IGF-1 Activates c-Abl to Regulate PTPα Tyrosine Phosphorylation and Cell Migration

by

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B.Sc., University of British Columbia, 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

Master of Science

in

THE FACULTY OF GRADUATE STUDIES
(Experimental Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

June 2011

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Abstract

Protein tyrosine phosphorylation is an important cellular mechanism that regulates intracellular signalling pathways to control many cellular activities, including proliferation, growth and differentiation. Tyrosine phosphorylation is a reversible process regulated by protein tyrosine kinases and protein tyrosine phosphatases (PTPs) which add and remove phosphate groups from target proteins, respectively. PTPα, a receptor-type member of the classical subfamily of PTPs, functions to activate Src family kinases (SFKs). In integrin signaling, PTPα-activated SFKs act in conjunction with focal adhesion kinase (FAK) to phosphorylate PTPα at a tyrosine residue (Tyr789) near its C-terminus to enable PTPα-dependent promotion of cell migration. Recently, our lab also found that Tyr789 of PTPα is phosphorylated in response to various growth factor stimuli, including IGF-1. However, the kinase and mechanism underlying this growth factor-stimulated phosphorylation and its functional importance in cellular responses were unknown. I investigated IGF-1-dependent phosphorylation of PTPα in mouse embryo fibroblasts lacking the SFKs Src, Yes, and Fyn (SYF cells) and in the SH-SY5Y neuroblastoma cell line. I found that the tyrosine kinase c-Abl was responsible for IGF-1-induced PTPα Tyr789 phosphorylation, and that IGF-1 activated c-Abl. Furthermore, PTPα was found to exist in a constitutive multi-protein complex with RACK1 and the IGF-1R. IGF-1 stimulation resulted in RACK1-dependent recruitment of the kinase c-Abl and phosphorylation of Tyr789 of PTPα. IGF-1-induced migration of SYF cells was dependent upon PTPα Tyr789, and that of SH-SY5Y cells was dependent upon both c-Abl activity and PTPα. My study has revealed an IGF-1-dependent signaling mechanism involving a RACK1-scaffolded multi-protein complex of IGF-1R, c-Abl, and PTPα that regulates c-Abl activity and PTPα tyrosine phosphorylation to promote fibroblast and neuroblastoma cell migration. This is distinct from the integrin-stimulated
SFK/FAK-dependent tyrosine phosphorylation of PTPα. Overall, my findings suggest that distinct receptor-mediated signaling pathways converge on PTPα to regulate its phosphorylation and promote cell migration. As aberrant IGF-1/IGF-1 receptor signaling is associated with tumourigenesis and metastasis of several types of cancers, further investigation into the role of IGF-1-regulated PTPα phosphorylation in neuroblastoma cells may reveal potential therapeutic targets for this pediatric tumour and also have broader relevance for the treatment of other types of cancer.
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Acknowledgements

I am deeply grateful to my supervisor Dr. Catherine Pallen, whose patience and understanding was crucial to the completion of this work. Her confidence in my ability and her invaluable guidance helped me immensely. Herself a tireless worker, she has always instilled in me the importance of dedication and hard work and not just the results.

I also want to thank my committee members, Dr. Poul Sorensen, Dr. Vincent Duronio, and Dr. Chinten Lim for their important suggestions and careful revisions of this thesis.

I would like to thank all the members of the Pallen lab for making my time in the lab an enjoyable and valuable experience. I would like to especially thank Dr. Lionel Samayawardhena who taught me the fundamental techniques in completing this thesis work. I would also like to thank the lab manager, Dr. Jing Wang, who helped me in several ways including procurement of necessary materials to complete my tasks.

I would also like to take this opportunity to thank my parents and my grandmother for their understanding and unconditional love and support especially during the trying times.
Chapter 1: Introduction

1.1 Cell regulation by reversible protein tyrosine phosphorylation

The regulation of cellular process is controlled by signalling events, of which there are several forms including hormonal signalling, second messenger signalling, electrochemical signalling and protein kinase cascades. This latter signal transduction pathway frequently involves a common post-translational modification termed protein tyrosine phosphorylation. The modification of protein tyrosine phosphorylation regulates proliferation, differentiation, migration and survival in cells. Protein tyrosine phosphorylation is controlled by two classes of enzymes: protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), which add and remove phosphate groups to proteins, respectively. Phosphorylation and dephosphorylation of specific tyrosine residues within a protein may induce changes in activity, stability, and cellular localization of proteins in addition to influencing protein-protein interactions. Hence, the dysregulation of PTKs and PTPs has been suggested to be a causal agent in the pathogenesis of many diseases including cancer. Although, historically, scientific research has been more focussed on elucidating the importance of PTKs, the role of PTPs in cellular processes is now emerging.

1.2 Protein tyrosine phosphatase superfamily

PTPs have acquired new found recognition as their importance in regulating the levels of phosphorylation of proteins involved in several cellular processes keeps growing (Tonks and Neel, 1996; Mustelin and Tasken, 2003). Altogether, 107 human PTP genes have been discovered so far (Alonso et al., 2004) and have been classified into four families based on
amino acid sequences of their catalytic domains, substrate specificities and cellular localization (Fig. 1).

Class I of the PTP superfamily, the largest of the superfamily, comprises 38 tyrosine-specific “classical” PTPs (of which all have mouse analogs) and 61 “dual specific” protein phosphatases (DSPs). The 38 strictly tyrosine-specific “classical” PTPs can be further divided into transmembrane, receptor-like enzymes (RPTPs) represented in the human genome by 21 genes and the intracellular, non-receptor PTPs (NRPTPs), represented in the human genome by 17 genes (Alonso et al., 2004). Most of the RPTPs have tandem catalytic domains (Andersen et al., 2001). However the 61 DSPs, of which VH1 is the prototype member, are more diverse and can be further subdivided into several subgroups: the MAP kinase phosphatases (MKPs), “atypical” DSPs, slingshot PTPs, PRLs, CDC14 PTPs, PTENs, and myotubularins. The MKPs are characterized by dual phosphothreonine and phosphotyrosine specificity in addition to containing a CH2 region and other MAP kinase targeting motifs (Bordo and Bork, 2002). MKPs are specific for dephosphorylating ERK, Jnk, and p38 (Keyse, 1998; Saxena and Mustelin, 2000). The “atypical” DSPs lack specific MAP kinase targeting motifs and tend to be smaller in size (less than 250 amino acid residues in length) (Alonso et al., 2003). The CDC14 PTP subfamily is responsible for the dephosphorylation and inactivation of cyclin dependent kinases (Cdns) towards the end of mitosis through targeting the phosphothreonine in the activation loop of the Cdns (Visintin et al., 1998). The PRLs are poorly understood but have been found to be overexpressed in a number of human cancers (Bessette et al., 2008). The Slingshots dephosphorylate cofilin and thus play a role in the regulation of actin dynamics (Huang et al., 2006). The last two subgroups of DSPs, the PTENs and myotubularins, target inositol phospholipids (Wishart and Dixon, 2002).
The class II PTPs are also cysteine-based PTPs and, in the human genome, the entire family consists of one small phosphatase, the low molecular weight protein tyrosine phosphatase (LMPTP) encoded by the ACP1 gene (Alonso et al., 2004). Class II PTPs are involved in the regulation of capsule polysaccharide synthesis in bacteria (Bottini et al., 2002). Human LMPTP and its variants, on the other hand, have been shown to play important roles in the pathogenesis of rheumatoid arthritis, diabetes, Alzheimer’s disease and asthma (Bottini et al., 2002; Alonso et al., 2003). The class III cysteine-based PTPs in humans are comprised of the cell cycle regulators CDC25A, CDC25B, and CDC25C. They dephosphorylate inhibitory phosphothreonine and phosphotyrosine residues of the CdkS at N-terminal motifs to activate these kinases during cell cycle progression (Honda et al., 1993). In addition to the cysteine-based PTPs, there are also aspartate-based PTPs and members of this family function in cellular development and nuclear morphology (Tootle et al., 2003; Satow et al., 2002; Siniossoglou et al., 1998).

PTP domains interact with other proteins and/or phospholipids to facilitate the regulation and subcellular localization of the PTPs to substrates and cellular compartments (Manning et al., 2002). PTPs have several domains commonly found in PTKs such as DNA binding, PH, and FERM domains that function to bind DNA molecules, lipids and cytoskeleton/plasma membrane interface elements, respectively (Wishart and Dixon, 1998). Interestingly, SH2 and SH3 protein-protein interaction domains, while common in PTKs are rare in PTPs. Only two PTPs (SHP1 and SHP2) contain SH2 domains, while SH3 domains are absent from the PTP family (Alonso et al., 2004). PTPs also have a number of domains not found in PTKs such as CRAL/TRIO (Sec14p homology), FYVE, and mRNA capping domains that aid in binding small lipophilic molecules, phosphatidylinositol 3-phosphate and mRNA molecules, respectively. Overall, the
number and types of domains found in PTPs and the combination of these domains in individual members provide for a diverse family of proteins.

1.3 Protein tyrosine phosphatase alpha (PTPα)

PTPα was identified in 1990 (Kaplan et al., 1990; Matthews et al., 1990; Sap et al., 1990), and mapped to chromosome 2 in the murine genome (Sap et al., 1990) and chromosome 20 (20p13) in the human genome (Rao et al., 1992). PTPα is a widely expressed receptor protein tyrosine phosphatase (RPTP) which is highly expressed in the brain (Sap et al., 1990; Kaplan et al., 1990; Krueger et al., 1990; Matthews et al., 1990).

1.3.1 Structure of PTPα

Human PTPα is a transmembrane molecule of 802 amino acids that consists of a short, glycosylated extracellular domain, a single-pass transmembrane domain, and an effector cytoplasmic region, which comprises the bulk of the molecule. The cytoplasmic region of PTPα has two tandem catalytic domains, a common feature of RPTPs (Fig. 2). The extracellular region of PTPα is unusual amongst the RPTPs - it is relatively short and does not contain cell-adhesion-like structural motifs (such as immunoglobulin (Ig) and fibronectin (FN)-III domains) found in other RPTPs, limiting the molecules that PTPα interacts with through this domain. Indeed, the only interaction known that uses the extracellular domain is between PTPα and contactin in neuronal cell differentiation (Zeng et al., 1999). In addition, the extracellular region of PTPα undergoes extensive post-translational modification through N- and O-linked glycosylation which results in a mature 130 kDa protein (Daum et al., 1994).

As mentioned, PTPα, like most RTPs, contains two tandem catalytic domains in the cytoplasmic region. While the majority of the catalytic activity of PTPα is associated with the
membrane proximal (D1) domain, the membrane distal (D2) domain of PTPα displays some activity towards the low molecular weight substrate \( p \)-nitrophenyl phosphate (pNPP) but not towards phosphotyrosyl peptide substrates (Lim et al., 1997; Wu et al., 1997). It has been suggested that the D2 domain has regulatory functions and plays a role in protein-protein interactions to facilitate substrate recognition or to target PTPα to protein complexes (Pallen, 2003). This hypothesis is supported by the finding that the D2 domain interacts with the PDZ domain of PSD-95 protein that plays a role in the regulation of N-methyl-D-aspartate (NMDA) receptor activity (Lei et al., 2002). Also, the D2 domain of PTPα can interact with the N-terminal region of the D1 domain of PTPα in trans and form homo- and hetero-dimers with PTPα and other RPTP molecules, respectively (Bilwes et al., 1996; Jiang et al., 2000; Tertoolen et al., 2001). Though the known interactions of the D2 domain of PTPα are not phosphotyrosine dependent, its structure has remained conserved through evolution, suggesting complex functional involvement in PTPα signalling. Possibly, this domain may act upon cellular substrates that are yet to be identified.

### 1.3.2 Cellular substrates of PTPα

#### 1.3.2.1 Src family kinases (SFKs)

The Src family of protein kinases (SFKs) comprise a family of PTKs with important roles in several signalling pathways, particularly those initiated by cell surface receptors. These receptors include receptor tyrosine kinases (Abram and Courtneidge, 2000), G-protein-coupled receptors (Ma and Huang, 2002), and cytokine receptors (Mustelin, 1994). The SFK family, of which Src is the prototype member, is comprised of eight other tyrosine kinases that are structurally related: Yes, Fyn, Lyn, Hck, Lck, Blk, Fgr and Yrk (Parsons and Parsons, 2004).
However, while Src, Fyn, and Yes are ubiquitously expressed, the other SFKs have a more restricted tissue and cell type expression (Thomas et al., 1997). Src remains in an inactive state in unstimulated cells and is activated in response to various stimuli, leading to downstream effects including activation of members of the mitogen-activated protein kinase (MAPK) pathways, DNA synthesis, cytoskeletal rearrangement and cell migration (Brown and Cooper, 1996). Src contains an N-terminal myristoylation motif for plasma membrane association, an SH3 domain that binds proline-rich sequences, an SH2 domain that binds phosphotyrosine, a kinase domain, and a C-terminal regulatory tail (Fig. 3). In the inactive state, Src is phosphorylated on Tyr527 in its C-terminal regulatory tail and this phosphotyrosine residue binds the SH2 domain in an intramolecular interaction (Matsuda et al., 1990). An interaction between the SH3 domain and the linker region located between the SH2 domain and the kinase domain also acts to repress the activity of Src (Xu et al., 1997). These two intramolecular interactions work in concert to maintain Src in a “closed”, inactive conformation.

Src may be activated by two mechanisms, the dephosphorylation of Tyr527 (Shenoy et al., 1992; Zheng et al., 2000) and/or interaction with an SH2 domain-containing ligand resulting in the displacement of the C-terminal tail from the Src-SH2 domain (Songyang et al., 1993). PTPα and other PTPs have been shown to catalyze Src Tyr527 dephosphorylation to activate Src (Zheng et al., 1992; den Hertog et al., 1993; Ponniah et al., 1999; Su et al., 1999). Studies conducted with rat embryonic fibroblasts and P19 embryonal carcinoma cell lines demonstrated that overexpression of PTPα reduced the Src Tyr527 phosphorylation in these cells (Zheng et al., 1992; der Hertog et al., 1993). Conversely, a high level of Src Tyr527 phosphorylation is exhibited by fibroblasts from PTPα knockout mice (Ponniah et al., 1999; Su et al., 1999). PTPα
was also shown to enhance the kinase activity of Src and other SFKs, such as Fyn, and Yes (Bhandari et al. 1998; Harder et al., 1998; Ponniah et al., 1999; Su et al., 1999).

PTPα has been implicated in mediating cellular functions through the activation of SFKs. One such function is the regulation of several intracellular proteins such as focal adhesion kinase (FAK), Crk-associated substrate (p130Cas) and the adaptor protein paxillin (Su et al., 1999; Zeng et al., 2003; Chen et al., 2006). In integrin signalling, fibronectin-induced tyrosine phosphorylation of FAK and paxillin was restored in PTPα-null fibroblasts by the re-introduction of PTPα (Chen et al., 2006) suggesting that PTPα acts as an upstream regulator of FAK and paxillin. As SFKs, target substrates of PTPα, are required for optimal and full FAK phosphorylation (Salazar and Rozengurt, 2001) it is likely that PTPα-mediated SFK dephosphorylation is the responsible mechanism behind PTPα regulation of FAK activity (Ponniah et al., 1999; Su et al., 1999). Also, overexpression of PTPα which increases SFK activation (as described above), has also been shown to increase the tyrosine phosphorylation of FAK and p130Cas (Harder et al., 1998).

1.3.2.2 Kv1.2 potassium channel

Kv channels, a distinct and ubiquitously expressed family of potassium ion channels, play an important role in the regulation of action potentials, neurotransmitter and hormone release, cellular volume regulation, and cellular proliferation (Pongs, 1995; Martens et al., 1999; Yi et al., 2001). The activity of these ion channels is modulated by the action of PTKs, such as SFKs, and PTPs. The m1 muscarinic acetylcholine receptor enhances PTK activity to promote phosphorylation of the voltage-gated shaker-related potassium channel Kv1.2 after carbachol treatment through a signalling pathway stimulated by the protein kinase C activator phorbol 12-
myristate 13-acetate (PMA) (Huang et al., 1993). In human embryonic kidney (HEK) 293 cells stably expressing the m1 receptor and the potassium channel Kv1.2, stimulation of the m1 muscarinic acetylcholine receptor resulted in the PKC-dependent tyrosine phosphorylation of PTPα (Tsai et al., 1999). Also, after m1 muscarinic acetylcholine receptor stimulation, PTPα associates with the Kv1.2 ion channel and counters the suppressive PTK-dependent tyrosine phosphorylation of Kv1.2, leading to activation of this ion channel (Tsai et al., 1999).

Recently, the relationship between PTPα and Kv ion channels has been examined further. In contrast to shaker-related Kv ion channels, PTPα was shown to catalyze the dephosphorylation of the shab-related potassium ion channel Kv2.1 resulting in the inhibition of its activity in Schwann cells (Tiran et al., 2006). In this instance, the absence of PTPα corresponded with reduced Src activity and, unexpectedly, with strong Kv2.1 activity. As PTPα was found to constitutively associate with the ion channel, this interaction is presumed responsible for restricting access of additional regulatory proteins to Kv2.1 and/or inducing a conformational change in the protein that inhibits its activity (Tiran et al., 2003, 2006). Although it appears that PTPα regulates the activity of Kv potassium ion channels, further investigations are warranted to elucidate the underlying mechanisms responsible for differences observed with the various channel subtypes.

1.3.3 Regulation of PTPα

1.3.3.1 Dimerization of PTPα

Both receptor PTKs and receptor PTPs, as transmembrane molecules, possess diverse extracellular domains that serve as ligand-binding domains. Indeed, ligand binding induces dimerization in PTKs that regulates receptor catalytic activation (Jiang and Hunter, 1999;
Studies involving CD45, a receptor PTP, in which the extracellular and transmembrane domains of CD45 were replaced by those of the EGF receptor (EGFR), showed that even though the chimeric protein was capable of initiating downstream signalling typical of CD45, EGF ligands induced dimerization of the protein and led to its inactivation (Desai et al., 1993). This suggested for the first time that PTPs like CD45 may be subject to regulation by the process of dimerization. Furthermore, Bilwes et al. (1996) found that PTPα-D1 crystalized as a dimer, and showed that the amino-terminal segment of each monomer forms a helix-turn-helix structural wedge that interacts with the active site of the opposing monomer. It was posited that this stereochemical arrangement disabled the ability of PTPα to bind phosphotyrosine-containing substrates and rendered the phosphatase catalytically inactive (Bilwes et al., 1996). The existence of such PTPα dimers was also confirmed in unstimulated “resting” living cells overexpressing tagged PTPα by fluorescence resonance energy transfer microscopy (Tertoolen et al., 2001) and by chemical crosslinking studies (Blanchetot et al., 2002).

However, dimerization does not always result in inhibition of PTPs. Examination of the effects of dimerization in single cysteine mutants of the extracellular domain demonstrate that forced dimerization through disulphide bridge formation using Cys137 inhibited PTPα activity while disulphide bridge formation through Cys 135 did not. This was attributed to the nature of the rotational coupling within the dimers. Thus, dimerization of PTPα leads to inhibition of its phosphatase activity only if the rotational coupling exhibited by the dimers allows the ‘wedge’ of one monomer to insert into the catalytic site of the other monomer (Jiang et al., 1999). It is crucial that the structure of this N-terminal wedge remains intact as structural disturbances such as those induced by point mutations could disrupt the inhibition induced by dimerization in PTPs (Majeti et al., 1998; Jiang et al., 1999). Interestingly, sites of serine phosphorylation such as
Ser180 and Ser204 reside near the dimer interphase and their phosphorylation has been suggested to regulate PTPα dimerization (Tracy et al., 1995).

Recently, oxidation has been identified as a causal agent in the dimerization of RPTPs. The cytoplasmic domains of RPTPs have been shown to contribute to dimer formation. In the case of PTPα, PTPα dimers are present in cells in either open (active) or closed (inactive) conformation. Oxidation induces conformational changes in the membrane distal (D2) domain of PTPα accompanied with changes in the rotational coupling of the dimer (Blanchetot et al., 2002; Groen et al., 2008). This stabilizes the dimer in a closed conformation (Groen et al., 2008). However, despite the progress made in understanding the role of dimerization in PTPs further research is required to more clearly define the functions of PTPα dimers.

1.3.3.2 Tyrosine phosphorylation of PTPα

Approximately 20% of endogenous PTPα is constitutively tyrosine phosphorylated in NIH 3T3 mouse fibroblasts (den Hertog et al., 1994). The site of tyrosine phosphorylation was identified as Tyr789 in the C-terminus of PTPα by phosphopeptide mapping techniques using \[^{32}\text{P} \] -orthophosphate-labelled PTPα and site-directed mutagenesis (den Hertog et al., 1994). Src was suggested to be the kinase responsible for this tyrosine phosphorylation \textit{in vivo} as transient overexpression of Src and PTPα in HEK 293 cells increased tyrosine phosphorylation at this site (den Hertog et al., 1994). Studies using a non-phosphorylatable mutant of PTPα (PTPα-Y789F) demonstrated that tyrosine phosphorylation of PTPα does not affect its intrinsic phosphatase activity (Su et al., 1996). The sequence C-terminal to Tyr789 (Y\textsuperscript{789}ANF) fits the consensus binding site for the SH2 domain (YXNX) of the adaptor protein Grb2, and Grb2 has been shown to associate with PTPα \textit{in vivo} (den Hertog et al., 1994; Su et al., 1994). The association between
PTPα and Grb2 results in a steric hindrance effect rendering PTPα-bound Grb2 unable to bind Son of sevenless (Sos), the downstream effector in the Grb2-Ras pathway, resulting in PTPα-mediated inactivation of the Grb2-Ras pathway (den Hertog et al. 1996; Su et al., 1996).

PTPα tyrosine phosphorylation was recently proposed to enable PTPα-mediated dephosphorylation of Src-Tyr527 during mitosis and play a role in Src activation. Zheng et al. (2002) discovered that overexpression of wild-type PTPα resulted in ~30% decrease in the level of Src tyrosine phosphorylation \textit{in vivo} and a three-fold increase in Src-specific kinase activity while overexpression of mutant PTPα (Y789F) did not affect either the Src phosphotyrosine level or kinase activity. This suggested that without the Tyr789 residue PTPα is unable to act on Src \textit{in vivo} (Zheng et al., 2000). This result was corroborated by \textit{in vitro} kinase assays that showed that pre-incubation of immunoprecipitated Src with immunoprecipitated wild-type PTPα resulted in a significant increase in Src kinase activity compared to pre-incubation of Src with immunoprecipitated PTPα (Y789F) (Zheng et al., 2000). Based on these observations, Zheng et al. (2000) proposed a phosphotyrosine displacement model in which phosphorylated-Tyr789 of PTPα binds to the Src-SH2 domain and displaces pTyr527, thereby rendering it more accessible to PTPα catalytic activity.

Although the experimental results of Zheng et al. (2000) show that PTPα-Tyr789 phosphorylation is required for Src dephosphorylation and activation, there are certain critical issues that need to be addressed. It was suggested that the binding between PTPα and Src will be stabilized upon PTPα-mediated Src-Tyr527 dephosphorylation after its displacement from Src-SH2 by pTyr789 of PTPα (Zheng et al., 2000). The reduced Src dephosphorylation and reduced binding between the catalytically-inactive PTPα (C433S/C723S) mutant and Src is consistent with the above displacement model (Zheng et al., 2000). In contrast, another study has shown...
that catalytically active PTPα with the Y789F mutation still dephosphorylates Src (Chen et al., 2006). PTPα-mediated Src activation was inhibited by expression of the Src-SH2 domain suggesting that the SH2 domain competed with full length Src to bind PTPα at pTyr789 (Zheng et al., 2000). However, the possibility exists that inhibition of PTPα-mediated Src activation was due to the protection of the pTyr527 by the exogenously expressed SH2 domain.

Subsequent work by the same authors (Zheng et al., 2001) suggests that PTPα-pTyr789-mediated displacement of Src-pTyr527 during mitosis is dependent on hyperphosphorylation of serine residues in the juxtamembrane region of PTPα. It was shown that serine hyperphosphorylation of PTPα resulted in differential reduction of the binding affinity of PTPα for the Grb2-SH2 domain compared to the Src-SH2 domain as binding between PTPα and Grb2 was reduced during mitosis while binding between PTPα and Src was increased. Recently, however, it was shown that binding of PTPα and Src during mitosis is dependent upon PP2A-mediated dephosphorylation of Ser204 in the juxtamembrane region of PTPα (Vacaru and den Hertog, 2010). Moreover, it was demonstrated that the binding between PTPα and Src is not mediated by pTyr-SH2 interactions as Src bound PTPα (Y789F) in mitotic cells (Vacaru and den Hertog, 2010). Therefore, although it is evident that PTPα acts as an upstream activator of Src in mitosis, the role of tyrosine (and serine) phosphorylation of PTPα in Src activation during this process requires further investigation.

The role of tyrosine phosphorylation in PTPα has been investigated for other signalling events/mechanisms. It was demonstrated that PTPα undergoes tyrosine phosphorylation in response to integrin stimulation in mouse embryonic fibroblasts (MEFs) (Chen et al. 2006). Phosphorylation of PTPα at Tyr789 upon integrin engagement with fibronectin was dependent on the activity of SFKs and, although tyrosine phosphorylation of PTPα was not required for
SFK activation, Src dephosphorylation was severely inhibited in PTPα-null fibroblasts transfected with catalytically inactive PTPα (Chen et al., 2006). Hence, PTPα-catalyzed SFK activation was responsible for SFK-catalyzed tyrosine phosphorylation of PTPα at Tyr789 upon integrin stimulation (Chen et al., 2006). Furthermore, FAK was also shown to play an important role in SFK-dependent PTPα tyrosine phosphorylation in this system (Chen et al., 2006).

Recent investigation of the roles of other extracellular factors in the stimulation of PTPα Tyr789 phosphorylation through the activation of receptor-based signalling pathways has revealed that growth factors such as insulin-like growth factor 1 (IGF-1) and acidic FGF, and small molecule stimuli such as phorbol 12-myristate 13-acetate (PMA) and lysophosphatidic acid (LPA), increase tyrosine phosphorylation of PTPα. However, the underlying mechanisms behind these phenomena remain undetermined (Chen et al., 2009). PTPα Tyr789 phosphorylation was increased upon activation of SFKs in T cells where it regulates the dephosphorylation of the SFK Fyn, and plays an important role in T cell receptor signalling and CD44-mediated cell spreading (Maksumova et al., 2007). Also, PTPα oxidation has been shown to be a major regulatory mechanism of PTPα signalling in cells. For example, H₂O₂ treatment of MEFs suppressed tyrosine phosphorylation of PTPα (Hao et al., 2006). However, the underlying mechanism responsible for this also remains unknown.

1.3.3.3 Serine phosphorylation of PTPα

As mentioned in sections 1.2.3.1 and 1.2.3.2, regulated serine phosphorylation of PTPα has been suggested to play a role in PTPα dimerization (Tracy et al., 1995) and Src dephosphorylation and activation in mitosis (Zheng et al., 2001; Vacaru and den Hertog, 2010). In the process of mitosis the characterization of PTPα serine phosphorylation regulation has been
a subject of active investigation. Recently, two models have been proposed to assign functions to the PTPα serine residues during mitosis. In the first model, hyperphosphorylation of two serine residues, Ser180 and Ser204, in the juxtamembrane domain of PTPα was suggested to decrease the affinity of PTPα for the Grb2-SH2 domain and increase the binding of PTPα to the Src-SH2 domain leading to Src dephosphorylation and activation (Zheng et al., 2001). Protein kinase C delta (PKCδ) (Brandt et al., 2003) and CaMKIIα (Bodrikov et al., 2008) have been identified as putative serine kinases responsible for PTPα phosphorylation in this proposed model. In the second model, decreased PTPα and Grb2 association was found to be dependent on PP2A-mediated dephosphorylation of Ser204 (Vacaru and den Hertog, 2010). The latter model suggests that phosphorylation of Ser204 inhibits Src binding to PTPα and during mitosis Ser204 is dephosphorylated by PP2A. This is followed by the binding of Src to a site/region other than PTPα pTyr789. The resulting close proximity of PTPα and Src leads to PTPα-mediated dephosphorylation and activation of Src (Vacaru and den Hertog, 2010). Although serine phosphorylation is regarded as an important regulator of PTPα activity and function, the mechanism(s) behind this regulation remains to be elucidated.

1.3.4 Cellular functions

PTPα, as a well characterised and widely expressed activator of SFKs, has been shown to play important in several cellular and physiological processes. In both PTPα-null cell and mouse model systems, PTPα was linked to the regulation of N-methyl-D-aspartate (NMDA) receptor activity, voltage-gated potassium ion channel activity, integrin signalling, mitosis, myelination and T-cell activation and mast cell degranulation.
PTPα catalyzes the SFK-mediated phosphorylation and activation of N-methyl-D-aspartate (NMDA) receptor in brain (Lei et al., 2002; Petrone et al., 2003; Le et al., 2006). In addition to activation of Kv1.2 potassium channels in response to m1 muscarinic acetylcholine receptor signalling, PTPα plays an important role in neurotransmitter release at pre-synaptic nerve endings (Tsai et al., 1999). In response to integrin engagement, PTPα has been shown to influence the integrin-induced cell changes such as spreading, focal adhesion formation, cytoskeletal rearrangement and cellular migration (Zeng et al., 2003; Chen et al., 2006). PTPα has also been implicated as a positive regulator of cell cycle progression and mitosis (Zheng et al., 2001; Su et al., 1996). Additionally, PTPα is required for the activation of the T-cell receptor and is a positive regulator of CD44-mediated T-cell spreading (Maksumova et al., 2005; Maksumova et al., 2007).

Investigation of the role of PTPα in FcεRI (high-affinity receptor for the Fc region of immunoglobulin E (IgE))-mediated mast cell activation and IgE-dependent allergic responses in mice demonstrated that PTPα functions as a negative regulator of bone marrow-derived mast cells (BMMCs) degranulation and IgE-dependent anaphylaxis. PTPα-null BMMCs exhibit hyperdegranulation and increased cytokine and cysteiny1 leukotriene secretion, and enhanced IgE-dependent anaphylaxis is observed in PTPα-null mice (Samayawardhena and Pallen, 2010). In summary, PTPα is involved in multiple cellular and physiological roles which are defined by cell/tissue types and signal transduction pathways.

1.3.4.1 PTPα and cell migration

Orchestrated movement of cells in a particular direction is required for immune responses, wound healing and tissue development, among other cellular processes, and is termed
cell migration. Recently, PTPα has been shown to have an important role in the migration of several cell types.

Tyrosine phosphorylation of PTPα is required for optimal cell spreading, cytoskeletal rearrangement and focal adhesion formation in response to engagement of receptor integrins by ECM components. The catalytic activity of PTPα is required for fibronectin-stimulated SFK activation (Su et al., 1999; Zeng et al., 2003), following which the multiprotein complex of SFK and SFK-activated FAK catalyze the tyrosine phosphorylation of PTPα (Chen et al., 2006). Tyrosine phosphorylation of PTPα and assembly of the activated SFK-FAK multiprotein complex then initiates downstream integrin signalling events to influence cellular migration. Fibronectin-stimulated PTPα-null MEFs have reduced migration compared to wildtype MEFs and overexpression of a non-phosphorylatable mutant form of PTPα, PTPα-(Y789F), in PTPα-null cells cannot restore this migration response (Chen et al., 2006). As integrin signalling regulates the actin cytoskeleton and formation of focal adhesion complexes through the activity of the Rho GTPases such as Rho, Rac and Cdc42, it was suggested that PTPα is either an upstream activator or a downstream effector of these GTPases (Chen at al., 2006). This is also supported by the work of by Herrera Abreu et al. (2008). Also, phosphorylation of Gab2, a Fyn SFK substrate, and defective Rho GTPase signalling in PTPα-null bone marrow mast cells (BMMCs) were identified as key causes for reduced cell migration of these cells (Samayawardhena and Pallen, 2008).

Mast cells migrate and polarize in response to the c-Kit ligand, stem cell factor (SCF). SCF binding to c-Kit promotes c-Kit phosphorylation at Tyr567/569 and Tyr719, and SFK activation, both of which are impaired in PTPα-null BMMCs. As pre-treatment of wild-type BMMCs with a general SFK inhibitor, SU6656, resulted in a comparable reduction of the
phosphorylation of the c-Kit receptor at Tyr567/569 and Tyr719 to that observed in PTPα-null BMMCs, it was suggested that PTPα-mediated SFK activation was required for SCF-stimulated c-Kit phosphorylation (Samayawardhena and Pallen, 2008). These authors further demonstrated that PTPα was required for SCF-induced Fyn mediated mast cell spreading, polarization, and cell migration (Samayawardhena and Pallen, 2008).

PTPα-dependent Fyn activation was also found to be important for cell spreading in T-cells (Maksumova et al., 2007). PTPα may have a role in T-cell migration as well, as cell spreading is an important early determinant of cell migration (Ngalim et al., 2010). PTPα plays an important role in cell migration of several cell types and further characterization of this role of PTPα may prove beneficial for understanding the dynamics of cell migration of normal and tumour cells.

1.4 IGF-1 signalling

Proper development of an organism is dependent upon proper regulation of its cellular growth and differentiation. The regulation of these processes is regulated, in part, by a composite family of pleiotropic hormones - insulin and insulin-like growth factors (IGF-1 and IGF-2), their respective receptors, insulin receptor (IR) and insulin-like growth factor receptor (IGF-1R), and additional extracellular binding proteins called insulin-like growth factor binding proteins (IGFBPs) (LeRoith et al., 2001; Rosenfeld, 2005). However, there exists a ‘division of labour’ among the insulin and IGF ligands, and their respective receptors (Werner et al., 2008). The insulin/insulin growth factor family can be compartmentalized and, while the insulin/insulin receptor (IR) pathway controls glucose uptake and the metabolism of carbohydrates, lipids and proteins in cells (Siddle et al., 2001), insulin-like growth factors (IGFs) and insulin-like growth
factor receptors (IGFRs) are responsible for the promotion of cellular growth, survival, differentiation and migration (Siddle et al., 2001; Werner et al., 2008; Adams et al., 2000, Yakar et al., 2005). In addition, extensive research in the last decade has implicated IGF-1 signalling to play a crucial role in the development and progression of cancer (Baserga et al., 1997; Belfiore et al., 1999; Andrews et al., 2001; Sciacca et al., 2002; Zhao et al., 2004; Kasprzak et al., 2006; Armakolas et al., 2010; Sichani et al., 2010). For the purpose of this study, IGF-1/IGF-1R signalling will be discussed in greater detail.

1.4.1 Components of IGF-1 signalling: IGFs, IGF-BPs and IGF-1R

The IGF signalling system is comprised of two insulin-like ligands (IGF-1 and IGF-2), two cell surface receptors (IGF-1R and IGF-2R), and a family of six high affinity insulin-like growth factor binding proteins (IGFBPs). The range of biological functions exhibited by the IGF system is dictated by the interactions between different IGF ligands and IGF receptors, between ligands and the IGF binding proteins and between the IGF binding proteins and the IGF receptors (Fig. 4).

1.4.1.1 Insulin-like growth factors (IGFs)

The insulin-like growth factors (IGFs) are highly homologous, short, single-chain polypeptides 7 to 8 kD in size. Their molecular structure consist of the A- and B-chains, the receptor binding domains, the C-peptide bridge between the A- and B-chains that directs preferential binding to IGF-1R, the D-domain, with a role in promoting the binding affinity of IGF-2 to receptors, and the E-domain. The polypeptides are derived from pre-propeptides like insulin. However, in insulin processing post-translational modifications leads to the cleavage of the C-peptide bridge, whereas IGF-1 and -2 retain the C-peptide bridge. In addition, post-
translational modification leads to the cleavage of the C-terminal E-domain in mature IGF-1 and -2 (Rinderknecht and Humbel, 1978; Denley et al., 2005). Both IGF-1 and IGF-2 are expressed in high concentration during embryogenesis in mice. Levels of IGF-1 remain high in systematic circulation after birth while the levels of IGF-2 decrease. Indeed, IGF-2 is only expressed in the exchange tissue of the brain in adult mice (Baker et al., 1993). In humans, IGF-1 is more mitogenic and growth-promoting in normal and cancer cells than IGF-2 (Neely et al., 1991). A major source of IGF-1 production is the liver where its synthesis is predominantly regulated by growth hormone and insulin (Boni-Schnetzler et al., 1991; Gianotti et al., 1998). However, IGF-1 is also synthesized in most other body tissues and, as such, is implicated in both endocrine and autocrine/paracrine activities (LeRoith et al., 2001). Conversely, IGF-2 synthesis is growth hormone independent and, though detectable in serum in human adults, its expression is highest during fetal development (Tabano et al., 2010; Tang et al., 2006). Located on chromosome 11p15.5, the IGF-2 gene is transcribed from four different promoters and is subject to genetic imprinting. IGF-2 functions as a hormone mostly during the gestation period but has also been implicated in the pathogenesis of hypoglycemia (Tabano et al., 2010; Baxter et al., 1995).

1.4.1.2 Insulin-like growth factor binding proteins (IGFBPs)

Insulin-like growth factor binding proteins (IGFBPs) are a family of six conserved, secreted proteins that bind IGF ligands with high affinities. Six IGFBPs, IGFBP-1 to -6, have been characterized in humans, and their genes have been localized to different chromosomes. IGFBP-1 and -3 map to chromosome 7, IGFBP-4 and IGFBP-6 map to chromosomes 17 and 6, respectively, and IGFBP-2 and -5 are located on chromosome 2 (Firth and Baxter, 2002; Kelley et al., 2002; Wood et al., 2005). With relatively low molecular weights, the IGFBPs have a
conserved N-terminal domain responsible for the high affinity binding of IGFs, a variable linker
domain known as the L-domain, and a highly conserved cysteine rich C-terminal domain
containing a nuclear localization signal (NLS) that coordinates intermolecular interactions with
other proteins. The affinity of IGFBP binding to the IGFs is regulated by several post-
translational modifications, such as glycosylation, phosphorylation, and proteolysis, which target
the L-domain of the IGFBP molecules (Duan et al., 1999; Maures and Duan, 2002; Clemmons,
2001). IGFBPs sequester IGFs, more so IGF-1 than IGF-2, and inhibit their activity as well as
regulating their physiological concentrations. The half-life of the IGFs in blood plasma is
increased by binding IGFBPs. In addition, IGFBPs transport IGFs to target tissues prevent cross-
binding of the IGFs to the insulin receptor (Jones and Clemmons, 1995; Guler et al., 1987;
Rajaram et al., 1997). IGFBPs are regulated by a number of proteins that bind and inactivate
them, thereby increasing the bioavailability of IGFs. These binding proteins include components
of the extracellular matrix (ECM), transmembrane proteins such as α5β1 integrin (Margot et al.,
1989) and the TGFβ receptor (Morales, 1997), and certain intracellular and nuclear proteins such
as retinoic acid receptor-α and importin-β (Schedlich et al., 2007). Furthermore, IGFBPs are
degraded by proteases such as thrombin, plasmin and cathepsins (Conover et al., 1995; Fowlkes,
1997).

1.4.1.3 Insulin-like growth factor receptors (IGF-1R and IGF-2R)

The IGFs mediate their biological effects through insulin-like growth factor receptor type
1 (IGF-1R) and type 2 (IGF-2R). IGF-1R belongs to the family of receptor tyrosine kinases. It is
a hetero-tetramer comprising two extracellular α subunits critical for IGF ligand binding, and
two transmembrane β subunits responsible for transducing signals intracellularly. The
conformation of the IGF-1R is β-α-α-β. The extracellular region of IGF-1R, specifically the α subunits, is comprised of six structural domains that establish a binding motif to accommodate the ligand molecules. This consists of two β helices (designated L1 and L2) separated by a cysteine-rich region (CR) and connected to three fibronectin type III domains. The CR is responsible for binding IGF-1 (Favelyukis et al., 2001). The β subunits have a hydrophobic sequence towards the N-terminal side which forms the transmembrane domain of the mature IGF-1R receptor. In addition, the juxtamembrane domains of the β subunits have an Asn-Pro-X-Tyr sequence known to function in receptor internalization and a tyrosine kinase catalytic domain (Luo et al., 1999; Sorensen et al., 2004).

Ligand binding induces a conformational change in the receptors followed by auto-phosphorylation of tyrosine residues in the activation loop within the tyrosine kinase catalytic domain (Luo et al., 1999). Phosphorylation of three specific tyrosine residues in this activation loop results in full activation of the catalytic activity of the receptor (Fig. 5). Initial phosphorylation occurs at Tyr1135 which is then followed by auto-phosphorylation of Tyr1131 and Tyr1136. As IGF-1R transitions from an unphosphorylated state to monophosphorylated (pTyr1135), biphosphorylated (pTyr1135 and pTyr1131) and triphosphorylated states (pTyr1135, pTyr1131, and pTyr1136) it becomes increasingly stable and enzymatically active (Favelyukis et al., 2001). Phosphorylation of these tyrosine residues triggers a major conformational change in the activation loop within the tyrosine kinase domain permitting ATP and substrate binding to the catalytic domain (Munshi et al., 2002). Signalling from the activated IGF-1R is dependent upon phosphorylation of intracellular substrates. IGF-1R activates several signalling pathways including the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and the MAPK/Erk pathway (Section 1.4.3). IGF-2R is different from IGF-1R in both structural and
functional aspects. IGF-2R, also designated as the mannose-6-phosphate receptor, is a monomeric glycoprotein that binds mannose-6-phosphate, IGF-2 and lysosomal proteins. However, IGF-2R lacks a cytoplasmic signalling domain and its sole function is to bind and internalize IGF-2 molecules for degradation, thus limiting the bioavailability of IGF-2 (Tarrago et al., 1999; McCusker and Novkofski, 2003). The IGF-1R forms the backbone of IGF-1 signalling and aberrant IGF signalling has been implicated in the pathogenesis of several cancers. As such, it presents an attractive target for anti-cancer therapies.

1.4.2 IGF-1R: a major drug target

IGF-1R has been found to be overactive and vastly expressed in cancer cells. Over the last decade it has been the subject of several cancer studies and the target of many experimental anti-cancer drug therapies. As evidenced by several in vivo studies, blocking IGF-1R activity induces apoptosis of cancer cells, and inhibits tumour cell invasion and metastasis (Hopfner et al., 2006; Lee et al., 2007; Haluska et al., 2006). Indeed, strategies that target IGF-1R expression are more potent in inhibiting cancer progression than strategies that target its downstream signalling or its tyrosine kinase activity (Sachdev et al., 2003; Macaulay et al., 2001), possibly owing to the fact that downregulation of IGF-1R expression results in complete inhibition of its function.

IGF-1R and its function have been targeted using a variety of methods. Most of the methods used block ligand/receptor interactions, interfere in its synthesis and/or expression, or inhibit its tyrosine kinase activity. Blocking the ligand/receptor interaction has been conventionally conducted using IGF-1R neutralizing antibodies. One of the most commonly used and extensively studied anti-IGF-1R antibodies is α-IR3 which competes with IGF-1 for IGF-1R
binding (Van Wyk et al, 1985) and has been shown to suppress the growth of human rhabdomyosarcoma cells in vivo (Kalebic et al., 1994). However, a disadvantage of IGF-1R blocking antibodies is that IGF-1R is overexpressed in cancer cells in many cases and under such conditions α-IR3 may act as an IGF-1 mimic to induce IGF-1R activity if its tyrosine kinase activity remains intact. For example, NIH-3T3 cells overexpressing human IGF-1R displayed an increased response to α-IR3 and facilitated the activation of downstream signalling (Kato et al., 1993). Another disadvantage of using monoclonal antibodies such as α-IR3 is that their large size prohibits optimal access to cancer cells in vivo, especially in targeting the central regions of solid tumours (Russell et al., 1992; Larsson et al., 2005). Recently, single-chain antibodies, such as scFv-Fc, have been found to be more successful in this respect, however some IGF-1R signalling still persists (Sachdev et al., 2003).

During the previous decade, other strategies were developed to block IGF-1R. A dominant-negative IGF-1R transfected into MDA-MB-435 metastatic breast cancer cells is secreted extracellularly causing a bystander effect. This inhibits IGF-1-stimulated invasion through collagen, and metastasis to the lungs, livers, lymph nodes and lymph vessels was significantly decreased in cells expressing dominant-negative IGF-1R compared to the vector control (Dunn et al., 1998). Similarly, truncated soluble IGFIR inhibited autophosphorylation of endogenous IGF-1R, resulting in the inhibition of tumorigenesis in syngeneic rats (D’Ambrosio et al., 1996). These techniques were successful in blocking IGF-1R activity but efficient drug uptake and administration in cells were not ideal. Also antisense RNA specifically targeting IGF-1R induced regression in tumorigenesis in cells in vivo and ex vivo (Resnicoff et al., 2004; Andrews et al., 2001).
Small molecule inhibitors are another strategy to interfere with IGF-1R activity. This has been used extensively in IGF-1/IGF-1R related cancer studies as this approach facilitates greater bioavailability of IGF-1R inhibiting agents compared to monoclonal antibodies or antisense oligonucleotides and is easier to administer (Larsson et al., 2005; Gable et al., 2006). Small molecular inhibitors compete with ATP molecules for access to the catalytic active site and prevent phosphorylation of intracellular substrates by IGF-1R. However, most first-generation IGF-1R inhibitors were found to have substantial reactivity with the very similar insulin receptor and resulted in adverse secondary effects (Larsson et al., 2005). Recently, the development of second-generation molecular inhibitors, such as pyrrol[2,3-d] pyrimidine and cyclolignan PPP, specifically interact with IGF-1R to neutralize its kinase activity (Garcia-Echeverria et al., 2004; Girnita et al., 2004). Substrate-competitor small molecule inhibitors such as AG 538 tyrphostin bioisostere prevented IGF-1 receptor autophosphorylation and the activation of its downstream targets PKB and Erk2 in intact cells (Blum et al., 2003).

1.4.3 IGF-1 signal transduction

Ligand binding to the α-subunits of IGF-1R leads to the phosphorylation of three specific tyrosine residues in the activation loop within the tyrosine kinase domain in the β-subunits and causes the activation of IGF-1R catalytic activity. This further leads to the subsequent phosphorylation of other tyrosine residues in the IGF-1R β subunits which provides docking sites for IGF-1R substrates that initiate downstream signalling mechanisms (Migliaccio et al., 1997). The initiation of two key signalling pathways, the MAPK/Erk pathway and the PI3K/Akt pathway, begins with the binding of two sets of adapter proteins, the Src-homology domain-containing adapter protein Shc and the insulin receptor substrates (IRSs), to the IGF-1R β
subunit (Migliaccio et al., 1997; Butler et al., 1998). Although there are three isoforms of Shc, all gene splice variants, and four isoforms of IRS proteins, bind to the same docking site, phosphorylated Tyr950, on the IGF-1R β subunits via their phosphotyrosine-binding (PTB) domains (Craparo et al., 1995; Dey et al., 1996; Tartare-Deckert et al., 1995; Xu et al., 1999). IGF-1R then phosphorylates these proteins on tyrosine. Tyrosine phosphorylated Shc binds to growth factor receptor-bound protein-2 (Grb2), an adaptor protein that then cooperates with the guanine nucleotide exchange factor Son of sevenless (Sos) to activate the GTP-binding protein Ras by the catalytic replacement of GDP with GTP (El-Shewy et al., 2006; Xu et al., 1999). Activated Ras leads to the activation of Raf which then leads to the phosphorylation of MAPK/Erk kinases, MEK1 and MEK2, and the downstream phosphorylation of extracellular signal-regulated kinases-1 and -2 (Erk 1and Erk 2) (De Meyts et al., 1994; LeRoith et al., 2003). Activated MAP kinases activate other intracellular proteins such as transcription factors and other kinases responsible for proliferative and differentiative effects in cells (Su and Karin, 1996).

IGF-1R-activated IRS adapter proteins couple IGF-1R to the phosphatidylinositol-3-kinase (PI3K) pathway. Activation of the PI3K pathway requires the binding of the p85 and p110 subunits of PI3K to IRS promoting the conversion of phosphatidylinositol-4,5-biphosphate (PIP$_2$) to phosphatidylinositol-3,4,5-triphosphate (PIP$_3$) (Sun et al., 2006). This activates the phosphoinositide-dependent kinase-1 (PDK1), which in turn is responsible for activating the serine/threonine kinase Akt. Akt performs the dual role of activating or inhibiting downstream effectors via phosphorylation. Akt inhibits negative regulators of cellular metabolism, cell survival, and proliferation, such as glycogen synthase kinase-3 (GSK3), the Bcl-2 family member Bad, and the cell cycle inhibitor p27, respectively (Leinninger et al., 2004; Duarte et al.,
Act also activates the mammalian target of rapamycin (mTOR) by inactivating its negative regulator tuberous sclerosis complex-2 (TSC2) by phosphorylation. mTOR activates the 4E-binding protein-1 (4EBP1) and S6 kinase (S6K), leading to enhanced protein synthesis and cell growth (Nave et al., 1999; Chenal et al., 2008). In addition to the activation of the MAPK and PI3K pathways, several other proteins which bind directly to the intracellular domains of the activated IGF-1R are activated in response to IGF-1 stimulation and facilitate downstream signalling. These include members of the Crk family of proteins which are implicated as a link between the activation of IGF-1R and the integrin signalling pathway. Crk proteins form complexes with focal adhesion proteins such as paxillin, p130Cas and FAK, which coincides with increased tyrosine phosphorylation of these proteins in response to IGF-1 (Casamassima and Rozengurt, 1998; Leventhal et al., 1997). The 14.3.3 proteins, a unique family of cytoplasmic proteins, bind to activated IGF-1R and function in neurotransmitter biosynthesis, cell cycle progression and cellular survival upon IGF-1 stimulation (Zha et al., 1996; Aitken et al., 1995). Another signalling molecule that was recently shown to directly associate with IGF-1R is the apoptosis signal-regulated kinase (ASK)-1. IGF-1R can directly phosphorylate and inhibit ASK-1 activity, which is involved in the regulation of the jun-N-terminal kinase (Jnk), although this pathway remains to be properly characterized (Krause et al., 2001; Kim et al., 2001; Aikin et al., 2004).

Recently, IGF-1R has been implicated in JAK/STAT signalling. The janus kinases (JAKs)/signal transducers and activator of transcription (STAT) pathway is activated in response to cytokine receptor activation by ligand binding (Kisseleva et al., 2002). As cytokine receptors lack intrinsic enzymatic activity, JAKs bind to the cytoplasmic domains of these receptors leading to the trans-autophosphorylation of JAKs. This facilitates the creation of phosphorylated
tyrosine docking sites on the receptor-bound JAKs for molecules such as the STATs which become activated upon tyrosine phosphorylation. Tyrosine phosphorylated STATs dimerize and translocate to the nucleus where they function as transcription factors to regulate transcription of genes that influence growth, survival and differentiation of cells (Kisseleva et al., 2002; Vinkemeier et al., 1998). Activation of IGF-1R has been shown to lead to the activation of STATs (namely STAT-1 and -3) in a number of studies (Yadav et al., 2005; Takahashi et al., 1999; Zong et al., 2000; Ebong et al., 2004), and JAKs are thought to be involved in this process (Zong et al., 2000; Gual et al., 1998; Yadav et al., 2005). However, investigations into IGF-1-stimulated JAK/STAT signaling are still in the early stages and this pathway requires further characterization.

Dysregulation of IGF-1/IGF-1R signalling has been associated with the pathogenesis of many cancers in the past two decades (Kablebic et al., 1994; Karrnieli et al., 1996; Kim et al., 1996; Hankinson et al., 1998; Girnita et al., 2000; Sachdev et al., 2003; Zhao et al., 2004; Petricoin et al., 2007; Tornkvist et al., 2008). IGF-1R overexpression and enhanced activation of IGF-1 mediated signalling pathways have been observed in several cancers, such as breast (Schillaci et al., 2006), colon (Bauer et al., 2007), liver (Kong et al., 2008), and prostate carcinomas (Kawada et al., 2006) as well as several types of melanomas (Girnita et al., 2006; Leroith and Roberts, 2003). Aberrant IGF-1R signalling promotes the transformation of normal cells as cells with downregulated expression of IGF-1R and IGF-1R-related signalling components were immune to the transformative effects of various oncogenes (Sell et al., 1994; Valentinis et al., 1994; Coppola et al., 1994). IGF-1R activity is crucial for anchorage-independent growth, a property uniquely associated with malignant cells (Baserga, 1999; Urbanska et al., 2007). Overexpression of IGF-1R and activation of its downstream pathways
result in anti-apoptotic effects in cancer cells (Resnicoff et al., 1994; 1994b; Reiss et al., 1998; Sachdev et al., 2003), and enhance cell motility and metastasis in many cancers (Nolan et al., 1997; Reiss et al., 2001; Jackson et al., 2001), especially breast cancer cells. For example, Dunn et al. (1998) demonstrated that a soluble form of fragmented IGF-1R inactivates IGF-1R function and inhibits the motility of MDA-MB-435 breast cancer cells. Additionally, IGF-1R was identified as a novel upstream activator of endogenous Abelson kinase-1(Abl-1) in breast cancer cells (Srinivasan et al., 2008). Abl kinases such as c-Abl (Abl-1) are active in breast cancer cells and responsible for enhancing the invasive and metastatic properties of these cells (Srinivasan and Plattner, 2006). In MDA-MB-435 breast cancer cells, IGF-1-induced cell cycle progression, cellular proliferation growth and migration is dependent upon c-Abl activity (Srinivasan et al., 2008; Srinivasan and Plattner, 2006; Wang et al., 2007).

Several interactions between IGF-1R and oncogenes have been described in cancer progression. It has been suggested that the promoter of IGF-1R is targeted by oncogenes in transformed cells resulting in a drastic increase in the level of transcription of IGF-1R. This also leads to IGF-1R being constitutively active (Kim et al., 1996). Oncogenes such as c-myb and hepatitis B virus X protein target the IGF-1R promoter and function in this manner (Kim et al., 1996). Certain oncogenes, such as Src, catalyze phosphorylation of the cytoplasmic β subunit of IGF-1R leading to constitutive activation of downstream signalling (Werner and Le Roith, 2000). Conversely, certain tumour suppressors, such as p53 and WT1 (Wilm’s tumour 1 gene) act to downregulate IGF-1R expression and function and loss of function of these tumour suppressors in cancer cells results in activation of IGF-1/IGF-1R signalling (Girnita et al., 2000; Lee et al., 2003; Werner et al., 1993; Gerald et al., 1995).
1.5 PTPs and PTPα in IGF-1 signalling

IGF-1R signalling involves PTPs that catalyze reversible phosphorylation of its substrates and downstream signalling components. PTPs were first recognized to function in insulin and insulin-like growth factor signalling during studies of insulin and IGF-1 resistance. It has been suggested that PTPs function to counter the redundancy of IGF-1 signalling arising from the highly similar IR and IGF-1R receptors. Though the substrates directly targeted and activated by IR and IGF-1R are similar, various PTPs may promote different downstream signalling pathways through regulation of different proteins (O’Connor, 2003; O’Connor et al., 1997).

During the past decade several PTPs have been demonstrated to be involved in IGF-1 signalling. PTP1B, a non-transmembrane, intracellular “classical” PTP can modulate tyrosine phosphorylation of the IGF-1R. For example, PTP1B-deficient mouse embryonic cells exhibit enhanced IGF-1R phosphorylation (Buckley et al., 2002). In these cells, IGF-1 stimulated activation of the MAPK/Erk and PI3K/Akt pathways were increased and these cells displayed enhanced cell migration and survival (Buckley et al., 2002). It was suggested that PTP1B plays a negative regulatory role in IGF-1 signalling. Conversely, the SH2 domain-containing PTP Shp2, also a non-transmembrane intracellular “classical” PTP, enhances MAPK activity upon IGF-1 stimulation in fibroblasts and often functions in complex with IRS proteins (Araki et al., 2003). Shp2 has also been implicated in linking integrin and IGF-1 signalling (Clemmons and Maile, 2005).

Recently, it was demonstrated that IGF-1 stimulation of MEFs enhanced Tyr789 phosphorylation in PTPα (Chen et al., 2009). As this tyrosine phosphorylation event is at least partially required for PTPα-dependent cell migration, it is posited that IGF-1-stimulated PTPα
Tyr789 phosphorylation may be critical in IGF-1-stimulated cell migration of normal and tumour cells. However, the mechanism behind this phenomenon is unknown and remains to be investigated.

1.6 Hypothesis

It is well known that migration is a fundamental cell process that is influenced by interactions between the cell and its environment; the latter includes other cells, the extracellular matrix (ECM), and a variety of extracellular soluble growth factors such as IGF-1. Improperly regulated cell migration is a hallmark of cancer metastasis. In fact, in cases of childhood cancers such as neuroblastoma, IGF-I-stimulation is known to modulate many aspects of neuroblastoma biology, including motility and invasiveness. However, little is known about the underlying mechanisms. As discussed earlier, our lab has found that PTPα is a positive regulator of cell migration in several cell systems. Previous investigations have shown that PTPα is phosphorylated at Tyr789 in response to stimulation with fibronectin and IGF-1, and while the fibronectin-stimulated PTPα Tyr789 phosphorylation has been identified as an essential requirement for integrin-induced migration of MEFs, the role of IGF-1-stimulated PTPα Tyr789 phosphorylation in cellular migration is unknown. I hypothesize that IGF-1-stimulated PTPα Tyr789 phosphorylation is a key event in IGF-1-stimulated migration of normal and tumour cells, and possibly in the invasion and metastasis of tumour cells. This hypothesis will be investigated through the following specific aims:

1) To elucidate the IGF-1 signalling mechanism that is responsible for enhancing PTPα Tyr789 phosphorylation
2) To investigate the importance of IGF-1 induced PTPα Tyr789 phosphorylation in tumour cell migration, specifically neuroblastoma cell migration
Figure 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-specificity PTPs which recognize phosphorylated serine and threonine residues as well as phosphotyrosine. Examples of members in each PTP class are shown. Abbreviations used: FN (fibronectin type III repeat), Ig (immunoglobulin-like repeat), D1 and D2 (tandem protein tyrosine phosphatase [PTP] domains), SH2 (Src-homology 2 domain), PEST (proline, glutamic acid, serine and threonine rich domain), CH2 (Cdc25 homology region 2), C2 (protein kinase C conserved region 2), PBM (PDZ binding motif), CAAX (prenylation motif),
FERM (4.1 protein, ezrin, radixin, moesin homology), Sec14p (Sec14p homology), and EyA D2 (EyA domain 2).
Figure 2. PTPα structural domains and interacting proteins.
The structure of PTPα is comprised of a short, glycosylated extracellular domain, a transmembrane domain and a cytoplasmic region containing two tandem catalytic domains (D1 and D2). Ser180 (S180) and Ser204 (S204) in the juxtamembrane region of PTPα can be phosphorylated by protein kinase C (PKC) or by CaMKIIα, while Tyr789 (Y789) tyrosine phosphorylation can be catalyzed by Src family kinases (SFKs) in integrin signaling. The D1 domain provides the majority of the catalytic activity and is responsible for the dephosphorylation of Src and potentially the Kv1.2 potassium channel. Other PTPα- interacting proteins include neuronal cell membrane molecules such as contactin, NCAM 140, and CHL1 and intracellular proteins such as Grb2 and Src.
Figure 3. Activation of Src and Src family kinases (SFKs).

(A) Src and other SFKs are maintained in an inactive closed conformation through intramolecular interactions between the SH2 domain and the phosphorylated Tyr527 (Y527) residue, 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 and/or by dephosphorylation of Y527 by PTPs such as PTPα, PTP1B, SHP1, and SHP2. The subsequent autophosphorylation of Src at Y416 in the kinase domain results in full kinase activation. Adapted from Pallen, 2003.
Figure 4. The insulin receptor family and related ligands.
The insulin-like growth factor 1 receptor (IGF-1R) shares significant homology with the insulin receptors, IR-A and IR-B. Heterodimers of IGF-1R and either IR-A or IR-B can form during protein processing inside the cell. IGF-2R, also known as the mannose-6-phosphate (M6P) receptor, has high affinity for binding IGF-2, but is a non-signaling receptor and functions as a “sink” that regulates the bioavailability of IGF ligands for binding to the IGF-1R. Most of the biological activities of the IGF ligands are mediated through IGF-1R. The IGFs are stabilized in the serum by a series of six insulin-like growth factor binding proteins (IGFBPs) indicated as BPs above. Adapted from Rowinsky et al., 2007.
Figure 5. Schematic diagram of the IGF-1R and its major signalling pathways.

IGF-1R is a heterotetradimer comprised of two extracellular α subunits, which contain cysteine-rich ligand-binding domain, and two β subunits with an extracellular and a transmembrane domain, and a cytoplasmic region containing a tyrosine kinase domain and C-terminal domain. Upon ligand binding, IGF-1R undergoes receptor cross-linking and autophosphorylation at several tyrosine residues, leading to the creation of multiple docking sites for the adaptor proteins IRS(1/2) and Shc. IRS binding leads to the activation of phosphoinositol 3’ kinase (PI3K) resulting in activation of Akt to mediate increased protein synthesis, cell growth, and survival. Shc binding to IGF-1R results in activation of the RAS/MAP kinase pathway leading to increased cell proliferation. Adapted from Zha and Lackner, 2010.
Chapter 2: Materials and methods

2.1 Cells

Wildtype (PTPα\(^{+/+}\)) and PTPα-null (PTPα\(^{-/-}\)) mouse embryonic fibroblasts (MEFs) have been described previously (Zeng et al., 2003) and were derived from embryos of wild-type mice or PTPα knockout mice, respectively and spontaneously immortalized. Cells from passages 32-40 were used in this study. The SYF (Src\(^{-/-}\)-Yes\(^{-/-}\)-Fyn\(^{-/-}\), FAK\(^{+/+}\), and FAK\(^{-/-}\) mouse embryo fibroblast cell lines were obtained from the American Type Tissue Collection (www.atcc.org). The cell lines were grown in Dulbecco’s modified Eagles’ medium (DMEM) containing 10% fetal bovine serum, penicillin and streptomycin. The SH-SY5Y neuroblastoma cell line, also from the American Type Tissue Collection (www.atcc.org), were grown in (1:1) DMEM/Nutrient Mixture F-12 (DMEM/F-12) Media supplemented with 10% fetal bovine serum, penicillin and streptomycin.

2.2 Antibodies and reagents

Antibodies raised against PTPα and phosphoTyr789-PTPα have been described previously (Zeng et al., 2003; Chen et al., 2006). The following antibodies were also used: anti-phosphotyrosine (4G10), phospho-Ser473-Akt, and Akt, all from Upstate Biotechnology, Inc.; IGF-1Rβ and c-Abl (K-12) from Santa Cruz Biotechnology; phospho-p44/42 Erk1/2, Erk1/2, RACK1, and anti-VSVG-Agarose beads from Cell Signaling Technology, Inc. Human recombinant IGF-1 was from Sigma, SU6656 was from EMD Biosciences and Protein A/G Plus-Agarose beads were from Santa Cruz Biotechnology. Imatinib (cat # I-5508) and nilotinib (cat # N-8207) were obtained from LC Laboratories (Woburn, MA).
2.3 Expression of exogenous PTPα

2.3.1 Adenoviral infection

Wildtype- or Y789F-PTPα-expressing adenovirus was obtained from stocks prepared previously (Chen et al., 2006) and used to infect PTPα−/− MEFs. MEFs were grown to 75-80% confluence and incubated with virus (~10,000 viral particles/cell) in 1 ml DMEM containing 10% FBS in a 10 cm dish at 37°C with occasional rocking. After incubation (90 minutes), DMEM containing 10% FBS (9 ml) was added and the cells were cultured for a further 24 hours.

2.3.2 Transient transfection

The pXJ41 vectors containing VSVG-tagged wild-type or mutant (C433/723S) PTPα were generated previously (Bhandari et al., 1998). For transient transfections, SYF cells were cultured to ~40%-45% confluence, washed once in serum-free media and transfected with the appropriate plasmids (5µg DNA/10 cm dish) using Lipofectamine™ 2000 (Invitrogen). The vectors (5µg) and Lipofectamine™ 2000 (Invitrogen) were each diluted in 0.1 ml serum-free DMEM at 37°C for 5 minutes, combined and incubated for 15 minutes at 37°C. Serum-free DMEM (0.8 ml) was added to the DNA:Lipofectamine mix and added drop-wise to cells. After incubating the cells for five hours, DMEM containing 10% FBS was added and the cells cultured for 24 h before further manipulation.

2.3.3 siRNA transfection

SYF cells were cultured to ~20%-25% confluence in 6 cm plates and washed once with serum-free media. RACK1 siRNA (mouse, ON-TARGETplus SMARTpool L-062125-00-0005, Dharmaco, Chicago, IL), c-Abl siRNA (mouse, ON-TARGETplus L-040285-00-005,
Dharmacon, Chicago, IL), scrambled control siRNA (siCONTROL Non-Targeting siRNA Pool number #2 D-001206-14-20, Dharmacon, Chicago, IL) and the RNAiMAX RNA transfection reagent (Invitrogen) were each diluted in 0.25 ml of serum-free DMEM, incubated for 5 min at 37°C, combined and incubated for a further 15 minutes at 37°C. Serum-free DMEM (0.5 ml) was added to the complex mix and the diluted siRNA (20 nM) was added drop-wise to the cells. After incubating the cells for 10 hours, 3.5 ml of DMEM containing 10% FBS was added and the cells were cultured for 32 hrs. Cells were serum-starved for 16 hours before growth factor stimulation (see section 2.4). SH-SY5Y neuroblastoma cells were transfected with 15nM human PTPα siRNA (ON-TARGETplus SMARTpool L-080089-01-0050, Dharmacon, Chicago, IL) following the same protocol.

2.4 Cell stimulation, lysis and immunoblotting

Cells were grown to ~ 80-90% confluency and serum starved for 20 hr prior to stimulation. The cells were washed once with serum-free DMEM and stimulated with 100 ng/ml insulin-like growth factor-1 (IGF-1) (Sigma) for the indicated times. In some experiments, the cells were pre-treated for 1 hr with SU6656 (2 µM or 10 µM), for 3 hr with imatinib (10 µM or 20 µM), or nilotinib (2.5 µM or 10 µM) in serum-free medium prior to IGF-1 stimulation. Cells were washed twice with ice-cold PBS and lysed with RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM Na3VO4, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin) and the lysates clarified by centrifugation at 12,000 rpm for 20 min. Protein concentration was determined with the Bio-Rad Protein Determination Reagent (Bio-Rad Laboratories). Samples containing 60 µg of protein (for c-Abl) or 25 µg of protein (all other proteins) were resolved by SDS-PAGE and
electrotransferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% bovine serum albumin (BSA) or 5% skim milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBST) for 1 hr at room temperature. Primary antibodies were diluted in PBST containing 5% BSA and incubated with the membranes overnight at 4°C. The membranes were washed twice with PBST and incubated with horseradish peroxidase-conjugated secondary antibody diluted in PBST for 1 hr at room temperature. The membranes were washed three times with PBST and the antibody-bound proteins detected with enhanced chemiluminescence (ECL).

2.5 Immunoprecipitation

Cells were lysed with modified RIPA lysis buffer lacking sodium deoxycholate and SDS, passed through a 25 gauge syringe and clarified by centrifugation at 12,000 rpm for 20 minutes. Protein concentration was determined as described in section 2.4 and 1.5 mg of lysate was precleared with 35 µl of Protein A/G Agarose beads (Santa Cruz Biotechnology) for one hour at 4°C. The precleared lysates were incubated with the appropriate antibody (10 µl) for one hour at 4°C, protein A/G agarose beads (80 µl) added and incubated a further 20 hours. The immunoprecipitates were washed three times in modified RIPA lysis buffer, bound proteins eluted with 40 µl of Laemmli sample buffer (5x: 10% SDS, 312.5 mM Tris-Cl, pH 6.8, 50% glycerol, 0.01% bromophenol blue, 25% β-mercaptoethanol) and subjected to electrophoresis and immunoblotting.
2.6 In vitro kinase assay

c-Abl was immunoprecipitated as described in section 2.5 using c-Abl-specific antibody (K-12). The immunoprecipitated c-Abl from 1000 µg cell lysate was incubated with 10 µg of recombinant Crk (Millipore), kinase buffer (20 mM HEPES, pH 7.1, 150 mM NaCl, 1% Triton X-100, 200 µM sodium vanadate, 10% glycerol, 1 mM MgCl₂, 2 mM MnCl₂, 1 mM phenylmethylsulfonyl fluoride, 2mM dithiothreitol and 10 µg/ml aprotinin) and 100µM ATP in a total volume of 40 µl for 30 min at 37°C. The reactions were centrifuged, stopped with Laemmlili sample buffer (10 µl) and the supernatant subjected to immunoblotting with anti-phosphotyrosine (4G10) antibody to detect tyrosine phosphorylated recombinant Crk. The proteins bound to A/G beads in the pellets were eluted with 40 µl of Laemmlili sample buffer and subjected to electrophoresis and immunoblotting.

2.7 Migration assays

MEFs were serum starved overnight, trypsinized and resuspended in serum-free DMEM containing 0.5% BSA. Cells (8x10⁴/100 µl) were resuspended in DMEM containing 0.5% BSA, placed into the top chamber of a Transwell insert (8.0 µm) with the underside of the membrane coated with 10 µg/ml fibronectin. The insert was placed into the chamber containing serum-free DMEM with 0.5% BSA, minus or plus 100 ng/ml IGF-1. The Transwells were incubated for 90 min at 37°C. Cells on the upper side of the membrane were removed by wiping with a cotton bud, and the cells on the bottom side of the membrane were fixed in methanol for 30 min at 4°C and stained with Giemsa solution (Sigma). The cells were visualized by light microscopy (Leica DM400B with Q-Imaging Retiga 1300I camera, 40X magnification) and cells were counted from
ten field of views. SH-SY5Y cells (8x10^4/100 µl/insert) were incubated for 6 hr in the Transwell chambers.

2.8 Data analysis

The Quantity One program (Bio-Rad Laboratories) was used to determine relative intensities of protein bands through densitometry. PTPα and c-Abl phosphorylation levels were quantified by normalizing the intensities of the phosphorylated protein bands to the intensities of PTPα and/or c-Abl protein bands. The association of RACK1 and c-Abl was measured in arbitrary units by normalizing the intensities of co-immunoprecipitated c-Abl to the intensities of the immunoprecipitated RACK1. Data are shown as the mean ± standard deviation and the $p$ values were determined using the student’s t-test.
Chapter 3: IGF-1-stimulated tyrosine phosphorylation of PTPα-Tyr789: identification of the responsible tyrosine kinase

3.1 Rationale

Our lab demonstrated that fibronectin-stimulated integrin signaling regulates PTPα-Tyr789 phosphorylation in fibroblasts, increasing it from the basal level detected in unstimulated (suspension) cells (Chen et al., 2006). While investigating if other stimuli might play a role in regulating PTPα-Tyr789 phosphorylation it was discovered that IGF-1 also stimulates the tyrosine phosphorylation of PTPα (Chen et al., 2009). However, the responsible kinase and mechanism that mediated this IGF-1-induced increase in PTPα-Tyr789 phosphorylation was unknown. Therefore, the first objective of this project was to identify the IGF-1-stimulated PTPα tyrosine kinase. I thus investigated if the kinases responsible for integrin-induced PTPα-Tyr789 phosphorylation, namely SFKs and FAK (Chen et al., 2006), or kinases that play important roles in known IGF-1-stimulated downstream pathways, play a role in IGF-1-stimulated PTPα-Tyr789 phosphorylation.

3.2 IGF-1-stimulated PTPα-Tyr789 phosphorylation is independent of the activity of Src family kinases

Upon integrin engagement, SFKs associate with β integrins (Obergfell et al., 2002; Arias-Salgado et al., 2003) and FAK (Schaller et al., 1994, 1999) while PTPα can associate with α, integrin subunits (von Wichert et al., 2003). This proximity is proposed to allow PTPα to catalyze the activation of SFKs that occurs in a manner independent of PTPα-Tyr789 (Chen et al., 2006). Activated SFKs then phosphorylate and activate FAK (Schaller et al., 1999) and the
ensuing active SFK-FAK complex phosphorylates PTPα-Tyr789 (Chen et al., 2006). The requirement for SFKs and FAK for the phosphorylation of PTPα-Tyr789 was demonstrated using mouse embryonic fibroblasts (MEFs) lacking Src, Yes, and Fyn (SYF cells) or FAK (FAK−/−) (Chen et al., 2006). In these cell types, the integrin-stimulated increase in PTPα-Tyr789 phosphorylation is abolished.

In order to determine whether IGF-1-stimulated PTPα-Tyr789 phosphorylation was also SFK dependent, SYF cells were serum-starved and treated with IGF-1. Even in the absence of the SFKs Src, Yes, and Fyn, the IGF-1-stimulated increase in PTPα-Tyr789 phosphorylation remained intact (Fig. 6A, lanes 1 and 2). IGF-1 treatment also induced the phosphorylation of Erk1/2 (Fig. 6A, third panel), MAP kinases that function in IGF-1 signalling in fibroblasts, confirming the efficacy of the IGF-1 stimulation.

To determine if other SFKs (other than Src, Yes, and Fyn) that might be expressed in fibroblasts have a role in IGF-1-stimulated PTPα-Tyr789 phosphorylation, SYF cells were treated with the SFK inhibitor SU6656 at a concentration (2 µM) at which SFKs are inhibited (Blake et al., 2000). The IGF-1-stimulated increase in PTPα-Tyr789 phosphorylation remained unaffected by the SU6656 treatment (Fig. 6A and 6B). SYF fibroblasts were also treated with a higher concentration of 10 µM SU6656. Although this reduced the IGF-1-stimulated increase in PTPα-Tyr789 phosphorylation, it is possible that this is due to the non-specific inhibition of non-SFK kinases in the system at this higher SU6656 concentration (Blake et al., 2000). To confirm the efficacy of the SU6656 treatment, wild-type MEFs were treated with this inhibitor and the IGF-1-stimulated PTPα-Tyr789 phosphorylation was determined. In the absence of IGF-1, SU6656 reduced the basal level of PTPα-Tyr789 phosphorylation to an extent equivalent to that observed in SFK-deficient SYF cells (Fig. 6C). However, despite the SU6656 treatment, the
wild-type MEFs showed a two-fold increase in IGF-1-stimulated PTPα-Tyr789 phosphorylation (Fig. 6C). This is similar to the IGF-1-stimulated fold-increase in PTPα-Tyr789 phosphorylation in SYF cells (Fig. 6A and B) but very different from the 10-20% increase observed in IGF-1-stimulated MEFs in the absence of SU6656 (Fig. 6C). Together, these observations indicate that SFKs mediate considerable basal phosphorylation of PTPα-Tyr789, but are not required for IGF-1-stimulated PTPα-Tyr789 phosphorylation.

3.3 IGF-1-stimulated PTPα-Tyr789 phosphorylation is independent of the activity of focal adhesion kinase (FAK)

Integrin-induced PTPα-Tyr789 phosphorylation requires the activity of FAK (Chen et al., 2006). To determine if FAK is likewise required for IGF-1-stimulated PTPα-Tyr789 phosphorylation, serum-starved wild-type and FAK-deficient (FAK−/−) MEFs were treated with IGF-1. As shown in Fig. 7, IGF-1 treatment of FAK−/− MEFs led to an increase in PTPα-Tyr789 phosphorylation to a level comparable to that in IGF-1 treated wild-type cells. The efficacy of the IGF-1 stimulation was confirmed by the IGF-1-induced phosphorylation of Erk 1/2 (Fig. 7). As observed with SYF cells, the level of PTPα-Tyr789 phosphorylation in serum starved and unstimulated FAK−/− cells was below the basal level observed in wild-type cells under similar conditions (Fig. 7). These experiments show that SFKs and FAK play a role in maintaining a high basal level of PTPα-Tyr789 phosphorylation in fibroblasts, but do not play a role in IGF-1-stimulated PTPα-Tyr789 phosphorylation.
3.4 IGF-1-stimulated PTPα-Tyr789 phosphorylation is independent of the activity of mammalian target of rapamycin (mTOR) and p70 S6 kinase 1 (S6K1)

Among the IGF-1 signalling pathways involved in IGF-1-stimulated phosphorylation of focal adhesion proteins that are key to cell migration, PI3 kinase (PI3K) and MAP kinase pathways have already been shown not to be required for the IGF-1-stimulated increase in tyrosine phosphorylation of PTPα (Chen et al., 2009). However, recently, the mammalian target of rapamycin (mTOR)/S6 kinase pathway was shown to regulate the IGF-1-induced tyrosine phosphorylation of the focal adhesion proteins FAK, paxillin, and Cas – all players in cellular migration (Liu et al., 2008). Since PTPα plays an important role in cellular migration (Chen et al., 2009) and like FAK it can localize to focal adhesions (Lammers et al., 2000), I investigated whether the phosphorylation of PTPα in response to IGF-1 requires mTOR activity.

SYF cells were treated with or without IGF-1 in the absence or presence of rapamycin, a specific mTOR inhibitor (Brown et al., 1994; Kunz et al., 1993), at the same concentration shown to inhibit IGF-1-stimulated FAK phosphorylation (Liu et al., 2008). No reduction in IGF-1-stimulated PTPα-Tyr789 phosphorylation was observed in cells pre-treated with rapamycin (Fig. 8A and B). Since S6K1 is a downstream substrate of mTOR and its phosphorylation/activation is mTOR-dependent (Jefferies et al., 1997), the cell lysates were also probed with phospho-S6K1 antibody to analyze the efficacy of the rapamycin treatment. The observed downregulation of S6K1 phosphorylation (Fig. 8A) confirmed the effectiveness of the rapamycin treatment. Thus, the data from these experiments led to the conclusion that IGF-1-stimulated PTPα-Y789 phosphorylation is independent of the activity of mTOR.
3.5 IGF-1-stimulated PTPα-Tyr789 phosphorylation is dependent on the activity of Abelson kinase 1 (c-Abl)

A recent study revealed another mechanism by which IGF-1 can regulate the tyrosine phosphorylation of FAK (Kiely et al., 2009). As previously reported, IGF-1 treatment of MCF-7 breast carcinoma cells activates the tyrosine kinase c-Abl (Srinavasan et al., 2008). Kiely et al. (2009) demonstrated that activated c-Abl phosphorylates the scaffolding protein RACK1 (receptor for activated protein kinase C) to promote and stabilize the interaction of RACK1 and FAK. Interestingly, this induces FAK tyrosine dephosphorylation, a key step in the dynamic cycles of FAK phosphorylation and dephosphorylation that are essential for cell migration (Guvakova et al., 1999; Kiely et al., 2005; Manes et al., 1999; Mauro et al., 1999). These findings demonstrating that c-Abl is an IGF-1-stimulated kinase prompted me to investigate whether IGF-1-stimulated PTPα-Tyr789 phosphorylation is dependent upon the activity of c-Abl.

SYF fibroblasts were treated with two well-known c-Abl inhibitors; imatinib and nilotinib. Imatinib, a derivative of 2-phenylaminopyrimidine, was developed as a Bcr-Abl inhibitor for the treatment of chronic myelogenous leukemia (CML) (Heinrich et al., 2000; Carroll et al., 1997). This inhibitor competitively binds to the kinase domain and restricts access of the substrate (Buchdunger et al., 2000). Nilotinib, a hydrochloride monohydrate salt, is a second generation Bcr-Abl inhibitor. It binds to and stabilizes the inactive conformation of the kinase domain preventing ATP binding and kinase activation (Weisberg et al., 2005). Imatinib can target the tyrosine kinase domains of c-Abl, stem cell receptor (c-Kit), and platelet-derived growth factor receptor (PDGF-R) (Heinrich et al., 2000; Buchdunger et al., 2000). Nilotinib has
a higher specificity for c-Abl and a 20-fold increased potency for specifically inhibiting this kinase compared to imatinib (Weisberg et al., 2005).

SYF cells were pre-treated with or without imatinib and nilotinib for three hours and then stimulated with or without IGF-1. PTPα-Tyr789 phosphorylation upon IGF-1-stimulation under these conditions was determined. As shown in Figs. 9A and B, IGF-1-stimulated PTPα-Tyr789 phosphorylation was suppressed by imatinib and nilotinib in a dose-dependent manner. While imatinib at 10 µM essentially prevented IGF-1-induced PTPα-Tyr789 phosphorylation, nilotinib at 2.5 and 10 µM actually reduced PTPα-Tyr789 phosphorylation to levels below that in serum-starved, unstimulated cells (Fig. 9B). These results indicate that c-Abl is indeed required for IGF-1-induced phosphorylation of PTPα, and that this kinase also participates in maintaining a basal level of PTPα tyrosine phosphorylation.

3.6 IGF-1-stimulated PTPα-Tyr789 phosphorylation is inhibited as a result of silencing c-Abl expression

As another approach to investigate c-Abl as the candidate PTPα kinase, SYF fibroblasts were subjected to c-Abl siRNA treatment to down-regulate the expression of c-Abl. SYF cells were transfected with or without a scrambled (control) or c-Abl targeting siRNA pool and then the cells were stimulated with or without IGF-1. The PTPα-Y789 phosphorylation was then determined. In SYF cells, where c-Abl expression was silenced (Fig. 10, top panel), the IGF-1-stimulated PTPα-Y789 phosphorylation was completely inhibited (Fig. 10, middle panel). On the other hand, SYF cells that were treated with scrambled siRNA or without any siRNA showed a significant increase in PTPα-Y789 phosphorylation upon IGF-1 stimulation (Fig. 10, middle
panel). The lysates were also probed with anti-PTPα to confirm the presence of comparable amounts of PTPα (Fig. 10, bottom panel).

3.7 IGF-1- activates c-Abl

If IGF-1 acts via c-Abl to induce PTPα-Tyr789 phosphorylation, then one would predict that IGF-1 activates the kinase activity of c-Abl. Indeed, the IGF-1R was recently identified as an upstream activator of Abl kinase in breast cancer cells (Srinivasan et al., 2008). However, this has not been established in fibroblasts. To investigate this, serum-starved SYF fibroblasts were pre-treated with or without nilotinib at a concentration (2.5 µM) shown to be effective in inhibiting c-Abl activity (Fig. 9), and then stimulated with or without IGF-1. Cell lysates were immunoprecipitated with anti-c-Abl antibody and the immunoprecipitates were probed with phosphoTyr245-c-Abl antibody. The phosphorylation of c-Abl at Tyr245 is associated with its activation by growth factors in breast cancer cells (Brasher et al., 2000). IGF-1 stimulation led to dramatically increased c-Abl phosphorylation, indicative of enhanced activity of c-Abl. Furthermore, this was prevented by pre-treatment with nilotinib (Fig. 11A).

In a complementary approach, c-Abl was directly assayed for kinase activity by incubating c-Abl immunoprecipitates with recombinant Crk (a c-Abl kinase substrate, Ren et al., 1994) in an in vitro kinase reaction (described in Materials and Methods section). After 30 minutes, the mixtures were placed on ice to stop the reactions, and the bead-bound Abl was separated from recombinant Crk by centrifugation. The supernatants containing Crk were probed with anti-phosphotyrosine antibody to determine Crk tyrosine phosphorylation, while the pellets were probed for c-Abl to verify equivalent amounts of the kinase. IGF-1-induced a readily apparent increase in Crk phosphorylation that was blocked by nilotinib pre-treatment (Fig. 11B),
indicating that IGF-1 stimulates c-Abl activation. Additionally, in order to confirm that nilotinib treatment does not affect the activation of IGF-1R in presence of IGF-1, IGF-1R was immunoprecipitated under all the above conditions and the tyrosine phosphorylation status of IGF-1R was determined using the anti-phosphotyrosine antibody 4G10 (Fig. 12). These immunoprecipitates were also probed with anti-IGF-1Rβ (Fig. 12).

Together, the results from the above experiments demonstrate that IGF-1 stimulates c-Abl activity that is required for IGF-1-stimulated phosphorylation of PTPα, and that the c-Abl inhibitors imatinib and nilotinib inhibit IGF-1-induced PTPα-Y789 phosphorylation.

3.8 Summary

The present study identifies c-Abl as a kinase required for the IGF-stimulated increase in PTPα-Tyr789 phosphorylation. Our previous studies demonstrated that the inhibition of PI3K and MAP kinase pathways did not affect IGF-1-stimulated PTPα-Tyr789 phosphorylation (Chen et al., 2009). In addition, my present results demonstrate that the IGF-1-stimulated increase in PTPα-Tyr789 phosphorylation is not dependent on IGF-1-activated kinases downstream of PI3K/Akt such as mTOR and S6K. Therefore PI3K/Akt activities are not required for IGF-1-induced PTPα-Tyr789 phosphorylation. Likewise, the SFKs and FAK that play an important role in integrin-stimulated PTPα-Tyr789 phosphorylation are not required for IGF-1-induced PTPα-Tyr789 phosphorylation. Instead, IGF-1 stimulated PTPα-Tyr789 phosphorylation seems to be dependent on a distinct kinase, c-Abl.

c-Abl is a non-receptor tyrosine kinase that can localize to both nucleus and cytoplasm. It is known to regulate cell growth, survival and cellular migration. As described in the ‘Introduction’ section, c-Abl can be activated by disruption of several intramolecular inhibitory
interactions and/or by phosphorylation. c-Abl is activated by several growth factors. Growth factor induced c-Abl activation was first observed in quiescent fibroblasts stimulated with PDGF (platelet-derived growth factor) (Plattner et al., 1999). Ligand engagement leads to phosphorylation of tyrosine residues in the juxtamembrane region of the PDGF receptor (PDGFR) which then bind SFKs (Plattner et al., 1999; Furstoss et al., 2002). c-Abl forms a complex with the SFK-bound PDGFR which leads to SFK-induced c-Abl phosphorylation and activation (Stanglmaier et al., 2003; Plattner et al., 2004). In addition, PDGF was also shown to increase c-Abl activation by enhancing PLCγ activity (Plattner et al., 2003). PLCγ is required for PDGF-induced S1P (sphingosine 1 phosphate) accumulation in cells (Olivera et al., 1999). Therefore, it has been proposed that PDGF-induced S1P accumulation may cause S1P receptors to activate the Gi protein/Src pathway that allows c-Abl activation (Veracini et al., 2006; Sirvent et al., 2008). EGF (epidermal growth factor) has also been shown to activate c-Abl by engaging members of the EGF receptor (EGFR) family (Jones et al., 2006). However, the EGFRs, do not associate with SFKs and the EGF-stimulated activation of c-Abl, in breast cancer cells, is believed to be induced by direct binding of the c-Abl through its SH2 domain to a phosphotyrosine site on the receptor, thus stabilizing the open conformation of c-Abl (Sirvent et al., 2008; Srinivasan and Plattner, 2006; Bromann et al., 2004). In fact, a high-affinity binding site for Abl-SH2 was found in all the members of the EGFR family by a recent protein microarray study (Jones et al., 2006). The TGF-β (transforming growth factor β) receptor can activate c-Abl, in mesenchymal cells, by utilizing PAK2 (Wilkes and Leof, 2006). Interestingly, IGF-1 was recently shown to induce the tyrosine phosphorylation of c-Abl in a SFK-independent manner, thus activating c-Abl in breast cancer cells (Srinivasan et al., 2008). The results described in this chapter demonstrate that IGF-1 also activates c-Abl in (SYF) fibroblasts. In my
studies, the observations that IGF-1 stimulates c-Abl activation in SYF fibroblasts and in the presence of the SFK inhibitor SU6656 suggest that SFKs are dispensable for IGF-mediated c-Abl activation. This is consistent with the findings of Srinivasan et al. (2008).

Inhibition of c-Abl activity by pre-treatment with c-Abl inhibitors and by silencing its expression reduces or prevents IGF-1-stimulated PTPα-Tyr789 phosphorylation. This indicates that PTPα is downstream of c-Abl. However, the mechanism by which c-Abl phosphorylates PTPα in response to IGF-1 still remains unclear. Recently, it was shown that the association of FAK, a key focal adhesion protein, with the IGF-1R complex is required for FAK phosphorylation and dephosphorylation in response to IGF-1 and that this is regulated by c-Abl (Kiely et al., 2009). PTPα, in accord with its role in migration, can also localize to focal adhesions like FAK (Lammers et al., 2000) and therefore its IGF-1-induced phosphorylation may be co-ordinated by a similar mechanism. Thus, the molecular interactions involved in IGF-1-stimulated and c-Abl-dependent PTPα-Tyr789 phosphorylation were investigated and are described in the next chapter.

IGF-1 plays key roles in cellular proliferation, survival, migration, and tumour progression. Aberrant IGF-1 signalling imparts enhanced motility and invasiveness to cancer cells (Ryan and Goss, 2008). As PTPα plays a role in cellular migration (Chen et al., 2009), it is important to understand the role of IGF-1-stimulated PTPα-Tyr789 phosphorylation and the mechanism by which this occurs. This will enhance our understanding of cancer cell migration, and may prove informative for the development of targeted molecular therapies.
SYF cells were serum starved and pretreated with or without 2 or 10 µM SU6656 for 1 hour before treatment without (-) or with (+) IGF-1 for 10 min. (A) The cell lysates were immunoblotted 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). In the top panel, the arrow indicates PTPα, and the arrowhead indicates a reactive 175 kDa band representing an unknown non-PTPα phosphoprotein. (B) PTPα Y789 phosphorylation was determined from three independent experiments as in A. 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-treated cells determined relative to that. Data are shown as mean ± S.D. Asterisks denote a significant difference (p<0.05) from serum-starved, untreated cells. (C) To confirm the efficacy of SU6656 (2 µM) treatment, wild-type (PTPα+/+) cells were treated as in (A) and cell lysates were immunoblotted with phosphosite-specific PTPα Y789 antibody followed by PTPα antibody. The arbitrary densitometric units of PTPα phospho-Y789 per amount of PTPα are shown at bottom.
Figure 7. IGF-1-stimulated PTPα tyrosine phosphorylation is focal adhesion kinase (FAK) independent.

Wild-type (FAK+/-) and FAK-/- fibroblasts were serum starved and then treated without (0) or with 100 ng/ml IGF-1 for 10 min (10). The cell lysates were immunoblotted 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 8. IGF-1-stimulated PTPα phosphorylation in SYF fibroblasts is not affected by rapamycin.

SYF cells were serum-starved and pre-treated with (A) 0.1 μM rapamycin for 2 hours prior to treatment with or without 100ng/ml IGF-1 for 10 min. The cell lysates were immunoblotted with phosphosite-specific PTPα Y789 antibody followed by PTPα antibody or phospho-S6 antibody. The arrow indicates PTPα, and the arrowhead indicates a reactive 175 kDa band. (B) 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-treated cells determined relative to that and the bars show the mean ± S.D. The asterisks indicate a significant difference (p≤0.05) with serum-starved, untreated cells (n=3).
Figure 9. IGF-1 stimulates Abl-dependent phosphorylation of PTPα-Tyr789.

(A) SYF fibroblasts were serum-starved, pretreated with or without the c-Abl inhibitors imatinib mesylate (Imat) or nilotinib (Nilot) for 3 hours at the concentrations indicated (µM), and treated without (-) or with (+) 100ng/ml IGF-1 for 10 min. Lysates were probed for phospho-Y789-PTPα and PTPα. (B) 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, IGF-1+Imat, and IGF-1+Nilot treated cells determined relative to that. Data are shown as means ± S.D. The asterisks indicate a significant difference (p<0.02) from serum-starved IGF-1 treated cells (n=4).
Figure 10. Silencing c-Abl abolishes IGF-1-stimulated PTPα-Tyr789 phosphorylation.

SYF cells were transfected with or without (none) the indicated siRNAs (scramb, scrambled). After 48 hours of incubation, the last 16 hours of which involved serum starvation, the cells were treated without (-) or with (+) 100ng/ml IGF-1 for 10 min. Lysates were probed for c-Abl, phospho-Y789-PTPα, and PTPα.
Figure 11. IGF-1 activates c-Abl.

SYF cells were serum-starved, pre-treated for 3 hours with or without 2.5 μM nilotinib (Nilot), and then treated without (-) or with (+) 100ng/ml IGF-1 for 10 min. (A) c-Abl immunoprecipitates (IP) were probed with anti-phospho-Tyr245-c-Abl antibody and anti-c-Abl antibody. (B) c-Abl IPs were assayed in an in vitro kinase (IVK) assay with the substrate Crk. Reactions were stopped and immunoprobed for Crk tyrosine phosphorylation (4G10 antibody) and for the presence of c-Abl.
Figure 12. Nilotinib does not affect IGF-1Rβ phosphorylation.
SYF fibroblasts were serum starved overnight and pretreated with Nilotinib (Nilot, 10 μM) for 3 hours before IGF-1 stimulation (10 min, 100ng/ml). Immunoprecipitates were prepared with anti-IGF-1Rβ antibody and probed with anti-phosphotyrosine (4G10) and anti-IGF-1Rβ antibodies.
Chapter 4: Identification of a novel PTPα/IGF-1R/c-Abl signalling complex

4.1 Rationale

IGF-1/IGF-1R signalling plays an important role in the regulation of cell growth, proliferation, invasion, and migration. Therefore, characterization of IGF-1R-activated cellular pathways is important for the development of treatments that could counteract IGF-1/IGF-1R dysregulation in diseases such as cancer. Although the investigations described in Chapter 3 allowed me to conclude that IGF-1-stimulated PTPα-Tyr789 phosphorylation is dependent on the activation of c-Abl, the mechanism underlying this event was unknown.

IGF-1R is a transmembrane, ligand-activated tyrosine protein kinase that consists of two alpha and two beta subunits that are linked by disulfide bridges. Ligand engagement leads to a conformational change in the intracellular beta subunits that causes the stimulation of the tyrosine kinase activity. Following the increase in the intrinsic kinase activity, the autophosphorylation of several tyrosine residues of the IGF-1R takes place and this leads to the phosphorylation of associated proteins such as IRS and Shc (Torres-Aleman, 2005; Shelton et al., 2004; Kuemmerle., 2003). I hypothesized that PTPα might be phosphorylated in response to IGF-1-stimulation in a similar manner. Therefore, I investigated whether the IGF-1-stimulated PTPα-Tyr789 phosphorylation results from the association of PTPα with the activated IGF-1R.

4.2 PTPα associates with IGF-1-receptor (IGF-1R)

PTPα is rapidly phosphorylated (within 2 min) in response to IGF-1 treatment (Chen et al., 2009). Also, PTPα is a receptor-type protein tyrosine phosphatase which, like the IGF-1R, is located at the plasma membrane. Taken together, these considerations led to the notion that
PTPα might be positioned as a potential IGF-1R substrate through its association with the IGF-1R.

4.2.1 IGF-1Rβ associates with endogenous PTPα and heterologously expressed wild-type PTPα in SYF fibroblasts

IGF-1Rβ immunoprecipitates prepared from lysates of SYF fibroblasts were probed for associated PTPα. As shown in Fig. 13A (very faint band, lane 1), endogenous PTPα co-immunoprecipitated with IGF-1Rβ from these cells. SYF fibroblasts were also transfected with VSVG (vesicular stomatitis virus glycoprotein)-tagged wild-type and catalytically inactive mutant forms of PTPα. When IGF-1Rβ was immunoprecipitated from these cells, the heterologously expressed PTPα was detected in the immunoprecipitates (Fig. 13A, lanes 3 and 4; Fig. 13B). Probing with anti-phosphoTyr789-PTPα antibody revealed that although IGF-1-stimulation induced the tyrosine phosphorylation of PTPα (Fig. 13A, lanes 3 and 4), it had little effect on the association of PTPα with IGF-1Rβ (Fig. 13B, top panel). The IGF-1R was not detectably dephosphorylated by PTPα, as the tyrosine phosphorylation of IGF-1R in the cell lysates and in IGF-1Rβ immunoprecipitates remained unchanged in the presence of wild-type PTPα or catalytically inactive PTPα (Fig. 13B, bottom two panels; Fig. 13C). This indicates that PTPα activity is directed downstream of IGF-1R and does not affect the IGF-1-mediated PTPα Tyr789 phosphorylation. The association of PTPα and IGF-1Rβ was also confirmed by the detection of IGF-1Rβ in reciprocal immunoprecipitations of VSVG-tagged PTPα (Fig. 13D). The reciprocal immunoprecipitation of PTPα also confirmed that the association between PTPα and IGF-1Rβ was not dependent on IGF-1 stimulation (Fig. 13D, lanes 4-7), nor upon PTPα catalytic activity (Fig. 13D, lanes 6 and 7).
4.2.2 IGF-1Rβ associates with mutant forms of transfected PTPα lacking either catalytic domain

In order to identify the region of PTPα that is required for its interaction with the IGF-1R, VSVG-tagged mutant forms of PTPα lacking either the membrane proximal catalytic domain (D1) or lacking the membrane distal catalytic domain (D2) and C-terminal tail region (Fig. 14A) were transfected into SYF fibroblasts and subjected to immunoprecipitation with the VSVG antibody. This allowed the specific immunoprecipitation of VSVG-tagged PTPα and not endogenous PTPα. The tagged wild-type and mutant forms of PTPα were confirmed to be expressed in the cells and successfully immunoprecipitated (Fig. 14B, left panel). As shown in Fig. 14B (right panel), IGF-1Rβ was detected in the VSVG immunoprecipitates from these transfected cells. Therefore, the deletion of either catalytic domain of PTPα had no apparent effect on the association of PTPα with IGF-1Rβ.

4.3 PTPα constitutively associates with IGF-1R independent of IGF-1R catalytic activity

Previous experimental results have shown that the association between PTPα and IGF-1Rβ is independent of IGF-1 stimulation. This was confirmed in another approach using IGF-1R null mouse embryonic fibroblasts stably transfected with a catalytically inactive form of IGF-1R (K1003N) having a point mutation of a critical lysine residue in the ATP-binding site of this kinase. These cells were a kind gift from Drs. P. Sorensen and C. Tognon (BC Cancer Research Centre, Vancouver). IGF-1R null cells and these cells re-expressing either wild-type or inactive mutant (K1003N) IGF-1R were serum-starved and treated with or without IGF-1 before being lysed and subjected to immunoprecipitation with anti-IGF-1Rβ antibody. As shown in Fig. 15,
PTPα was detectable in IGF-1R immunoprecipitates from IGF-1R null fibroblasts re-expressing wild-type IGF-1R (Fig. 15, lanes 1 and 2), but was not present in immunoprecipitates from the parental IGF-1R null cells (Fig. 15, lanes 3 and 4). PTPα was also detected in immunoprecipitates from the cells re-expressing the catalytically inactive form of IGF-1R, irrespective of IGF-1-stimulation (Fig 15, lanes 5 and 6). The immunoprecipitates were also probed with anti-IGF-1Rβ antibody to confirm the presence of the IGF-1R (Fig. 15, bottom panel). PTPα was detected in IGF-1R immunoprecipitates from SYF cells as well (Fig. 15, lanes 7 and 8). Indeed, more PTPα co-precipitated with IGF-1R in these cells, suggesting that SFKs compete with the IGF-1R for PTPα binding. These results also suggest that the association between PTPα and IGF-1R is independent of IGF-1 binding to its receptor.

4.4 PTPα forms a novel signalling complex with IGF-1R and RACK1

My results demonstrate that PTPα associates with the IGF-1R however whether the two proteins associate directly or indirectly is unclear. During the course of these studies, Kiely et al. (2009) reported that the association between the IGF-1R and FAK, a protein that localizes to focal adhesions like PTPα, is mediated via the scaffolding protein RACK1. RACK1 constitutively associates with the IGF-1R. Interestingly, the RACK1-mediated association of the IGF-1R with FAK is regulated by c-Abl (Kiely et al., 2009). In view of my findings that c-Abl is the IGF-1-activated kinase responsible for PTPα-Tyr789 phosphorylation I investigated whether the interaction of the IGF-1R and PTPα is mediated by RACK1, and whether such a signalling complex facilitates the IGF-1-stimulated c-Abl-dependent tyrosine phosphorylation of PTPα.
4.4.1 PTPα and IGF-1Rβ constitutively associate with RACK1

To determine if PTPα and the IGF-1R associate with RACK1, lysates of serum-starved or IGF-1-stimulated SYF cells were subjected to immunoprecipitation with anti-RACK1 antibody. The immunoprecipitates were probed for PTPα and IGF-1Rβ. PTPα was detected in RACK1 immunoprecipitates, and the association of PTPα and RACK1 appeared to be independent of IGF-1 stimulation (Fig. 16, top panel). Consistent with other reports (Hermanto et al., 2002; Kiely et al., 2002, 2005), the IGF-1R was also constitutively associated with RACK1 (Fig. 16, second panel). To investigate if c-Abl activity was required for the association of PTPα and IGF-1R with RACK1, serum-starved SYF fibroblasts were pretreated with nilotinib before IGF-1 stimulation (Fig. 16, lane 3). This c-Abl inhibitor was previously shown to abolish the IGF-induced c-Abl-dependent tyrosine phosphorylation of PTPα by inhibiting c-Abl activity (Chapter 3, Sections 3.5 and 3.7). Nilotinib pretreatment did not affect the association of PTPα and IGF-1R with RACK1 (Fig. 16, lane 3, top two panels), suggesting that the complex between PTPα, IGF-1R, and RACK1 is not regulated by the activity of c-Abl. Inhibition of c-Abl activity is reported to abolish the association between RACK1 and FAK (Kiely et al., 2009), therefore, the RACK1 immunoprecipitates were probed with anti-FAK antibody to validate the efficacy of the nilotinib treatment (Fig. 16, lane 3, third panel). These immunoprecipitates were also probed with anti-RACK1 to confirm the presence of RACK1 (Fig. 16, bottom most panel). Together, these results demonstrate that PTPα, along with IGF-1R, associates with RACK1. The formation of this signalling complex is independent of IGF-1 stimulation and not subject to regulation by the activity of c-Abl.
4.4.2 c-Abl associates with PTPα, IGF-1Rβ, and RACK1

The inhibition of c-Abl activity does not affect the association of PTPα and IGF-1R with RACK1. To investigate whether c-Abl participates in this signalling complex, SYF cells were pretreated with or without nilotinib and then stimulated with or without IGF-1, and subjected to immunoprecipitation with anti-c-Abl antibody. The c-Abl immunoprecipitates were probed with anti-PTPα. Although the association of PTPα with c-Abl was observed in both the unstimulated (Fig. 17A, lane 1) and IGF-1-stimulated cells (Fig. 17A, lane 2), the association between the two proteins was completely abolished by pre-treatment with nilotinib (Fig. 17A, lane 3). These immunoprecipitates were probed for c-Abl (Fig. 17A, bottom panel), confirming that it was immunoprecipitated under all conditions. Reciprocal immunoprecipitation using anti-PTPα antibody confirmed that the association of c-Abl with PTPα was independent of IGF-1-stimulation (Fig. 17B, top panel), and almost completely abolished with nilotinib pretreatment (Fig. 17B, top panel). These immunoprecipitates were also probed for PTPα (Fig. 17B, bottom panel). In addition, SYF cells, pretreated with or without nilotinib and then stimulated with or without IGF-1, were also subjected to immunoprecipitation with anti-RACK1 antibody. The RACK1 immunoprecipitates were probed with anti-PTPα, anti-IGF-1Rβ, and anti-c-Abl antibodies (Fig. 17C and D). A constitutive association was observed between RACK1, PTPα, and IGF-1R, however the association of c-Abl with RACK1 was increased upon IGF-1 stimulation and abolished by nilotinib pretreatment (Fig. 17D, middle panel). The association of c-Abl with RACK1 was quantified from several experiments, and as shown in Fig. 17E, the association between c-Abl and RACK1 was reproducibly and significantly enhanced by about fifty percent by IGF-1-stimulation. Together, these results demonstrate the existence of a novel
signalling complex in which PTPα and the IGF-1R associate with RACK1, and c-Abl recruitment to this complex is increased upon IGF-1-stimulation.

**4.5 Silencing RACK1 inhibits IGF-1-stimulated PTPα-Tyr789 phosphorylation**

My results suggest that RACK1 plays a fundamental role in IGF-1-stimulated PTPα-Tyr789 phosphorylation. As IGF-1 stimulation increases the recruitment of c-Abl kinase to RACK1, this might explain how IGF-1-activated c-Abl facilitates RACK1-associated PTPα-Tyr789 phosphorylation. To investigate if RACK1 plays an essential role in IGF-1-stimulated c-Abl-dependent PTPα-Tyr789 phosphorylation, RACK1 expression was silenced using siRNA. As shown in Fig. 18, SYF cells were transfected with or without a siRNA pool targeting the expression of RACK1 or with a scrambled non-specific pool of siRNA, and the lysates were then probed with anti-phosphoTyr789-PTPα antibody. In the case of cells where RACK1 expression was silenced (Fig. 18, lanes 3 and 4, top panel), the IGF-1-stimulated increase in PTPα-Tyr789 phosphorylation was completely abolished (Fig. 18, lanes 3 and 4, middle panel). The expression of PTPα in all the lysates was verified by probing with anti-PTPα antibody (Fig. 18, bottom panel).

**4.6 Silencing RACK1 greatly reduces the association of c-Abl and IGF-1R with PTPα**

In other experiments, the effect of silencing RACK1 expression on the formation of the IGF-1R/PTPα/c-Abl signalling complex was examined. The SYF cells were transfected with RACK1-targeting siRNA or scrambled siRNA, and stimulated with or without IGF-1 in the presence or absence of nilotinib. The siRNA-mediated silencing of RACK1 was confirmed by
probing the cell lysates with anti-RACK1 antibody (Fig. 19A). PTPα immunoprecipitates were prepared from the cell lysates and probed with anti-IGF-1Rβ (Fig. 19B, top panel) and anti-c-Abl (Fig. 19B, middle panel) antibodies. In SYF cells treated with scrambled siRNA the association between PTPα and the IGF-1R remained intact irrespective of IGF-1-stimulation (Fig. 19B). Nilotinib pretreatment did not affect the association between PTPα and the IGF-1R (Fig. 19B, top panel), but abolished the association of PTPα with c-Abl (Fig. 19B middle panel). However, in SYF cells where RACK1 expression was silenced, the association between PTPα and the IGF-1R was greatly reduced (Fig. 19B, top panel) and IGF-1-stimulation of these cells did not induce an association between PTPα and the IGF-1R (Fig. 19B, top panel). In addition, the association between PTPα and c-Abl was completely abolished under all conditions upon silencing of RACK1 expression (Fig. 19B, middle panel). The blots were stripped and probed for PTPα (Fig. 19B, bottom panel). Taken together, these results suggest that RACK1 plays an essential role in the formation of the IGF-1R/PTPα/c-Abl signalling complex.

4.7 Summary

The results described in this chapter demonstrate that PTPα associates with the IGF-1R. This association appears to be constitutive, as it is not affected by IGF-1-stimulation. This indicates that neither the catalytic activity of the IGF-1R nor its tyrosine phosphorylation, with the latter mediating IGF-1R association with many other proteins, is required for its association with PTPα. This was confirmed by demonstrating that a catalytically inactive mutant form of IGF-1R (K1003N) was able to interact with PTPα. Likewise, the catalytic activity of PTPα was not required for interaction with the IGF-1R. Indeed, the deletion of the D1 catalytic domain of PTPα, the domain responsible for the majority of the phosphatase activity (Lim et al., 1997), did
not prevent PTPα-IGF-1R binding. Likewise, the deletion of the D2-tail region of PTPα did not affect the association with the IGF-1R, indicating that neither catalytic domain is specifically required for the interaction. However, it is possible that the presence of either catalytic domain is sufficient for the association with IGF-1R.

Further investigation revealed that other proteins are components of the PTPα-IGF-1R complex. Immunoprecipitation experiments and immunoprobing demonstrated that the scaffolding protein RACK1, c-Abl, and FAK are associated with one another and with PTPα and the IGF-1R. The IGF-1R has previously been shown to constitutively associate with RACK1 (Hermanto et al., 2002; Kiely et al., 2002, 2005). Their association is essential for the IGF-1-stimulated c-Abl-dependent tyrosine phosphorylation of key focal adhesion proteins (Kiely et al., 2009). The results of the experiments conducted here show for the very first time that RACK1 also associates with PTPα. Moreover, this association appears to take place irrespective of IGF-1-stimulation. Since RACK1 constitutively associates with the inactive K1003N form of IGF-1R (Kiely et al., 2002), it is possible that the association of IGF-1R with PTPα is mediated via RACK1. Indeed, silencing RACK1 expression abolishes the interaction of PTPα and IGF-1R, demonstrating that RACK1 is required for the association of IGF-1R and PTPα.

RACK1 is a scaffolding protein that interacts with many proteins that modulate IGF-1R signal transduction (Sklan et al., 2006). It interacts with several proteins simultaneously through its seven WD (tryptophan-aspartate) motifs and co-ordinates the recruitment and release of these proteins in response to IGF-1-stimulation (Sklan et al., 2006). The WD motif-containing protein binding domain of RACK1 mediates the association, localization and activity of several receptor and cytosolic phosphatases (Kiely et al., 2002; O’Connor at al., 1997, 2003). One such phosphatase is protein tyrosine phosphatase µ (PTPµ), a receptor PTP that is closely related to
PTPα (Blanchetot and Hertog, 2000; Zondag et al., 2000). RACK1 interacts with PTPµ at a region within the conserved catalytic domain of RPTPs and colocalizes with PTPµ at cell-cell contacts and adhesion junctions (Mourton et al., 2001). This raises the possibility that RACK1 may interact with PTPα in a similar manner.

Although probing the anti-RACK1 immunoprecipitates shows that the association between RACK1 and IGF-1R and PTPα remains unaffected by nilotinib treatment, suggesting that this association is not regulated by the activity of c-Abl, IGF-1 induced the increased association of c-Abl with the IGF-1R/RACK1/PTPα complex. This is consistent with the findings of a recent study where the increased activation of IGF-1R downstream signalling molecules, such as Ras, enhanced the association of c-Abl with RACK1 (Huang et al., 2008). Therefore, the increased recruitment of c-Abl to the RACK1 signalling complex in response to IGF-1 may be important to facilitate IGF-1-stimulated PTPα tyrosine phosphorylation. Although the existence of a signalling complex in which RACK1 mediates the association of PTPα with IGF-1R and recruits IGF-1 activated c-Abl may explain how IGF-1-induced PTPα-Tyr789 phosphorylation occurs, outstanding questions remain. It is not yet clear whether there exists a direct association between IGF-1R and PTPα (in addition to the one facilitated by RACK1), or whether this association requires the involvement of additional signalling proteins. Additionally, we now know that IGF-1 activates c-Abl and enhances its recruitment to the IGF-1R/RACK1/PTPα complex, however the underlying mechanism behind this remains unresolved.

The results described in this chapter demonstrate the existence of a novel IGF-1R/RACK1/PTPα signalling complex that enables IGF-1-stimulated PTPα-Tyr789 phosphorylation. In order to elucidate the significance of this signalling mechanism, investigations were carried out into the functional importance of the IGF-1-stimulated c-Abl dependent PTPα Tyr789 phosphorylation
with respect to IGF-1-stimulated cellular functions such as cell migration in normal and tumour cells, as described in the next chapter.
Figure 13. PTPα associates with the IGF-1 receptor.
SYF cells were untransfected or transfected with VSVG-tagged PTPα or inactive mutant PTPα (VSVG-PTPα-CS). The cells were serum-starved and stimulated with or without IGF-1 for 2 min. (A) Immunoprecipitates (IP) were prepared with (+Ab) or without (–Ab) anti-IGF-1Rβ antibody and probed with phosphosite-specific PTPα Y789 antibody (top) followed by anti-IGF-1Rβ (bottom). (B) IGF-1Rβ immunoprecipitates were prepared, and a portion was probed for PTPα and reprobed for IGF-1Rβ (top two panels). Another portion was probed for phosphotyrosine and reprobed for IGF-1Rβ (top two panels). (C) Cell lysates were probed with anti-VSVG antibody to detect heterologous PTPα (top), and with anti-phosphotyrosine (middle) and anti-IGF-1Rβ (bottom). The arrow in the middle panel depicts a band that comigrates with IGF-1Rβ. (D) Cell lysate (Lys, lane 1) or anti-VSVG immunoprecipitates (lanes 2–7) were probed for IGF-1Rβ (top) and for PTPα (bottom panel).
Figure 14. Neither PTPα catalytic domain alone is essential for association with IGF-1Rβ.

(A) Schematic of VSVG-tagged wild type PTPα and mutant forms of PTPα, containing either only the D1 catalytic domain (PTPα-D1ΔD2) or the D2 catalytic domain (PTPα-ΔD1D2). (B) SYF fibroblasts were transfected with VSVG-tagged wild type PTPα or mutant forms of PTPα and serum-starved, then stimulated with IGF-1 for 10 min. Immunoprecipitates (IP) were prepared with anti-VSVG antibody and a portion was probed for VSVG (left panel). Another portion of these immunoprecipitates was probed for IGF-1Rβ (right panel). The positions and sizes (kDa) of molecular weight markers are shown on the left of each panel.
Figure 15. PTPα associates with catalytically inactive IGF-1R.
IGF-1R null MEFs (R⁻) transfected with wild-type (R⁺), catalytically-inactive mutant IGF-1R (K1003N) or SYF fibroblasts were serum starved overnight and stimulated with IGF-1 (10 min, 100ng/ml). (A) Immunoprecipitates were prepared with anti-IGF-1Rβ antibody and probed with anti-PTPα, anti-RACK1, and anti-IGF-1Rβ antibodies. (B) Cell lysates were probed with anti-PTPα and anti-Actin antibodies. The above data are representative of the results of two experiments (n=2).
Figure 16. RACK1 associates with PTPα.

SYF fibroblasts were serum starved overnight and pretreated with nilotinib (Nilot, 2.5 μM) for 3 hours before IGF-1 stimulation (10 min, 100ng/ml). Immunoprecipitates were prepared with anti-RACK1 antibody and probed with anti-PTPα, anti-IGF-1Rβ, anti-FAK and anti-RACK1 antibodies. The above data are representative of the results of four experiments (n=4).
Figure 17. PTPα, IGF-1R, and c-Abl are complexed with the RACK1 scaffolding protein.

Immunoprecipitates (IPs) of (A) c-Abl, (B) PTPα, (C, D) and RACK1 were prepared from SYF fibroblasts that had been serum-starved overnight, pre-treated without (-) or with (+) 2.5 μM nilotinib (Nilot) for 3 hours and then treated without (-) or with (+) 100 ng/ml IGF-1 for 10 min. The IPs were probed as indicated. (E) The immunoblots from four independent experiments as in (D) were quantified, and the IGF-1-stimulated association of c-Abl with RACK1 is shown in the graph. The asterisk denotes a significant difference (p<0.05) from the serum-starved untreated cells. The association of c-Abl per unit of RACK1 was quantified in arbitrary units.
Figure 18. Silencing RACK1 inhibits IGF-1-stimulated PTPα-Tyr789 phosphorylation.

SYF cells were transfected with or without (none) the indicated siRNAs. The cells were incubated for 32 hours and serum starved for another 16 hours, then treated without (-) or with (+) 100 ng/ml IGF-1 for 10 min. Lysates were probed for RACK1, phospho-Y789-PTPα, and PTPα.
**Figure 19. Silencing RACK1 disrupts the association of the IGF-1Rβ and c-Abl with PTPα.**

SYF fibroblasts were transfected with 10 nM of control (scrambled, scr) or RACK1 siRNA and cultured for 32 hours. The cells were serum starved for 16 hours, pretreated with Nilotinib (Nilot, 2.5 μM) for 3 hours before IGF-1 stimulation (10 min, 100ng/ml). (A) Cell lysates were also probed with anti-RACK1 and anti-Actin. (B) Immunoprecipitates were prepared with anti-PTPα antibody and probed with anti-IGF-1Rβ, anti-c-Abl and anti-PTPα antibodies.
Chapter 5: Functional effects of IGF-1-mediated PTPα-Tyr789 phosphorylation

5.1 Rationale

Migration is a fundamental cell process that is controlled by finely orchestrated interactions between the cell and its environment; with the latter including other cells, the extracellular matrix (ECM), and a variety of extracellular soluble factors such as growth factors. Improperly regulated cell migration is implicated in several pathologies, notably cancer metastasis (Ulrich et al., 2009). Among childhood cancers, neuroblastoma accounts for 8-10% of all childhood cancers (Park et al., 2008). Patients with neuroblastoma have one of the lowest childhood cancer survival rates, and account for ~15% of pediatric cancer deaths. Despite the fact that tumour spread, or metastasis, is found in nearly half of all these patients at diagnosis, little is known about the factors that determine neuroblastoma migration and invasion (Park et al., 2008). Improved understanding of the conditions and molecular mechanisms that promote these metastatic properties is essential for the development of targeted therapies to inhibit neuroblastoma progression and spreading, and to control this often lethal disease.

Our lab has found that PTPα is a positive regulator of cell migration in response to engagement of receptor integrins by ECM components (Zeng et al., 2003; Chen et al., 2006). However, the role of PTPα in mediating IGF-1-stimulated cell migration was unknown. IGF-1 plays key roles in cell proliferation, survival, migration, and tumour progression and modulates many aspects of neuroblastoma biology, including motility and invasiveness (Meyer et al., 2001; Puglianiello et al., 2000). Therefore, investigations were carried out to determine if PTPα is involved in IGF-1-
mediated cellular migration of normal and tumour cells and, in this context, to test the functional significance of IGF-1-stimulated PTPα tyrosine phosphorylation.

5.2 PTPα-Tyr789 is required for IGF-1-stimulated fibroblast migration

To assess the role of PTPα in IGF-1-stimulated migration of fibroblasts, wild-type and PTPα−/− mouse embryonic fibroblasts were utilized for Transwell migration assays. As shown in Fig. 20A, the migration of wild-type MEFs to the underside of Transwell chambers, coated with fibronectin, in the assays was enhanced by 30% by IGF-1-stimulation. Consistent with an earlier report (Zeng et al., 2003), the migration of PTPα−/− MEFs was impaired. The migratory ability of unstimulated PTPα−/− cells was decreased by ~40% with respect to wild-type MEFs (Fig. 19A). IGF-1-stimulation enhanced PTPα−/− MEF migration by only 17% (Fig. 20A). This indicates that almost half of the IGF-1-dependent migration ability of the MEFs depends on the presence of PTPα. PTPα was re-introduced into PTPα−/− MEFs via adenoviral infection, resulting in the restoration of the migratory ability of these cells with or without IGF-1-stimulation (Fig. 20A). IGF-1-stimulation induced a 29% increase in the migration of these cells. In contrast, re-introduction of a mutant form of PTPα lacking Tyr789 (Y789F) failed to restore the migratory ability of PTPα−/− cells. IGF-1-stimulation of these cells enhanced migration by only 18% (Fig. 20A). Taken together, these data suggest that Tyr789 phosphorylation is required for PTPα-dependent IGF-1-stimulated cell migration. In order to confirm the re-expression and appropriate phosphorylation of PTPα in PTPα−/− cells, aliquots of all cell types were lysed and examined for PTPα protein expression via Western blotting (Fig. 20B).
5.3 Neuroblastoma migration is dependent on both the activity of c-Abl and PTPα

Investigation into IGF-1-stimulated PTPα Tyr789 phosphorylation in mouse embryonic fibroblasts revealed the role of a RACK1 signalling complex. In unstimulated MEFs (SYF cells), IGF-1Rβ and PTPα are complexed with RACK1. IGF-1 activates c-Abl and promotes c-Abl association with RACK1, with the former and possibly also the latter event resulting in IGF-1-induced tyrosine phosphorylation of PTPα. The human neuroblastoma cell line SH-SY5Y expresses IGF-1R and readily migrates in response to IGF-1 (Puglianiello et al., 2000), and therefore provides a good model system to validate and investigate the functional significance of these findings.

5.3.1 IGF-1-activates c-Abl dependent PTPα Tyr789 phosphorylation in SH-SY5Y cells

To establish whether IGF-1 activates c-Abl dependent phosphorylation of PTPα Tyr789, SH-SY5Y cells were grown to 85-90% confluence and serum-starved for 20 hours before being pre-treated with the c-Abl inhibitors imatinib and nilotinib. IGF-1-stimulation greatly increased the level of tyrosine phosphorylation of PTPα (Fig. 21, compare lanes 1 and 2) compared to the unstimulated neuroblastoma cells. In contrast, no IGF-1-stimulated PTPα-Tyr789 phosphorylation was detected in SH-SY5Y cells that were pre-treated with the c-Abl inhibitors (Fig. 21, lanes 3-6).

My previous experiments demonstrated that inhibition of c-Abl leads to inhibition of the IGF-1-stimulated PTPα Tyr789 phosphorylation and that IGF-1-stimulates c-Abl activation in SYF fibroblasts. This suggests that IGF-1-mediated c-Abl activation lies upstream of PTPα. To determine if PTPα has an effect on the IGF-1-stimulated activation of c-Abl, PTPα expression was ablated in SY-SH5Y cells using siRNA. Serum-starved SH-SY5Y cells were transfected
with scrambled siRNA or PTPα targeting siRNA and then stimulated with IGF-1. The cell lysates were probed with phospho-Tyr245-c-Abl antibody in order to determine the level of tyrosine phosphorylation of c-Abl, reflecting c-Abl activity, in response to IGF-1. In the case of neuroblastoma cells treated with scrambled siRNA, the level of c-Abl phosphorylation increased in the presence of IGF-1 (Fig. 22, lane 2, top panel). This IGF-1-stimulated increase in c-Abl phosphorylation was abolished when neuroblastoma cells were pretreated with nilotinib (Fig. 22, lane 3, top panel). This is consistent with what was observed in SYF cells where IGF-1-induced c-Abl activation was inhibited in the presence of nilotinib. The neuroblastoma cells that were treated with PTPα-targeting siRNA also displayed a similar pattern of c-Abl phosphorylation under these conditions, as the level of c-Abl phosphorylation increased in the presence of IGF-1 (Fig. 22, lane 5, top panel) and was abolished with nilotinib pretreatment in these cells (Fig. 22, lane 6, top panel). This suggests that IGF-1 activates c-Abl in SH-SY5Y neuroblastoma cells and that PTPα indeed functions downstream of c-Abl in the IGF-1 signalling pathway as it has a minimal role role in IGF-1-mediated c-Abl phosphorylation. The lysates from these cells were also probed for c-Abl (Fig. 22, second panel) and actin (Fig. 22, third panel). Additionally, these lysates were probed with anti- PTPα antibody in order to confirm PTPα knockdown (Fig. 22, fourth panel). Furthermore, these samples were probed with anti-IGF-1R and anti-RACK1 (Fig.22, bottom two panels) to confirm that there was no change in the expression of these signalling molecules believed to play a regulatory role in c-Abl activation (Kiely et al., 2009).

SH-SY5Y cells express src family kinases (SFKs). SFKs have been attributed to increase the level of c-Abl tyrosine phosphorylation in response to IGF-1 in other cell systems (Stanglmaier et al., 2003). Although IGF-1-stimulation activates c-Abl in SFK-deficient SYF fibroblasts, it remains possible that in SH-SY5Y cells, the role of SFKs may not be dispensable. To determine
if SFKs facilitate c-Abl activation in response to IGF-1, serum-starved SH-SY5Y cells were stimulated with or without IGF-1 following pretreatment with or without the SFK inhibitor SU6656. The level of IGF-1-stimulated tyrosine phosphorylation of c-Abl was then determined. As shown in Fig. 23, neuroblastoma cells not pretreated with SU6656 exhibited the characteristic increase in c-Abl tyrosine phosphorylation in response to IGF-1 stimulation (Fig. 23, lanes 1 and 2, top panel). Pretreatment with nilotinib inhibited the IGF-1-induced activation of c-Abl (Fig. 23, lane 3, top panel), confirming the efficacy of nilotinib treatment. In neuroblastoma cells pretreated with SU6656, the IGF-1-stimulated increase in c-Abl tyrosine phosphorylation was still observed (Fig. 23, lane 5, top panel) and was abolished in the presence of nilotinib (Fig. 23, lane 6, top panel). This demonstrates that there exists a mechanism independent of SFK activity that activates/phosphorylates c-Abl in response to IGF-1 in SH-SY5Y cells.

5.3.2 IGF-1R/RACK1/PTPα signalling complex present in SH-SY5Y cell model system

To determine if PTPα exists in a signalling complex with IGF-1R and RACK1, serum-starved SH-SY5Y neuroblastoma cells, pretreated with or without nilotinib and stimulated with or without IGF-1, were subjected to immunoprecipitation with anti-PTPα antibody. Probing the immunoprecipitates demonstrated that IGF-1Rβ and RACK1 associated with PTPα irrespective of IGF-1-stimulation in SH-SY5Y cells (Fig. 24, lane 1 and 2, top two panels). Furthermore, these results also suggest that the complex between IGF-1R, RACK1 and PTPα is not regulated by the activity of c-Abl since pretreatment with the c-Abl inhibitor, nilotinib, did not affect the association of RACK1 and IGF-1R with PTPα (Fig. 24, lane 3, top two panels). The PTPα immunoprecipitates were also probed with anti-FAK antibody (Fig. 24, third panel). Nilotinib inhibits the activation of c-Abl and leads to the dissociation of
FAK from the IGF-1R/RACK1 signalling complex (Kiely et al., 2009). The absence of FAK in the immunoprecipitates from cells pretreated with nilotinib confirms the efficacy of nilotinib treatment (Fig. 24, lane 3, third panel). In addition, the immunoprecipitates were probed with anti-PTPα in order to confirm that PTPα was immunoprecipitated (Fig. 24, bottom panel). Together, these results demonstrate that IGF-1R, RACK1 and PTPα constitutively associate in a signalling complex in SH-SY5Y cells. This is consistent with previous results showing that PTPα constitutively associates with IGF-1Rβ and RACK1 in SYF cells.

5.3.3 Neuroblastoma cell migration is prevented by c-Abl inhibitors or by PTPα silencing

The human neuroblastoma cell line SH-SY5Y expresses IGF-1R and readily migrates in response to IGF-1 (Puglianiello et al., 2000). In order to assess the functional importance of the role of PTPα in the IGF-1-stimulated cellular migration of neuroblastoma cells, ablation of PTPα expression in SH-SY5Y neuroblastoma cells was carried out. Cells were transfected with scrambled or PTPα-targeting siRNA and cultured for forty-eight hours. During the last sixteen hours of this period the cells were serum-starved. The cells were then pretreated with or without the indicated concentrations of nilotinib and placed in Transwell inserts to determine IGF-1-stimulated migration abilities. The migration of scrambled siRNA-treated SH-SY5Y cells was stimulated two-fold by IGF-1 (Fig. 25A). The siRNA-mediated ablation of PTPα reduced the basal (in the absence of IGF-1) migration ability of the cells by 40%, and IGF-1 promoted a much smaller enhancement (17%) of the migration of these cells (Fig. 25A). Overall, silencing PTPα expression resulted in about a 2.5-fold reduction in IGF-1-stimulated SH-SY5Y cell migration (Fig. 25A, compare the 2nd and 7th bars). The efficacy of PTPα silencing (>90%) was confirmed by immunoblotting cell lysates (Fig. 25B and C). Therefore, not only does PTPα play
an important role in mediating IGF-1-stimulated increase in neuroblastoma migration, it also has a role in maintaining an optimal basal level of migration in these cells.

To investigate if c-Abl activity was required for SH-SY5Y cell migration, the scrambled and PTPα siRNA treated cells were pre-treated with increasing concentrations of nilotinib prior to assessing migration. IGF-1-stimulated migration of control cells expressing PTPα and of cells with defective PTPα expression declined in a dose-dependent manner after nilotinib treatment (Fig. 25A). The IGF-stimulated migration of control SH-SY5Y cells expressing PTPα was reduced by treatment with 2.5-5 µM nilotinib, although the stimulatory effect of IGF-1 was not completely abolished. Only treatment with 10 µM nilotinib was able to inhibit the migration of the control SH-SY5Y cells to a lower extent than that observed in the absence of IGF-1 (Fig. 25A). In contrast, the IGF-1-stimulated migration of cells lacking PTPα was abolished by 2.5 µM nilotinib, and higher concentrations of nilotinib (5-10 µM) inhibited migration to levels below that observed in the absence of IGF-1. Probing lysates of the control and PTPα-siRNA-treated cells treated with nilotinib confirmed the effective silencing of PTPα by at least 90% (Fig. 25B and C). These results demonstrate that both c-Abl activity and PTPα are important for optimal IGF-1-stimulated neuroblastoma cell migration, and that in the absence of PTPα any residual c-Abl activity is not sufficient to promote a migratory response to IGF-1.

5.4 Summary

IGF-1 signalling induces the migration of several types of cells, including tumour cells (Leroith and Roberts, 2003). The IGF-1-induced migration of mouse embryonic fibroblasts (MEFs) is inhibited by approximately fifty percent if PTPα is not expressed, therefore it can be concluded that PTPα is involved in mediating this response. Also, IGF-1-induced MEF
migration specifically requires PTPα with an intact Tyr789 phosphorylation site as only the reintroduction of wild-type, and not the Y789F mutant, restored the defective migration of PTPα-null cells. This finding suggests that IGF-1-stimulated tyrosine phosphorylation of PTPα, which is independent of SFK/FAK activity, plays an important role in IGF-1-induced cell migration. This is in contrast with integrin-stimulated SFK/FAK dependent PTPα-Tyr789 phosphorylation which is required for integrin-induced cellular migration (Chen et al., 2006). In the case of integrin signalling, phosphorylation of PTPα-Tyr789 occurs downstream of FAK activation and mediates cytoskeletal rearrangement, focal adhesion formation, and cell migration (Chen et al., 2006). However, IGF-1 also induces changes in the tyrosine phosphorylation status of FAK and other focal adhesion molecules and promotes F-actin reorganization and cell migration. This suggests that several different stimuli utilize distinct upstream signalling mechanisms to induce the phosphorylation of focal adhesion proteins such as FAK and PTPα, however, post phosphorylation, these proteins function in conserved downstream signalling pathways that lead to cell migration.

SH-SY5Y cells express type 1 and type 2 IGF receptors and are known to experience morphological and functional neuronal differentiation upon the ligand engagement of these receptors. It is for this reason that they are used as a model for studying the neuronal development (Pahlman et al., 1990) and more importantly for studying the interactions between the IGF system and neuroblasts (Cianfarani et al., 1996; Martin and Feldman, 1993; Sumantram and Feldman, 1993). Insulin-like growth factors (IGFs) exert anti-apoptotic effects on these cells (Matthews and Feldman, 1996) and also increase the migratory capacity of these cells by inducing actin polymerization and protrusions in the leading edge of these cells (Lauffenburger and Horwitz, 1996; Leventhal et al., 1997). The PI-3 kinase pathway is implicated in facilitating
the IGF-1-stimulated neuroblast migration (Waters and Pessin, 1996; Kim et al., 1997). However, recent evidence has shown that inhibition of the PI-3 kinase pathway reduces but does not completely inhibit IGF-1-induced SH-SY5Y migration (Puglianiello et al., 2000). This suggests that there may be alternate or additional pathways activated in response to IGF-1 that contribute to the migration of these cells. The results described in this chapter show that IGF-1-stimulation leads to the activation of a novel c-Abl/PTPα-dependent signalling pathway that enhances migration of SH-SY5Y cells.

In SH-SY5Y cells, IGF-1-stimulated tyrosine phosphorylation of PTPα is observed to be dependent on the IGF-1-induced activity of c-Abl which is similar to what I observed with SYF fibroblasts. Also, as in SYF fibroblasts, IGF-1R, RACK1 and FAK were shown to associate with PTPα in SH-SY5Y neuroblastoma cells. Together, this suggests that RACK1 acts as an essential scaffolding protein that co-ordinates and regulates the function (phosphorylation) of PTPα in response to IGF-1 in a c-Abl dependent manner. Recent findings by others have shown that IGF-1-stimulated c-Abl/RACK1 dependent modulation of FAK phosphorylation leads to an increase in cell migration in other systems (Guvakova and Surmacz, 1999; Genua et al., 2009; Kiely et al., 2006; Kiely et al., 2009). In view of this, it is possible that IGF-1-stimulated c-Abl/RACK1 dependent tyrosine phosphorylation of PTPα mediates IGF-1-induced cell migration in SH-SY5Y cells. Therefore, investigating the role of the Tyr789 residue of PTPα in IGF-1-induced cell migration comprises an important next step.
Figure 20. PTPα-Tyr789 is required for IGF-1-stimulated cell migration. 
(A) Wild-type (PTPα<sup>+/+</sup>) and PTPα<sup>−/−</sup> fibroblasts and PTPα<sup>−/−</sup> fibroblasts infected with adenovirus (AdV) expressing wild-type PTPα (WT) or mutant PTPα-Y789F (Y789F) were utilized for Transwell migration assays. The bars represent the mean number of migrated cells/field of view ± S.D. in 10 microscopic fields from triplicate experiments (*p<0.05, **p<0.005; IGF-1-treated vs. untreated) (B) The cells were analyzed for PTPα expression and PTPα-Tyr789 phosphorylation.
Figure 21. IGF-1 stimulates Abl-dependent phosphorylation of PTPα-Tyr789 in neuroblastoma cells.

Serum-starved SH-SY5Y cells were pretreated without (-) or with (+) the c-Abl inhibitors imatinib mesylate (Imat) or nilotinib (Nilot) as indicated for 3 hours (µM) then treated with or without IGF-1 (100 ng/ml) for 10 min. Lysates were probed for phospho-Y789-PTPα and PTPα.
Figure 22. Phosphorylation of c-Abl in neuroblastoma cells is reduced by silencing of PTPα.

SH-SY5Y cells were transfected with 15 nM control (scrambled, scr) or PTPα siRNA and cultured for 32 hours then serum-starved for another 16 hours. The cells were pretreated for 3 hours with or without the Abl inhibitor nilotinib (Nilot, 2.5 µM), then stimulated (+) or not (-) with IGF-1 (10 min, 100 ng/ml). Cell lysates were probed with anti-phospho-Tyr245-c-Abl, anti-c-Abl, anti-actin, anti-IGF-1Rβ, anti-RACK1 and anti-PTPα. The above data are representative of two experiments (n=2).
Figure 23. IGF-1-stimulated c-Abl activation is SFK independent in neuroblastoma cells.

SH-SY5Y cells were serum-starved and incubated for 3 hours without (-) or with (+) the Abl inhibitor nilotinib (Nilot, 2.5 µM). During the last one hour of this period the cells were pretreated as indicated with 2 µM SU6656 before treatment without (-) or with (+) 100 ng/ml IGF-1 for 10 min. The cell lysates were probed with anti-phospho-Tyr245-c-Abl antibody and reprobed with anti-c-Abl. The above data are representative of two experiments (n=2).
Figure 24. IGF-1Rβ, RACK1 and FAK associate with PTPα in neuroblastoma cells. SH-SY5Y cells were serum-starved overnight and pretreated with Nilotinib (Ni, 2.5 μM) for 3 hours before IGF-1 stimulation (10 min, 100ng/ml). Immunoprecipitates were prepared with anti-PTPα antibody and probed with anti-IGF-1Rβ, anti-RACK1, anti-FAK, and anti-PTPα. The above data are representative of the results of two experiments (n=2).
Figure 25. Neuroblastoma cell migration is inhibited by nilotinib and silencing of PTPα.

SH-SY5Y cells were transfected with 15 nM of control (scrambled, scr) or PTPα siRNA, cultured for 32 hours and serum-starved for another 16 hours. The cells were then incubated for 3hrs without or with the indicated concentration (µM) of the c-Abl inhibitor nilotinib (Nilot). (A) Cells were placed in Transwell inserts and IGF-1-stimulated migration (100 ng/ml IGF-1, bottom chamber) was assessed after 6 hours by counting the number of cells that migrated to the underside of the insert membrane. The bars represent the mean number of migrated cells/field ± S.D. in 10 microscopic fields from triplicate experiments (*p<0.05, **P<0.005, untreated vs. IGF-1-stimulated). (B) Aliquots of the
above treated cells were stimulated (+) or not (-) with IGF-1 (10 min, 100 ng/ml) and probed for PTPα and actin. (C) The graph displays the mean PTPα expression relative to actin ± S.D. from three experiments.
Chapter 6: Discussion

I have demonstrated that the IGF-1-stimulated increase in tyrosine phosphorylation of PTPα is dependent on the kinase activity of c-Abl. IGF-1-stimulated tyrosine phosphorylation of PTPα utilizes a signalling mechanism that is distinct from integrin-induced tyrosine phosphorylation of PTPα in that the activities of SFKs and FAK are dispensable for the former event. Furthermore, I have shown that PTPα associates with the IGF-1R and this association is dependent neither on the catalytic activity nor the tyrosine phosphorylation of either of the two proteins. The IGF-1R/PTPα complex also comprises the scaffolding protein RACK1. IGF-1-stimulation increases the activation of c-Abl and its association with the constitutive IGF-1R/RACK1/PTPα complex, which may explain the increase in PTPα tyrosine phosphorylation upon IGF-1-stimulation. In addition, I have shown that PTPα plays an important role in IGF-1-stimulated mouse embryonic fibroblast cell migration, which requires an intact Tyr789 phosphorylation site of PTPα. Similarly, the IGF-1-induced migration of SH-SY5Y neuroblastoma cells was demonstrated to be dependent on the presence of PTPα and the activity of c-Abl. In SH-SY5Y cells, IGF-1-stimulation also increased the activation of c-Abl and its association with the IGF-1R/RACK1/PTPα complex and lead to an increase in PTPα-Tyr789 phosphorylation. However, whether this IGF-1-stimulated c-Abl/RACK1-dependent tyrosine phosphorylation of PTPα mediates the IGF-1-induced cell migration of SH-SY5Y cells remains undetermined.

6.1 IGF-1 stimulates c-Abl activation

My experiments show that IGF-1 stimulates c-Abl tyrosine phosphorylation and activation in SYF fibroblasts and in SH-SY5Y neuroblastoma cells. However, precisely how the
ligand-activated IGF-1R tyrosine kinase effects c-Abl activation is unknown. Structure-function studies indicate that several intramolecular interactions maintain c-Abl in an inactive, closed conformation (Fig. 25). These include interactions of the SH3 domain with proline-rich (PXXP) stretches in the linker (Barila and Surperti-Furga, 1998); the SH2 domain with the C-terminal lobe of the kinase domain (Pluk et al., 2002); the myristoylation region with a hydrophobic pocket in the C-terminal lobe (Hantschel et al., 2003) and the Cap with the SH3-SH2 connector (Nagar et al., 2006). The opening of this conformation is known to play an important role in the activation of c-Abl. In this respect, interaction with SH3- and SH2-binders have been shown to induce catalytic activation (Hantschel and Superti-Furga, 2004). In addition, c-Abl is also regulated by phosphorylation (Fig. 25). Among several tyrosine phosphorylation sites clustered at the N-terminus of c-Abl, phosphorylation of Tyr245 and Tyr412 are required for c-Abl activation (Brasher and Van Etten, 2000; Dorey et al., 2001). Also, c-Abl activity is regulated by phosphorylation on serine and threonine residues (Hanstchel et al., 2003). Phosphorylation of unidentified serine residues in the Cap region of c-Abl and of Ser637 and Ser638 in the kinase domain also lead to opening of the inactive conformation of c-Abl (Nagar et al., 2006; Jung et al., 2008). In the following, I propose and discuss four mechanisms that could be involved in IGF-dependent c-Abl activation and PTPα Tyr789 phosphorylation.

6.1.1 IGF-1 stimulated activation of c-Abl is SFK-independent

Tyrosine phosphorylation of c-Abl is increased in response to IGF-1 in SYF fibroblasts lacking the SFKs Src, Yes, and Fyn. Furthermore, IGF-1-stimulated c-Abl-dependent phosphorylation of PTPα in these cells was unaffected by the SFK inhibitor SU6656. Likewise, the IGF-1-stimulated tyrosine phosphorylation of c-Abl occurred in the presence of SU6656 in
SH-SY5Y cells. These findings indicate that IGF-1-stimulated c-Abl activation takes place independent of SFKs in these cell types. This is supported by the findings of Srinivasan et al. (2008) that suggest that IGF-1-stimulated c-Abl activation is independent of SFK activity in breast cancer cells.

6.1.2 IGF-1-stimulated activation of c-Abl by direct association of c-Abl with IGF-1R

It is possible that the mechanism of IGF-1-stimulated c-Abl activation may be similar to the mechanism of EGF-stimulated c-Abl activation. Using protein microarrays it was recently discovered that all EGF receptor (EGFR) family members contain a high-affinity binding site for the SH2-domain of c-Abl (Jones et al., 2006), which facilitates specific protein interactions between the activated EGFR and c-Abl and is suggested to result in the opening of the inactive conformation of c-Abl in response to EGF stimulation (Sirvent et al., 2008). This is also supported by the works of Srinivasan and Plattner (2006) and Jones et al. (2006) that show that c-Abl associates with EGFRs in several cell lines. Hence, the IGF-1R could function in a manner similar to the members of the EGFR family. IGF-1R activation, upon IGF-1 stimulation, may create an Abl-SH2-binding site on IGF-1R that recruits and stabilizes the c-Abl kinase in an open and active conformation. Since IGF-1R is constitutively associated with PTPα and RACK1, the association of IGF-1R with activated c-Abl may therefore re-localize c-Abl from the cytoplasmic pool to the IGF-1R/ PTPα/RACK1 complex. The close proximity afforded by this interaction could then allow activated c-Abl to phosphorylate PTPα (Fig. 26A). This hypothesis is supported by my earlier observation of enhanced association of c-Abl with RACK1 upon IGF-1-stimulation in SYF fibroblasts. One appropriate way to determine whether IGF-1R associates with c-Abl to facilitate IGF-1-induced PTPα Tyr789 phosphorylation would be to carry out siRNA-mediated
RACK1 knockdown in SYF fibroblasts and SH-SY5Y cells, followed by immunoprecipitation of IGF-1R. If the enhanced association of c-Abl with the complex is independent of RACK1 then I would expect to find c-Abl in these immunoprecipitates.

### 6.1.3 IGF-1-stimulated activation of c-Abl by IGF-1-induced PLCγ activation

Increased PLCγ activity, which depletes the levels of its substrate PIP2 (phosphatidylinositol 4,5-bisphosphate) in vivo, was recently shown to facilitate PDGFR-stimulated c-Abl activation (Plattner et al., 2003; Sirvent et al., 2008). Interestingly, PIP2 modulates the activity of actin binding proteins and focal contact proteins in lipid rafts (Rohatgi et al., 1999; Raucher et al., 2000; Pollard and Borisy, 2003). This in turn affects the structure of F-actin, and F-actin is known to interact with the C-terminal domain of c-Abl and inhibit its activity (Woodring et al., 2001). It is suggested that a decrease in PIP2 levels alleviates the inhibitory effect of F-actin on c-Abl and thus allows PDGF-stimulated c-Abl activation (Sirvent et al., 2008). Lipid rafts, characterized as cholesterol-rich and detergent-resistant membrane microdomains, play an important role in regulating growth factor signal transduction across the plasma membrane (Simons and Toomre, 2000; Anderson, 1998; Brown and London, 1998). IGF-1-activated IGF-1R in lipid rafts activates PLCγ in neuronal cells like SH-SY5Y cells (Huo et al., 2003; Baserga et al., 1997; Chattopadhyay and Carpenter, 2002; Yamaguchi et al., 2009). Therefore it is possible that IGF-1R activation leads to the PLCγ-mediated depletion of PIP2 to facilitate the activation of c-Abl in close proximity to PTPα, as a population of PTPα is also localized to lipid rafts (Maksumova et al., 2005; Vacaresse et al., 2008). Activated c-Abl kinase could then phosphorylate PTPα by interacting with the IGF-1R/RACK1/PTPα complex (Fig. 26B). Targeting PLCγ activity by using specific PLCγ inhibitors such as U73122 (Lorenzo et al.
2002), or targeting PLCγ expression by siRNA knockdown, would be a useful approach to determine if PLCγ is required for IGF-1-stimulated c-Abl activation and consequently, for IGF-1-stimulated c-Abl dependent PTPα tyrosine phosphorylation.

6.1.4 IGF-1-stimulated activation of c-Abl by IGF-1-induced Ras activation

Lastly, it is also possible that IGF-1-stimulated c-Abl-dependent PTPα Tyr789 phosphorylation may be accomplished through the IGF-1-induced activation of Ras. The ligand-induced activation of IGF-1R activates Ras via the Shc/Grb2/Sos signalling complex, and it was recently discovered that enhanced Ras activity also promotes the association of c-Abl with RACK1 in fibroblasts (Huang et al., 2008). Therefore, IGF-1-induced Ras activation could be the signalling event that leads to the activation of c-Abl and its recruitment to the IGF-1R/RACK1/PTPα complex where it catalyzes the tyrosine phosphorylation of PTPα (Fig. 26C). Determining the effects on IGF-1-dependent phosphorylation of c-Abl and PTPα after treatment of SYF fibroblasts and SH-SY5Y cells with specific Ras inhibitors, such as farnesylthiosalicylic acid (Gana-Weisz et al., 2002) or dominant-negative mutant Ras (N17), would be one way to test this hypothesis.

6.2 Role of RACK1 in IGF-1-stimulated PTPα-Tyr789 phosphorylation

In this study, I have shown that IGF-1-stimulated tyrosine phosphorylation is dependent on RACK1. This may be because RACK1 facilitates the association between PTPα and IGF-1R, as also demonstrated by my experiments. RACK1 comprises seven internal Trp-Asp40 (WD40) repeats that give it an extended ‘flat-disk’ shape with several interaction sites above, below and around its circumference (Sklan et al., 2006). This enables it to interact with multiple proteins, as for example in this case PTPα and IGF-1R, and form signalling complexes (Sondek and
Siderovski, 2001; Chen et al., 2004). Certain RACK1-signalling complexes play important roles in several cellular functions like transcription of proteins (Quan et al., 2006), cAMP synthesis (Yarwood et al., 1999), cell growth (Chang et al., 2001), and immune responsiveness (Zhu et al., 1998). In fact, RACK1 also associates with other protein tyrosine phosphatases, such as PTP1B and PTPµ (Chen, Chen, and Chuang, 2004; Mourton et al., 2001).

Although much remains unknown about the interaction of RACK1 with PTP1B, Mourton et al. (2001), in a recent study, showed that PTPµ, a close relative of PTPα (Blanchetot and den Hertog, 2000), specifically interacts with RACK1 at the plasma membrane. The antisense-mediated down-regulation of PTPµ expression in mink lung epithelial cells demonstrated that in the absence of PTPµ, RACK1 could not localize to the plasma membrane and points of cell-cell contact (Mourton et al., 2001). This indicates that PTPµ specifically recruits RACK1 to the plasma membrane. Moreover, Mourton et al. (2001) demonstrated that unlike some RACK1-protein liaisons where interaction is facilitated by conformational changes in the RACK1 structure brought on by phosphorylation of its tyrosine residues (Sklan et al., 2006), the association between PTPµ and RACK1 was mediated by protein-protein interactions not dependent on phosphotyrosine. My experimental results, where the PTPα/RACK1 association is constitutive, support a similar conclusion. I therefore propose that PTPα recruits RACK1, in a similar manner to PTPµ, as a scaffolding protein that facilitates the association of PTPα with IGF-1R at the plasma membrane. Upon IGF-1-stimulation, the association of c-Abl with RACK1 is enhanced and this leads to downstream signalling events such as IGF-1-induced Tyr789 phosphorylation.

Additionally, Mourton et al. (2001) demonstrated that RACK1 interacts with membrane proximal catalytic D1 domain of PTPµ. This may be the case with PTPα as well since the D1
domain of PTPα is highly homologous to PTPµ-D1. My results so far show that the association of PTPα with RACK1/IGF-1R can be mediated by the presence of either of the two catalytic domains (D1 or D2) of PTPα. Since the precise binding sequence within D1 or D2 that mediates the PTPα-RACK1 interaction remains undetermined, future investigations using wild-type and truncated mutants of PTPα and RACK1 in *in vitro* studies such as GST-pull downs and scanning peptide arrays and *in vivo* studies such as immunoprecipitations of epitope-tagged proteins, would prove useful in characterizing the PTPα-RACK1 interaction.

### 6.3 Role of PTPα in IGF-1R/ RACK1/ c-Abl signalling

IGF-1 plays key roles in cell proliferation, survival, migration, and tumour progression (Ryan and Goss, 2008). IGF-1 has also been shown to modulate many aspects of neuroblastoma biology including motility and invasiveness (Meyer et al., 2001; Puglianiello et al., 2000). My results show that PTPα plays a major role in IGF-1-mediated cell migration of normal and tumour cells, since its absence halves the migratory ability of both fibroblasts and neuroblastoma cells. I have demonstrated that IGF-1-stimulated tyrosine phosphorylation of PTPα is required for IGF-1-induced migration in mouse embryonic fibroblasts. Additionally, my results show that IGF-1-stimulated migration of neuroblastoma cells is dependent on both the presence of PTPα and the activity of c-Abl. Since c-Abl is required to catalyze tyrosine phosphorylation of PTPα, at Tyr789, present in the constitutive IGF-1R/RACK1/PTPα signalling complex in response to IGF-1 in these cells, it is possible that IGF-1-stimulated RACK1/c-Abl dependent PTPα-Tyr789 phosphorylation may also be a requirement of IGF-1-induced neuroblastoma migration.

Another key component of the IGF-1R/RACK1/PTPα signalling complex is FAK. Both PTPα and FAK are involved in focal adhesions (Lammers et al., 2000; Liu et al., 2008) which are
multi-protein complexes consisting of ECM molecules, integrin receptors, intracellular signalling complexes and cytoskeletal proteins formed during cell migration (Hynes, 2002; Mitra et al., 2005). Recently, it was discovered that the association between FAK and RACK1 is required for IGF-1R mediated modulation of FAK phosphorylation (Kiely et al., 2009). Therefore, it is possible that the interaction between PTPα and FAK, facilitated by the IGF-1R/RACK1/c-Abl complex, plays an important role in IGF-1-stimulated cell migration. In fact, in integrin signalling, fibronectin stimulation leads to the activation of FAK which is essential for catalyzing tyrosine phosphorylation of PTPα at Tyr789 that in turn is required for cell migration (Chen et al., 2006). My investigations have shown that FAK is dispensable for IGF-1-stimulated PTPα-Tyr789 phosphorylation. However, this does not exclude the possibility that PTPα plays a role in IGF-1-mediated FAK activity.

Several key observations from this study and from other research (Kiely et al., 2005; 2009; Lynch et al., 2005; Chen et al., 2006) suggest that IGF-1 and integrin signalling converge at the level of FAK and PTPα activity, despite having distinct initial mechanisms. While in integrin signalling, fibronectin stimulation promotes the association of PTPα with α integrins and its catalytic activity is involved in the activation of SFKs and FAK which then catalyze the phosphorylation of PTPα on Tyr789 (Chen et al., 2006), in IGF-1 signalling, PTPα-Tyr789 phosphorylation is not dependent on FAK but may subsequently modulate FAK phosphorylation and activity in response to IGF-1. Furthermore, it may well be that RACK1 regulates the nature of the PTPα and FAK interaction depending on the stimuli received by the cell, since RACK1 associates both with integrins (Kiely et al., 2006; Liliental and Chang, 1998; Buensuceso et al., 2001) and IGF-1R (Kiely et al., 2009). Therefore, it remains to be determined whether fibronectin stimulation leads to the recruitment of RACK1, along with PTPα, to integrin. This
may provide evidence for a model whereby PTPα links activated integrin complexes to the non-canonical activation of IGF-1R effectors downstream of the receptor that influence cellular migration. Conversely, fibronectin stimulation may induce integrin-dependent sequestering of PTPα from RACK1 complexes leading to downstream integrin signalling exclusive of signalling from the IGF-1R/RACK1/PTPα complex.
Figure 26. Structure and regulation of c-Abl.

(A) c-Abl comprises a variable N-terminus, which is followed by a SH3 and a SH2 domain, a linker sequence that connects the SH2 domain to the kinase domain and the catalytic core. Proline-rich stretches (PXXP), a DNA-binding domain (DNA BD), three nuclear localization sequences (NLS), one nuclear export sequence (NES) and G-(monomeric) and F-actin binding motifs (Actin BD) are present in the C-terminus. Tyrosine residues that undergo phosphorylation in response to growth factor activation have been indicated. (B) The inactive ‘closed’ conformation of c-Abl is facilitated by several intra-molecular interactions: SH3 domain with the PXXP motif in the linker sequence, SH2 domain with the C-terminal lobe and the Cap with the SH3-SH2 connector. ‘Opening’ of the conformation leads to c-Abl activation. Phosphorylation of tyrosine residues Tyr245 (Y245) in the linker sequence and Tyr412 (Y412) in the activation loop, in response to growth factor stimulation, stabilizes c-Abl in an open and active confirmation. (Adapted from Sirvent et al., 2008)
Figure 27. Possible mechanisms of IGF-1-stimulated c-Abl dependent PTPα Tyr789 phosphorylation.

Recruitment of the active c-Abl to the IGF-1R/RACK1/PTPα complex is key to IGF-1-stimulated, c-Abl-dependent PTPα Tyr789 phosphorylation and may be mediated by one of three possible mechanisms: (A) IGF-1-mediated creation of an Abl-SH2-binding site on the IGF-1R that recruits and activates c-Abl to the IGF-1R/RACK1/PTPα complex.
directly. (B) IGF-1-stimulated, PLCγ-dependent c-Abl activation and recruitment. (C) IGF-1-stimulated, Ras-dependent c-Abl activation and recruitment.
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