. In integrin signaling, PTPα has dual roles in affecting both early and late signaling events (see Section 1.4.2). PTPα overexpression in fibroblasts can induce cell transformation, suggesting an oncogenic potential of PTPα (Zheng *et al.*, 1992). On the other hand, PTPα may have a tumour suppressive function in breast cancer (Ardini *et al.*, 2000), and is involved in the anti-tumourigenic actions of the somatostatin analogue TT-232 (Stetak *et al.*, 2001). In mitosis, PTPα is required for mitotic activation of Src (Zheng *et al.*, 2001). In brain, PTPα is an important regulator of NMDA receptor signaling (Lei *et al.*, 2002; Le *et al.*, 2006) and PTPα knockout mice exhibit defects in NMDA receptor-associated processes including learning and memory (Petrone *et al.*, 2003; Skelton *et al.*, 2003). In T cell signaling, PTPα is implicated in the regulation of Fyn activity prior to T cell receptor (TCR) stimulation and in TCR-stimulated thymocyte proliferation (Maksumova *et al.*, 2005). Taken together, these findings indicate that PTPα functions as an important regulator of many physiological processes, and its exact role is dependent on cell type and signaling pathway.

1.3 Integrin Signaling

1.3.1 Overview: Integrins and Integrin Signaling

Integrins comprise a large family of cell surface receptor proteins that play fundamental roles in mediating cell and extracellular matrix (ECM) interactions. They are composed of non-covalently linked heterodimers of α and β subunits. In mammals, 18 α subunits and 8 β subunits have been identified which combine with each other in an overlapping yet selective manner to form 24 different integrin receptors (Hynes, 2002). Most integrins can bind to a wide range of ECM proteins, and conversely, the same ligand may be recognized by more than one integrin (Ruoslahti *et al.*, 1987; Humphries, 1990). Despite this complexity, most integrin-ligand recognitions are based on a similar mode of molecular interaction (Humphries *et al.*, 2006). The best characterized example is the RGD (Arg-Gly-Asp) tripeptide motif, which is found in many extracellular matrix proteins including fibronectin and vitronectin, and is the site for integrin recognition and interaction (Pierschbacher *et al.*, 1984). Both the α and the β subunits of integrins are involved in ligand specificity and binding. Despite the seemingly redundant presence of so many α and β subunits and their combinations to form receptors, it is believed that each integrin has a specific function. This is supported by the distinct phenotypes presented by different integrin knockout mice (Hynes, 2002). The great number of integrins thus may allow precise, cell type-specific regulation of a wide range of cell behaviors in response to different extracellular conditions.

Both integrin α and β subunits are type I transmembrane proteins, characterized by large extracellular domains and short cytoplasmic domains (with the exception of the integrin

β4 subunit). Signaling through integrins is bi-directional as they can mediate both "inside-out" and "outside-in" pathways. On the one hand, the "inside-out" signaling refers to the regulation of integrin affinity towards ECM ligands mediated by intracellular signaling events (Hynes, 2002). On the other hand, the classical "outside-in" signaling is initiated at the cell surface when an integrin binds to its respective ligand and transduces the signals into the cell to control cell behavior. Unlike growth factor receptors, integrins are devoid of enzymatic activity and therefore rely on the recruitment of proteins and formation of signaling complexes for subsequent signal transduction. Indeed, the cytoplasmic tail of integrins is known to bind to a wide range of signaling (adaptors, kinases, and others) and cytoskeletal structural proteins. Through these interactions, integrins evoke both chemical and mechanical signals to control cell behavior.

As the sensor of the extracellular environment, integrins regulate important cell processes including proliferation, survival, and migration. The major signaling pathways utilized by integrins include the mitogen-activated protein kinase (MAPK) pathway involved in cell proliferation, the phosphoinositide-3 kinase (PI3K)-mediated pathway that is key to cell survival, and signaling via the Rho family of small GTPases to promote cell migration. Since each integrin possesses specific functions, the exact signaling outcomes are dependent on the cell type and the nature of the integrin-ligand interaction.

1.3.2 Central Molecules Mediating Integrin Signaling: FAK and Src

Focal adhesion kinase (FAK) is a non-receptor PTK that is activated by most integrins (Giancotti *et al.*, 1999). FAK, as suggested by its name, is localized to focal adhesions,

which are the contact points between the cell and the underlying substratum and the sites of integrin-ECM interactions (Schaller *et al.*, 1992). Integrin stimulation induces FAK phosphorylation at Tyr-397 (Y397) creating a binding site for the SH2 domains of Src and Fyn SFKs (Schaller *et al.*, 1994). The association between phospho-FAK Y397 and the Src SH2 domain in part promotes Src activation by displacing Src phospho-Y527 and disrupting this inhibitory intramolecular interaction. The full activation of Src is achieved by PTP-mediated dephosphorylation of Src at Y527, followed by autophosphorylation of Src at Y416. The active Src in turn phosphorylates FAK at Y576 and Y577 in the catalytic domain to achieve full FAK activation, and at Y925 to recruit the downstream SH2-containing adaptor protein Grb2 (Calalb *et al.*, 1995; Schlaepfer *et al.*, 1996). Subsequent phosphorylation of FAK-associated proteins such as p130Cas and paxillin by FAK and/or Src leads to phosphorylation-dependent recruitment of further downstream signaling proteins. Ultimately, a large signaling protein complex is formed at the focal adhesions, with the FAK/Src complex acting as a central coordinator of integrin signaling.

The majority of integrin-regulated processes, including cell spreading, migration, survival, and proliferation are dependent on integrin signaling through FAK (Wierzbicka-Patynowski *et al.*, 2003) (Figure 1.4). Src-dependent phosphorylation of FAK at Y925 can activate the MAPK signaling cascade by recruiting the adaptor protein Grb2, which is complexed with the Ras activator Sos (Schlaepfer *et al.*, 1994; Schlaepfer *et al.*, 1996). For some integrins, the recruitment and phosphorylation of the adaptor protein Shc by Src and/or Fyn also contribute to the Grb2-Sos recruitment and MAPK activation (Wary *et al.*, 1996). FAK, via its phospho Y397 site, can directly associate with the SH2 domain of

the regulatory p85 subunit of PI3K to activate cell survival signaling (Chen *et al.*, 1996). Likewise, FAK and Src can activate the Rho family of small GTPases via multiple pathways to regulate cellular actin cytoskeleton structures (see Section 1.3.3.2). Taken together, these evidence indicate that FAK and Src-SFKs are indeed central signaling molecules that link integrin activation to multiple downstream signaling events.

1.3.3 Rearrangement of the Cytoskeleton

A major integrin-regulated process is the rearrangement of the actin cytoskeleton, a fundamental mechanism driving cell spreading and migration. Changes in this cytoskeletal structure can be visualized by immunofluorescent staining for filamentous actin (F-actin). Plating serum-starved, suspended fibroblasts on the immobilized ECM protein, fibronectin, is known to induce a series of actin cytoskeletal rearrangements (Clark *et al.*, 1998). Once the cells have adhered to the substratum, they begin to flatten and spread out through the formation of large membranous protrusions (Figure 1.5A). At this stage, F-actin is concentrated at the membrane of the leading edge of the cell, forming structures known as filopodia and lamellipodia. Filopodia are characterized by rod-like projections filled with bundles of parallel filaments, and lamellipodia are structures of web-like actin sheets. During the process of active spreading, these membrane protrusions are engaged in a dynamic movement referred as ruffling. Once cells are fully spread, F-actin is arranged in bundles that run across the center core of the cell and terminate at focal adhesions. These cytoplasmic actin bundles are known as stress fibers, and they function to support cell shape and account for cell contractility.

Integrin signaling is also implicated in the cytoskeletal reorganization events of cell migration, an important process for embryonic morphogenesis, tissue repair, and immune surveillance, and in tumorigenesis and cancer progression (Guo *et al.*, 2004; Eble *et al.*, 2006). Similar to cell spreading, cell migration requires extensive remodeling of the cellular actin cytoskeleton (Figure 1.5B). Once signals to migrate are received, the cells polarize with filopodia and lamellipodia leading at the front edge, followed by formation of new focal adhesion contacts. Concurrently, cells must detach at the rear end, or trailing edge, from substrate adhesion sites, while the forward movement is driven by cell body retraction. The remodeling of the cytoskeleton requires the dynamic disassembly and assembly of actin networks and is regulated by the Rho family of small GTPases.

1.3.3.1 Rho Family of Small GTPases

The Rho family of small GTPases are the major players involved in the regulation of cellular cytoskeleton networks. They belong to the Ras GTPase superfamily and are small guanine nucleotide-binding proteins of around 21kD. About 20 members of the Rho family of small GTPases have been identified in mammals (Wherlock *et al.*, 2002).

1.3.3.1.1 Regulation

The activity of the Rho family of small GTPases is regulated by the binding of guanine phosphates, either GDP or GTP (Figure 1.6) (Kaibuchi *et al.*, 1999). In their GDP-bound form, the Rho GTPases are inactive, and during activation, GDP is replaced with GTP. The active GTP-bound Rho GTPases are recognized by their specific downstream

effector proteins. This interaction between effectors and active Rho GTPases activates the effector protein function to affect actin polymerization and promote cytoskeletal reorganization. The activation of Rho GTPases is transient and is reversed when GTP is hydrolyzed to GDP by their intrinsic GTPase activity. The cycling between GDP- and GTP-bound forms of Rho GTPases is controlled by three groups of regulatory proteins: GDIs (GDP dissociation inhibitors), GEFs (guanine nucleotide exchange factors), and GAPs (GTPase activating proteins). The GDIs specifically bind to the GDP-bound Rho GTPases and prevent their translocation to the plasma membrane where the Rho GTPases function to interact with and activate effector proteins. The GEFs positively regulate Rho GTPases by catalyzing the GDP to GTP exchange. The GAPs, by contrast, accelerate the intrinsic GTPase activity of the Rho GTPases to enhance GTP hydrolysis. To date, more than 50 GEFs and 40 GAPs have been identified and the list continues to expand (Raftopoulou *et al.*, 2004).

1.3.3.1.2 Function

The majority of functional studies on the Rho GTPases have focused on three members, RhoA, Rac1, and Cdc42, which are responsible for regulating different features of the actin cytoskeleton (Figure 1.6). The functions of each of these Rho GTPases were determined in early studies involving microinjection of these proteins into Swiss 3T3 fibroblasts. This revealed that RhoA is responsible for the formation of stress fibers and focal adhesions (Paterson *et al.*, 1990; Ridley *et al.*, 1992a), while Rac1 and Cdc42 induce formation of lamellipodia and filopodia, respectively (Ridley *et al.*, 1992b; Kozma *et al.*, 1995). The formation of these cytoskeletal structures is mediated by

specific Rho GTPase effector proteins. RhoA induces stress fiber formation by activating effectors such as Rho-kinase (ROCK) and mDia. These in turn target several other proteins, including LIM kinases to induce actin polymerization, and myosin light chain and myosin phosphatases to increase myosin phosphorylation (Kaibuchi *et al.*, 1999; Burridge *et al.*, 2004). Interestingly, although Rac1 and Cdc42 are responsible for the formation of different actin structures, they appear to utilize some common effectors, such as p21-activated kinase (PAK). PAK is a serine/threonine kinase that upon activation by binding to active Rac1 or Cdc42, phosphorylates LIM kinases to induce actin polymerization and myosin kinases to reduce myosin phosphorylation (Figure 1.7) (Raftopoulou *et al.*, 2004; Zhao *et al.*, 2005). As more and more effectors are identified for each Rho GTPase, it remains to be determined how different effector functions are integrated to lead to particular cytoskeletal structures and modulate their dynamic nature.

1.3.3.2 Regulation of Rho GTPases by Integrins

Integrin stimulation of cells induces remodeling of the actin cytoskeleton into structures that resemble those formed upon RhoA, Rac1, and Cdc42 activation. The activation of each of these Rho GTPases by integrins was confirmed using the Rho GTPase activity assays developed by Ren *et al.* (Ren *et al.*, 1999; del Pozo *et al.*, 2000; Cox *et al.*, 2001). Several integrin-regulated signaling proteins have been implicated in the regulation of Rho GTPase activities through their actions on the GEF, GDI, and/or GAP regulatory proteins (Figure 1.4). The lipid product formed upon PI3K activation, phosphatidylinositol-3,4,5-triphosphate (PIP3), can recruit GEFs such as Vav, P-Rex1, and SWAP-70 to the plasma membrane where they can catalyze the GDP to GTP

exchange and activate the Rho GTPases (Welch *et al.*, 2003). FAK/Src-dependent phosphorylation of p130Cas and paxillin and subsequent recruitment of Crk is involved in the recruitment of DOCK180 GEF (Kiyokawa *et al.*, 1998). Src can catalyze tyrosine phosphorylation of Rho GTPase regulatory proteins Vav and p190RhoGAP thereby increasing their respective GEF and GAP activities towards the Rho GTPases (Crespo *et al.*, 1997; Arthur *et al.*, 2000). It is well established that integrin signaling may temporally and spatially utilize and coordinate different Rho GTPases to regulate cell spreading and migration (Schwartz *et al.*, 2000; Ridley *et al.*, 2003). In addition, integrin signaling may also directly regulate downstream effectors of the Rho GTPases. One such example is the Rac1/Cdc42 effector protein PAK. PAK is found in a complex with the PAK-interacting exchange factor (Pix), the adaptor protein Nck, and paxillin-kinase linker (PKL) (Turner *et al.*, 1999) (Figure 1.7). PKL contains a paxillin-binding site, which can interact with the leucine-rich domain (LD)-4 of paxillin and subsequently recruit the whole complex to focal adhesions (West *et al.*, 2001). It is proposed that PAK is properly localized to focal adhesions via this Nck-PAK-Pix-PKL complex where PAK may serve as an adaptor protein (Obermeier *et al.*, 1998; Daniels *et al.*, 1999). Disruption of the PKL and paxillin interaction can lead to impaired cytoskeletal reorganization and cell migration (Turner *et al.*, 1999; West *et al.*, 2001). Although the timing, regulation, and exact signaling mechanism(s) of the recruitment of this protein complex to paxillin remain elusive, it demonstrates how integrin signaling may regulate Rho GTPase signaling pathways at different levels to affect cellular actin cytoskeleton structures.

1.4 PTPs in Integrin Signaling

1.4.1 Overview

Integrin stimulation by ligand binding induces early activation of FAK and the Src/Fyn SFKs, which then initiate a cascade of downstream tyrosine phosphorylation events. The coordinated PTK-mediated phosphorylation and PTP-mediated dephosphorylation of multiple integrin signaling molecules are critical not only in propagating signaling, but also in achieving reversible and dynamic integrin-mediated cellular events. Several PTPs including PTPα (see Section 1.4.2), PTP-PEST, PTEN, SHP2, and PTP1B have been implicated in integrin signaling. The importance of each of these PTPs in integrin signaling is reflected by the altered actin cytoskeleton and migration in PTP knockout and/or overexpressing cell systems.

PTP-PEST is a non-receptor PTP (Figure 1.1) that interestingly, when deleted or overexpressed, can lead to impaired cell migration (Angers-Loustau *et al.*, 1999; Garton *et al.*, 1999). One of the major substrates of PTP-PEST is p130Cas, which is a direct target of the FAK/Src complex (Garton *et al.*, 1999). A recent study has additionally demonstrated a direct action of PTP-PEST in dephosphorylating two Rho GTPase regulatory proteins, Vav2 and p190RhoGAP, to respectively decrease Rac1 and increase RhoA activities (Sastry *et al.*, 2006). These results indicate that PTP-PEST can act on multiple targets to regulate different and opposing aspects of the cell migration process, and thus explain the impaired migratory potential observed in cells with either null or excessive PTP-PEST protein expression. PTEN is a dual-specific phosphatase (Figure 1.1) that is able to dephosphorylate phosphotyrosyl proteins and lipids. PTEN knockout cells

exhibit increased cell migration, while PTEN overexpression has the opposite effect on cell migration (Tamura *et al.*, 1998; Liliental *et al.*, 2000). These results clearly indicate a negative role of PTEN in cell migration and it is proposed that PTEN downregulates Rac1 and Cdc42 activities (Liliental *et al.*, 2000). The exact mechanism(s) by which PTEN regulates cell migration is however still unclear. SHP2 is a ubiquitously expressed non-receptor PTP that contains two SH2 domains (Figure 1.1). SHP2 knockout fibroblasts exhibit impaired cell spreading and migration on fibronectin, with increased focal adhesion and stress fiber formation (Yu *et al.*, 1998). Molecular analysis revealed reduced integrin-induced src activation, FAK, p130Cas, and paxillin phosphorylation, and MAPK activation in SHP2 knockout fibroblasts (Oh *et al.*, 1999). SHP2 may positively regulate integrin signaling by inhibiting the recruitment of Csk, a negative regulator of SFKs, to the plasma membrane where SFKs are localized (Zhang *et al.*, 2004). SHP2 may also downregulate RhoA activity by an unknown mechanism (Schoenwaelder *et al.*, 2000). It is likely that SHP2 acts on multiple targets to regulate integrin signaling and cell migration. PTP1B is another non-receptor PTP (Figure 1.1) that is implicated in integrin signaling. PTP1B performs both positive and negative roles in integrin signaling by activating SFKs (Liu *et al.*, 1998) and by dephosphorylating p130Cas, respectively (Liu *et al.*, 1996; Liu *et al.*, 1998). Results obtained from PTP1B knockout mouse embryonic fibroblasts supported a positive role of PTP1B in integrin signaling, indicating that its action in SFK activation is dominant (Cheng *et al.*, 2001). Taken together, these examples demonstrate how multiple PTPs mediate tyrosine dephosphorylation of different molecules of the integrin signaling pathway, and how

depending on the target, they positively and/or negatively regulate integrin signaling events.

1.4.2 PTPα in Integrin Signaling

PTPα is a ubiquitously expressed RPTP that possesses activating phosphatase activity towards several members of the SFKs (see Section 1.2.2.1). Src and Fyn are important regulators of the integrin signaling cascade and are rapidly activated following integrin stimulation. The activation of Src/Fyn involves the disruption of inhibitory intramolecular interactions and dephosphorylation of Src/Fyn at Y527 to stabilize the open active conformation. The role of PTPα in integrin signaling was established in studies utilizing fibroblasts isolated from PTPα$^{-/-}$ mice (Ponniah *et al.*, 1999; Su *et al.*, 1999; Zeng *et al.*, 2003; Chen *et al.*, 2006). Functional analysis revealed that PTPα is involved in integrin-induced cell migration, as PTPα knockout cells exhibited reduced migration in a cell monolayer wound-healing assay and in an assay of cell haptotaxis towards fibronectin (Zeng *et al.*, 2003; Chen *et al.*, 2006). Close examination of the integrin-regulated changes in fibroblast morphology by immunofluorescent staining for F-actin and vinculin, a focal adhesion localized protein, showed that PTPα$^{-/-}$ cells have delayed spreading and focal adhesion formation following fibronectin stimulation as compared to wildtype cells. At the molecular level, integrin-induced Src/Fyn activation was impaired in PTPα knockout fibroblasts, indicating that PTPα indeed functions early in integrin signaling by dephosphorylating and activating Src/Fyn.

Another major integrin signaling defect observed in PTP$\alpha^{-/-}$ fibroblasts is impaired FAK autophosphorylation at Y397. Although the precise mechanism leading to the initial FAK Y397 phosphorylation upon integrin stimulation is not clear, the maximal activation of FAK is dependent on Src/Fyn-catalyzed phosphorylation of FAK at Y576 and Y577 in the catalytic domain, which subsequently promotes FAK autophosphorylation at Y397 (Calalb *et al.*, 1995). The requirement for PTPα in integrin-induced Src/Fyn activation hence explains the impaired FAK Y397 autophosphorylation in PTPα knockout cells. Collectively, these results indicate that PTPα functions proximal to integrins to affect activation of the central signaling molecules Src/Fyn and FAK, which subsequently mediate integrin-regulated processes including cell spreading and migration.

Interestingly, recent work from our lab has discovered a new role of PTPα in integrin signaling, which involves the Y789 site of PTPα (Chen *et al.*, 2006). We showed that phosphorylation of PTPα at Y789 is positively regulated by integrin signaling. This occurs in a Src/Fyn/Yes- and FAK-dependent manner, as integrin stimulation failed to induce PTPα Y789 phosphorylation in Src/Fyn/Yes triple knockout (SYF) and FAK single knockout cells. The functional significance of this event was investigated through rescue experiments utilizing the re-expression of wildtype-, catalytically inactive-, or Y789F-PTPα in PTPα knockout cells. Re-introduction of wildtype but not catalytically inactive PTPα restored several defects observed in integrin-stimulated PTPα knockout cells, including impaired Src/Fyn and FAK activation, cell spreading, focal adhesion formation, and cell migration. Surprisingly, reintroduction of Y789F mutant PTPα into PTPα knockout cells effectively induced Src/Fyn and FAK activation, but failed to

restore the cell spreading, focal adhesion formation, and cell migration defects. It was originally proposed that PTPα Y789 phosphorylation may function to directly recruit Src for dephosphorylation and activation (Zheng *et al.*, 2000). However, our results indicated that PTPα Y789 phosphorylation is not required for Src activation in integrin signaling. More importantly, we demonstrated that in addition to the phosphatase domain, the Y789 motif is essential for PTPα to regulate integrin-mediated cytoskeletal reorganization events for cell spreading and migration.

We proposed that PTPα has dual roles in integrin signaling (Figure 1.8). Following integrin stimulation, PTPα acts proximal to the integrins to affect Src/Fyn and FAK activation. The active Src/Fyn and/or FAK in turn phosphorylate and activate multiple downstream signaling molecules, with one of these being PTPα. Phosphorylation of PTPα at Y789 is then involved in and is required for subsequent cytoskeletal rearrangement processes by a mechanism that remains to be determined.

1.5 Hypotheses

Although the studies described above have revealed a role for PTPα Y789 phosphorylation in integrin signaling, little is known about the regulation and function of phospho-PTPα in this and other signaling systems. **I hypothesize that in addition to integrin signaling, phosphorylation of PTPα at Y789 is dynamically regulated in other signaling pathways, such as those induced by growth factors.** A specific aim of this study was thus to determine if cellular stimuli other than the integrin ligand fibronectin induce the phosphorylation of PTPα at Y789, and if so, investigate whether

this was dependent on SFK activity. Since PTPα Y789 phosphorylation is required for integrin-induced cytoskeletal reorganization, **I hypothesize that phospho-Y789 of PTPα mediates important signaling events in cytoskeletal reorganization processes.** A second specific aim of this study was to investigate integrin signaling molecules acting downstream of PTPα phospho-Y789, and to identify immediate signaling targets/effectors of phosphotyrosyl-PTPα.

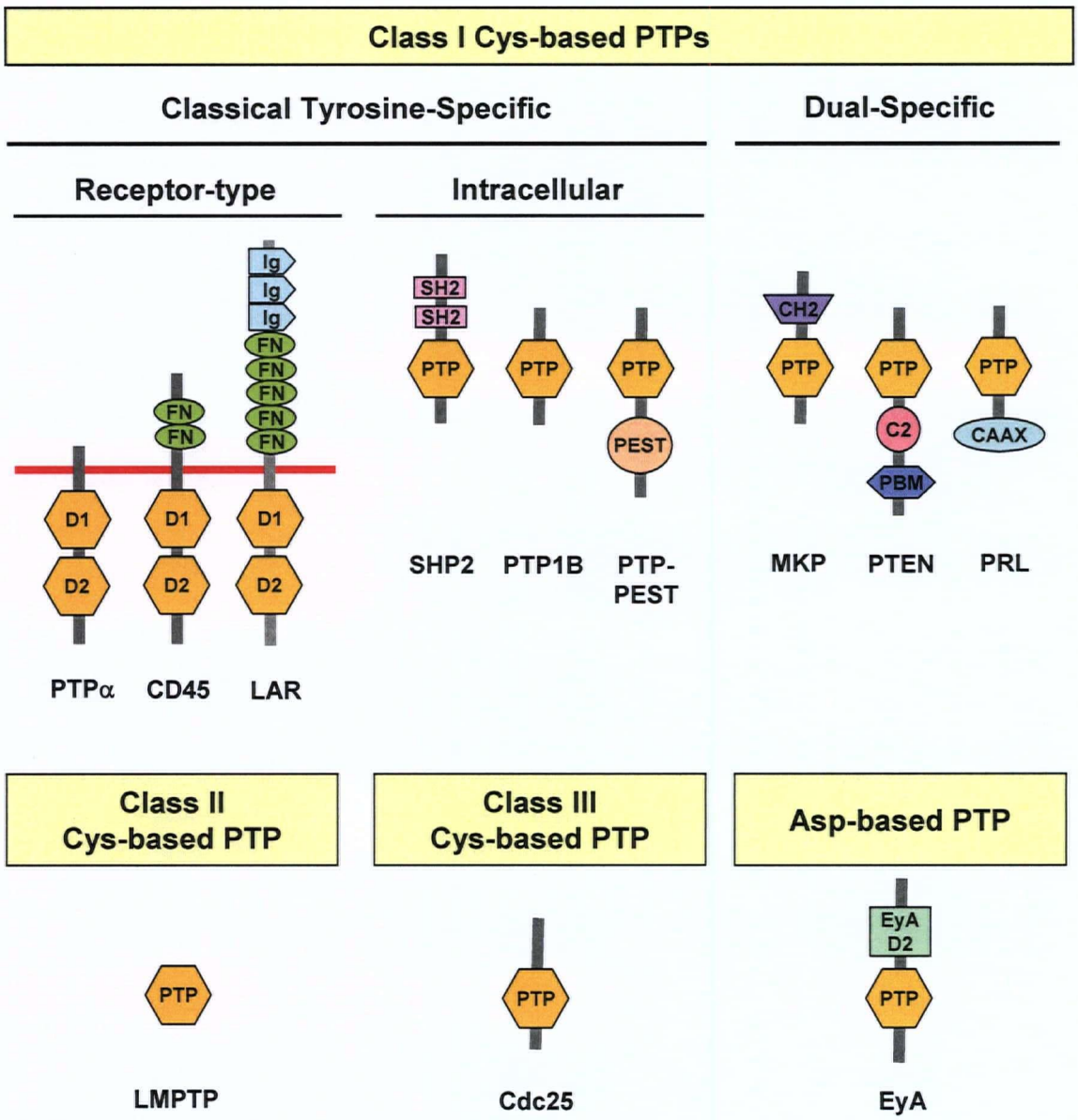

Figure 1.1 Protein tyrosine phosphatase (PTP) superfamily. The PTP superfamily is divided into four classes: Class I Cys-based PTPs, Class II Cys-based low molecular weight PTP (LMPTP), Class III Cys-based Cdc25 PTPs, and Asp-based EyA PTPs. The Class I Cys-based PTPs is further subdivided into tyrosine-specific receptor-like and intracellular PTPs, and dual-specific PTPs. Examples of members each PTP class are shown in the figure. Abbreviations used: FN (fibronectin-like), Ig (immunoglobulin-like), D1 and D2 (tandem PTP domains), CH2 (Cdc25 homology region 2), C2 (protein kinase C conserved region 2), PBM (PDZ binding motif), and EyA D2 (EyA domain 2).

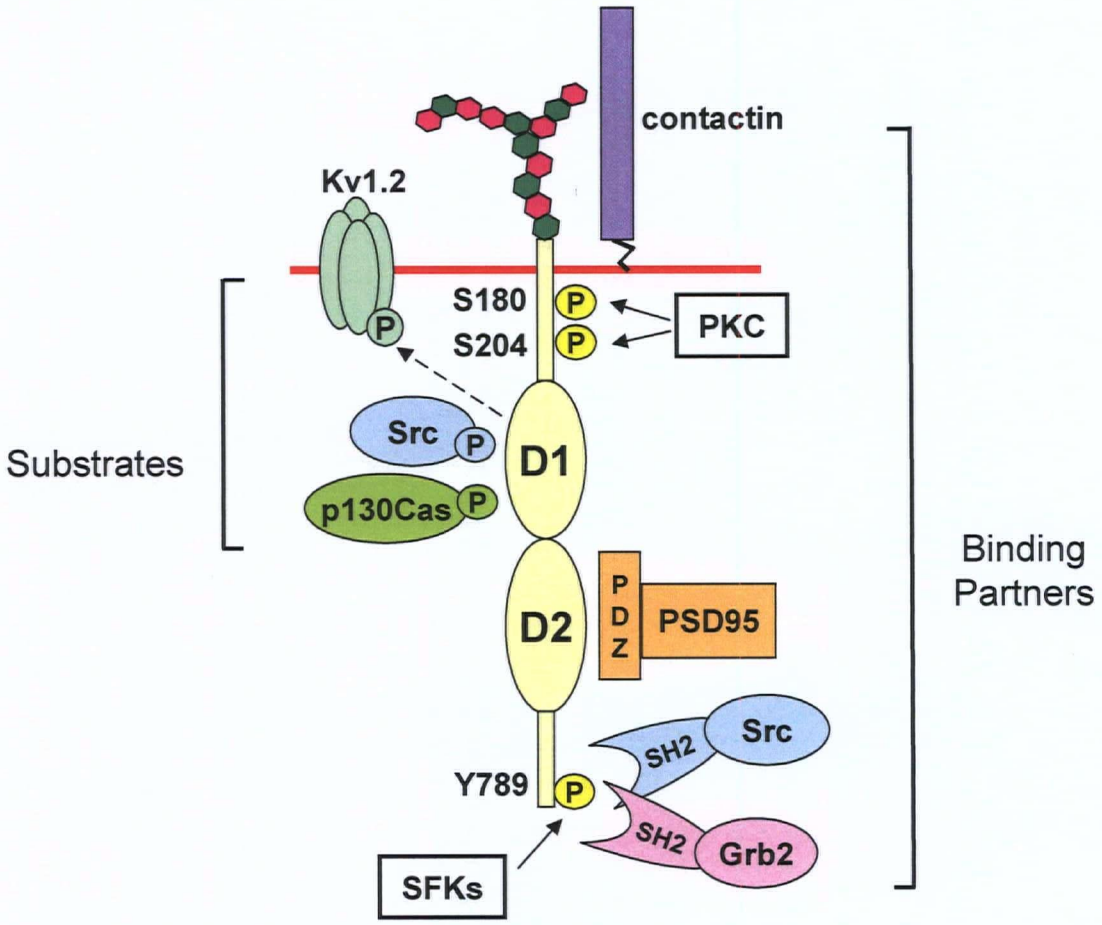

Figure 1.2 PTPα structural domains and interacting proteins. PTPα contains a short, highly glycosylated extracellular domain, a transmembrane domain, and a cytoplasmic domain containing two tandem catalytic domains (D1 and D2). PTPα is serine phosphorylated at S180 and S204 by protein kinase C (PKC), and tyrosine phosphorylated at Y789 by Src family kinases (SFKs). The D1 domain is the major catalytic domain which dephosphorylates Src, p130Cas, and potentially the Kv1.2 potassium channel. Several cellular proteins including PSD95, Src, and Grb2 are known to interact with PTPα, while the extracellular domain of PTPα can form a complex with contactin.

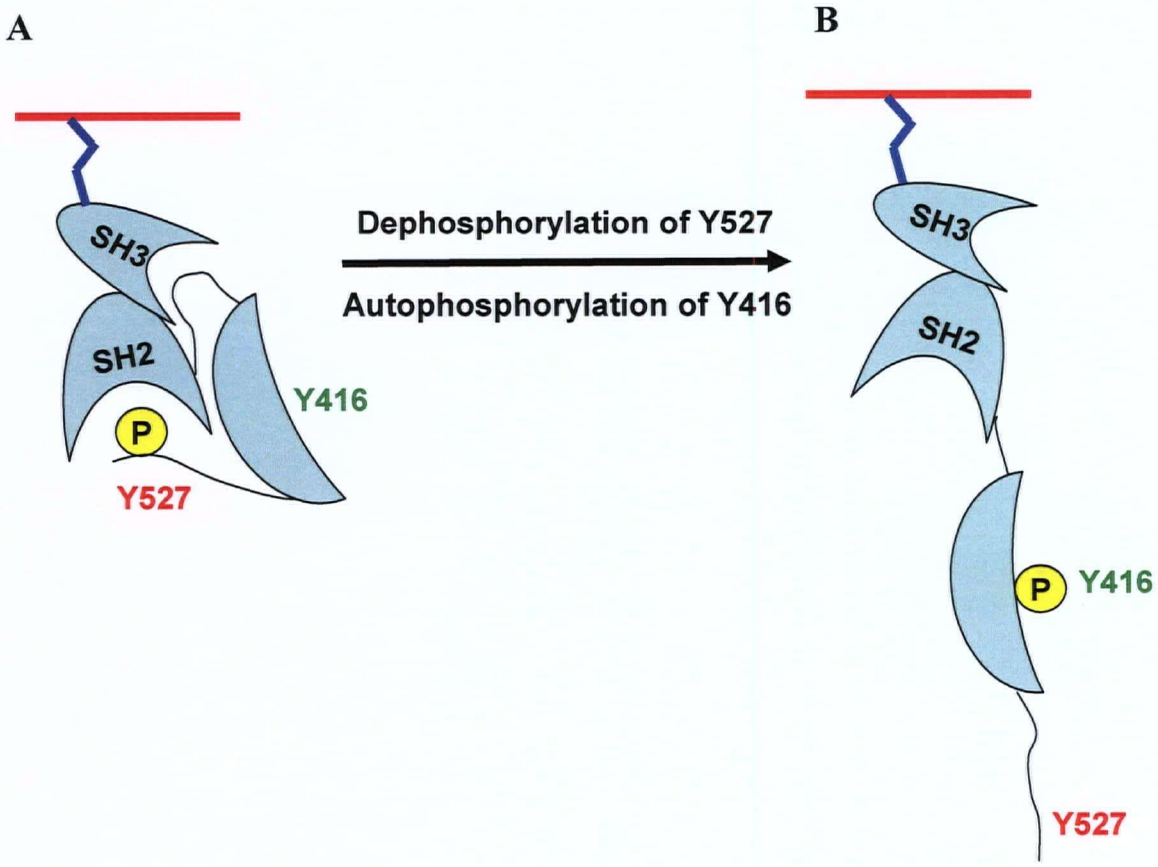

Figure 1.3 Activation of Src and Src family kinases (SFKs). **(A)** Src and other SFKs are maintained in an inactive closed conformation by intramolecular interactions between the SH2 domain and the phosphorylated Y527 motif and between the SH3 domain and the linker region that connects the SH2 and the kinase domains. **(B)** Activation of SFKs is achieved by disruption of one or more of these inhibitory intramolecular interactions, followed by dephosphorylation of Y527 by PTPs such as PTPα, PTP1B, SHP1, and/or SHP2. The subsequent autophosphorylation of Src at Y416 in the kinase domain results in full kinase activation.

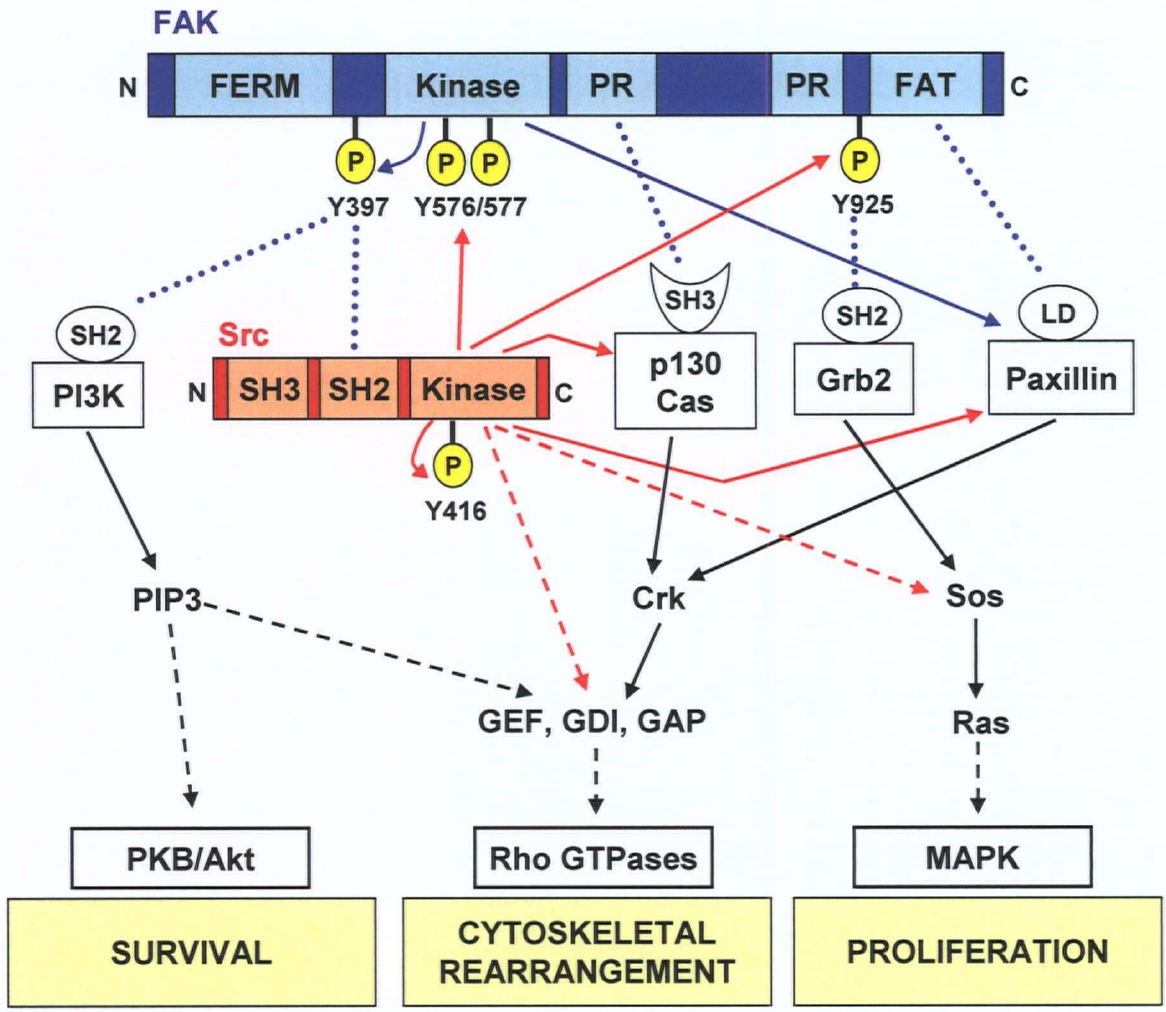

Figure 1.4 Integrin signaling through the FAK/Src complex. Integrin activation by ligand binding induces the early activation of FAK and Src, which function as central signaling molecules to recruit, phosphorylate, and/or activate downstream signaling molecules. This results in signal cascades leading to the activation of PKB/Akt, the Rho family of small GTPases, and MAPK, which ultimately regulate cell survival, cytoskeletal rearrangement required for cell migration, and cell proliferation. The blue dotted lines indicate specific protein interactions with FAK. The blue arrows indicate signaling events transduced from FAK, whereas the red arrows indicate those transduced by FAK-bound Src. Abbreviations used: FERM (protein 4.1, ezrin, radixin, moesin domain), PR (proline-rich domain), FAT (focal adhesion targeting domain), LD (leucine-rich domain), GEF (guanine nucleotide exchange factor), GDI (GDP dissociation inhibitor), and GAP (GTPase activating protein).

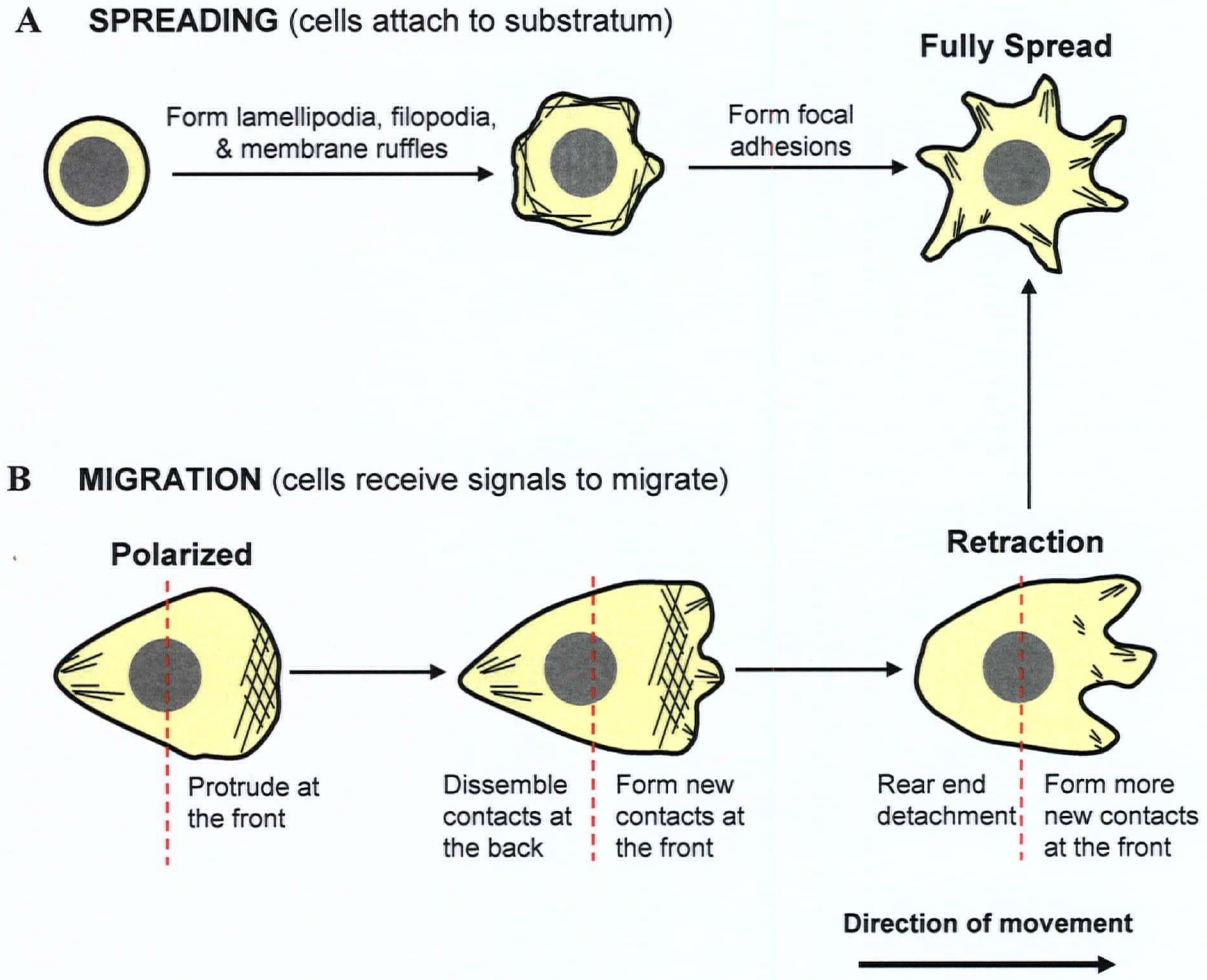

Figure 1.5 Cytoskeletal rearrangements in cell spreading and migration. (A) Upon attachment of cells to the substratum through interaction with extracellular matrix components, they begin to spread out through formation of lamellipodia, filopodia, and membrane ruffles. At the end of the spreading process, cells then form focal adhesion contacts with the underlying substratum. **(B)** When cells receive signals to migrate towards a stimulus, they become polarized with formation of lamellipodia and membrane ruffles at the protruding front. New focal adhesion contacts are formed at the leading edge of the migrating cells, while the cells dissemble the focal contacts at the rear end. Subsequent detachment of cells at the rear end and retraction of the cell body drive forward cell movement.

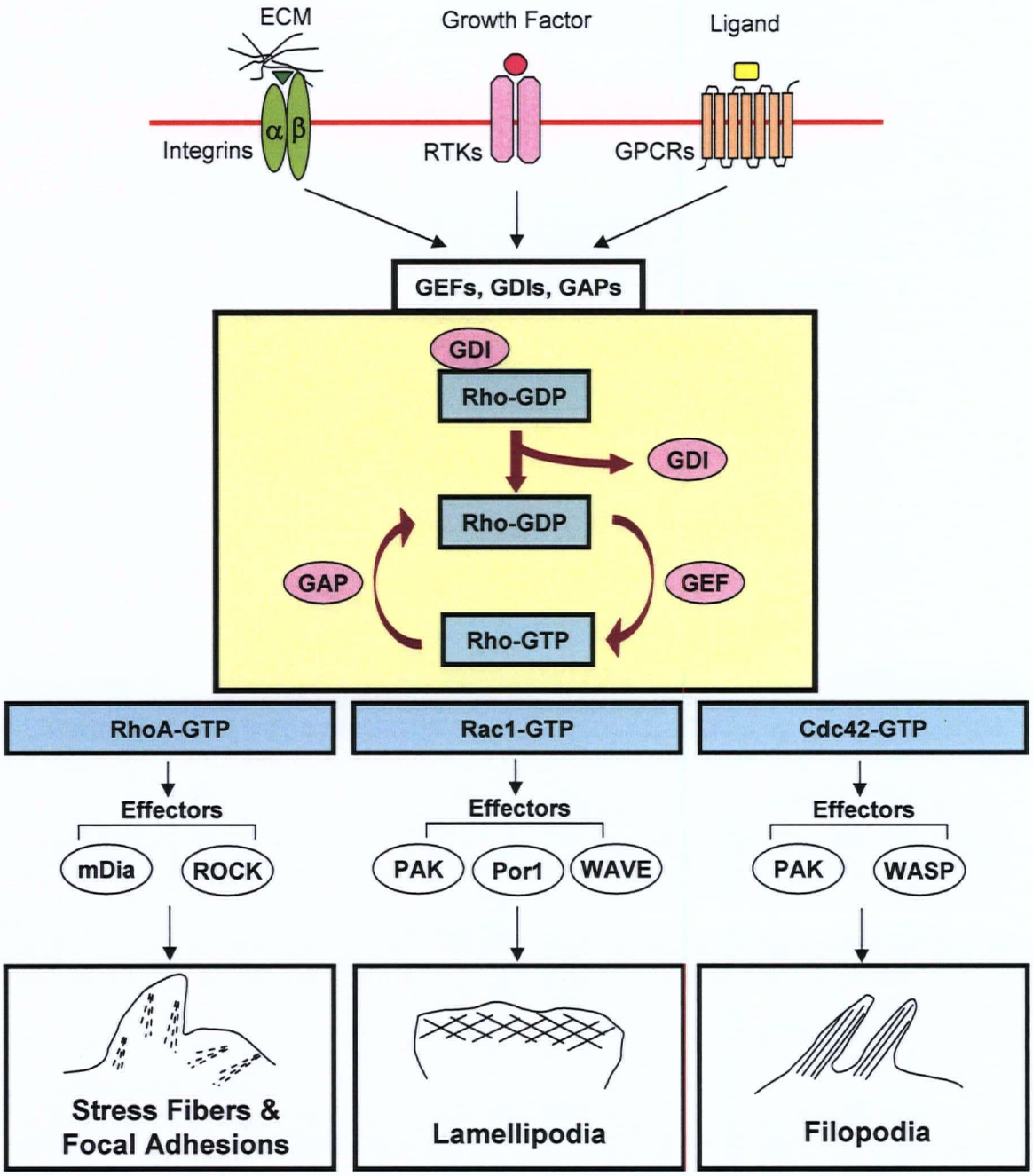

Figure 1.6 Regulation and function of Rho family GTPases. Activation of cell surface receptors such as integrins, receptor tyrosine kinases (RTKs), and G protein-coupled receptors (GPCRs) can activate the Rho family of small GTPases by regulating the functions and/or activities of guanine nucleotide exchange factors (GEFs), GDP-dissociation inhibitors (GDIs), and GTPase-activating proteins (GAPs). The active GTP-bound Rho GTPases, namely RhoA, Rac1, and Cdc42, interact with and activate their downstream effectors, leading to formation of stress fibers and focal adhesions, lamellipodia, and filopodia, respectively.

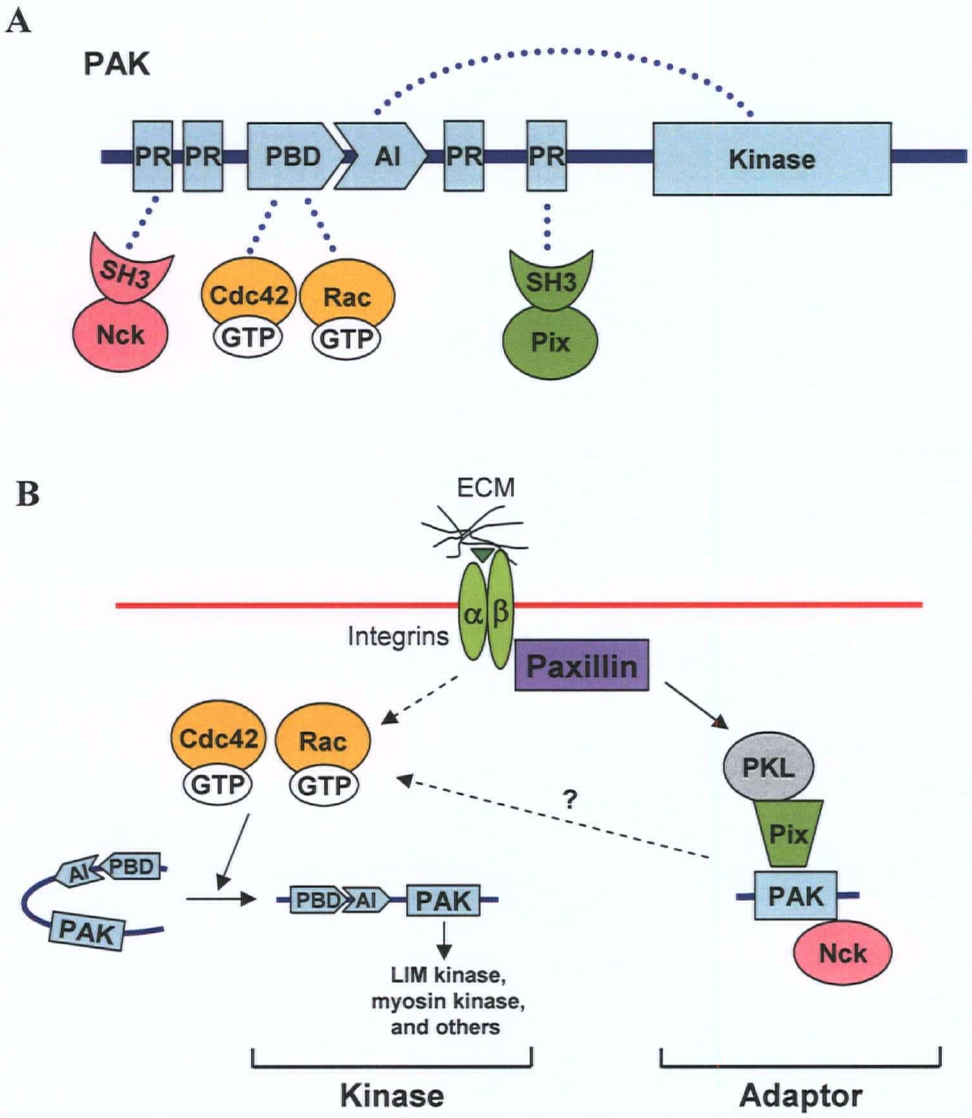

Figure 1.7 Dual roles of p21 activated kinase (PAK). **(A)** Schematic representation of PAK structural domains. PR, proline-rich domain; PBD, p21-binding domain; AI, autoinhibitory domain. Blue dotted lines indicate specific interactions with PAK. **(B)** Integrin engagement induces activation of Rho GTPases such as Cdc42 and Rac. The active GTP-bound Cdc42 and/or Rac in turn activate the kinase activity of PAK by releasing PAK from its intramolecular (kinase-AI) inhibitory interaction. The active PAK subsequently phosphorylates various downstream proteins to regulate cytoskeletal reorganization. In addition, integrin can recruit PAK, in the complex of PKL-Pix-PAK-Nck, to focal adhesions via the interaction between paxillin and PKL. In this complex, PAK functions as an adaptor protein. The precise function of the PKL-Pix-PAK-Nck complex remains to be determined.

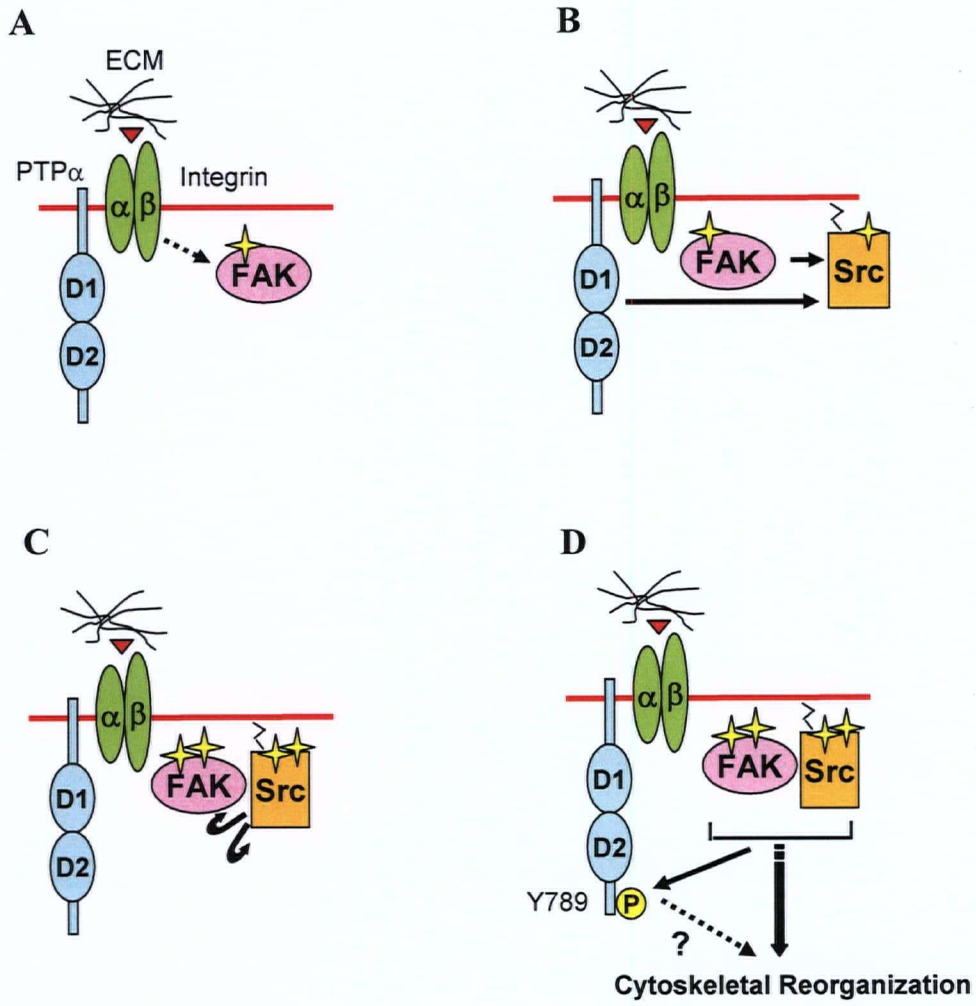

Figure 1.8 Dual roles of PTPα in integrin signaling. **(A)** Integrin stimulation by ligand binding (for example, activation of α5β1 by fibronectin [FN]) induces an early activation and autophosphorylation of FAK at Y397. **(B)** FAK binding to Src and PTPα dephosphorylation of Src Y527 induce Src activation. **(C)** The active Src autophosphorylates itself at Y416 and phosphorylates FAK at several other residues to achieve full Src/FAK activation. **(D)** The fully active Src/FAK complex then recruits, phosphorylates, and/or activates downstream signaling molecules leading to reorganization of the cellular actin cytoskeleton, and cell spreading and migration. One of the downstream targets of the Src/FAK complex is PTPα. Src/FAK-mediated PTPα Y789 phosphorylation regulates the cytoskeletal reorganization events by an unidentified mechanism. The single star indicates partially activated kinases, while double stars indicate fully activated kinases.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Lines and Cell Culture

Spontaneously immortalized and established PTPα$^{+/+}$, PTPα$^{-/-}$, KP PTPα$^{-/-}$, and Src$^{-/-}$ mouse embryonic fibroblast cell lines (Zeng *et al.*, 2003; Chen *et al.*, 2006) were used between passages 30 to 40. SYF (src$^{-/-}$, fyn$^{-/-}$, and yes$^{-/-}$) and Src$^{+/+}$ (src$^{+/+}$, fyn$^{-/-}$, and yes$^{-/-}$) mouse embryonic fibroblast cell lines were obtained from the American Type Tissue Collection (Manassas, VA, USA). All the cell lines were cultured in high glucose Dulbecco's modified Eagle medium (DMEM) (Invitrogen Canada) containing 10% fetal bovine serum (FBS) (Invitrogen Canada), and penicillin and streptomycin (Invitrogen Canada).

2.2 Antibodies

2.2.1 Primary Antibodies

Rabbit antiserum raised against PTPα was previously described (Lim *et al.*, 1998) and was used at a 1:1000 dilution for immunoblotting. The following antibodies were obtained commercially and were used for immunoblotting at a 1:1000 dilution unless specified otherwise: horse radish peroxidase (HRP)-conjugated anti-phosphotyrosine antibody clone PY20 (BD Biosciences, San Jose, CA, USA), rabbit anti-phospho-Thr202/Tyr204-p44/42 ERK antibody (Cell Signaling Technology Inc., Danvers, MA, USA), rabbit anti-p44/42 ERK antibody (Cell Signaling Technology Inc., Danvers, MA,

USA), mouse anti-EGF receptor antibody (Stressgen Bioreagents, Victoria, BC), rabbit anti-IGF-IRβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-Rac1 antibody (Stressgen Bioreagents, Victoria, BC), rabbit anti-phospho-Tyr397-FAK antibody (Biosource International, Camarillo, CA, USA), mouse anti-FAK antibody (BD Biosciences, San Jose, CA, USA), rabbit anti-phospho-Ser144-PAK antibody (Cell Signaling Technology Inc., Danvers, MA, USA), and rabbit anti-PAK antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Mouse anti-Grb2 antibody (BD Biosciences, San Jose, CA, USA) was used at a 1:2000 dilution.

2.2.2 Phosphosite-specific PTPα Y789 Antibody

The phosphosite-specific PTPα Y789 antibody was custom-made by 21st Century Biochemicals (Marlboro, MA, USA). In brief, two rabbits were immunized five times with the phosphotyrosyl peptide CYIDAFSDpY^{789}ANFK (sequence confirmed by mass spectrometry) conjugated to keyhole limpet hemocyanin (KLH) to induce antibody production. Five production bleeds were collected during the course of immunizations. Serum sample from each bleed was sent to us and was evaluated by using the serum for immunoblotting cell lysates containing PTPα-phospho Y789 or unphosphorylatable mutant PTPα Y789F, or these forms of immunoprecipitated PTPα. The ELISA titer to the phosphopeptide and the non-phosphopeptide corresponding to the sequence of the antigen was determined by the company. Based on preliminary assessments, serum collected from one of the rabbits, rabbit #8890, was used for subsequent antibody purification. To ensure specificity, the serum was subjected to multiple rounds of

immunodepletion by passage through an affinity column of immobilized non-phosphopeptide. This was followed by affinity purification using a column with the phosphopeptide antigen as ligand. The specificity of the antibody was tested by immunoblotting (as described in Chapter 3), and this antibody was used at a 1:1000 dilution.

2.2.3 Secondary Antibodies

In most applications, HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were obtained from Sigma and were used at 1:3333 and 1:10000 dilutions, respectively. In the study of PTPα and Grb2 association, the HRP-conjugated goat anti-mouse secondary antibody used for Grb2 immunoblotting was obtained from GeneTex, Inc. (San Antonio, TX, USA) and was used at a 1:10000 dilution.

2.3 Expression of Exogenous PTPα using an Adenovirus Expression System

Expression of wildtype- or Y789F- PTPα in PTPα$^{-/-}$ mouse embryonic fibroblasts was achieved using the AdEasy vector system (Qbiogene, Inc., CA, USA). The PTPα-expressing adenovirus was previously generated as described (Chen *et al.*, 2006) and was used to infect QBI-293A cells to prepare a viral stock for use in experiments. After the cells exhibited a complete cytopathic effect, the viral particles were harvested through four freeze (100% ethanol filled with dry ice) / thaw (37°C water bath) cycles. The virus was purified by ultracentrifugation at 30,000 rpm for 16 hr at 14°C in a continuous CsCl gradient, followed by desalting using a PD-10 Sephadex™ G-25 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in filter-sterilized HEPES buffered saline (21

mM HEPES, 137 mM NaCl, 5 mM KCl, 0.73 mM Na_2HPO_4, and 5.6 mM dextrose pH7.1). Glycerol was added to a final concentration of 15% and the purified adenovirus was stored in aliquots at -80°C.

The amount of virus needed to express PTPα at a level approximately 5 times that of endogenous PTPα was determined. The fibroblast cells were first incubated with the desired amount of virus diluted in a minimal volume of DMEM containing 10% FBS (1 ml per 10 cm culture dish) for 90 min. The plates were agitated gently every 30 min to ensure full coverage of the cells with the virus solution. After incubation, DMEM containing 10% FBS (9 ml per 10 cm culture dish) was added. The cells were incubated for another 24 hr to allow expression of the exogenous protein before further manipulations were carried out.

2.4 Cell Stimulation

2.4.1 Fibronectin Stimulation

Cells were grown to approximately 80-90% confluency and were serum starved in DMEM containing 0.2% FBS for 18-20 hr prior to stimulation. After trypsinization with 0.05% trypsin-EDTA, the cells were washed once with serum-free DMEM and kept in suspension in DMEM with 0.1% BSA for 1.5-2 hr at 37°C. Stimulation was carried out by plating the cells on fibronectin (FN)- or poly-L-lysine (PLL)-coated dishes for the indicated times. FN- or PLL-coated dishes were prepared on the previous day by incubating the plates with 15 μg/ml of FN (Chemicon) or 20 μg/ml of PLL (Sigma) in

PBS overnight at 4°C on a platform shaker. Prior to cell plating, the coated dishes were washed once with PBS, covered with serum-free DMEM, and kept in the 37°C incubator.

2.4.2 Growth Factor Stimulation

Cells were grown to approximately 80-90% confluency and were serum starved in completely serum-free DMEM for 20 hr prior to stimulation. Following a quick wash with serum-free DMEM, the cells were stimulated with 100 ng/ml of insulin-like growth factor-1 (IGF-1) (Somagen Diagnostic, Edmonton, AB), 100 ng/ml of epidermal growth factor (EGF) (Sigma), 100 ng/ml of acidic fibroblast growth factor (aFGF) (Sigma), 150 nM of phorbol 12-myristate 13-acetate (PMA) (Sigma), or 10 μM of oleoyl-L-α-lysophosphatidic acid (LPA) (Sigma) for the indicated times.

2.5 Rho GTPase Activation Assays

2.5.1 RhoA Activation Assay

RhoA activation assays were performed according to Ren *et al.* (1999) with minor modifications. Cells were lysed in magnesium lysis buffer (25 mM HEPES pH7.5, 150 mM NaCl, 1% Igepal, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 25 mM NaF, and 1 mM Na_3VO_4) and scraped off the plate. The lysates were centrifuged at 14,000 g for 15 min. 1 mg of supernatant protein was used in a pull-down assay with 30 μl of a 50% (*vol/vol*) slurry of GST-Rhotekin-RBD beads (see Section 2.5.2). After 1 hr incubation at 4°C on a rotator, the samples were then centrifuged at 13,000 rpm for 30 sec. Unbound proteins were removed by washing the pellet three times with 500 μl lysis buffer, and the proteins that were associated with the

glutathione beads were released by adding 40 µl of 2X SDS sample buffer (62.5 mM Tris-HCl pH6.8, 20% glycerol, 2% SDS, 0.08% bromophenol blue, and β-mercaptoethanol). The samples were boiled for 5 min, centrifuged briefly, and stored at -20°C or loaded onto SDS-PAGE.

2.5.2 Production and Purification of GST-tagged Rhotekin-RBD Proteins

The *E. coli* bacteria transformed with the pGEX4T1 plasmid encoding GST-tagged Rhotekin-RBD fusion proteins were a gift from E. Manser (Institute of Molecular and Cell Biology, Singapore). Luria broth (LB) supplemented with 100 µg/ml ampicillin was used as the culture medium. An initial 50 ml of medium inoculated with bacteria stock was incubated overnight in a 37°C shaker (250 rpm). An aliquot of this pre-culture was added to 500 ml of fresh LB culture medium at a 1:50 dilution, and this was grown for about 1 hr (until the OD_{600} reached ~0.2). Iso-propyl-thio-β-D galactopyranoside (IPTG) was then added at a final concentration of 0.15 mM to induce protein production during culture in a shaker at room temperature for ~16 hr (until the OD_{600} reached ~1.8). The bacteria were pelleted by centrifugation at 6,000 rpm at 4°C for 10 min, and were resuspended and lysed in 10 ml lysis buffer (50 mM Tris-HCl pH7.6, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonylfluoride (PMSF), 20 µg/ml aprotinin, and 1% Triton X-100), followed by sonication. The lysates were then centrifuged at 12,000 rpm at 4°C for 20 min to clear the cell debris. To affinity purify the GST-RBD fusion proteins by adherence to glutathione beads, the supernatant was incubated with 1 ml of 50% GST-Bind™ Resin (Novagen, EMB Biosciences Inc., Madison, WI, USA) for 4 hr at 4°C on a rotator. The beads were washed three times with

8 ml/wash of lysis buffer and once with lysis buffer lacking Triton X-100. The bead pellet was resuspended in 0.5 ml of stock buffer (50 mM Tris-HCl pH7.6, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml aprotinin, 0.5% Triton X-100, and 10% glycerol) and stored in aliquots at -80°C.

2.5.3 Rac1 Activation Assay:

Rac1 activation assays were performed using the StressXpress Rac1 Activation Kit (Stressgen Bioreagents, Victoria, BC) according to the manufacturer's protocol. In brief, cells were lysed with the lysis buffer from the kit and scraped off the plate. The lysates were incubated on ice for 5 min and then were centrifuged at 13,000 rpm for 15 min. 1 mg of total supernatant protein was added to 20 μg of purified GST-tagged PAK-PBD fusion protein and an immobilized glutathione disc (supplied by the manufacturer). After incubation for 1 hr at 4°C on a rotator, the samples were centrifuged at 7,200 g for 30 sec. After three washes each with 400 μl of lysis buffer, the proteins that had bound to GST-PAK-PBD and been co-precipitated by the glutathione beads were released by the addition of 50 μl of 2X SDS sample buffer. The samples were boiled for 5 min, briefly centrifuged, and stored at -20°C or loaded onto SDS-PAGE.

2.6 Cell Lysis

Prior to lysis, cells were washed twice with ice-cold PBS. RIPA (radioimmune precipitation assay) lysis buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% SDS, 2 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) was used for all

experiments with the exception of the Rho and Rac GTPase activation assays (see Section 2.5). Cell lysates were cleared by centrifugation at 13,000 rpm for 20 min. Protein concentration was determined by Bradford protein assay (Bio-Rad Laboratories Canada) with absorbance measured at 595 nm.

2.7 Immunoprecipitation

Immunoprecipitations with anti-PTPα antiserum were carried out using 1 mg (for PTPα tyrosine phosphorylation) or 200 μg (for PTPα-Grb2 association) of cell lysate protein. The lysates were pre-cleared with 20 μl Protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1.5 hr. All incubations were carried out at 4°C on a rotator. After a brief centrifugation, the pre-cleared lysates were then incubated overnight with the immunoprecipitation antibody. The immunocomplex was precipitated by incubation with 40 μl of Protein A/G agarose beads for 1 hr. After centrifugation at 5,000 rpm for 4 min, the pelleted beads were washed four times (3 x 1 ml wash with RIPA lysis buffer and 1 x 1 ml wash with detergent-free lysis buffer). After the final wash, 20 μl of 2X SDS sample buffer was added to dissociate the immunocomplex from the beads and the samples were boiled for 5 min. The samples were immediately resolved by SDS-PAGE or stored at -20°C.

2.8 Immunoblotting

Appropriate amounts of protein samples were resolved by SDS-PAGE and then transferred from the gel onto a poly vinylidene fluoride (PVDF) membrane at 100V for 70 min. The membrane was blocked by incubation with 5% skim milk or 2% bovine

serum albumin (BSA) in PBST buffer (PBS with 0.1% Tween-20) at room temperature for 1 hr. Primary antibodies were diluted in PBST containing 1% BSA and incubated with the membrane at 4°C overnight. The membrane was then washed three times for 10 min each with PBST and incubated with HRP-conjugated secondary antibody diluted in PBST containing 1% BSA for 1 hr at room temperature. After another three washes of 10 min each with PBST, the bound antibody was detected using chemilumiscent ECL reagents.

2.9 PTPα Peptide Affinity Chromatography

2.9.1 Sample Preparation

FN-stimulated (30 min) PTPα$^{-/-}$ mouse embryonic fibroblasts were lysed in modified RIPA lysis buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and scraped off the plate. The samples were incubated on ice for an additional 20 min to promote complete lysis and then centrifuged at 13,000 rpm for 20 min to remove cell debris. After determining the protein concentration of the supernatants, the samples were diluted to 1 mg/ml with detergent-free lysis buffer such that the final Triton X-100 concentration of the lysates was 0.5%.

2.9.2 Peptide Affinity Chromatography

The two affinity matrices used here were generated by 21st Century Biochemicals (Marlboro, MA, USA) for the purification of phospho-PTPα Y789 antibody (see Section 2.2.2). Beads were conjugated to the PTPα peptide CYIDAFSDY^{789}ANFK or to the

phosphotyrosyl version of this peptide. Approximately 0.8 ml bed volume of each peptide-coupled bead was available and was transferred to a chromatography column (Econo-column) (Bio-Rad Laboratories Canada). The columns were equilibrated by washing with 10 ml of detergent-free lysis buffer. 4 ml of the diluted protein lysate (equivalent to 4 mg of protein) was added to each column, and the column was capped and incubated overnight at 4°C on a rotator. The beads were allowed to settle for 30 min before the flow-through was collected. The columns were washed with 10 ml of lysis buffer containing 0.05% Triton X-100, and then with 4 ml of detergent-free lysis buffer. Bound protein was eluted with 5 ml 0.1M glycine-HCl pH2.5. The first 4 ml of eluate was collected in a 15-ml tube containing 293.2 µl of 1M Tris pH9.5 for neutralization. The eluate was concentrated to a final volume of 250 µl using an Amicon Ultra-15 Centrifugal Filter Device with a nominal 10 kD molecular mass limit (Millipore, Billerica, MA, USA).

Samples (20 µl of the starting cell lysates, 30 µl of the column flow-throughs, and 30 µl of the column eluates) were diluted with an equal volume of 2X SDS sample buffer and boiled for 5 min. The samples were then loaded on a 10% SDS-PAGE. After resolution, the proteins were transferred from the gel to a PVDF membrane at 100V for 70 min. Total protein was visualized with Memcode reversible PVDF membrane stain (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's protocol. The membranes were de-stained according to the manufacturer's protocol prior to subsequent immunoblotting for Grb2. In another analysis of the column eluates, 30 µl and 10 µl of each eluate were diluted with an equal volume of 2X SDS sample buffer and boiled for 5

min. The samples were loaded on a 12% SDS-PAGE. After resolution, part of the gel (30 μl loaded/lane) was stained with SimplyBlue SafeStain (Invitrogen Canada). The proteins on the other half of the gel (10 μl loaded/lane) were transferred to a PVDF membrane, which was subsequently immunoblotted for phosphotyrosine.

2.10 Data Analysis

The densitometric intensities of immunoblotted protein bands were determined using the Quantity One program (Bio-Rad Laboratories). The PTPα and PAK phosphorylation levels were quantified by normalizing the densitometric units of phosphorylated protein to those of total PTPα or PAK. The Grb2 and PTPα association was quantified by normalizing the densitometric units of co-immunoprecipitated Grb2 to those of immunoprecipitated PTPα. Data are shown as the mean ± standard deviation. The *p* values were determined using the student's t-test.

CHAPTER 3

REGULATION OF PTPα Y789 PHOSPHORYLATION

3.1 Rationale

PTPα is constitutively tyrosine phosphorylated at Tyr-789 (Y789) located in its intracellular C-terminal tail (den Hertog *et al.*, 1994). Due to the unavailability of a phosphosite-specific antibody for this site, studies in our lab on the regulation of PTPα tyrosine phosphorylation in integrin signaling have relied on immunoprecipitation of PTPα, followed by immunoblotting with anti-phosphotyrosine antibody and then with anti-PTPα antibody (as shown in Figure 3.1A). Although Y789 is the only known site of tyrosine phosphorylation in PTPα, the results obtained using this method can only reflect the overall tyrosine phosphorylation status of PTPα, and cannot provide direct evidence that it occurs at Y789. This has been indirectly confirmed in our cell system by a lack of tyrosine phosphorylation immunoblotting signal when PTPα Y789 is mutated to phenylalanine (Y789F) (Chen *et al.*, 2006), assuming that the mutation does not induce conformational changes and interfere with tyrosine phosphorylation on other potential sites. To obtain a reasonable signal, the immunoprecipitation procedure requires preparation of at least 800 μg of cell lysate protein and the process is time-consuming. Due to these reasons, a phosphosite-specific PTPα Y789 antibody was custom-made by 21st Century Biochemicals (Marlboro, MA, USA).

3.2 Characterization of Phosphosite-specific PTPα Y789 Antibody

The antibody was produced in rabbits immunized with a phosphotyrosyl peptide (CYIDAFSDpY^{789}ANFK) corresponding to the amino acid sequence surrounding the PTPα Y789 phosphorylation site. The serum collected from the immunized animals was evaluated for its reactivity towards the antigen and a non-phosphorylated version of the same peptide, and by its performance on immunoblotting towards phospho PTPα and mutant PTPα Y789F. The antibodies were then purified from the selected serum batch by a series of immunodepletion and immunopurification steps (carried out by 21st Century Biochemicals, Marlboro, MA, USA and by Dr. Jing Wang, Pallen lab) to ensure its specificity towards phosphorylated PTPα.

The purified antibody was used for immunoblotting of 40 μg of cell lysate protein at a 1:1000 dilution. Both the phosphosite-specific PTPα Y789 antibody and an antibody directed to PTPα recognized a protein of ~170 kD. Although this is bigger than the predicted ~130 kD mass of PTPα, it is consistent with reported observations from our lab and others, and is due to the extensive glycosylation of PTPα (Daum *et al.*, 1994). The phosphosite-specific PTPα Y789 antibody did not detect any non-specific signals corresponding to the predicted or observed molecular weight of PTPα in lysates from either PTPα$^{-/-}$ cells or PTPα$^{-/-}$ cells expressing mutant PTPα Y789F. The antibody was then used to validate previous findings reported by our lab on the integrin-induced tyrosine phosphorylation of PTPα (Chen *et al.*, 2006). Mouse embryonic fibroblasts were serum starved, detached from the substratum, and maintained in suspension for ~1 hr prior to plating on dishes coated with the integrin ligand, fibronectin (FN). Consistent

with the FN-induced increase in tyrosine phosphorylation of PTPα observed in PTPα immunoprecipitates from these cells (Figure 3.1A), immunoblotting of total cell lysates with the phosphosite-specific antibody likewise demonstrated that plating the cells on FN for 15 min induced PTPα Y789 phosphorylation (Figure 3.1B). This was observed in both the $PTP\alpha^{+/+}$ cells and also in $PTP\alpha^{-/-}$ cells with reintroduced wildtype PTPα.

Min Chen in our lab previously demonstrated that at least one of three SFKs (Src, Fyn, and Yes) is involved in and is required for PTPα Y789 phosphorylation following FN stimulation. These experiments involved the immunoprecipitation and anti-phosphotyrosine immunoblotting of PTPα from cells lacking one or more of these SFKs. As another test of the utility of the phosphosite-specific antibody, these experiments were repeated. Instead of immunoprecipitating PTPα, the cell lysates were directly probed with the phosphosite-specific PTPα Y789 antibody. As shown in Figure 3.2, integrin-induced PTPα Y789 phosphorylation was not impaired in $Src^{-/-}$ ($Src^{-/-}$ / $Fyn^{+/+}$ / $Yes^{+/+}$) or $Src^{+/+}$ ($Src^{+/+}$ / $Fyn^{-/-}$ / $Yes^{-/-}$) cells, but was defective in SYF ($Src^{-/-}$ / $Fyn^{-/-}$ / $Yes^{-/-}$) cells. These results are consistent with those obtained by immunoblotting PTPα immunoprecipitates for phosphotyrosine (Chen *et al.*, 2006).

In summary, the feasibility and specificity of the custom-made phosphosite-specific PTPα Y789 antibody were confirmed. The antibody was specific for phospho-PTPα Y789 with no recognition of non-phosphorylated PTPα Y789 or other proteins of similar size. Immunoblotting cell lysates with this antibody provided a clean and strong signal, and thus eliminates the requirement for a prior PTPα immunoprecipitation step in the

analysis of PTPα tyrosine (Y789) phosphorylation. Furthermore, previous findings on the regulation of PTPα Y789 phosphorylation by integrin signaling were confirmed with the use of this antibody.

3.3 Regulation of PTPα Y789 Phosphorylation by Other Signaling Pathways

Phosphorylation of PTPα at Y789 has been shown to be functionally involved in PTPα-regulated processes including the mitotic activation of Src (Zheng *et al.*, 2001) and integrin-induced cytoskeletal reorganization (Chen *et al.*, 2006). In these events, the phosphorylation of PTPα at Y789 is tightly controlled to ensure proper timing for relayed downstream signaling events. Modulation of PTPα Y789 phosphorylation has also been reported in cells treated with H_2O_2 (model for oxidative stress), although the functional significance of this remains to be determined (Hao *et al.*, 2006b). It is not known whether other signaling pathways, such as those induced by growth factors, also utilize PTPα as part of the signaling cascade through modulation of its phosphorylation status. With the PTPα Y789 phosphosite-specific antibody available and its specificity confirmed, extracellular activators of different signaling pathways were tested for their potential roles in regulating PTPα Y789 phosphorylation. These activators include epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), acidic fibroblast growth factor (aFGF), lysophosphatidic acid (LPA), and phorbol myristyl acetate (PMA).

Serum-starved PTPα$^{+/+}$ mouse embryonic fibroblasts were treated with the indicated stimulus and the cell lysates were immunoblotted with the phosphosite-specific PTPα Y789 antibody. Since all the stimuli investigated here are activators of the ERK signaling

pathway, ERK phosphorylation at T202/Y204 was assayed to ensure proper stimulation of the cells. As shown in Figure 3.3A, while ERK phosphorylation was significantly diminished in the serum-starved cells, PTPα Y789 phosphorylation remained readily detectable. Overnight serum withdrawal did not appear to alter cellular PTPα Y789 phosphorylation, since this was similar in level to that of cells actively growing in medium supplemented with full serum (data not shown). Stimulation of cells with 100 ng/ml of EGF produced no change in PTPα Y789 phosphorylation, although as expected, it dramatically induced ERK T202/Y204 phosphorylation (Figure 3.3A). The presence of the EGF receptor (EGFR) in this fibroblast cell line was also confirmed by immunoblotting.

In contrast to EGF, IGF-1 stimulation enhanced PTPα Y789 phosphorylation at all timepoints examined (Figure 3.3A, top panel, arrow). Interestingly, an additional band was detected in the IGF-1 stimulated samples by immunoblotting with the phosphosite-specific PTPα Y789 antibody (Figure 3.3A, top panel, arrowhead). This reactive band migrated slightly above the major PTPα band and co-migrated with the 175 kD protein molecular weight standard. To verify whether this additional band corresponded to a more highly phosphorylated form of PTPα that was produced in response to IGF-1 stimulation, mouse embryonic fibroblasts lacking PTPα ($PTP\alpha^{-/-}$) were serum starved and stimulated with IGF-1 for 2 min. Immunoblotting with the phosphosite-specific PTPα Y789 antibody revealed the presence of the same reactive 175 kD band even in PTPα-deficient cells (Figure 3.3B). This demonstrates that the additional band does not represent a hyperphosphorylated form of PTPα. The detection of this unexpected 175 kD

band in both PTP$\alpha^{+/+}$ and PTP$\alpha^{-/-}$ cell samples indicates either a lack of specificity of the phosphosite-specific PTPα Y789 antibody, or the specific recognition of another protein possessing a similar phosphotyrosine-containing microsequence. The 175 kD molecular mass, and the early detection of this reactive band 2 min after IGF-1 (but not other stimuli, see below) stimulation, raise the possibility that it represents the protein IRS1. IRS1 or insulin receptor substrate 1 is one of the major substrates for the activated IGF-1 receptor and insulin receptor, and is extensively tyrosine phosphorylated upon IGF-1 or insulin stimulation (Myers *et al.*, 1993). Preliminary alignment of the PTPα amino acid sequence surrounding its Y789 phosphorylation site with the IRS1 amino acid sequence showed substantial sequence similarity with a region of IRS1 containing the Y1220 (Y1229 for human IRS1) phosphorylation site (PTPα: $_{782}$YIDAFSD*p*YANFK$_{793}$; IRS1: $_{1213}$SSEDLSN*p*YASIS$_{1224}$; 50% similarity). The identity of the immunoreactive band as IRS1 has not yet been verified due to the lack of a good IRS1 antibody for immunoblotting cell lysates, but could be attempted in future by immunoprecipitation of IRS1, followed by immunoblotting with the anti-PTPα phosphosite-specific antibody.

Densitometry analysis of four sets of data obtained from IGF-1 stimulated PTP$\alpha^{+/+}$ fibroblasts revealed an overall 35% increase in PTPα Y789 phosphorylation upon IGF-1 stimulation (1.39±0.19, p=0.026 at 2 min; 1.37±0.16, p=0.019 at 10 min; 1.23±0.12, p=0.033 at 30 min) (Figure 3.4). The increase is detectable as early as 2 min following IGF-1 stimulation while ERK phosphorylation was only moderately induced at this time point. The PTPα Y789 phosphorylation level remained high at 10 min post-stimulation and appeared to decrease slightly at 30 min post-stimulation.

Besides EGF and IGF-1, regulation of PTPα Y789 phosphorylation by another growth factor, aFGF, was also investigated. Fibroblast growth factors (FGF) comprise a large family of around 20 polypeptides that bind to the four FGF receptors, FGFR1-4, in an overlapping manner (Klint *et al.*, 1999). aFGF (or FGF-1) and bFGF (or FGF-2) are the two best-characterized members and are ubiquitously expressed (Hughes *et al.*, 1993). While the expression pattern of each FGFR is yet to be determined, a previous study has demonstrated that mouse embryonic fibroblasts express FGFR1 and are responsive to aFGF stimulation (Kilkenny *et al.*, 2003). Therefore, aFGF was used in this study. Stimulation of cells with 100 ng/ml of aFGF did not appear to significantly alter PTPα Y789 phosphorylation while greatly inducing ERK T202/Y204 phosphorylation (Figure 3.5). Similarly, stimulation of cells with PMA, a PKC activator, and LPA, a bioactive lysophospholipid mediator for G protein coupled receptors, yielded no visible change in PTPα Y789 phosphorylation level (Figure 3.5).

Of the five stimuli investigated here, only IGF-1 treatment led to a significant change in PTPα Y789 phosphorylation. The high level of basal PTPα phosphorylation that is unaffected by overnight serum withdrawal may limit the unphosphorylated PTPα that is available for phosphorylation upon stimulation, and also explain the moderate increase (35%) in PTPα phosphorylation with IGF-1 stimulation. It is well known that growth factor receptor signaling often works in concert with integrin receptor signaling, with significant crosstalk between the two systems ensuring optimal downstream signaling activation (Comoglio *et al.*, 2003). In the case of IGF-1 receptor signaling, blocking αVβ3 integrin occupancy inhibits IGF-1 receptor activation and attenuates IGF-1-induced

cell actions including DNA and protein synthesis and migration (Jones *et al.*, 1996; Zheng *et al.*, 1998). Since integrin signaling is required for the full activation of the IGF-1 receptor signaling and we have previously shown that integrin stimulation may induce PTPα Y789 phosphorylation, the involvement of integrin signaling in IGF-1-induced PTPα Y789 phosphorylation was investigated. Serum-starved PTPα$^{+/+}$ mouse embryonic fibroblasts were detached from the culture dish by minimal trypsinization and were kept in suspension for 1 hr to downregulate integrin signaling. The cells were then plated on FN- or PLL-coated dishes in the absence or presence of IGF-1 for 15 min. In the absence of IGF-1, the cellular PTPα Y789 phosphorylation level was reduced in suspended cells, and was increased upon integrin stimulation with FN; however, plating the cells on PLL, a non-integrin-mediated interaction, had a minimal effect on PTPα phosphorylation (Figure 3.6). Interestingly, the addition of IGF-1 induced PTPα Y789 phosphorylation regardless of the integrin activation state. These results indicate that IGF-1-induced PTPα Y789 phosphorylation is an integrin-independent event.

Integrin-induced PTPα Y789 phosphorylation is impaired in SYF cells (Src$^{-/-}$ / Fyn$^{-/-}$ / Yes$^{-/-}$) (Figure 3.2 and Chen *et al.*, 2006), indicating that at least one member of the Src, Fyn, and Yes SFKs is involved in and required for this process. Since IGF-1 may induce PTPα Y789 phosphorylation independently from integrin signaling, the requirement of Src, Fyn, and Yes in IGF-1-induced PTPα Y789 phosphorylation was investigated. Serum-starved PTPα$^{+/+}$ and SYF mouse embryonic fibroblasts were stimulated with IGF-1 for 2 min and immunoblotted with the phosphosite-specific PTPα Y789 antibody. As shown in Figure 3.7A, the basal PTPα Y789 phosphorylation level was considerably

lower in serum-starved SYF cells than in PTP$\alpha^{+/+}$ cells. This was not due to a greater sensitivity of the SYF cells towards serum withdrawal since a similarly lower phosphorylation of PTPα was observed in SYF versus PTP$\alpha^{+/+}$ (wildtype) fibroblasts growing in media supplemented with full serum (data not shown). These observations implicate a role of Src, Fyn, and/or Yes in maintaining the basal PTPα Y789 phosphorylation level. However, the residual phosphorylation detected in SYF cells also indicates the involvement of other cellular kinases. Despite the lack of Src, Fyn, and Yes kinases, the stimulation of SYF cells with IGF-1 resulted in an increase in PTPα Y789 phosphorylation (Figure 3.7A and B, and Figure 3.8). This is in contrast to integrin signaling where FN-induced PTPα Y789 phosphorylation was completely abolished in SYF cells (Figure 3.2). Although IGF-1 stimulation results in a similar level of PTPα phosphorylation in both cell lines, the fold increase is greater in SYF cells than in PTP$\alpha^{+/+}$ cells when compared to the corresponding levels in serum-starved cells. My previous results showed that only IGF-1, but not EGF, aFGF, LPA, or PMA, can positively regulate PTPα Y789 phosphorylation in PTP$\alpha^{+/+}$ fibroblasts (Figures 3.3, 3.4, and 3.5). The lack of stimulation may be due to the high basal PTPα phosphorylation level in the PTP$\alpha^{+/+}$ fibroblasts. Since the SYF cells have a relatively low basal PTPα phosphorylation level that apparently allows a greater response to stimulation, the SYF cells were also used to investigate the regulation of PTPα Y789 phosphorylation by EGF, aFGF, LPA, and PMA.

Serum-starved SYF cells were treated with the indicated stimulus for 10 min (Figure 3.7B). Densitometry analysis of the results from four such experiments showed a ~ two-

fold (98%) increase in PTPα Y789 phosphorylation upon IGF-1 stimulation (1.98±0.30, *p=0.007*) (Figure 3.8), compared to the ~35% increase (1.37±0.16, *p=0.019*) (Figure 3.4) in PTPα$^{+/+}$ fibroblasts. Surprisingly, aFGF stimulation of SYF cells also induced about a two-fold increase (1.83±0.31, *p=0.013*), while LPA and PMA stimulation resulted in a moderate increase (1.40±0.07, p=0.001 for LPA; 1.24±0.14, p=0.039 for PMA) in PTPα Y789 phosphorylation. EGF stimulation did not yield a statistically significant change in PTPα Y789 phosphorylation level (1.36±0.24, p=0.061) (Figure 3.8). The results from SYF cells clearly indicate the dispensable role of Src, Fyn, and Yes in PTPα Y789 phosphorylation induced by IGF-1, aFGF, LPA, and PMA. The different results obtained from PTPα$^{+/+}$ and SYF cells with respect to aFGF, LPA, and PMA stimulation is therefore indeed likely to be due to the different basal levels of PTPα phosphorylation in the two cell lines. In unstimulated PTPα$^{+/+}$ fibroblasts, the majority of the cellular PTPα may already be tyrosine phosphorylated such that stimulation with positive regulators such as IGF-1 only results in a minimal further increase, or in the cases with aFGF, LPA, and PMA, in no detectable changes. On the other hand, PTPα phosphorylation is relatively low in unstimulated SYF cells with more unphosphorylated PTPα available for phosphorylation upon stimulation. The observation that IGF-1 stimulation results in a similar final level of PTPα Y789 phosphorylation in PTPα$^{+/+}$ and SYF cells (Figure 3.7A) further supports the notion that PTPα phosphorylation content is perhaps closer to saturation in serum-starved, unstimulated PTPα$^{+/+}$ cells than in SYF cells. Alternatively, it cannot be ruled out that other cellular kinases are upregulated in SYF cells to compensate for the lack of Src, Fyn, and Yes kinases, and that these other kinases may phosphorylate PTPα at Y789.

In summary, among the five stimuli investigated here, IGF-1 was identified as the only positive regulator of PTPα Y789 phosphorylation in PTPα$^{+/+}$ cells. Although the full activation of the IGF-1 receptor and many consequent downstream signaling events require the cooperation of integrin signaling, which has also been shown to positively regulate PTPα tyrosine phosphorylation, IGF-1 appears to induce PTPα phosphorylation independently of integrin activation. In line with this, while integrin-induced PTPα phosphorylation requires the SFKs Src, Fyn, and/or Yes, IGF-1-induced PTPα phosphorylation is intact in SYF cells that lack these kinases. These results indicate that different kinases are utilized to phosphorylate PTPα in integrin and IGF-1 signaling. The studies involving SYF cells, which have a relatively low basal level of phosphorylated PTPα, further revealed aFGF and to a lesser extent, LPA and PMA, to be inducers of PTPα tyrosine phosphorylation. Although aFGF, LPA, and PMA failed to stimulate PTPα Y789 phosphorylation in wildtype PTPα$^{+/+}$ fibroblasts, it remains to be determined whether they may regulate PTPα phosphorylation in other cell lines that have low basal PTPα tyrosine phosphorylation.

3.4 Discussion

Constitutive phosphorylation of PTPα at Y789 was reported more than ten years ago (den Hertog *et al.*, 1994), but little is known about the regulation and the role of this modification in PTPα function. In the absence of a phosphosite-specific PTPα antibody, studies of regulators of PTPα Y789 phosphorylation were not easily performed due to the requirement for large amounts of cell samples for immunoprecipitation and subsequent immunoblotting with phosphotyrosine antibody. Here, a phosphosite-specific PTPα Y789

antibody was generated and characterized, and its specificity was confirmed. The antibody does not recognize the non-phosphorylated form of PTPα, nor does it recognize other proteins of similar size, with the exception of a ~175 kD phosphoprotein present in IGF-1-stimulated samples. The application of this antibody also yields reproducible results in support of earlier findings on the regulation of PTPα tyrosine phosphorylation in integrin signaling.

An estimated 20% of cellular PTPα is tyrosine phosphorylated in the unstimulated, serum-starved NIH3T3 mouse fibroblast cell line (den Hertog *et al.*, 1994). Whether the same basal level of PTPα phosphorylation occurs in other cell lines is unknown. PTPα appears to be relatively highly phosphorylated at Y789 in wildtype ($PTP\alpha^{+/+}$) mouse embryonic fibroblast cells growing in medium supplemented with full serum. Surprisingly, this high basal PTPα tyrosine phosphorylation level was not reduced in serum-starved cells. These results indicate that the molecular machinery controlling PTPα Y789 phosphorylation, specifically the appropriate kinases and phosphatases, was not subject to regulation by the withdrawal of serum and consequent cessation of cell proliferation and entry into a state of quiescence. In accord with this, although PTPα Y789 phosphorylation is involved in Src activation in mitosis, no alterations in PTPα tyrosine phosphorylation content were observed during mitosis (Zheng *et al.*, 2001). It has been proposed that all tyrosine phosphorylated PTPα is bound by the adaptor protein Grb2 (den Hertog *et al.*, 1994; Su *et al.*, 1994). Thus the phosphorylated Y789 residue of PTPα may be inaccessible to the corresponding phosphatase and therefore is protected from dephosphorylation.

Compared to wildtype PTP$\alpha^{+/+}$ cells, the reduced PTPα Y789 phosphorylation level in serum-deprived SYF cells clearly indicates an important role of Src, Fyn, and/or Yes in phosphorylating PTPα. Indeed, Src has been demonstrated to have *in vitro* kinase activity towards PTPα, and transient co-expression of Src and PTPα in 293T cells results in increased PTPα Y789 phosphorylation (den Hertog *et al.*, 1994). Together, this evidence indicates that Src is a PTPα kinase *in vivo*. In addition to Src, Fyn and/or Yes can phosphorylate PTPα Y789 upon integrin stimulation of Src$^{-/-}$ cells (Chen *et al.*, 2006). However, another study has produced conflicting results demonstrating a strong PTPα Y789 phosphorylation in SYF cells that cannot be further induced with the introduction of constitutively active Src, which indicates that SFKs are not involved in PTPα tyrosine phosphorylation (Hao *et al.*, 2006b). Although the same study revealed a reduced PTPα Y789 phosphorylation level in cells treated with the SFK inhibitor PP1, the authors proposed that the inhibition was mediated through the inactivation of other cellular kinases, such as c-Abl, by PP1. Results from the present study support the role of Src, Fyn, and/or Yes in maintaining basal PTPα Y789 phosphorylation level, while these kinases are dispensable for the enhanced PTPα phosphorylation induced by IGF-1, aFGF, LPA, and PMA. Although the kinases responsible have yet to be identified, it is clear that PTPα may be tyrosine phosphorylated by cellular kinases other than Src, Fyn, and Yes.

Depending on the signaling pathway initiated, different kinases may be utilized to catalyze PTPα tyrosine phosphorylation. Using integrin signaling as an example, integrin stimulation greatly induces Src activation through PTPα phosphatase-dependent dephosphorylation of the inhibitory Y527 residue of Src. In turn, the active Src then

phosphorylates PTPα at Y789 (Chen *et al.*, 2006). In fact, Src is one of the earliest kinases activated in integrin signaling and its activation is crucial for downstream signaling events. During integrin signaling, Src is recruited to the signaling complex at the plasma membrane where PTPα is also localized. Furthermore, both Src and PTPα have been shown to interact with integrins, Src with the integrin β3 subunit (Obergfell *et al.*, 2002) and PTPα with the integrin αv subunit (von Wichert *et al.*, 2003). These interactions thus position Src and PTPα in close proximity where Src can phosphorylate PTPα. In the present study, IGF-1, and potentially aFGF, LPA, and PMA, have been identified as positive regulators of PTPα Y789 phosphorylation. It is well established that growth factor signaling often cooperates with integrin signaling to achieve full effects, thus it was originally proposed that IGF-1 may induce PTPα Y789 phosphorylation via components of the integrin signaling pathway. However, the results indicate that IGF-1 can induce PTPα Y789 phosphorylation independently of integrin activation and in the absence of the Src, Fyn, and Yes SFKs that are required for integrin-induced PTPα phosphorylation. Interestingly, an analogous situation was observed with stimulus-dependent tyrosine (Y397) phosphorylation of FAK. Integrin stimulation induces SFK-dependent FAK Y397 phosphorylation. However, while LPA can also readily induce FAK Y397 phosphorylation, this process does not require SFKs (Salazar *et al.*, 2001). This example demonstrates that SFKs are indeed key signaling molecules in the integrin signaling pathway, but that alternate SFK-independent pathways may be utilized by other activators to achieve the same signaling event.

IGF-1 signals through the IGF type I receptor, IGF-IR, which presents at the cell surface as $\alpha 2\beta 2$ tetramers. Unlike the integrins which lack catalytic activity, ligand binding to IGF-IR induces conformational changes and activates its receptor kinase activity. Autophosphorylation of the receptor itself and subsequent phosphorylation of immediate downstream signaling proteins, namely the IRS proteins, initiate signaling events involving the PI3K-mediated cell survival pathway and the MAPK-mediated cell proliferation pathway. The fact that IGF-1-induced PTPα tyrosine phosphorylation is detectable as early as 2 min after stimulation, a time at which ERK1/2 phosphorylation is only moderately induced, indicates that the responsible kinase is activated early in the signaling pathway. The phosphosite-specific PTPα Y789 antibody unexpectedly recognizes another protein band at 175 kD which may represent the tyrosine phosphorylated IRS1 protein, suggesting a sequence and/or structural similarity between the phosphorylation sites of IRS1 and PTPα. As IRS1 is an early immediate substrate of the IGF-IR, this raises the possibility that the IGF-IR also phosphorylates PTPα Y789. While significant crosstalk occurs between signaling pathways initiated by integrins and growth factors, direct associations between growth factor receptors and integrins themselves have also been reported. IGF-IR was found to coimmunoprecipitate with the $\beta 1$ integrin subunit in human chondrocytes (Shakibaei *et al.*, 1999). Although integrin activation is not required for IGF-I-induced PTPα Y789 phosphorylation, the physical interaction of IGF-IR with integrins may allow a close association of IGF-IR and its immediate downstream signaling proteins with PTPα, since PTPα can also interact with integrins (i.e., the integrin αv subunit) (von Wichert *et al.*, 2003). The cellular kinases

and the precise IGF-1-stimulated signaling pathways leading to PTPα Y789 phosphorylation remain to be determined.

The present study investigated the regulation of PTPα Y789 phosphorylation in response to five different extracellular stimuli, IGF-1, EGF, aFGF, LPA, and PMA. IGF-1, EGF, and aFGF are growth factors that are respectively recognized by the receptor tyrosine kinases IGF-IR, EGFR, and FGFR. LPA binds to the cell surface G protein-coupled LPA receptors, while PMA is an analogue of diacylglycerol (DAG) that together with Ca^{+2} interacts with and activates PKC. Although different cell surface membrane-associated proteins are activated in each case, receptor transactivation and crosstalk occur between the different receptors with utilization of some common signaling molecules. For example, signaling through the EGFR, FGFR, and LPA receptor are known to induce PKC activation (van Corven *et al.*, 1989; Nishibe *et al.*, 1990; Peters *et al.*, 1992); activated IGF-IR, EGFR, and LPA receptor can all initiate the cell survival PI3K pathway (Giorgetti *et al.*, 1993; Laffargue *et al.*, 1999; Okano *et al.*, 2000); and as indicated by increased ERK T202/Y204 phosphorylation, all five stimuli are potent activators of the mitogenic MAPK signaling cascade. In contrast, the fact that only IGF-1 was found to positively regulate PTPα Y789 phosphorylation in wildtype $PTP\alpha^{+/+}$ fibroblasts reflects the specificity of this phosphorylation event in a particular signaling pathway. Interestingly, activation of Src phosphotransferase activity has been reported to occur in response to EGFR and LPA receptor signaling which might be expected to lead to increased PTPα Y789 phosphorylation upon ligand stimulation (Oude Weernink *et al.*, 1994; Luttrell *et al.*, 1996). However, the lack of altered PTPα tyrosine phosphorylation

observed upon EGF and LPA stimulation in wildtype fibroblasts demonstrates a specific regulation of PTPα tyrosine phosphorylation by IGF-1 that is independent of the action of Src.

The present study has provided new insights into the regulation of PTPα Y789 phosphorylation by signaling pathways other than those documented in integrin-stimulated signaling, and implicate a functional involvement of PTPα and its tyrosine phosphorylation status in IGF-1 signaling. Although only a moderate increase (35%) in PTPα Y789 phosphorylation was detected upon IGF-1 stimulation of wildtype PTPα$^{+/+}$ mouse embryonic fibroblasts, the results from SYF cells suggest that a higher induction is possible in other cell types with a low basal PTPα phosphorylation level. So far, the degree of PTPα tyrosine phosphorylation in other cell lines has not been established. Alternatively, a greater induction in PTPα Y789 phosphorylation may be achieved in cell lines overexpressing the IGF-IR. IGF-1 is a potent growth factor implicated in the pathogenesis of many diseases, including cancer. In fact, overexpression of the IGF-IR has been documented in a variety of human cancers including those of colon (Weber *et al.*, 2002), prostate (Krueckl *et al.*, 2004), breast (Resnik *et al.*, 1998), thyroid (Belfiore *et al.*, 1999), and others (Ouban *et al.*, 2003). It remains to be determined whether IGF-1 stimulation of these cancer cells induces PTPα tyrosine phosphorylation. Future studies will aim to investigate the signaling mechanism(s) and the functional significance of PTPα tyrosine phosphorylation in IGF-1 signaling.

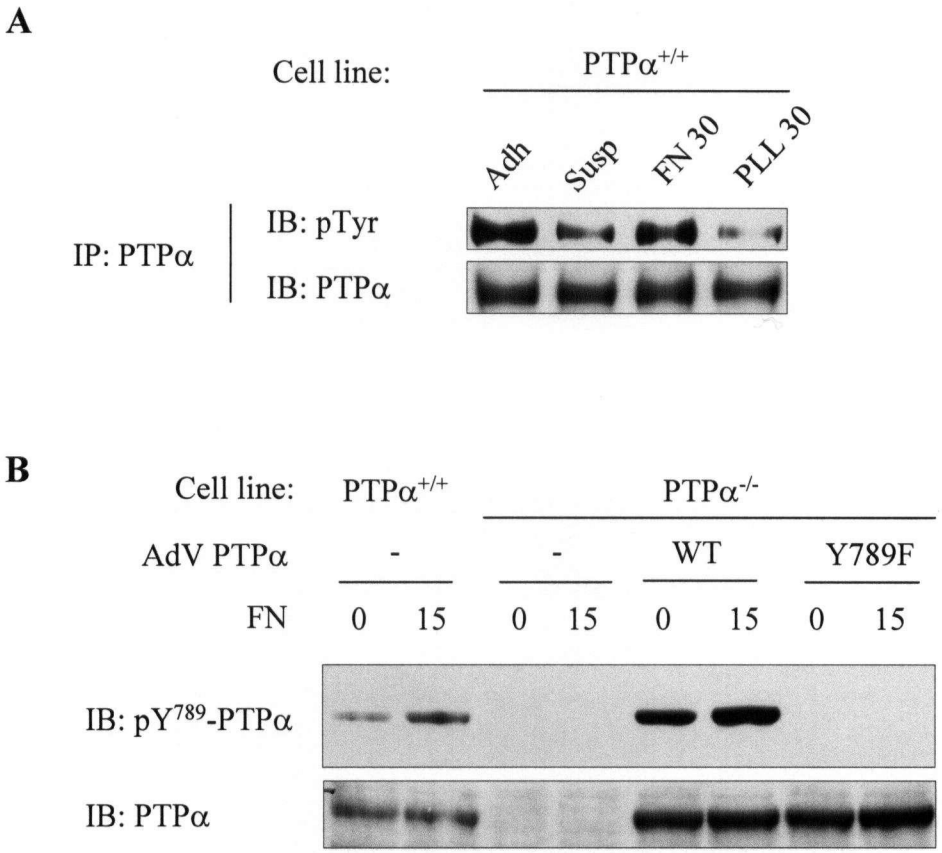

Figure 3.1 Integrin stimulation induces PTPα Y789 phosphorylation. (A) $PTP\alpha^{+/+}$ mouse embryonic fibroblasts growing on dishes (Adh) were trypsinized and kept in suspension for 1 hr (Susp), followed by plating on fibronectin (FN) or poly-L-lysine (PLL) for 30 min. Cell lysates were immunoprecipitated (IP) with PTPα antiserum, followed by immunoblotting (IB) for anti-phosphotyrosine (top panel) and then for PTPα (bottom panel) (170kD). **(B)** $PTP\alpha^{+/+}$, $PTP\alpha^{-/-}$, and $PTP\alpha^{-/-}$ cells re-expressing wildtype (WT) or Y789F mutant PTPα via an adenovirus expression system (AdV) were kept in suspension (0) or plated on FN for 15 min. Total cell lysates were immunoblotted with the phosphosite-specific PTPα Y789 antibody (top panel) and with PTPα antibody (bottom panel). This figure is representative of two individual experiments.

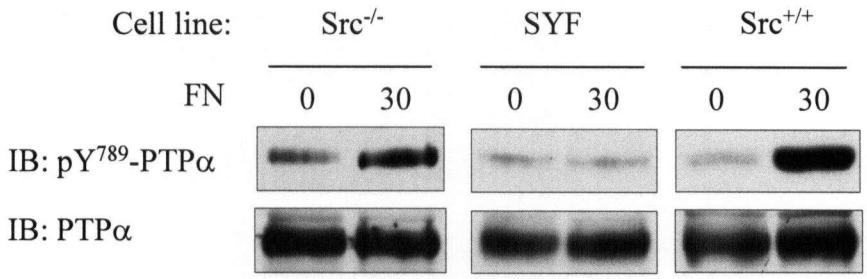

Figure 3.2 SFKs are required for integrin-induced PTPα Y789 phosphorylation. $Src^{-/-}$ ($src^{-/-}$, $fyn^{+/+}$, $yes^{+/+}$), SYF ($src^{-/-}$, $fyn^{-/-}$, $yes^{-/-}$), and $Src^{+/+}$ ($src^{+/+}$, $fyn^{-/-}$, $yes^{-/-}$) mouse fibroblasts were kept in suspension (0) or plated on FN for 30 min before harvesting. Cell lysates were immunoblotted (IB) with phosphosite-specific PTPα Y789 antibody (top panels) and with PTPα antibody (bottom panels). This figure was generated from a single experiment to confirm the finding described in Chen *et. al.* (2006).

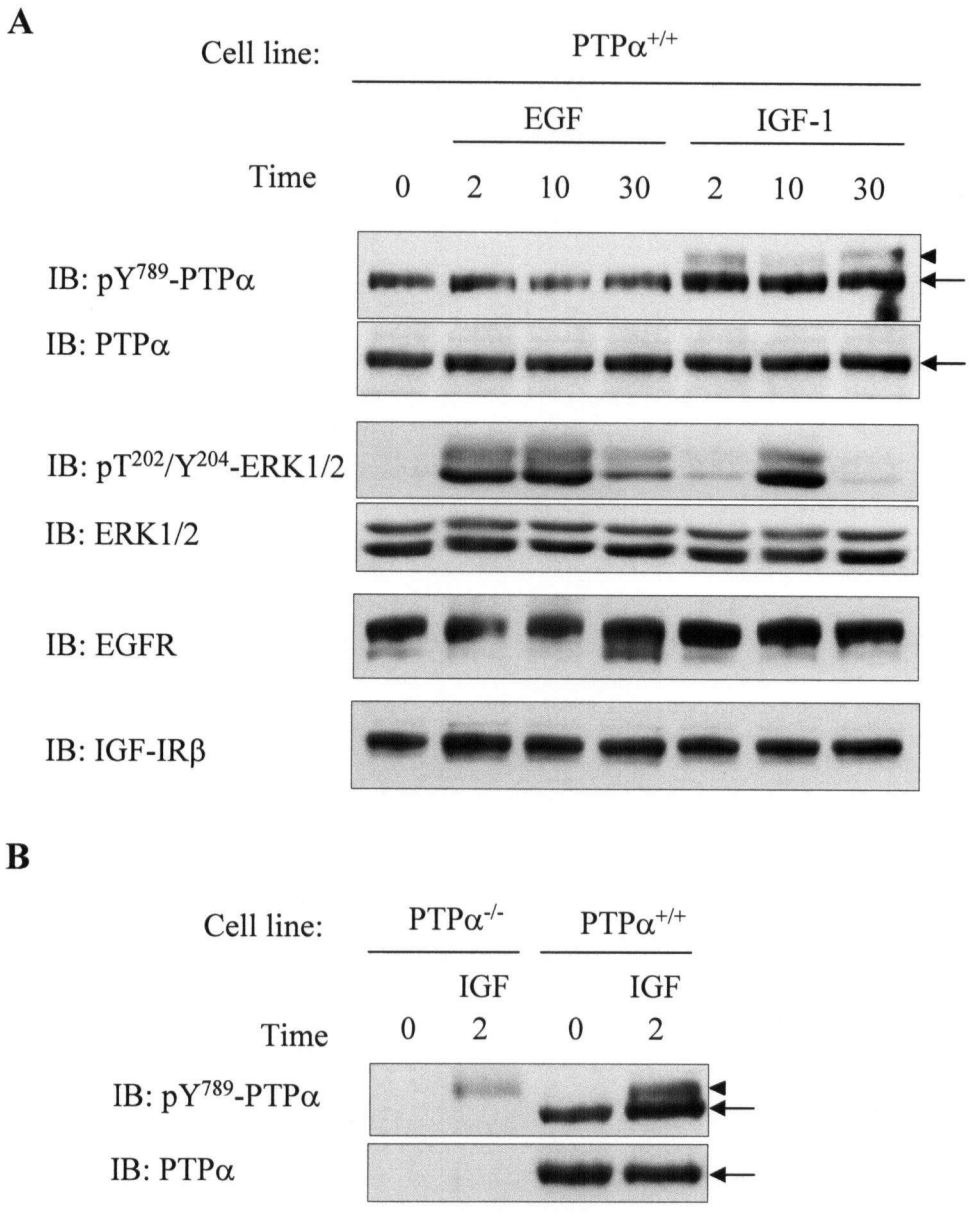

Figure 3.3 IGF-1, but not EGF, stimulates PTPα Y789 phosphorylation. (A) PTPα$^{+/+}$ fibroblasts were serum-starved (0) and stimulated with 100 ng/ml of EGF or IGF-1 for the indicated times. The cell lysates were immunoblotted (IB) with phosphosite-specific PTPα Y789 antibody followed by PTPα antibody (top two panels), and other indicated antibodies. The arrow indicates PTPα, and the arrowhead indicates a novel 175kD band. **(B)** PTPα$^{-/-}$ and PTPα$^{+/+}$ cells were serum-starved (0) and stimulated with 100 ng/ml of IGF-1 for 2 min. The cell lysates were immunoblotted with phosphosite-specific PTPα Y789 antibody (top panel) followed by PTPα antibody (bottom panel).

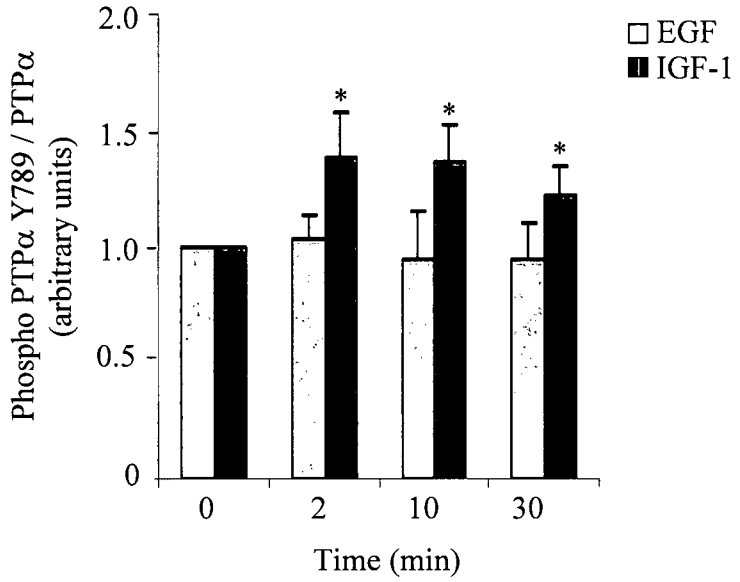

Figure 3.4 Quantitative analysis of PTPα Y789 phosphorylation stimulated by EGF and IGF-1. PTPα Y789 phosphorylation was determined from four independent experiments as in Figure 3.3A. The arbitrary densitometric units of PTPα phospho-Y789 per amount of PTPα were determined, with that from serum-starved cells taken as 1.0 and those from IGF-1- or EGF-treated cells determined relative to that. The asterisks indicate a significant difference ($p \leq 0.05$) with serum-starved, untreated cells.

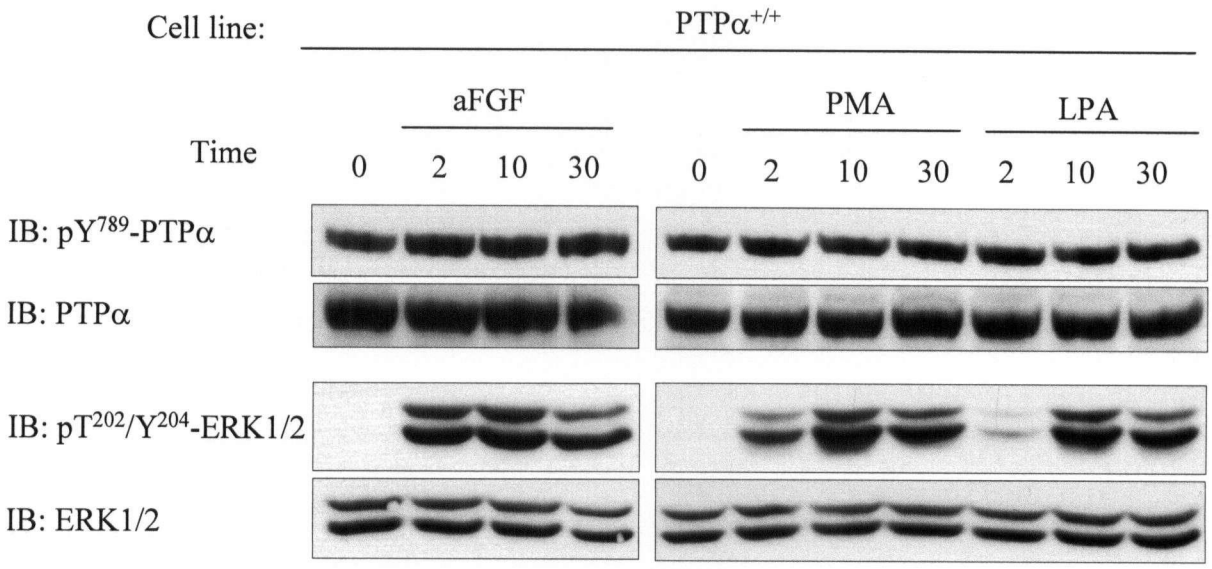

Figure 3.5 aFGF, PMA, and LPA do not stimulate PTPα Y789 phosphorylation. PTPα$^{+/+}$ fibroblasts were serum-starved (*0*) and then stimulated with 100 ng/ml of aFGF, 150 nM of PMA, or 10 μM of LPA for the indicated times. The cell lysates were immunoblotted (*IB*) with phosphosite-specific PTPα Y789 antibody followed by PTPα antibody (top two panels), or with phospho-ERK antibody followed by ERK antibody (bottom two panels).

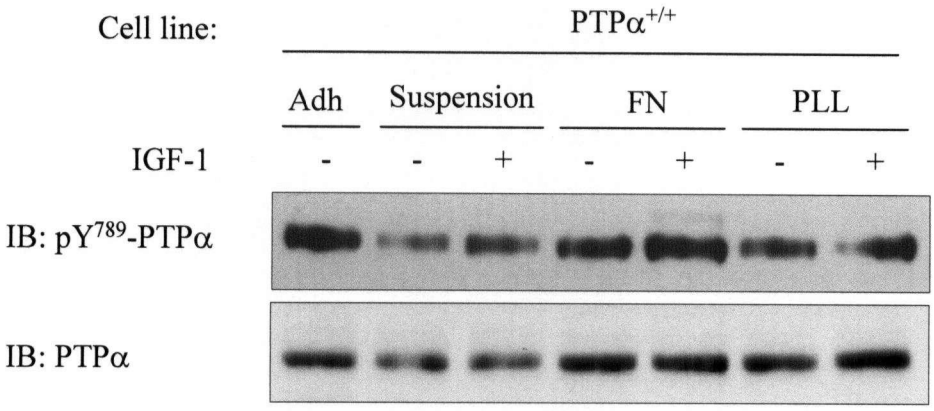

Figure 3.6 IGF-1-induced PTPα Y789 phosphorylation does not require integrin activation. $PTP\alpha^{+/+}$ fibroblasts growing on dishes (*Adh*) were trypsinized and kept in suspension for 1 hr. The suspended cells were left un-plated, or plated on FN or PLL for 15 min in the absence (-) or presence (+) of 100 ng/ml of IGF-1. The cell lysates were immunoblotted (*IB*) with the phosphosite-specific PTPα Y789 antibody (top panel) and with PTPα antibody (bottom panel). This figure is representative of two individual experiments.

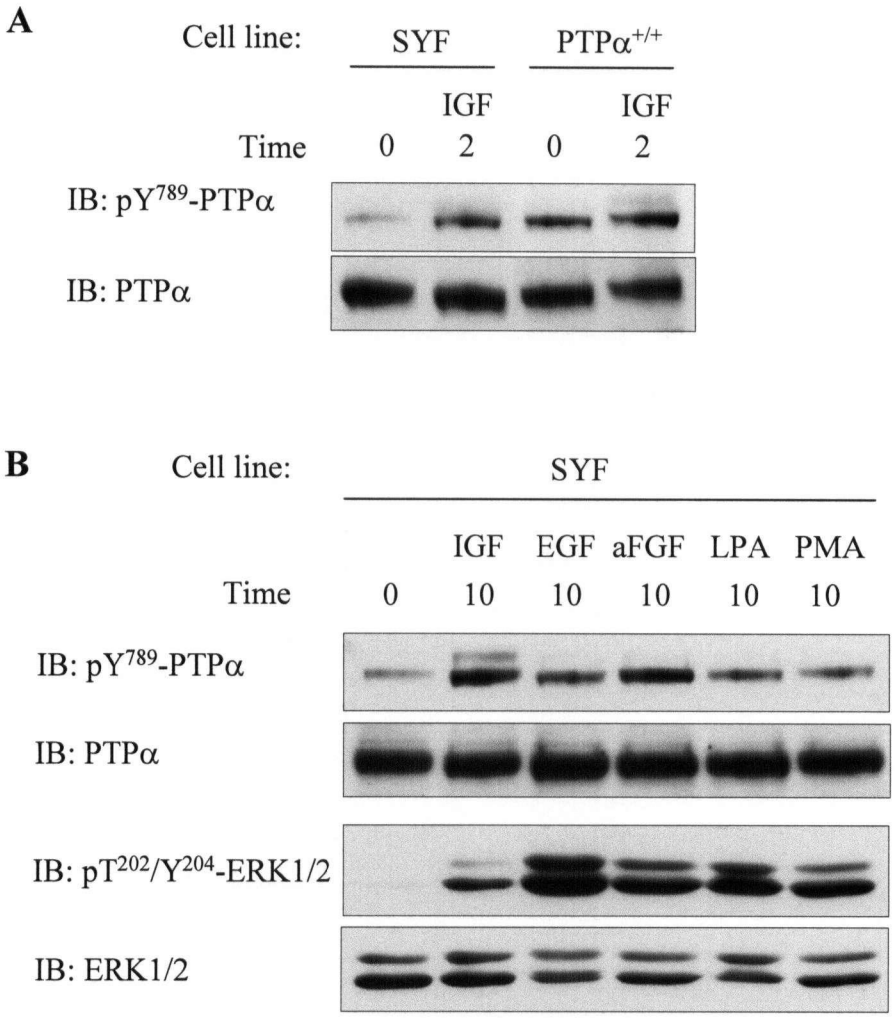

Figure 3.7 IGF-1, aFGF, LPA, and PMA, but not EGF, stimulate PTPα Y789 phosphorylation in SYF cells. **(A)** SYF ($src^{-/-}$, $fyn^{-/-}$, $yes^{-/-}$) and $PTP\alpha^{+/+}$ fibroblasts were serum starved (*0*) and then stimulated with 100 ng/ml of IGF-1 for 2 min. The cell lysates were immunoblotted (*IB*) with phosphosite-specific PTPα Y789 antibody and (top panel) with PTPα antibody (bottom panel). **(B)** SYF cells were serum starved (*0*) and then stimulated with 100 ng/ml of IGF-1, 100 ng/ml of EGF, 100 ng/ml of aFGF, 10 μM of LPA, or 150 nM of PMA for 10 min. The cell lysates were immunoblotted (*IB*) with phosphosite-specific PTPα Y789 antibody followed by PTPα antibody (top two panels), or with phospho-ERK antibody followed by ERK antibody (bottom two panels).

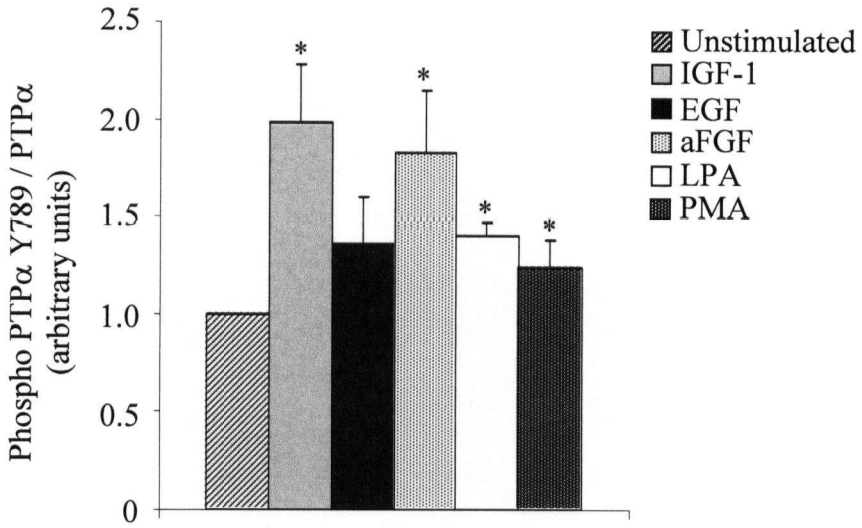

Figure 3.8 Quantitative analysis of PTPα Y789 phosphorylation stimulated by various factors in SYF cells. PTPα Y789 phosphorylation was determined from three independent experiments as in Figure 3.7B. The arbitrary densitometric units of PTPα phospho-Y789 per amount of PTPα were determined, with that from serum-starved cells taken as 1.0 and those from IGF-1-, EGF-, aFGF-, LPA-, or PMA-treated cells determined relative to that. The asterisks indicate a significant difference ($p \leq 0.05$) with serum-starved, untreated cells.

CHAPTER 4

THE ROLE OF PTPα Y789 PHOSPHORYLATION IN INTEGRIN-INDUCED CYTOSKELETAL REORGANIZATION SIGNALING EVENTS

4.1 Rationale

The regulation and function of PTPα Y789 phosphorylation in integrin signaling have recently been studied in our laboratory. As reported by Chen *et al.* (2006) and described in Chapter 3, the downregulation of integrin signaling induced by detaching cells from the substratum and maintaining them in suspension led to decreased PTPα Y789 phosphorylation, whereas integrin stimulation induced by plating the cells on fibronectin (FN) greatly increased PTPα Y789 phosphorylation. Functional characterization of this phosphorylation event showed that it is not required for PTPα to dephosphorylate and activate Src, with integrin-induced Src Y527 dephosphorylation and Y416 autophosphorylation occurring normally in PTPα$^{-/-}$ cells re-expressing mutant PTPα Y789F (Chen *et al.*, 2006). Likewise, integrin-induced Src-dependent phosphorylation of FAK Y397 and paxillin were not altered by PTPα Y789F mutation. Surprisingly, despite Src and FAK activation proceeding normally in PTPα$^{-/-}$ cells re-expressing mutant PTPα Y789F, immunofluorescent staining for F-actin and vinculin showed reduced integrin-induced stress fiber assembly and focal adhesion formation when compared to wildtype PTPα$^{+/+}$ cells or PTPα$^{-/-}$ cells re-expressing wildtype PTPα. Similarly, re-expression of mutant PTPα Y789F was unable to completely rescue the cell spreading, cell migration, and cytoskeletal reorganization defects observed in PTPα$^{-/-}$ fibroblasts, which implicates

a role for phosphorylation of PTPα at Y789 in these integrin signaling events (Chen *et al.*, 2006). However, the signals that are transduced by PTPα phospho-Y789 are unknown. In the present study, two approaches were taken to investigate the exact function of PTPα Y789 phosphorylation in integrin signaling. First, I investigated if signaling events that are critical for proper focal adhesion and stress fiber formation and cell spreading are dysregulated in PTPα$^{-/-}$ cells re-expressing mutant PTPα Y789F. Second, I aimed to identify proteins that interact with Y789-phosphorylated PTPα to empirically determine the signals directly transduced by the phospho-PTPα Y789 motif.

4.2 Role of PTPα and its Y789 Phosphorylation Status in Integrin-Stimulated Rho GTPase Activation

Cytoskeletal reorganization is a complex process that incorporates multiple signal transduction events involving numerous signaling molecules. The Rho family of small GTPases, notably RhoA, Rac1, and Cdc42, are key mediators of the remodeling of the cellular actin cytoskeleton in response to growth factor receptor and integrin stimulation (described further in Section 1.3.3.1). PTPα and its phosphorylation at Y789 may thus be involved in regulating the Rho GTPases to affect integrin-induced cytoskeletal reorganization. An *in vitro* method of determining the cellular activation status of Rho GTPases was first reported in 1999 (Ren *et al.*, 1999), and since then has been extensively used to determine the activities of many Rho GTPases. In brief, cell lysates are subjected to a pull-down assay with glutathione beads conjugated to a GST-fusion protein comprising the binding domain of the relevant Rho GTPase effector protein (Figure 4.1). The effector binding domain specifically recognizes the active GTP-bound

form of the Rho GTPase, which therefore is precipitated by the GST-fusion protein-coupled glutathione beads in the pull-down assay. The amount of precipitated Rho GTPase, representing the active GTP-bound form of the GTPase, is visualized by immunoblotting to provide a quantitative indication of the cellular activation status of the Rho GTPase of interest.

To investigate if PTPα and/or its tyrosine phosphorylation status are involved in regulating Rho GTPase activation, I first determined if the activities of specific Rho GTPases were dysregulated in PTPα$^{-/-}$ cells, and then investigated whether any such defects could be restored upon re-expression of wildtype or Y789F mutant PTPα.

4.2.1 RhoA

Two major defects observed in PTPα$^{-/-}$ cells and PTPα$^{-/-}$ cells re-expressing mutant PTPα Y789F are reduced focal adhesions and stress fiber formation (Zeng *et al.*, 2003; Chen *et al.*, 2006). RhoA is the best characterized Rho GTPase responsible for the assembly of these structures. Blocking RhoA function either by introducing the RhoA inhibitor *Clostridium botulinum* C3 exoenzyme (Barry *et al.*, 1997) or dominant-negative RhoA T19N (Clark *et al.*, 1998) inhibited integrin-induced stress fiber and focal adhesion formation, indicating an essential role of RhoA in the proper formation of these structures. The activity of RhoA in integrin-stimulated PTPα$^{+/+}$ and PTPα$^{-/-}$ mouse embryonic fibroblasts was investigated. The cells were trypsinized and maintained in a suspended state for 1-2 hr prior to plating the cells on FN to stimulate integrin signaling. As shown in Figure 4.2A (top panel), the suspended cells exhibited some basal RhoA activity,

which was then greatly stimulated by plating the cells on FN for 15 min. The cellular RhoA protein expression level (Figure 4.2A, bottom panel) was comparable between PTP$\alpha^{+/+}$ and PTP$\alpha^{-/-}$ cell lines and was not altered by integrin stimulation, indicating that the observed induction in RhoA activity was not due to an increase in RhoA protein expression. Densitometry analysis of the results from three independent experiments revealed that integrin stimulation induced a 1.66 (±0.41)-fold increase in the amount of active RhoA in PTP$\alpha^{+/+}$ cells when compared to the amount of basally active RhoA detected in the suspended PTP$\alpha^{+/+}$ cells (Figure 4.2B). Likewise, PTP$\alpha^{-/-}$ cells exhibited a 1.52 (±0.19)-fold increase in the amount of active RhoA upon integrin stimulation. There was no significant difference between the integrin-induced fold-activation of RhoA in the two cell lines ($p=0.6$). These results indicate that, although PTPα is involved in integrin-induced stress fiber and focal adhesion formation, PTPα is not required for integrin-induced RhoA activation.

4.2.2 Rac1

Another defect that was observed in PTP$\alpha^{-/-}$ cells and PTP$\alpha^{-/-}$ cells re-expressing mutant PTPα Y789F is impaired cell spreading on FN (Zeng *et al.*, 2003; Chen *et al.*, 2006). The cell spreading process is driven by the formation of lamellipodia at the cell periphery that promote the outwards extension of the cell membrane towards the substratum. Rac1 is the key mediator of the formation of lamellipodia (Ridley *et al.*, 1992b). Inhibiting Rac1 action by introducing dominant-negative Rac1 S17N can impair lamellipodia formation and cell spreading on FN (Price *et al.*, 1998). I therefore investigated the potential involvement of PTPα in regulating integrin-induced Rac1 activation.

In initial experiments to determine the relative Rac1 activity in adherent growing PTP$\alpha^{+/+}$ and PTP$\alpha^{-/-}$ cells, I observed almost no active Rac1 in PTP$\alpha^{-/-}$ cells, while a significant amount of active Rac1 was detected in wildtype PTP$\alpha^{+/+}$ cells (Figure 4.3A, top panel). The presence of Rac1 protein in this PTP$\alpha^{-/-}$ cell line was confirmed by immunoblotting the cell lysate for Rac1 (Figure 4.3A, bottom panel). Similar levels of Rac1 protein were found in PTP$\alpha^{+/+}$ and PTP$\alpha^{-/-}$ cells, indicating that the difference observed in Rac1 activity was not due to a difference in Rac1 protein expression. To investigate Rac1 activity upon integrin stimulation, PTP$\alpha^{+/+}$ and PTP$\alpha^{-/-}$ cells were trypsinized, maintained in a suspended state for 1-2 hr, and plated on FN. Surprisingly, integrin stimulation did not lead to detectable Rac1 activity in PTP$\alpha^{-/-}$ cells (Figure 4.3B, top panel), although the presence of Rac1 protein was readily detected in these cells (Figure 4.3B, second top panel). These results strongly suggested an involvement of PTPα in Rac1 activation in both adherent growing and integrin-stimulated states. To confirm this role of PTPα, wildtype PTPα was reintroduced into PTP$\alpha^{-/-}$ cells via an adenovirus expression system. Unexpectedly, PTP$\alpha^{-/-}$ cells re-expressing wildtype PTPα still exhibited undetectable Rac1 activity upon integrin stimulation (Figure 4.3B, top panel). Expression of the exogenous PTPα was confirmed by immunoblotting the cell lysate for PTPα. To ensure that the exogenous PTPα was functional, integrin-induced FAK autophosphorylation at Y397 was verified by immunoblotting with a phosphosite-specific FAK antibody. The role of PTPα in integrin-induced FAK autophosphorylation has been well characterized by Zeng *et al.* (2003) (see also Section 1.4.2). As shown in Figure 4.3B, integrin-induced FAK Y397 phosphorylation was reduced in PTP$\alpha^{-/-}$ cells when compared to that of wildtype PTP$\alpha^{+/+}$ cells. Reintroduction of wildtype PTPα into PTP$\alpha^{-/-}$ cells restored FAK

Y397 phosphorylation to a level comparable to that of wildtype PTP$\alpha^{+/+}$ cells, indicating that the exogenous PTPα was indeed catalytically active and functional. This rescue experiment was also attempted under conditions of different FN concentration, stimulation time, and exogenous PTPα expression level; however under these different experimental parameters, the re-expression of wildtype PTPα uniformly failed to restore the impaired Rac1 activity observed in PTP$\alpha^{-/-}$ cells (data not shown).

My results revealed that Rac1 activity was low to undetectable in PTP$\alpha^{-/-}$ fibroblasts in the adherent growing and the integrin-stimulated states. Surprisingly, the re-expression of functional wildtype PTPα in PTP$\alpha^{-/-}$ cells was unable to restore the defective Rac1 activity, indicating that the Rac1 defect was unlikely to be simply due to the absence of PTPα. An alternative explanation is that these cell lines exhibit different intrinsic levels of Rac1 activity, and this particular PTP$\alpha^{-/-}$ fibroblast cell line has a low Rac1 activity that is below the detection range of the activation assay. To explore this possibility, the Rac1 activity in another independently-derived PTP$\alpha^{-/-}$ mouse embryonic fibroblast cell line, denoted as KP PTP$\alpha^{-/-}$, was investigated. Interestingly, unlike the PTP$\alpha^{-/-}$ cell line used in the initial investigation that demonstrated very minimal Rac1 activity, the Rac1 activity in KP PTP$\alpha^{-/-}$ cells in an adherent growing state was readily detectable (Figure 4.4, top panel). These results reveal that there is highly variable basal Rac1 activity among different cell lines, and that this appeared to be independent of the presence or absence of PTPα. As the sensitivity of the Rac1 activation assay is limited, it still remains to be determined if PTPα is involved in integrin-induced Rac1 activation.

4.2.3 PAK

Due to the variations in basal Rac1 activity among different cell lines and the restricted sensitivity of the Rac1 GTPase activation assay, I was not able to determine if PTPα plays a role in integrin-induced Rac1 activation. PAK is a protein serine/threonine kinase that is activated by active Rac1. Microinjection of constitutively active mutant PAK into cells can induce the formation of lamellipodia and membrane ruffles (Sells *et al.*, 1997). Furthermore, the introduction of active PAK can partially restore the cytoskeletal defects observed in Rac1 knockout mouse embryonic fibroblasts (Guo *et al.*, 2006). These observations indicate that PAK is one of the main downstream effectors of Rac1. Therefore, as an alternative approach to directly analyzing Rac1 activity, I investigated the role of PTPα in integrin-induced PAK activation.

Upon stimulation, the association between PAK and its upstream regulator, such as active Rac1 or Cdc42, disrupts the inhibitory interaction between the PAK kinase and its autoinhibitory domains. This leads to kinase activation and subsequent autophosphorylation of PAK at several residues (Chong *et al.*, 2001). One of the earliest residues in PAK that is autophosphorylated is Ser-144 (S144). Cellular PAK activation status can thus be assayed by immunoblotting for phospho-S144 PAK. There are at least four PAK isoforms in mammals, with PAK1 being the best characterized member (Daniels *et al.*, 1999; Zhao *et al.*, 2005). As shown in Figure 4.5A (bottom panel), immunoblotting with PAK1 antibody revealed the presence of two PAK isoforms, the 68 kD PAK1 and the 62 kD PAK2. Immunoblotting with the phosphosite-specific PAK S144 antibody detected mainly the 68 kD PAK1 with very minimal signal detected for

the 62 kD PAK2 (Figure 4.5A, top panel). This may be due to a lower sensitivity of the phosphosite-specific antibody towards the PAK2 isoform or to much lower PAK2 phosphorylation compared to PAK1. The quantitative analysis thus only included the PAK1 isoform (Figure 4.5A, arrow). PTP$\alpha^{+/+}$ and PTP$\alpha^{-/-}$ fibroblasts were trypsinized and maintained in suspension for ~1 hr, followed by plating on FN to stimulate integrin signaling. As shown in Figure 4.5A, immunoblotting with the phosphosite-specific PAK S144 antibody revealed almost undetectable PAK S144 phosphorylation in suspended PTP$\alpha^{+/+}$ and PTP$\alpha^{-/-}$ cells. Plating the cells on FN for 5 min slightly induced PAK S144 phosphorylation in both cell lines, while it was greatly induced at 15 min and remained high at 30 min on FN. Although both PTP$\alpha^{+/+}$ and PTP$\alpha^{-/-}$ cells exhibited a similar trend of increased PAK phosphorylation during integrin stimulation, densitometric analysis revealed a significant ~55% reduction in PAK S144 phosphorylation level in PTP$\alpha^{-/-}$ cells at 15 min on FN compared to that in PTP$\alpha^{+/+}$ cells at this time (1.00±0.07 for PTP$\alpha^{+/+}$ cells at 15 min on FN; 0.45±0.07 for PTP$\alpha^{-/-}$ cells at 15 min on FN) (Figure 4.5B). These results indicated that integrin-induced PAK S144 autophosphorylation, and thus its activation, is impaired in PTP$\alpha^{-/-}$ cells.

To investigate the role of PTPα and PTPα tyrosine phosphorylation in integrin-induced PAK activation, wildtype or Y789F mutant PTPα were reintroduced into PTP$\alpha^{-/-}$ cells. The activity of the exogenous PTPα was confirmed by immunoblotting for phospho FAK Y397. As expected, re-expression of either wildtype or Y789F mutant PTPα in PTP$\alpha^{-/-}$ cells restored integrin-induced FAK Y397 phosphorylation to a level comparable to that of wildtype PTP$\alpha^{+/+}$ cells (Figure 4.6A, third panel from top). Interestingly, integrin-

induced PAK S144 phosphorylation in PTPα$^{-/-}$ cells re-expressing either wildtype or Y789F mutant PTPα was also restored to ~80% of that of wildtype PTPα$^{+/+}$ cells (0.82±0.10 for PTPα$^{-/-}$ cells re-expressing wildtype PTPα; 0.80±0.13 for PTPα$^{-/-}$ cells re-expressing Y789F PTPα) (Figure 4.6B). The PAK S144 phosphorylation level in PTPα$^{-/-}$ cells re-expressing Y789F mutant PTPα was not statistically different from that of PTPα$^{-/-}$ cells re-expressing wildtype PTPα or from PTPα$^{+/+}$ cells. These results indicate that although PTPα is required for the full activation of PAK in integrin signaling, phosphorylation of PTPα at Y789 is not involved in this process.

In summary, my results do not support a role for PTPα in integrin-induced RhoA activation. My investigation of the role of PTPα in integrin-induced Rac1 activation did not provide a conclusive answer. However, integrin-induced PAK autophosphorylation (activation) is impaired in PTPα$^{-/-}$ cells, and PAK is a main downstream effector of Rac1 as well as Cdc42. I demonstrated that re-expression of either wildtype or mutant PTPα Y789F in PTPα$^{-/-}$ cells restored PAK activation. Thus, this function of PTPα is not dependent on PTPα phosphorylation. The results of these studies of integrin-induced PAK autophosphorylation imply that PTPα may likewise be involved, in a phosphorylation-independent manner, in integrin-induced Rac1 and/or Cdc42 activation. The role of PTPα in Cdc42 activation is as yet undetermined, as I experienced difficulties with the anti-Cdc42 antibody that were not resolved. Although my present results indicate that PTPα likely mediates Rac1 activation to effect PAK autophosphorylation and activation in a manner independent of PTPα Y789 phosphorylation, there remains the possibility that PTPα tyrosine phosphorylation may have opposite effects on integrin-

induced Rac1 and Cdc42 activation that counteract one another to produce no overall alteration in PAK autophosphorylation.

4.3 Role of PTPα Y789 Phosphorylation in Recruitment of Signaling Proteins in Integrin Signaling

Phosphotyrosine is a well characterized protein-protein interaction motif that is extensively utilized in many signal transduction events. Two known phosphotyrosine-binding domains are the Src homology 2 domain (SH2) and the phosphotyrosine binding domain (PTB), which are found in a wide variety of proteins including adaptors, kinases, phosphatases, and many others (Schlessinger *et al.*, 2003). It is highly probable that integrin-induced phosphorylation of PTPα at Y789 results in the recruitment of downstream SH2- and/or PTB-containing signaling molecules that mediate integrin-induced signaling events such as cytoskeletal reorganization. Therefore, I aimed to 1) investigate the association between PTPα and its known binding protein Grb2 in integrin signaling, and 2) identify new binding proteins for Y789 phosphorylated PTPα.

4.3.1 PTPα and Grb2 Association in Integrin Signaling

Phosphorylation of PTPα at Y789 allows the binding of Grb2 or Src to this site through their SH2 domains (den Hertog *et al.*, 1994; Su *et al.*, 1994; Zheng *et al.*, 2000). On the one hand, in mitosis, the interaction between PTPα Y789 and Src is required for mitotic activation of Src through the displacement mechanism proposed by Zheng *et al.* (2000). On the other hand, phosphorylation of PTPα, and thus its potential to directly recruit Src, is not essential for integrin-stimulated Src activation (Chen *et al.*, 2006), indicating that

the important role of PTPα Y789 phosphorylation in integrin-induced cytoskeletal rearrangements is unlikely to be manifested through its function as a Src binding motif. Grb2 is a ubiquitously expressed adaptor protein and has been proposed to act as a negative regulator of PTPα-mediated signaling events by competing with Src and potentially with other SH2 containing signaling proteins for binding to phospho-Y789 of PTPα (Zheng *et al.*, 2000). The regulation and functional significance of PTPα and Grb2 association have not been examined in the context of integrin signaling. In the present study, I investigated whether phospho-Y789-dependent association of PTPα and Grb2 is regulated by integrin signaling.

As expected from the high PTPα phosphorylation content observed in adherent growing mouse embryonic fibroblasts (discussed in Chapter 3), immunoprecipitation of PTPα revealed the presence of Grb2 in the immunocomplex, confirming the known association of PTPα and Grb2 in adherent wildtype PTPα$^{+/+}$ cells (Figure 4.7A). Detaching the cells from the substratum and maintaining them in suspension caused PTPα and Grb2 association to decrease to ~78% (±6%) of the adherent level, whereas integrin stimulation by plating the cells on FN for 30 min greatly induced PTPα and Grb2 association to ~114% (±9%) of the adherent level (Figure 4.7B). The ~36% increase in PTPα-Grb2 association upon integrin stimulation is similar to the increase observed in PTPα Y789 phosphorylation (~36%) (Figure 3.1A) (Chen *et al.*, 2006). The requirement for residue Y789 in this association of Grb2 with PTPα was confirmed by the lack of Grb2 in a PTPα immunoprecipitate from PTPα$^{-/-}$ cells re-expressing mutant PTPα Y789F (Figure 4.8). Reintroduction of wildtype PTPα into PTPα$^{-/-}$ cells restored integrin-induced Grb2 and

PTPα association (Figure 4.8). These results demonstrate that the increase in PTPα Y789 phosphorylation upon integrin stimulation is accompanied by the recruitment of Grb2 to PTPα. Since PTPα Y789 phosphorylation is a functional signaling event in integrin actions, the concomitantly increased association with Grb2 suggests a positive role of Grb2 in PTPα-mediated integrin signaling events. It remains to be determined what signals are transduced downstream from the PTPα-Grb2 complex.

4.3.2 Identification of Binding Proteins for Y789 Phosphorylated PTPα

Besides Grb2 and Src, it is not known what other proteins might also interact with PTPα via the phosphorylated Y789 motif. To identify new binding proteins for Y789-phosphorylated PTPα, I utilized two peptide affinity columns that were generated by 21st Century Biochemicals (Marlboro, MA, USA) and that had been previously used for purification of the anti-phospho-Y789 PTPα antibody. The non-phosphopeptide affinity column comprised a peptide, CYIDAFSDYANFK, corresponding to the amino acid sequence of PTPα surrounding the Y789 site, while the phosphopeptide column comprised a phosphotyrosyl peptide of the same amino acid sequence. The two columns were used to affinity purify cellular proteins that specifically interact with the conjugated peptide. To minimize competitive binding from endogenous PTPα, lysate from integrin-induced PTPα$^{-/-}$ cells was used as the source of binding protein. An equal amount of lysate was loaded onto each affinity column, and the flow-through containing the unbound proteins, and the eluate containing the bound proteins, were collected and resolved by SDS-PAGE (see Section 2.9).

The resolved proteins were transferred to a PVDF membrane and were stained with Memcode reversible protein stain. As shown in Figure 4.9A (lane 1), the stain effectively revealed numerous protein bands in the initial cell lysate, confirming the staining procedure and the sensitivity of the stain. A comparison of the proteins present in the unbound fractions (flow-through) collected from the non-phosphopeptide and the phosphopeptide columns revealed no apparent difference in the overall staining profile between the two (Figure 4.9A, lanes 3 and 4). Interestingly, different protein staining patterns were detected in the eluates collected from the non-phosphopeptide and the phosphopeptide columns (Figure 4.9A, lanes 6 and 8), indicating that different subsets of cellular proteins had bound to each column. To validate the differential abilities of the columns to bind to phospho-Y789 interacting proteins, the same PVDF membrane was de-stained and immunoblotted for Grb2, a known binding protein for tyrosine phosphorylated PTPα. As shown in Figure 4.9B, Grb2 was exclusively present in the flow-through from the non-phosphopeptide column (Figure 4.9B, compare lanes 3 and 4), indicating that Grb2 did not interact with the non-phosphorylated peptide. In contrast, Grb2 was detected in the eluate from the phosphopeptide column (Figure 4.9B, lane 8) but not in that from the non-phosphopeptide column (Figure 4.9B, lane 6), in accord with its expected interaction with phospho-Y789 of PTPα. These results indicate that this approach is effective in purifying cellular proteins that specifically interact with the phosphorylated PTPα peptide, and thus is potentially useful in the isolation of novel PTPα binding proteins.

As shown in Figure 4.9A, two protein bands, corresponding to molecular masses of ~70 kD and ~56 kD, and a diffusely stained signal, likely representing multiple proteins of ~25-29 kD (lane 8, solid arrows), were uniquely present in the phosphopeptide column eluate. A band of ~53 kD (dashed arrow) was present in the phosphopeptide column eluate at a higher abundance than in the non-phosphopeptide column eluate (Figure 4.9A, compare lanes 6 and 8). Theses proteins may represent cellular proteins that interact with PTPα in a phospho-Y789-dependent manner. In an attempt to better visualize proteins in these column eluates, another aliquot of the same eluates were loaded on a 12% SDS-PAGE (instead of the 10% SDS-PAGE shown in Figure 4.9) in duplicate sets. After protein resolution, a part of the gel was directly subjected to protein staining (Figure 4.10A), while proteins on the other half of the gel were transferred to a PVDF membrane and immunoblotted for phosphotyrosine content (Figure 4.10B). As shown in Figure 4.10A, direct staining of the polyacrylamide gel for proteins revealed the presence of the same 70 kD, 56 kD, 53 kD, and 25-29 kD protein bands of interest as those observed in Figure 4.9A. The ~70 kD protein band that was only slightly visible on the protein-stained membrane (Figure 4.9A) was readily detected on the protein-stained polyacrylamide gel (Figure 4.10A) and was resolved to be comprised of two protein bands.

Immunoblotting for phosphotyrosine content revealed the presence of three major tyrosine phosphorylated proteins, corresponding to molecular masses of ~53 kD, ~40 kD, and ~37 kD, that were present at a higher abundance in the phosphopeptide column eluate than in the non-phosphopeptide column eluate (Figure 4.10B, dashed arrows). Two

faintly detectable tyrosine phosphorylated proteins with molecular masses of ~29 kD and ~27 kD were uniquely present in the phosphopeptide column eluate (Figure 4.10B, solid arrows). The 53 kD, 29 kD, and 27 kD proteins in the phosphotyrosine immunoblot matched the positions of some of the protein bands of interest identified in the protein-stained gel in Figure 4.10A, indicating that the latter are tyrosine-phosphorylated proteins (Figure 4.10, asterisks). Taken together, these results demonstrate that some cellular proteins were uniquely bound to the phosphopeptide column (as indicated by the solid arrows in Figures 4.9 and 4.10), while others appeared to preferentially bind to the phosphopeptide column with a higher affinity (as indicated by the dashed arrows in Figures 4.9 and 4.10). These proteins represent potential binding partners for Y789-phosphorylated PTPα.

In summary, I have investigated the role of PTPα Y789 phosphorylation in the recruitment of proteins potentially involved in mediating downstream signal transduction events. I showed that in addition to the increased PTPα Y789 phosphorylation, there is an increase in Grb2 recruitment to PTPα via the phospho-Y789 motif during integrin signaling. With the application of the peptide affinity purification approach, I have detected some protein bands of interest that have preferential binding affinity towards a tyrosine-phosphorylated peptide that is identical in amino acid sequence to the region flanking the PTPα Y789 phosphorylation site. These proteins may represent cellular proteins that interact with PTPα in a manner dependent on PTPα tyrosine phosphorylation. Future work will aim to determine the identities of these proteins by mass spectrometry and further validate the interactions *in vitro* and *in vivo*.

4.4 Discussion

The rearrangement of the cellular actin cytoskeleton is a fundamental mechanism required for cell migration in response to cell surface receptor signaling. It is well established that integrin stimulation induces an early activation of the central signaling molecules Src and FAK, which then initiate a cascade of tyrosine phosphorylation signaling events leading to the activation of the Rho family of small GTPases and the remodeling of the cellular actin cytoskeleton. Several PTPs, including PTPα, are implicated in integrin-induced cytoskeletal reorganization (see Section 1.4). The major phenotypic defects observed in PTPα knockout fibroblasts are delayed cell spreading and impaired migration towards FN (Zeng *et al.*, 2003). Recent work from our lab revealed dual functions of PTPα in integrin-induced cytoskeletal reorganization, namely its initial action to activate Src, and a second action downstream of Src/FAK that requires phosphorylation of PTPα at the Y789 site located in its intracellular C-terminal tail (Chen *et al.*, 2006). The Rho family of small GTPases are believed to be the key regulators of actin cytoskeleton remodeling and thus are likely candidates to be involved in integrin-induced PTPα-mediated cytoskeletal rearrangements. There are multiple ways by which PTPα may regulate Rho GTPase activation in integrin signaling. Although the precise signaling events linking integrins and the Rho family of small GTPases are poorly understood, the majority of the known Rho GTPase activation pathways are initiated from activated SFKs (including Src and Fyn) and FAK (see Section 1.3.3.2). PTPα thus may function to regulate integrin-induced Rho GTPase activation through its upstream action in mediating Src and FAK activation. A recent study has demonstrated that PTP-PEST, a cytosolic PTP, can directly dephosphorylate a Rho/Rac GEF, Vav2, and a Rho

GAP, p190-RhoGAP, to respectively upregulate Rac1 and downregulate RhoA (Sastry *et al.*, 2006). Besides Vav (Crespo *et al.*, 1997) and p190-RhoGAP (Fincham *et al.*, 1999), the activity of RhoGDI can also be regulated by tyrosine phosphorylation (DerMardirossian *et al.*, 2006). It is possible that PTPα may directly dephosphorylate and regulate the Rho GTPase regulatory proteins to affect Rho GTPase activation.

My investigation of the role of PTPα in integrin-induced Rho GTPase activation showed that PTPα is not required for integrin-induced RhoA activation. This result was somewhat unexpected since PTPα knockout fibroblasts exhibit delayed integrin-induced stress fiber and focal adhesion formation (Zeng *et al.*, 2003). It is well established that the processes of cytoskeletal rearrangement require the coordinated actions of different Rho GTPases whose activities are temporally and spatially regulated (Schwartz *et al.*, 2000; Ridley *et al.*, 2003). More specifically, although the formation of stress fibers and focal adhesions are promoted through the activation of RhoA, this occurs concomitantly with the downregulation of Rac1 activity at the sites of assembly. It is possible that PTPα may regulate other Rho GTPases to affect stress fiber and focal adhesion formation. Integrin stimulation was shown to induce an early, Src-dependent suppression of RhoA activity, followed by prolonged RhoA activation (Ren *et al.*, 1999; Arthur *et al.*, 2000). PTPα thus may participate in the early suppression of RhoA following integrin stimulation through its action on Src. However, this reported early inhibition of RhoA activity has been difficult to observe even in wildtype fibroblasts (Min Chen, Pallen lab, personal communication), and therefore has not yet been investigated for possible impairment in

PTP$\alpha^{-/-}$ cells. Alternatively, PTPα may regulate RhoA activity at later times beyond those investigated in this study.

My investigation of the role of PTPα in integrin-induced Rac1 activation has generated interesting but inconclusive results. In most cases, I failed to detect any Rac1 activity in the PTP$\alpha^{-/-}$ fibroblast cells in both the adherent growing and integrin-stimulated states. Reintroduction of wildtype PTPα into PTP$\alpha^{-/-}$ cells did not restore or induce detectable Rac1 activity. The findings that the ablation of Rac1 in mice is embryonically lethal (Sugihara *et al.*, 1998), and that Rac1-deficient mouse embryonic fibroblasts display a spindle-like cell shape (Guo *et al.*, 2006), indicate a critical role of Rac1 in many cell processes including maintaining an intact actin cytoskeleton. Thus in the present study, it is unlikely that the lack of detectable Rac1 activity in PTP$\alpha^{-/-}$ cells truly reflects the absence of functional Rac1. Instead, it may reflect a limit in the sensitivity of the Rac1 activation assay. In addition, I tested and observed a readily detectable Rac1 activity in another independently-derived PTP$\alpha^{-/-}$ fibroblast cell line (KP PTP$\alpha^{-/-}$). These observations indicate that each cell line may require and/or exhibit different levels of Rac1 activity despite having similar Rac1 protein expression. In another approach to investigate functional links between PTPα and Rac1, I demonstrated that PTPα, but not its tyrosine phosphorylation, is required for the full activation of the Rac1 effector PAK in integrin signaling. Besides Rac1, PAK can also be activated by another Rho GTPase, Cdc42 (Manser *et al.*, 1994). The activation of PAK can induce both Cdc42- and Rac1-mediated formation of filopodia and lamellipodia respectively, indicating that both GTPases utilize PAK in their signal transduction cascades (Sells *et al.*, 1997). Although I

cannot conclude whether the reduced PAK S144 autophosphorylation observed in the absence of PTPα is due to the action of PTPα on Cdc42 and/or Rac1, my results demonstrate that PTPα tyrosine phosphorylation is not required for Cdc42- and/or Rac1-mediated PAK autophosphorylation and activation in integrin signaling.

Both PTPα$^{-/-}$ fibroblasts and PTPα$^{-/-}$ fibroblasts re-expressing mutant PTPα Y789F exhibit delayed cell spreading on FN with impaired stress fiber and focal adhesion formation (Zeng *et al.*, 2003; Chen *et al.*, 2006). Unexpectedly, my results did not support a role for PTPα and/or its tyrosine phosphorylation status in integrin-induced activation of any of the three Rho GTPases (RhoA, Rac1, and Cdc42) that are key players in these cytoskeletal reorganization events. As discussed above, the temporal as well as the spatial regulation of the Rho GTPases are critical for their function in mediating cytoskeletal rearrangement. It is possible that PTPα may not be required for the full activation of these Rho GTPases, but may instead be involved in their localization to sites where the remodeling processes occur. Alternatively, PTPα may directly regulate signaling molecules that are downstream of the active Rho GTPases to mediate the signaling events requisite for cytoskeletal reorganization.

To determine what signals are directly transduced from phosphorylated Y789 of PTPα, I investigated the role of PTPα Y789 phosphorylation in recruiting downstream signaling molecules. The model in which tyrosine phosphorylation of one protein recruits other proteins, leading to formation of signaling complexes, has been well documented in many different signaling events and contexts. Our previous study revealed that in integrin

signaling, tyrosine phosphorylation of PTPα is detectable as early as 5 min following FN stimulation, indicating that it is an early integrin-induced signaling event (Chen *et al.*, 2006). The almost immediate phosphorylation of PTPα upon integrin stimulation thus may allow the timely recruitment of signaling molecules and/or complexes to PTPα for subsequent signaling events. I have demonstrated increased PTPα and Grb2 association upon integrin stimulation. Grb2 is a well known adaptor protein involved in various signal transduction events. Thus the observation that more Grb2 is recruited to PTPα during integrin signaling may suggest that proteins are recruited to PTPα through Grb2 to mediate certain integrin signaling events, such as those for cytoskeletal rearrangement. The inability of mutant Y789F PTPα to recruit Grb2 thus could explain the cytoskeletal reorganization defects observed in PTPα$^{-/-}$ cells re-expressing mutant PTPα Y789F.

Thus far, it is not known if the Grb2 association with PTPα is accompanied by the recruitment of other Grb2-associated proteins. I and others have failed to detect the Ras activator Sos in the PTPα and Grb2 complex (data not shown) (den Hertog *et al.*, 1994; Su *et al.*, 1996). On the one hand, it is proposed that Grb2 may negatively bind to and prevent further recruitment of other proteins to Y789-phosphorylated PTPα. On the other hand, my observation is supportive of a positive signaling interaction between PTPα and Grb2 in integrin signaling. Grb2 interacts with PTPα predominantly through its SH2 domain, whereas the two SH3 domains of Grb2 are known to bind to a variety of signaling proteins including many that are implicated in cytoskeletal organization. Two Rho GTPase GEFs, C3G and Vav, are among the proteins that can bind to the SH3 domains of Grb2 (Tanaka *et al.*, 1994; Ramos-Morales *et al.*, 1995). However, since my

results did not reveal a role of PTPα and/or its tyrosine phosphorylation in Rho GTPase activation, it is unlikely that tyrosine phosphorylation of PTPα functions to recruit a pool of Grb2 that is complexed with these Rho GTPase GEFs. Dynamin is another Grb2 SH3 domain-interacting protein that has been implicated in the regulation of Rac1 localization and function (Schlunck *et al.*, 2004). However, the binding of Grb2 to dynamin and PTPα was found to be mutually exclusive (den Hertog *et al.*, 1996).

Interestingly, Grb2 has also been shown to interact with the Rac1/Cdc42 effector PAK and the Cdc42 effector WASp through its SH3 domain (She *et al.*, 1997; Puto *et al.*, 2003). In both instances, the association with Grb2 was found to be constitutive. When cells are stimulated with the growth factor EGF, Grb2 along with the associated PAK or WASp are recruited to the tyrosine phosphorylated EGF receptor at the plasma membrane. It is proposed that Grb2 may function to translocate PAK and WASp from the cytosol to the plasma membrane where they can interact with their upstream activators and downstream effectors. Both PAK and WASp function downstream of the Rho GTPases, and are closely involved in the assembly of actin cytoskeleton networks (Daniels *et al.*, 1999; Miki *et al.*, 2003). Thus the recruitment of PAK and WASp to the activated receptor by Grb2 provides a direct link between growth factor receptors and the actin cytoskeleton. PTPα has been shown to colocalize with αv integrin during the early phase of cell spreading on FN (von Wichert *et al.*, 2003). It remains to be determined whether integrin-induced PTPα Y789 phosphorylation recruits a specific pool of Grb2 that is complexed to PAK and/or WASp, thus linking integrins to components of the actin polymerization machinery. My results have demonstrated that integrin-induced PAK

autophosphorylation does not require PTPα Y789 phosphorylation. Tyrosine phosphorylation of PTPα, however, may affect PAK localization through Grb2. Nonetheless, it cannot be ruled out that the increase in PTPα and Grb2 association is simply a consequence of an increase in the phosphotyrosine content on PTPα that attracts free cellular Grb2. In such a case, the complex between PTPα and Grb2 would not function as a signaling platform. It remains to be determined what signaling events are mediated downstream of PTPα and Grb2 association.

The peptide affinity purification assay has revealed several protein bands that represent novel binding proteins (direct and indirect) for Y789-phosphorylated PTPα. These include two proteins of ~70 kD, one protein of ~56 kD, one tyrosine phosphorylated protein of ~53 kD, and several proteins, tyrosine phosphorylated or not, of ~25-29 kD. The detection of some tyrosine phosphorylated proteins by this affinity purification procedure may lead to the identification of new PTPα substrates. Although future studies are required to characterize these proteins and their interactions with PTPα, my results favor a functional role of PTPα tyrosine phosphorylation in mediating protein-protein interactions for subsequent signal transduction events. In addition to integrins, one study has reported the colocalization of the focal adhesion protein paxillin with PTPα (Lammers *et al.*, 2000). This is abolished by PTPα Y789F mutation, suggesting an Y789-dependent association between PTPα and components of the focal adhesion complexes. Phosphorylation of PTPα upon integrin stimulation may thus position PTPα in close proximity to other focal adhesion proteins that are substrates for PTPα-mediated dephosphorylation. A potential candidate is p130Cas, which was shown to be an *in vivo*

substrate of PTPα (Buist *et al.*, 2000). The identification of binding proteins for Y789-phosphorylated PTPα will provide insights into its precise role in integrin-induced cytoskeletal reorganization.

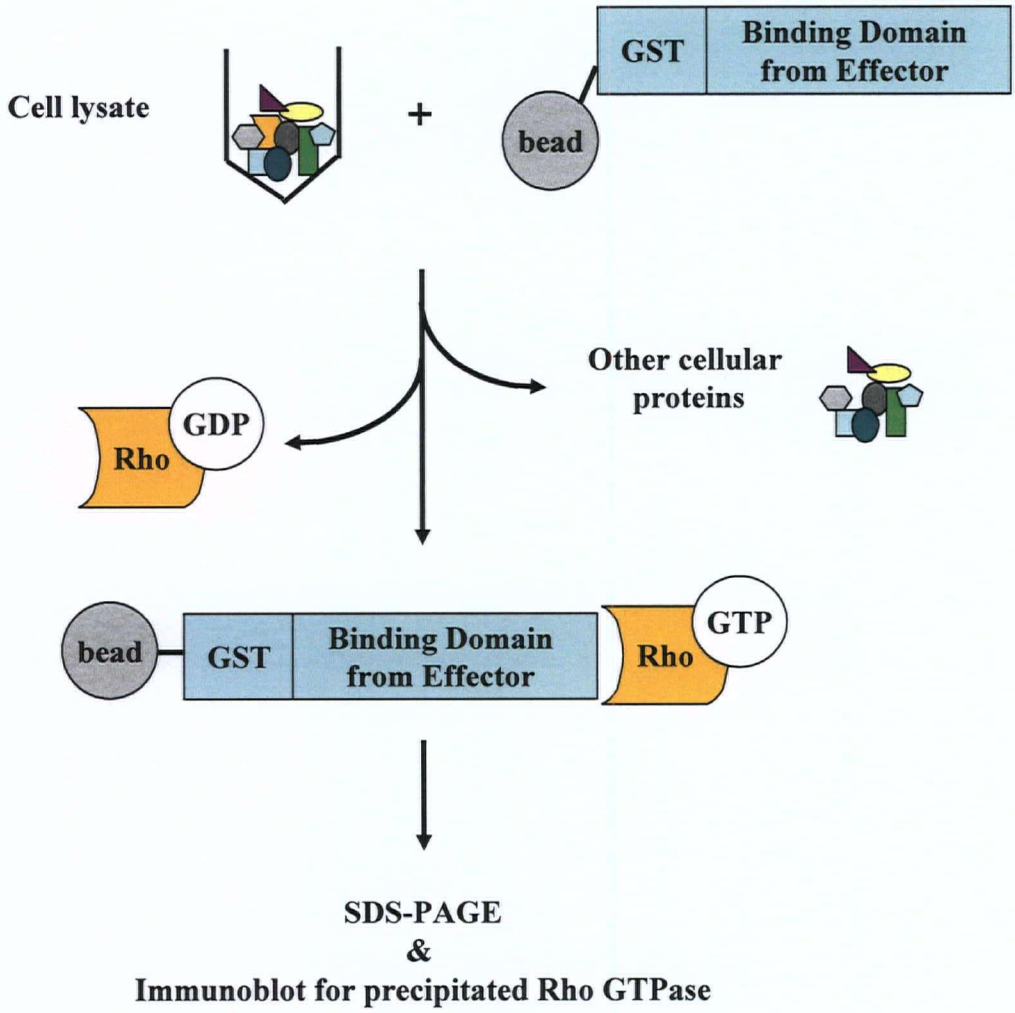

Figure 4.1 Rho GTPase activation assay. Lysate of a treated cell sample is incubated with a GST-fusion protein comprising the binding domain of the effector protein of the Rho GTPase coupled to glutathione beads. The binding domain used in the RhoA activation assay is the Rho-binding domain (RBD) of the Rho effector rhotekin, and for the Rac1 activation assay, is the p21-binding domain (PBD) of the Rac effector p21-activated kinase (PAK). Cellular proteins, including the inactive GDP-bound form of the Rho GTPase, that are not bound by the fusion protein are removed during the washing steps. The amount of bound Rho GTPase, representing the active GTP-bound form, is visualized by immunoblotting for the appropriate Rho GTPase.

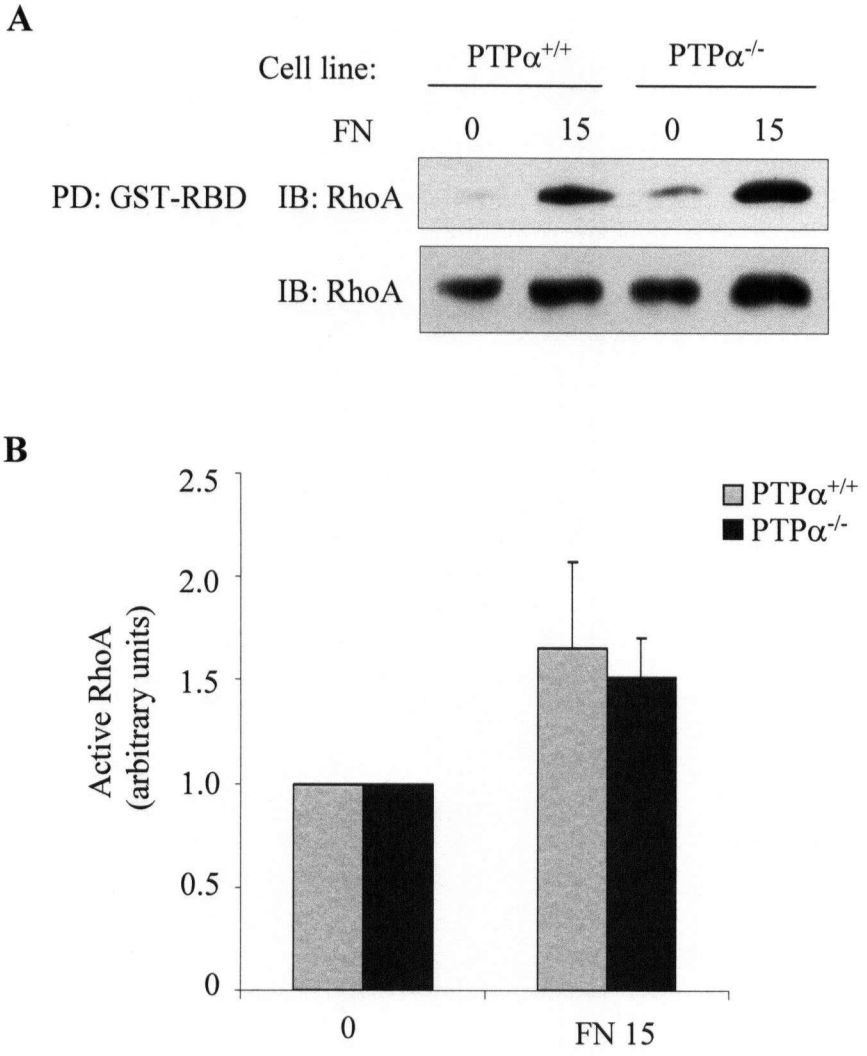

Figure 4.2 Integrin-induced RhoA activation is normal in PTPα$^{-/-}$ fibroblasts. **(A)** Serum-starved PTPα$^{+/+}$ and PTPα$^{-/-}$ fibroblasts were trypsinized and maintained in suspension (0), followed by plating on FN for 15 min (FN 15). To assay the RhoA activity, cell lysates were subjected to GST-Rho-binding domain (RBD) pull-down assays (PD), followed by immunoblotting (IB) for the amount of precipitated RhoA (RhoA-GTP) (top panel). Cell lysate was also immunoblotted for cellular RhoA content (bottom panel). **(B)** The cellular RhoA activity was determined from three independent experiments as in (A). The arbitrary densitometric units of active GTP-bound RhoA were determined, with that from serum-starved cells taken as 1.0 and that from FN-stimulated cells determined relative to the former.

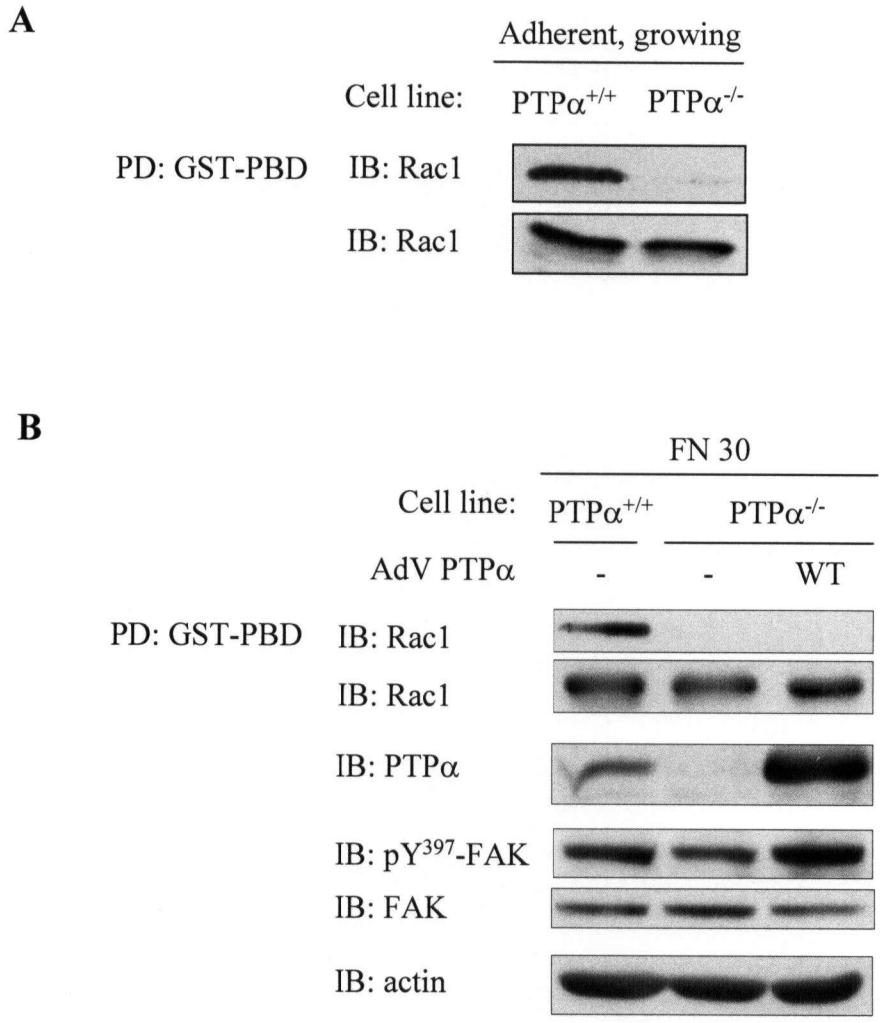

Figure 4.3 Rac1 activity in PTPα$^{+/+}$ and PTPα$^{-/-}$ fibroblasts. Cellular Rac1 activity was assayed by GST-p21-binding domain (PBD) pull-down assays (PD), followed by immunoblotting (IB) for the amount of precipitated Rac1 (Rac1-GTP) (A and B: top panels). In addition, cell lysates were immunoblotted for cellular Rac1 content (A: bottom panel; B: second top panel). **(A)** Rac1 activity was assayed in adherent growing PTPα$^{+/+}$ and PTPα$^{-/-}$ fibroblasts. **(B)** PTPα$^{+/+}$, PTPα$^{-/-}$, and PTPα$^{-/-}$ cells re-expressing wildtype (WT) PTPα introduced via an adenovirus expression system (AdV) were stimulated with FN for 30 min and were assayed for Rac1 activity. In addition, the cell lysates were immunoblotted for PTPα, phospho-FAK Y397, FAK, and actin. This figure is representative of at least three individual experiments.

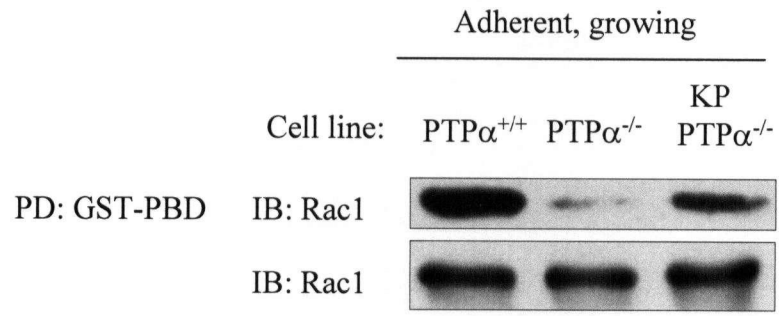

Figure 4.4 Rac1 activity in PTPα$^{+/+}$, PTPα$^{-/-}$, and KP PTPα$^{-/-}$ fibroblasts. The Rac1 activities in growing adherent PTPα$^{+/+}$ and PTPα$^{-/-}$ cell lines, and in another independently derived PTPα$^{-/-}$ (KP PTPα$^{-/-}$) cell line were determined by GST-p21-binding domain (PBD) pull-down assays (PD), followed by immunoblotting (IB) for the amount of precipitated Rac1 (Rac1-GTP) (top panel). In addition, the cell lysates were immunoblotted for Rac1 content (bottom panel).

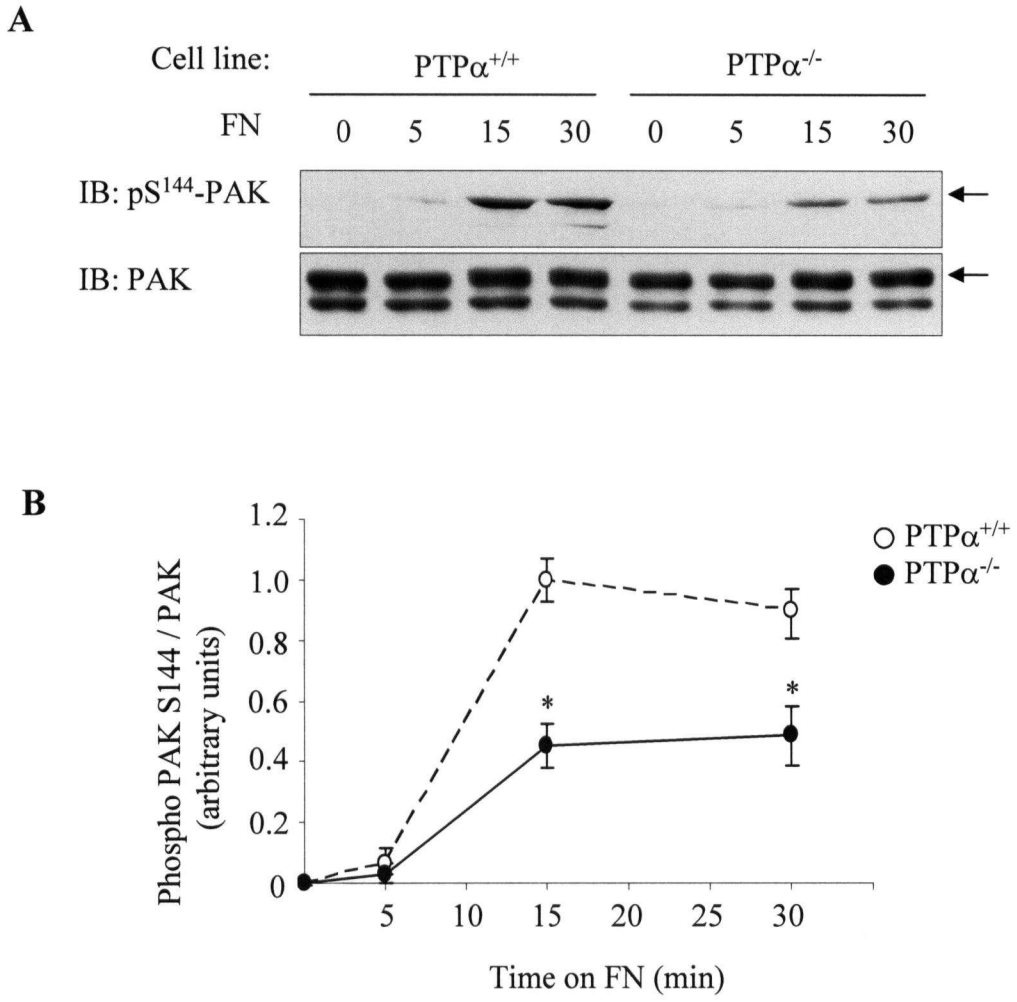

Figure 4.5 Integrin-induced PAK S144 autophosphorylation (activation) is impaired in PTPα$^{-/-}$ fibroblasts. (A) PTPα$^{+/+}$ and PTPα$^{-/-}$ fibroblasts were kept in suspension (0), followed by plating on FN for the indicated times. The cell lysates were immunoblotted for phospho PAK S144 (top panel) and for PAK (bottom panel). The arrow indicates the PAK1 isoform. **(B)** PAK S144 phosphorylation was determined from three independent experiments as in (A). The arbitrary densitometric units of PAK phospho-S144 per amount of PAK were determined, with that from FN15-stimulated PTPα$^{+/+}$ cells taken as 1.0 and others determined relative to that. The asterisks indicate a significant difference ($p \leq 0.05$) between PTPα$^{+/+}$ and PTPα$^{-/-}$ cells.

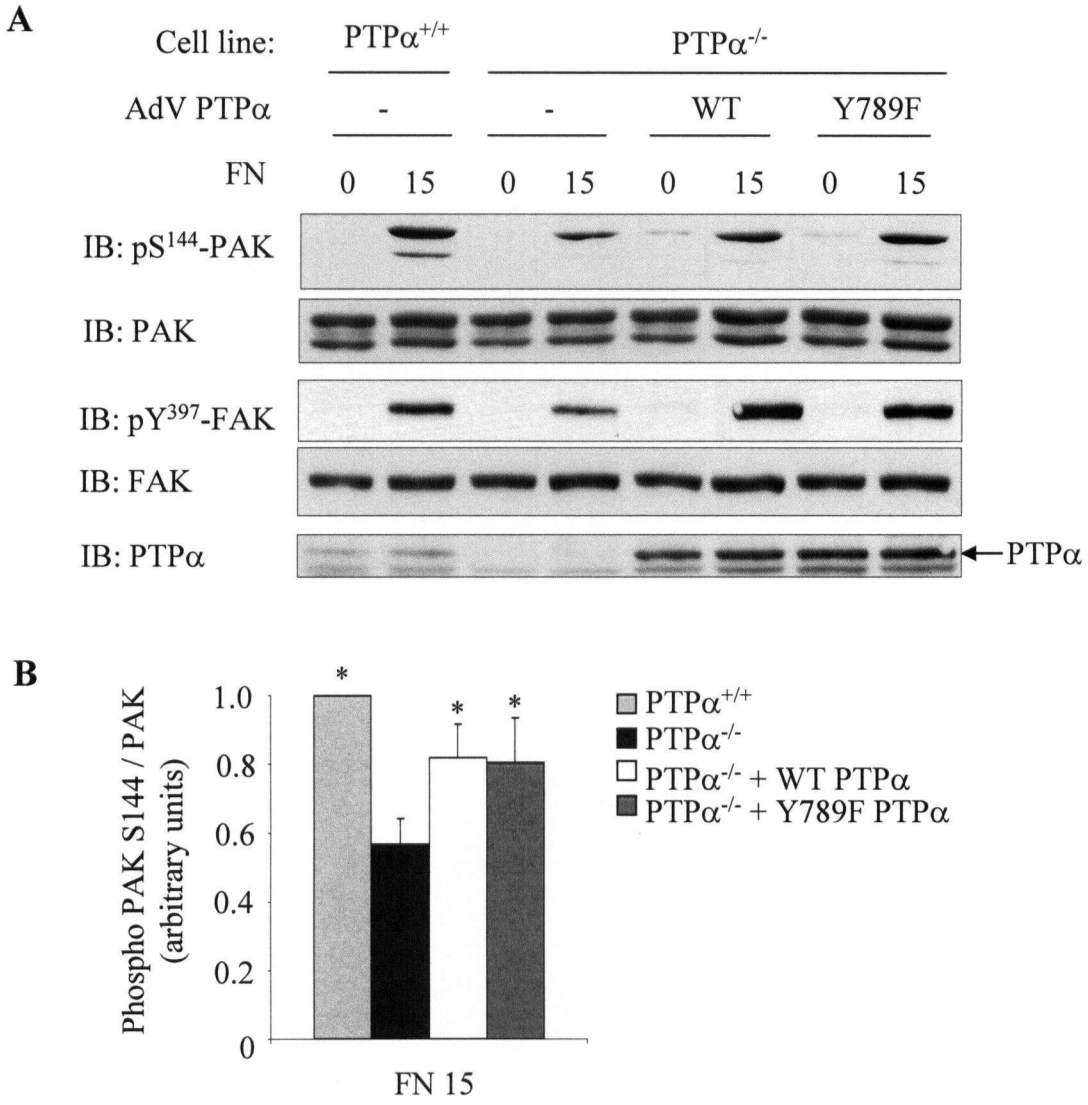

Figure 4.6 PTPα Y789 phosphorylation is not required for integrin-induced PAK autophosphorylation (activation). (A) PTPα$^{+/+}$, PTPα$^{-/-}$, and PTPα$^{-/-}$ cells re-expressing wildtype (WT) or Y789F mutant PTPα introduced via an adenovirus expression system (AdV) were kept in suspension (0), followed by plating on FN for 15 min. Cell lysates were immunoblotted (IB) for phospho-PAK S144, PAK, phospho-FAK Y397, FAK, and PTPα. **(B)** PAK S144 phosphorylation was determined from four independent experiments as in (A). The arbitrary densitometric units of PAK phospho-S144 per amount of PAK were determined, with that from FN15-stimulated PTPα$^{+/+}$ cells taken as 1.0 and that from PTPα$^{-/-}$, PTPα$^{-/-}$ cells re-expressing wildtype (WT) PTPα, or PTPα$^{-/-}$ cells re-expressing Y789F mutant PTPα determined relative to that. The asterisks indicate a significant difference ($p \leq 0.02$) with PTPα$^{-/-}$ cells.

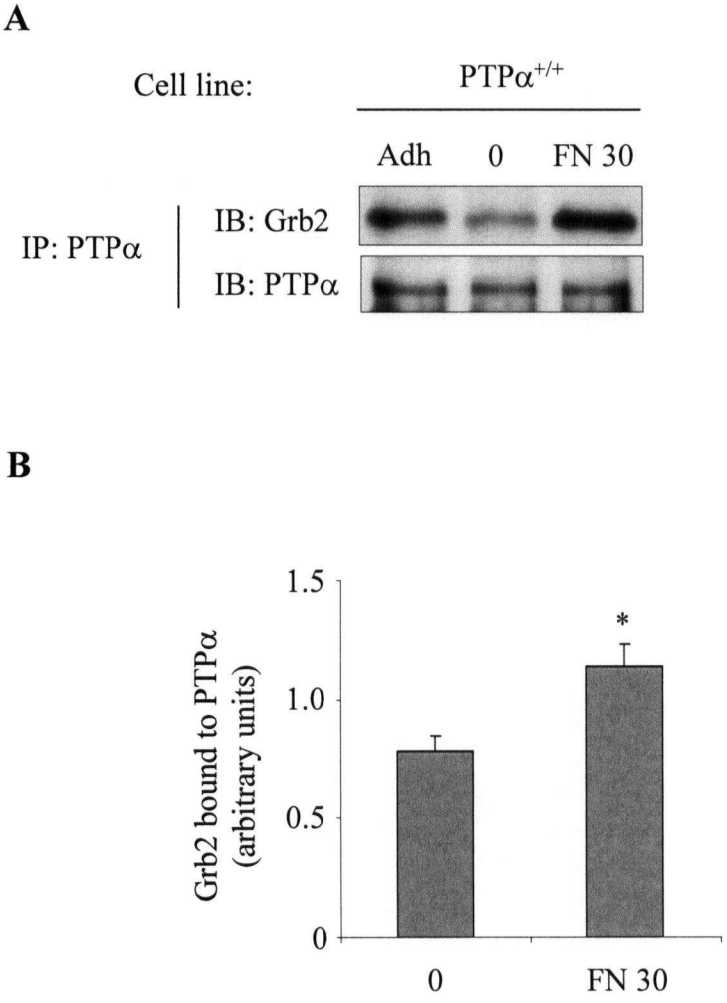

Figure 4.7 Integrin stimulation induces PTPα and Grb2 association. (A) PTPα$^{+/+}$ mouse embryonic fibroblasts growing on dishes (Adh) were trypsinized and kept in suspension for 1 hr (0), followed by plating on FN for 30 min. Cell lysates were immunoprecipitated (IP) with PTPα antiserum, followed by immunoblotting (IB) for associated Grb2 (top panel) and for total PTPα (bottom panel). **(B)** PTPα and Grb2 association was determined from four independent experiments as in (A). The arbitrary densitometric units of associated Grb2 per amount of PTPα were determined, with that from adherent (Adh) cells taken as 1.0 (not shown) and that from suspended or FN-stimulated cells determined relative to that. The asterisk indicates a significant difference ($p \leq 0.02$) with suspended cells.

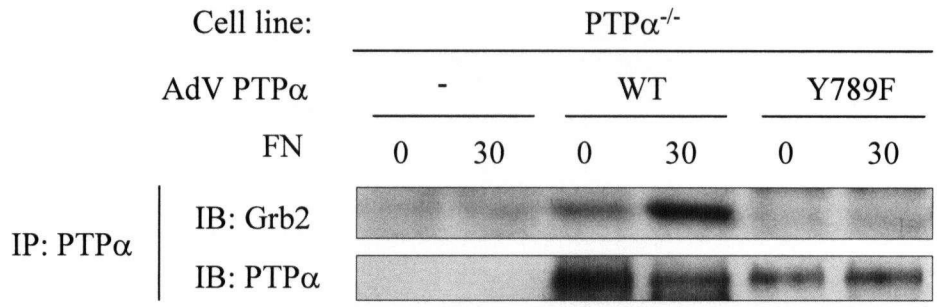

Figure 4.8 Grb2 association with PTPα is dependent on PTPα Y789. PTPα$^{-/-}$ and PTPα$^{-/-}$ cells re-expressing wildtype (WT) or Y789F mutant PTPα introduced via an adenovirus expression system (AdV) were kept in suspension (0), followed by plating on FN for 30 min. Cell lysates were immunoprecipitated (IP) with PTPα antiserum, followed by immunoblotting (IB) for associated Grb2 (top panel) and for PTPα (bottom panel). This figure is representative of three individual experiments.

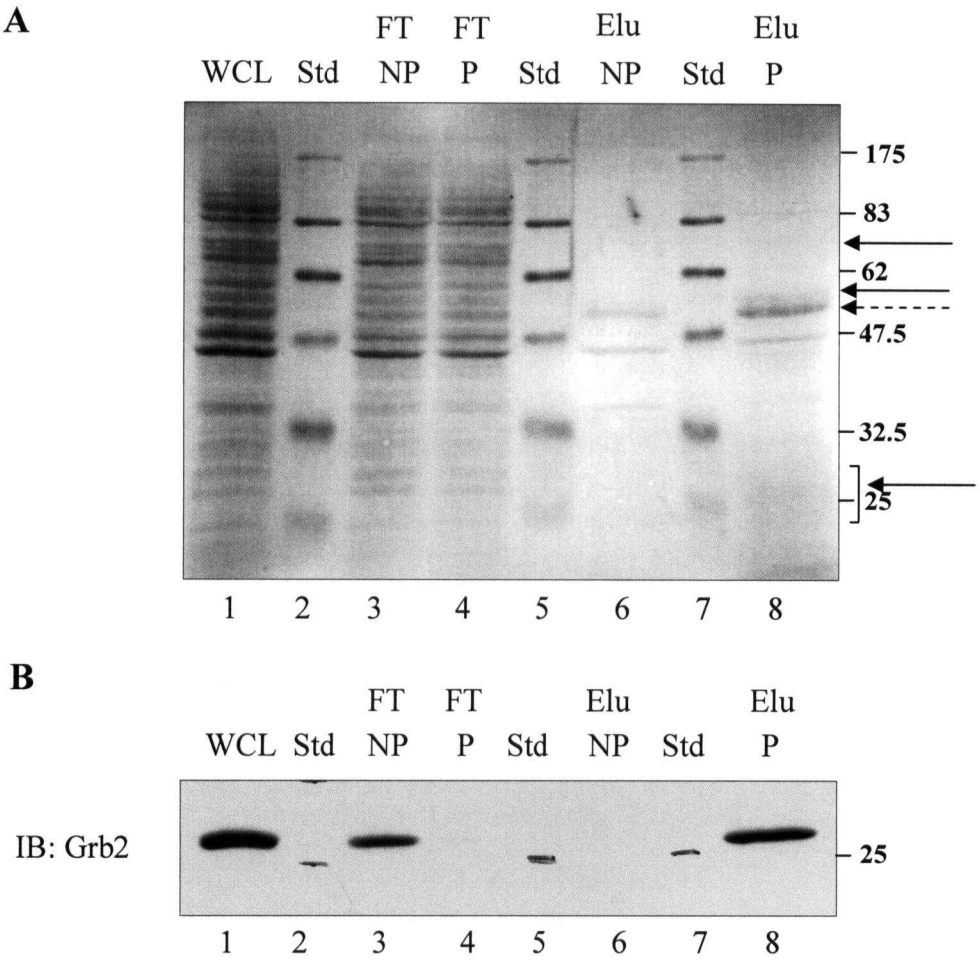

Figure 4.9 Affinity purification of potential binding proteins for Y789-phosphorylated PTPα. Cell lysate from FN30-stimulated PTPα$^{-/-}$ fibroblasts (WCL) (lane 1) was loaded onto the non-phosphopeptide (NP) or the phosphopeptide (P) affinity column. The flow-through (FT) (lanes 3 and 4) and eluate (Elu) (lanes 6 and 8) were collected from each affinity column, concentrated using Amicon Ultra-15 centrifugal filter, and aliquots resolved by SDS-PAGE. Some lanes were loaded with protein molecular weight markers (Std) (lanes 2, 5, and 7). The resolved proteins were transferred to a PVDF membrane. **(A)** The PVDF membrane was stained with Memcode reversible protein stain. The solid arrows indicate the positions of protein bands that are uniquely present in the eluate from the phosphopeptide column, whereas the dashed arrow indicates a protein band that is present at a higher amount in the eluate from the phosphopeptide column (lane 8) than from the non-phosphopeptide column (lane 6). **(B)** The PVDF membrane was de-stained and immunoblotted (IB) for Grb2. Lanes are as described for (A).

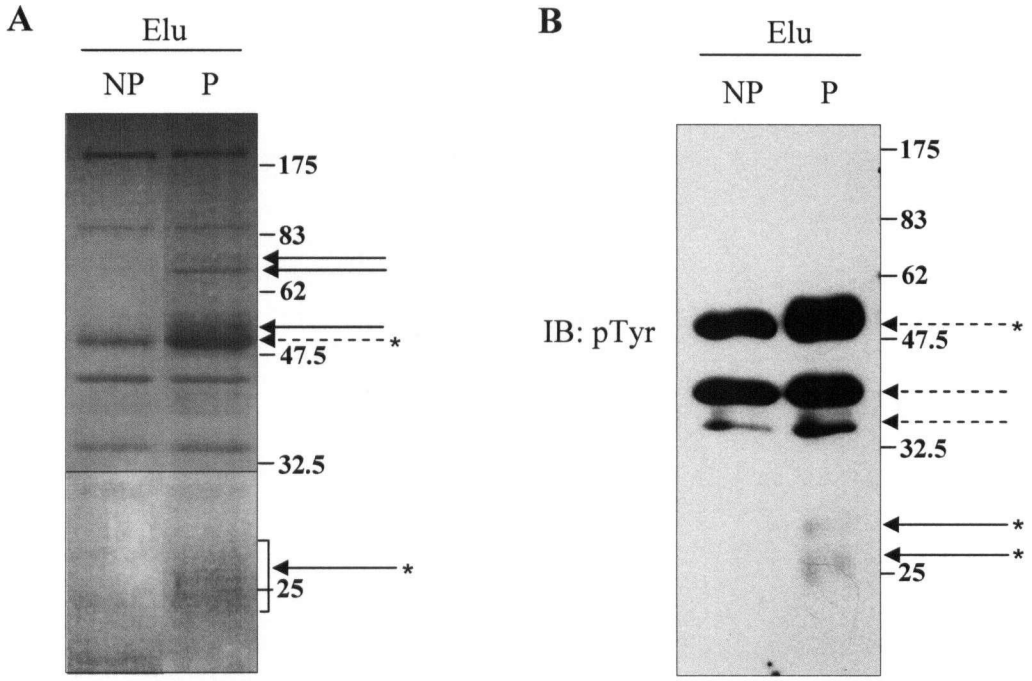

Figure 4.10 Affinity purification of potential binding proteins for Y789-phosphorylated PTPα. The eluates (Elu) collected from the non-phosphopeptide (NP) and the phosphopeptide (P) affinity columns were resolved by SDS-PAGE. **(A)** Part of the polyacrylamide gel containing the resolved proteins was stained with Coomassie G-250 protein stain. The bottom portion of the stained gel is shown adjusted to a higher intensity to improve visualization of stained proteins. **(B)** The resolved proteins on the other half of the gel piece were transferred to a PVDF membrane and were immunoblotted (IB) for phosphotyrosine. The solid arrows indicate the positions of protein bands that are uniquely present in the eluate from the phosphopeptide column, whereas the dashed arrows indicate protein bands that are present at a higher amount in the eluate from the phosphopeptide column than from the non-phosphopeptide column. The asterisks indicate protein bands that are detected both using the total protein stain in (A) and by phosphotyrosine immunoblotting in (B).

CHAPTER 5

GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 General Discussion

PTPα is a ubiquitously expressed, tyrosine phosphorylated RPTP that has been implicated in a variety of cell processes including integrin signaling, mitosis, neuronal differentiation and outgrowth, NMDA receptor signaling, and tumorigenesis (Zheng *et al.*, 1992; den Hertog *et al.*, 1993; Zheng *et al.*, 2000; Zeng *et al.*, 2003; Le *et al.*, 2006). Most of these functions of PTPα are linked to its ability to dephosphorylate and activate SFKs (Pallen, 2003). Altered PTPα Y789 phosphorylation has been reported to be associated with both integrin- and H_2O_2-induced signaling (Chen *et al.*, 2006; Hao *et al.*, 2006b). Furthermore, PTPα tyrosine phosphorylation has been functionally implicated in mitosis-associated Src activation by a displacement model proposed by Zheng *et al.* (2000) and in integrin-induced cytoskeletal reorganization, but not in Src activation, by an unidentified mechanism (Chen *et al.*, 2006). Little is known about the role of PTPα and its tyrosine phosphorylation status in other signaling pathways such as growth factor receptor signaling. As phospho-Y789 of PTPα is emerging as a functional signaling motif in PTPα-regulated processes, in the present study I have investigated the regulation of PTPα tyrosine phosphorylation in other signaling pathways including those induced by IGF-1, EGF, aFGF, LPA, and PMA. In addition, I have investigated signaling events transduced downstream of PTPα phospho-Y789 in order to unravel the precise

mechanism by which PTPα Y789 phosphorylation regulates integrin-induced cytoskeletal reorganization.

My investigation has identified IGF-1 as a positive regulator for PTPα Y789 phosphorylation in wildtype mouse embryonic fibroblasts, whereas aFGF and to a lesser extent, LPA and PMA, may induce PTPα Y789 phosphorylation in other cell systems. The findings demonstrate that tyrosine phosphorylation of PTPα is a downstream signaling event of activated IGF-IR and implicate a potential role of PTPα in IGF-IR signaling. Tyrosine phosphorylation of PTPα has been reported not to affect PTPα phosphatase activity but to instead be involved in mediating protein-protein interactions, specifically with SH2 containing proteins (Su *et al.*, 1996). Both Src and the adaptor protein Grb2 can interact with PTPα via the phosphorylated Y789 motif (den Hertog *et al.*, 1994; Su *et al.*, 1994; Zheng *et al.*, 2000). In mitosis, the regulated and alternative binding of Grb2 and Src to phospho-PTPα is critical for PTPα to dephosphorylate and activate Src (Zheng *et al.*, 2000). Studies on the effects of IGF-1 on Src activity and the role of the latter in IGF-1 signaling events have yielded conflicting results in different cell types. IGF-1 stimulation inhibits Src phosphotransferase activity in 293T and NIH3T3 cells (Arbet-Engels *et al.*, 1999) while increased Src activity was observed in IGF-1 stimulated 3T3-L1 preadipocytes (Boney *et al.*, 2001; Sekimoto *et al.*, 2003), neuroblastoma cells (Bence-Hanulec *et al.*, 2000), and colon cancer cell lines overexpressing IGF-IR (Sekharam *et al.*, 2003). In these cell lines, treatment with a Src inhibitor reduced IGF-1-induced mitogenic effects. One study has shown that PTPα knockdown in 3T3-L1 adipocytes using an antisense strategy significantly reduced

cellular Src activity, indicating the importance of PTPα in Src activation (Arnott *et al.*, 1999). It is therefore interesting to speculate that IGF-1 stimulation of these cell lines could induce PTPα Y789 phosphorylation and thus promote Src recruitment and activation.

Our lab has recently discovered a new role of PTPα Y789 phosphorylation in mediating integrin-induced cytoskeleton reorganization and cell migration (Chen *et al.*, 2006). Interestingly, the role of IGF-1 signaling in cytoskeletal reorganization and cell migration has been extensively documented in both normal and cancer cell lines. In SH-SY5Y neuroblastoma cells, treatment with IGF-1 leads to membrane ruffling with formation of large lamellipodia (Leventhal *et al.*, 1997; Kim *et al.*, 1998), while prolonged stimulation can enhance cell motility (Meyer *et al.*, 2001). Likewise, IGF-1 stimulation of MCF-7 breast cancer cells leads to remodeling of the actin fiber network with increased chemotaxis towards extracellular matrix proteins (Doerr *et al.*, 1996; Guvakova *et al.*, 1999; Zhang *et al.*, 2005). Other examples of IGF-1 induced cell motility have been reported with keratinocytes (Ando *et al.*, 1993), vascular smooth muscle cells (Pukac *et al.*, 1998), A2058 human melanoma cells (Stracke *et al.*, 1988), human colonic epithelial cells (Andre *et al.*, 1999), KM12L4 human colorectal carcinoma cells (Bauer *et al.*, 2005), and more. Multiple signaling pathways have been implicated in IGF-1-induced cell motility including those involving integrins, PI3K, and MAPK. The IGF-1-induced PTPα Y789 phosphorylation may represent a novel step by which IGF-1 modulates cellular cytoskeleton networks in these or other signaling cascades.

Thus far, it is not known what signaling events are transduced from tyrosine phosphorylated PTPα upon integrin or IGF-IR stimulation. In integrin signaling, PTPα Y789 phosphorylation is required for the remodeling of the actin cytoskeleton for subsequent cell spreading and migration processes. This function of PTPα is independent of its ability to recruit Src for activation since PTPα Y789F mutation did not affect integrin-induced Src activation but did delay cytoskeleton reorganization (Chen *et al.*, 2006). Although my investigation did not reveal a role of PTPα and/or its tyrosine phosphorylation in the activation of the three members of the Rho family of small GTPases, RhoA, Rac1, and Cdc42, that are the key mediators of cytoskeletal rearrangement, there are alternative pathways by which PTPα may affect actin cytoskeleton remodeling. I have demonstrated an increase in Grb2 recruitment to PTPα upon PTPα Y789 phosphorylation in integrin signaling. As an adaptor protein, Grb2 may recruit downstream signaling proteins such as PAK or other proteins to PTPα to mediate certain cytoskeletal reorganization events. In addition to Grb2, I have identified several cellular proteins, both those that are and are not tyrosine phosphorylated, as potential binding proteins for Y789-phosphorylated PTPα. The molecular identification of these proteins will provide further insights into the downstream signaling events transduced by the PTPα phospho-Y789 motif.

Both integrins and IGF-IR are cell surface receptors that act as sensors of the extracellular environment and transduce external signals into cells utilizing a cascade of tyrosine phosphorylation events to regulate cell behavior. The activated integrins initiate signal transduction events by utilizing Src-SFKs and FAK, whereas the IGF-IR itself

exhibits intrinsic tyrosine kinase activity. It is well established that integrins and growth factor receptors such as IGF-IR often work in concert to achieve full signaling activity. Although my investigation demonstrated that IGF-1-induces PTPα Y789 phosphorylation independently of integrin activation, and utilizes different cellular kinases from those involved in integrin-induced PTPα Y789 phosphorylation, it is conceivable that tyrosine phosphorylation of PTPα mediates similar downstream signaling events in both integrin- and IGF-IR-regulated signaling pathways. Stimulation of integrins or the IGF-IR can both induce cytoskeletal rearrangements in the cells. PTPα thus may serve as a common signaling molecule utilized in the integrin and the IGF-IR signaling cascades, where tyrosine phosphorylation of PTPα transduces signaling events leading to remodeling of the actin cytoskeleton. However, the functional significance of IGF-1-induced PTPα Y789 phosphorylation remains to be determined.

Taken together, the results of my investigation have provided new insights into the function of PTPα Y789 phosphorylation, particularly of its role in mediating integrin-induced cytoskeletal reorganization. They have also extended our current knowledge of regulators of PTPα Y789 phosphorylation to include the growth factor IGF-1, and potentially aFGF, LPA, and PMA. These findings implicate PTPα in IGF-IR signaling, and furthermore, in the genesis or progression of diseases linked to aberrant IGF-IR signaling such as human cancer. In addition, despite the involvement of PTPα in many signaling pathways and cell processes, alterations in PTPα mRNA or protein expression level have not been documented in human diseases except in late-stage colon cancer (Tabiti *et al.*, 1995). We and others have demonstrated that the function of PTPα can be

regulated at the level of post-translational modification by tyrosine phosphorylation. Dysregulation of PTPα Y789 phosphorylation thus may potentially be involved in the pathogenesis of some human diseases.

5.2 Future Directions

The mechanism and function of IGF-1-induced PTPα Y789 phosphorylation are not known. Future research effort thus will focus on 1) the identification of the cellular kinases responsible for IGF-1-induced PTPα Y789 phosphorylation using different kinase inhibitors and/or kinase knockout cell systems; and 2) the characterization of the functional impact of PTPα Y789F mutation on IGF-1-induced signaling events including cytoskeletal reorganization. In addition, elevated IGF-IR signaling has been documented in many types of human cancers (Resnik *et al.*, 1998; Weber *et al.*, 2002; Ouban *et al.*, 2003; Krueckl *et al.*, 2004), and it is therefore of interest to investigate whether IGF-1 stimulation of these cancer cells will lead to a substantial increase in PTPα Y789 phosphorylation. If so, this would suggest a role for PTPα Y789 phosphorylation in tumorigenesis and cancer progression. In order to elucidate the precise signaling function of PTPα Y789 phosphorylation, the identification of phospho-Y789 binding proteins is a priority. To this end, the identities of those proteins exhibiting greater binding affinity for the phospho(-Y789)peptide than for the non-phospho(-Y789)peptide in the affinity purification assay described in Chapter 4 (Section 4.3.2) will be determined by mass spectrometry. The *in vivo* interaction between PTPα and the identified protein(s), with emphasis on integrin-dependent regulation of this event, will be validated by co-immunoprecipitation.

CHAPTER 6

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APPENDIX

LIST OF ACHIEVEMENTS

PUBLICATION:

Chen, M., **Chen, S. C.,** and Pallen, C. J. (2006). "Integrin-Induced Tyrosine Phosphorylation of PTPα is Required for Cytoskeletal Reorganization and Cell Migration." J Biol Chem **281**(17): 11972-80.

ABSTRACTS:

Chen, S. C., Chen, M., and Pallen, C. J. Tyrosine Phosphorylation of Protein Tyrosine Phosphatase Alpha (PTPα) Is Required For Integrin-Induced Cytoskeletal Reorganization. ***[Poster Presentation]*** 6th Annual Student Research Forum, Child and Family Research Institute. June 15, 2006.

Chen, S. C., Chen, M., and Pallen, C. J. Integrin-induced Tyrosine Phosphorylation of Protein Tyrosine Phosphatase Alpha is Required for Cytoskeletal Reorganization. ***[Selected for Oral Presentation]*** Pathology Day 2006, Department of Pathology and Laboratory Medicine, UBC. May 26, 2006.

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SCHOLARSHIPS:

Michael Smith Foundation for Health Research Junior Graduate Studentship
BC Research Institute for Children's & Women's Health Graduate Studentship (declined)
David Hardwick Graduate Studentship
Graduate Entrance Scholarship