SHP-1 and PDK1 Form a Phosphotyrosine-Dependent Nucleo-Cytoplasmic Shuttling Complex: Implications for Differentiation.

A Thesis Submitted to the College of

Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Doctorate of Philosophy

in the Department of Psychiatry

University of Saskatchewan

Saskatoon

By

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ABSTRACT

SHP-1 is a protein tyrosine phosphatase that often targets the phosphatidylinositol 3'-kinase (PI3K)/Akt signalling pathway. PI3K/Akt signalling regulates cell growth and survival, proliferation and differentiation. Growth factor-stimulated PI3K phospholipid production at the plasma membrane helps to recruit 3'-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt, where PDK1 phosphorylates and activates the pro-survival kinase Akt.

Tyrosine phosphorylation of PDK1 may regulate its function and, perhaps more importantly, its nuclear localization. Yet, it is unclear how PDK1 is imported into the nucleus as it does not contain a nuclear localization signal (NLS), although it does contain a nuclear export signal (NES). Interestingly, several tyrosines in PDK1 are targets for Src kinase and are putative target motifs for SHP-1, which does have an NLS.

Hypothesis: SHP-1 and PDK1 form a tyrosine-dependent, nucleo-cytoplasmic shuttling complex.

Removal of serum from C6 glioma cell cultures induces a platelet-derived growth factor receptor (PDGFR)-sensitive redistribution of PI3K lipid kinase activity to the nucleus. PDK1 tyrosine phosphorylation and its association with SHP-1 are also increased, as is the accumulation of both SHP-1 and PDK1 in the nucleus. Site-directed mutagenesis of tyrosine residues in PDK1 reveals that tyrosine 9 (Tyr9) and Tyr376 are important for the interaction of PDK1 with SHP1, whereas Tyr333 and Tyr 373 are not. Using pharmacological and genetic manipulations, it was demonstrated that SHP-1 and PDK1 shuttle between the nucleus and cytoplasm, and that the C-terminal-expressed NLS of SHP-1 facilitates shuttling, while dephosphorylation of PDK1 Tyr9 and Tyr376 regulates the rate of PDK1 (and by virtue of association, SHP-1) export from the nucleus. The SHP-1/PDK1 complex, which is constitutive in most cell lines, is functionally relevant as indicated by its requirement for NGF-induced differentiation of preneuronal cells to a neuronal phenotype.

ACKNOWLEDGMENTS

I would like to extend my gratitude to my supervisor Dr. Darrell D. Mousseau who has provided me with the support to develop my skills as an independent researcher. I am grateful to have the opportunity to learn from his experience and appreciate the wisdom he has shared with me.

I would also like to thank the members of my advisory committee Dr. Lillian E. Dyck, Dr. XinMin Li and Dr. Deborah H. Anderson for their advice and support throughout my graduate studies at the University of Saskatchewan.

I would also like to express my appreciation for the members of the Mousseau Lab, (Susan)Xia Cao, Yunxiu Dai, Zelan Wei, Ji Qi, Di Zhang, Geraldine Gabriel, whom I have had the opportunity to work.

I would particularly like to express my gratitude and love towards Paul Dutchak; you have been there for me through it all. I would like to thank all my friends, class-mates and fellow graduate students, April Thorimbert, Jennifer Chlan, Shaun Labiuk, Alina Wood and Nicole Payette for their support. I am blessed to have friends as caring as these.

Finally, I would like to express my gratitude and love towards my parents, Boyd and Sharon Sephton, for their love and support over all the years. I am forever grateful for all they have given me. Thank you to my siblings Sheldon and Alysha Sephton both of whom have loved and supported me over the years. I am blessed to have you in my life.

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LIST OF ABBREVIATIONS

A/Ala	Alanine
Amp	Ampicillin
BCR	B cell antigen receptor
BDNF	Brain-derived neurotrophic factor
CD	Cluster of differentiation
CHO	Chinese hamster ovary
C/Cys	Cysteine
CL	Cardiolipin
CNS	Central nervous system
CRM1	Chromosomal region maintenance protein 1
CFS-1	Colony-stimulating factor 1
D/Asp	Aspartic acid
DAPI	4',6-Diamidino-2-Phenylindole
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
dYT	Double yeast and tryptone
E/Glu	Glutamic acid
EDTA	Ethylene-Diamine Tetraacetic Acid Disodium Salt
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EGTA	Ethylene Glycol Bis (2-Aminoethyl Ether)-N,N,N'N'-Tetraacetic Acid Disodium Salt
Erk	Extracellular signal-regulated kinase
EtOH	Ethanol
F/Phe	Phenylalanine
FcR	Fc receptor
FGFR	Fibroblast growth factor receptors
FRS	Fibroblast growth factor receptor substrate

LIST OF ABBREVIATIONS (cont.)

Gab1	Grb2-associated binder-1
GFP	Green fluorescent protein
GH	Growth hormone
G/Gly	Glycine
GS	Goat serum
GSK-3β	Glycogen synthase kinase-3 beta
HCl	Hydrochloric acid
HDAC	Histone deacetylase
H/His	Histidine
HEPES	N-2-Hydroethylpiperazine-N'-2-Ethane Sulfonic acid
HNTG	HEPES, NaCl, Triton X-100 and glycerol
HPR	Horseradish peroxidase
H_2O_2	Hydrogen peroxide
IB	Immunoblot
IGF-1	Insulin-like growth factor-1
I/Ile	Isoleucine
INF-1	Interferon-1
IP	Immunoprecipitation
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JAK	Janus kinase
Kan	Kanamycin
KCl	Potassium Chloride
KIR	Killer inhibitory receptor
L/Leu	Leucine
LMB	Leptomycin B
MAPK	Mitogen-activated protein kinase
MEK	Mitogen and extracellular signal-activated protein kinase kinase

LIST OF ABBREVIATIONS (cont.)

MEF	Mouse embryonic fibroblast
M/Met	Methionine
MHC	Major histocompatibility complex
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulfate
N/Asp	Asparagine
NaCl	Sodium Chloride
Na_2CO_3	Sodium Bicarbonate
NaF	Sodium Fluoride
NaOH	Sodium Hydroxide
NGF	Nerve growth factor
NES	Nuclear export sequence
NK	Natural killer
NLS	Nuclear localization signal
NPC	Nuclear pore complex
NPTP	Non-receptor protein tyrosine phosphatase
NRPTK	Non-receptor protein tyrosine kinase
OD	Optical density
P/Pro	Proline
PA	Phosphatidic acid
PAE	Porcine aortic endothelial
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDK1	3'-phosphoinositide-dependent protein kinase-1
PEG	Polyethylene Glycol

LIST OF ABBREVIATIONS (cont.)

PG	Phosphatidylglycerol
PH	Pleckstrin homology
PI	Phosphatidylinositol
3'-PI	3'-phosphoinositide
PIPES	1,4-Piperazine Diethane Sulfonic acid, Sodium Salt
$PI3,4P_2$	Phosphatidylinositol-3,4-*bis*phosphate
$PI3,4,5P_3$	Phosphatidylinositol-3,4,5-*tris*phosphate
PI3K	Phosphatidylinositol 3'-kinase
PIF	PDK1 interacting fragment
PKB	Protein kinase B
PMSF	Phenylmethylsulfonyl Fluoride
PPARγ	Peroxisome proliferator activated-receptor gamma (γ)
PP-1	Protein phosphatase-1
PP2A	Protein phosphatase 2A
PS	Phosphatidylserine
PTEN	phosphatase and tensin homologue deleted on chromosome 10
PTP	Protein tyrosine phosphatase
PV	Pervanadate
R/Arg	Arginine
RFP	Red fluorescent protein
RPTP	Receptor protein tyrosine phosphatase
RSK	p90 ribosomal S6 kinase
RT	Room temperature
RTK	Receptor tyrosine kinase
S/Ser	Serine
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
SHIP-1	Src Homology domain-containing inositol phosphatase-1

LIST OF ABBREVIATIONS (cont.)

SHIP-2	Src Homology domain-containing inositol phosphatase-2
SHP-1	Src homology 2 containing protein tyrosine phosphatase-1
SHP-2	Src homology 2 containing protein tyrosine phosphatase-2
SHPS-1	SH2 domain-containing protein tyrosine phosphatase substrate-1
siRNA	Small interfering RNA
STAT	Signal transducers and activators of transcription
SWD	Serum withdrawal
T/Thr	Threonine
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with Tween-20
TCL	Total cell lysates
TCR	T cell antigen receptor
TEMED	N,N,N',N'-Tetramethylenediamine
Tris	2-Amino-2-Hydroxymethyl-1,3-Propanediol
TrkA	Tropomycin kinase A receptor
TSA	Trichostatin-A
V/Val	Valine
Y/Tyr	Tyrosine
WT	Wildtype

1. REVIEW OF THE LITERATURE

1.1 Signal Transduction.

Normal cellular function is mediated by several factors including external signals or stimuli that most often act through membrane receptors. This transfer of information from the environment to the cell's interior is commonly referred to as signal transduction. Signal transduction pathways mediate the sensing and processing of stimuli and often depend on the balance between activation and inhibition of intracellular proteins.

1.1.2 Cellular tyrosine phosphorylation.

Tyrosine phosphorylation of cellular proteins regulates fundamental biochemical processes in cells exerting both positive and negative effects on signal transduction pathways. Post-translational modification of proteins by tyrosine phosphorylation is an evolutionarily conserved mechanism critical for regulating a variety of cell functions (Walton and Dixon, 1993). This dynamic process is regulated by the opposing actions of kinases and phosphatases that are often themselves regulated by phosphorylation. Protein tyrosine kinases catalyze tyrosine phosphorylation and protein tyrosine phosphatases catalyze tyrosine dephosphorylation of proteins. Deregulation of kinases or phosphatases contributes to several human pathologies, including neurodegeneration, cancer and diabetes (Tonks, 2006).

1.1.3 Receptor tyrosine kinases (RTKs).

Protein tyrosine kinases are a diverse group of proteins that exist throughout the cell either plasma membrane-bound or free within the cytoplasm. Receptor tyrosine kinases (RTKs) are a large family of transmembrane receptors that control

fundamental cellular processes including cell cycle, migration, metabolism, survival, proliferation, as well as differentiation (Hunter, 2000; Schlessinger, 2000). All RTKs contain an extracellular ligand-binding domain, a hydrophobic transmembrane domain, a cytoplasmic domain that contains a catalytic kinase segment, as well as regulatory sequences that are subjected to autophosphorylation and phosphorylation by other protein kinases (Hubbard et al., 1998; Hunter, 1998).

With the exception of the insulin receptor family of RTKs, all known RTKs including the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR), exist as monomers in the cell membrane (Lemmon and Schlessinger, 1994). A general mechanism for ligand-induced activation of receptor tyrosine kinases follows ligand binding to the extracellular domain and the induction of receptor dimerization (Lemmon and Schlessinger, 1994). Dimerization leads to activation of the kinase domain and autophosphorylation that, in turn, leads to generation of docking sites for SH2 (Src homology 2) domain containing proteins (Pawson and Scott, 1997). This results in the initiation of signal transduction pathways. The activation and function of RTKs are primarily regulated by the tyrosine phosphorylation state of the intracellular kinase domain. Inhibition of RTK signalling very often occurs through dephosphorylation by protein tyrosine phosphatases.

1.1.4 Non-receptor protein kinases (NRPKs).

Non-receptor protein kinases are located in the cytoplasm, nucleus or localized to the inner portion of the plasma membrane. They can be divided into protein tyrosine kinases and serine/threonine kinases. Protein tyrosine kinases are grouped into eight families: Src, Abl, Btk, Janus kinases (Jak), focal adhesion kinases (Fak), Fps, Csk and Syk (Hubbard and Till, 2000) and each family consists of several members. With the exception of homologous kinase domains and some protein-protein interaction domains, they have little in common structurally (Hubbard and Till, 2000). Protein tyrosine kinases have diverse roles in the regulation of cellular processes. For example, Src kinases are involved in cell growth and Fps kinases are involved in differentiation, while Abl kinases are involved in growth inhibition and Fak activity is associated with cell adhesion (Hubbard and Till, 2000).

Similarly, serine/threonine kinases play a role in several cellular processes (Cross et al., 2000). Members of this family include the mitogen-activated protein kinase (MAPK) family (includes ERK, p38, JNK) and the AGC kinase family (cAMP-dependent protein kinase, cGMP-dependent protein kinase and protein kinase C) specifically Akt (also known as protein kinase B: PKB), 3'-phosphoinositide-dependent protein kinase-1 (PDK1) and protein kinase C (PKC) (Cross et al., 2000).

Protein kinases are regulated through phosphorylation by kinases and dephosphorylation by phosphatases, however, unlike RTKs, dephosphorylation of NRPKs can lead to activation or inhibition of signalling. Some of these kinases will be discussed in more detail in subsequent sections that deal with specific signalling pathways.

1.1.5 Protein tyrosine phosphatases (PTPs).

Protein tyrosine phosphatases participate in diverse cellular processes to offset the effect of protein tyrosine kinase activity, *e.g.* RTKs. There are as many as 45 individual protein tyrosine phosphatase species, yet relatively little is known about their precise role in cellular function (Tonks, 2006) (Figure 1.1).

PTPs have at least one highly conserved catalytic domain of ~280 amino acids that contains an active site with the consensus sequence (I/V)HCXAGXXR(S/T)G (single-letter code for amino acids, where X represents any amino acid) (Dixon, 1995). They are inhibited by pervanadate, can hydrolyze *p*-nitrophenyl phosphate, they are insensitive to okadaic acid (an inhibitor of tyrosine kinases and serine/threonine phosphatases) and do not require metal ions during catalysis (Zhang, 1998; Zhang, 2002). Generally, PTPs display poor substrate specificity *in vitro*, suggesting that conditions for their activity *in vivo* must be tightly regulated in order to elicit signalling responses.

Regulation of PTP activity *in vivo* can occur through modulation of steady-state protein levels, post-translational modification (*i.e.* phosphorylation), dimerization and subcellular localization. Both the catalytic domain and non-catalytic segments of PTPs contribute to substrate specificity. Detailed enzyme studies and crystal structure analysis

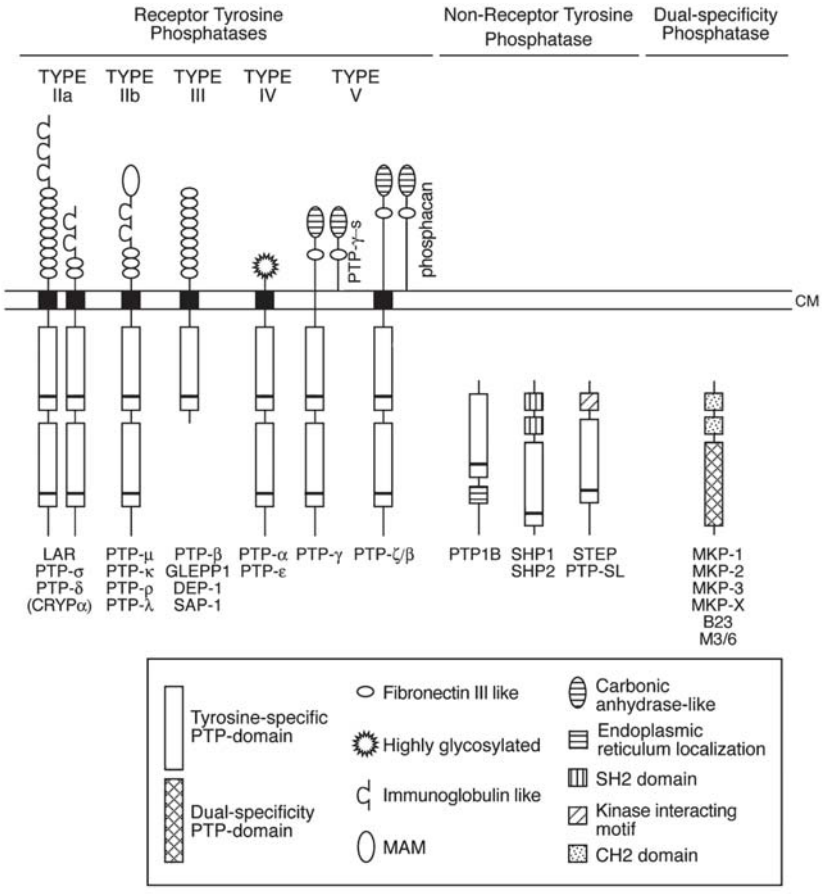

Figure 1.1: Major receptor protein tyrosine phosphatases (PTPs), non-receptor PTPs and dual-specificity phosphatases expressed in the nervous system. Src homology-2 (SH2); cell membrane (CM); Cdc 25 homology domain (CH2). (Birkhäuser Verlag AG, Basel, 2003, Cellular and Molecular Life Sciences, 60, S. Paul, and P.J. Lombroso, 2465-2482)

of several PTPs reveal that the active site contains a cysteine residue that is important for enzymatic activity, as mutation of this residue inactivates the enzymes. The cysteine residue functions in nucleophilic attack on the substrate phosphotyrosine residue, forming a transient phosphoenzyme intermediate. A nearby arginine residue within the active site helps to stabilize the enzyme-substrate interaction. A conserved aspartic acid facilitates this reaction by serving as a proton donor to the leaving phenolic oxygen. The reaction is terminated by the hydrolysis of the phosphoenzyme intermediate. Termination is also facilitated by the same aspartic acid through abstraction of a proton for the attacking water molecule. In contrast, the non-catalytic domain serves to target PTPs to specific proteins or intracellular compartments (Barford, 1999; Zhang, 1998; Zhang, 2002).

The PTP family consists of both tyrosine-specific and dual specificity phosphatases. Tyrosine-specific phosphatases hydrolyze only phosphotyrosine-containing proteins. In contrast, the dual specificity phosphatases can target proteins that contain both phosphotyrosine as well as phosphoserine or phosphothreonine residues. The tyrosine-specific phosphatases are further divided into two groups: the receptor-like PTPs and the non-receptor-like PTPs (Figure 1.1).

1.1.5.1 Receptor-like protein tyrosine phosphatases (RPTPs).

RPTPs function as an interface between the extracellular and intracellular environment of a cell and its intracellular signalling pathways. They usually possess two functional intracellular phosphatase domains (Alonso et al., 2004). Their extracellular domains contain motifs that are implicated in cell adhesion, although RPTPs are all highly variable. Most RPTPs are considered orphan receptors, as their mode of action and function is largely unknown (Alonso et al., 2004).

1.1.5.2 Non-receptor protein tyrosine phosphatases (NPTPs).

The NPTPs are further divided into two groups based on their substrate specificity. The first group includes the tyrosine-specific phosphatases, *i.e.* Src homology 2 (SH2) domain containing phosphatases (SHP), SHP-1 and SHP-2 (Zhao et al., 1995) (Figure 1.2). The second group includes dual specificity phosphatases such as

mitogen-activated protein kinase phosphatase (MKP-3) (Muda et al., 1996). In contrast to RPTPs, members of this family lack a transmembrane domain, possess a single phosphatase domain, and have multiple variable domains either in the N- or C-terminus.

There are a large number of protein tyrosine phosphatases with distinct substrate specificities and modes of regulation. SHP-1 is one such phosphatase that belongs to a subfamily of cytoplasmic protein tyrosine phosphatases referred to as Src homology 2 (SH2) domain containing phosphatases (SHP). During PubMed literature searches, one should not confuse SHP-1 with the atypical nuclear receptor, small heterodimeric partner (SHP), which is upregulated by the nuclear receptor transcription factor, Farnesoid X Receptor (FXR) (Denson et al., 2001). Other members of the protein tyrosine family of phosphatases include SHP-2 and the homologue of mammalian SHP-2, *Drosophila* Csw. SHP-1 and SHP-2 have nearly 55% overall sequence homology and 59% sequence homology within the two SH2 domains and the catalytic domain (Zhao et al., 1995) (Figure 1.2). SHP-1 and SHP-2 are also regulated in a similar manner (Zhao et al., 1995). However, SHP-1 is generally considered as a negative regulator of signal transduction and SHP-2 is considered to be a positive regulator of signal transduction (Adachi et al., 1996; Perkins et al., 1992).

1.2 Src homology-2 domain containing phosphatase-1 (SHP-1).

SHP-1, cloned in 1991 (Shen et al., 1991; Yi et al., 1991) and also known as PTP1C, SHP-PTP1, HCP, PTPN6, or SHP (Adachi et al., 1996), is expressed at high levels in peripheral tissues including the liver, spleen and thymus, with particularly high expression in hematopoietic cells (Yi et al., 1992). SHP-1 is also expressed in the central nervous system (CNS), *i.e.* in brain (cerebral cortex, hippocampus and cerebellum) and spinal cord (Jena et al., 1997; Massa et al., 2000).

1.2.1 SHP-1 structure and regulation of activity.

The human SHP-1 gene is located on chromosome 12p13-p12 and consists of 17 exons spanning 17 kb of DNA (Matsushita et al., 1999; Plutzky et al., 1992; Yi et al., 1992). There are three non-hematopoietic SHP-1 transcripts identified in various cell lines and shown to be transcribed from a common promoter (Banville et al., 1995). The

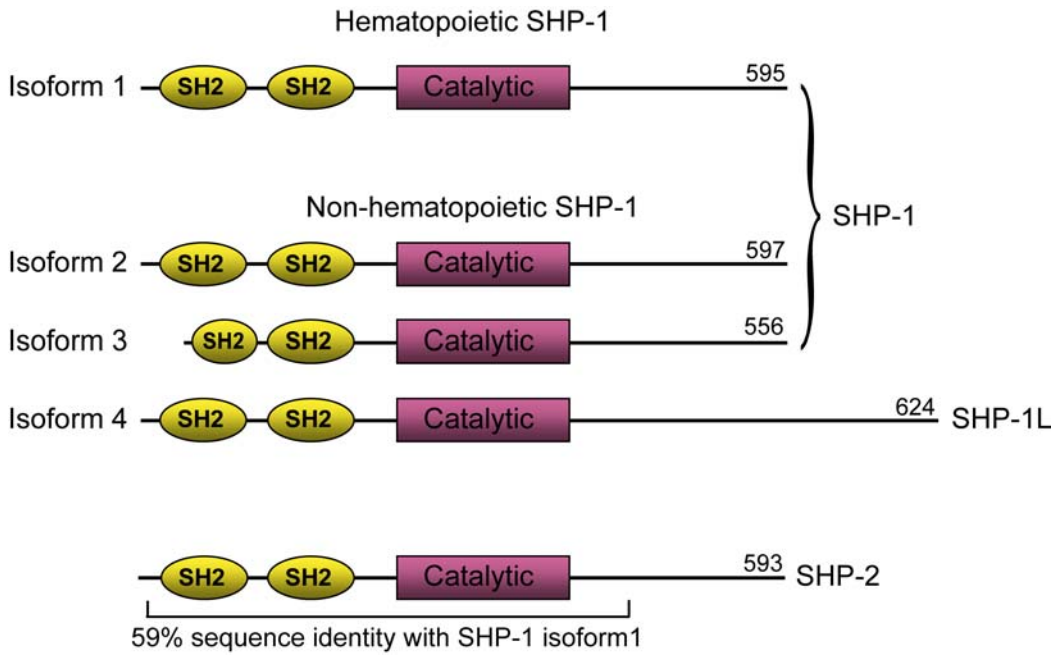

Figure 1.2: Protein structures of Src homology-2 domain containing phosphatase (SHP-1) isoforms and SHP-2. Splice variants of SHP-1 generate 4 gene products; 3 variants of SHP-1 with variations at the N-terminus of the protein and a long form of SHP-1 (SHP-1L) where the N-terminus is conserved, but the C-terminus is extended. The other tyrosine phosphatase, SHP-2 shares 59% sequence homology with SHP-1 isoform 1 within the two SH2 domains and the catalytic domain (Adapted from: Poole and Jones, 2005).

hematopoietic form of the SHP-1 transcript is initiated at a downstream promoter separated by 7 kb from the non-hematopoietic promoter. Activation of the downstream promoter is exclusive to cells of hematopoietic lineage (Banville et al., 1995). The hematopoietic cell-specific isoforms of SHP-1 and the non-hematopoietic cell-specific isoforms differ only by their alternative initiation sites and by three amino acids at the N-terminus (Banville et al., 1995) (Figure 1.2).

SHP-1 is comprised of two N-terminal SH2 domains, named after a conserved sequence region of the oncoprotein Src (Pawson, 1988; Sadowski et al., 1986), a central catalytic domain and a C-terminal domain (Yang et al., 1998) (Figure 1.2). As with other signalling molecules, protein-protein interactions are an integral part of the function of SHP-1. These interactions are mediated by domains such as SH2, SH3 and breakpoint cluster region domains, which recognize specific amino acid motifs in their interacting protein (Cohen et al., 1995). Binding domains resulting in interactions can mediate different events including catalyzing phosphorylation, bringing substrates to catalytic centres, linking signal transducers to upstream proteins, conformation changes or localizing protein complexes to cellular sub-regions. SH2 domains for example, bind specifically to tyrosine phosphorylated substrates. Most SH2 domains fall into one of two broad categories: class I SH2 domains prefer to bind substrates containing the consensus motif, pY-hl-hl-h (pY, phosphotyrosine; hl, hydrophilic amino acid; h, hydrophobic amino acid); and class III SH2 domains have a high affinity for the consensus motif, pY-h-X-h (pY, phosphotyrosine; h, hydrophobic amino acid; X, any amino acid) (Cohen et al., 1995; Pawson and Gish, 1992).

Analysis of the crystal structure of the C-terminus of SHP-1 has lead to the proposed model whereby the N-terminal SH2 domain regulates the phosphatase activity of the enzyme (Yang et al., 2003). In the “closed” or inhibited form, the N-terminal SH2 domain is in contact with the catalytic domain through charge-charge interactions. The C-terminal SH2 domain has little interaction with the N-terminal SH2 domain or the catalytic domain except through the connecting peptide backbone. In the inactive state, the N-terminal SH2 domain is in contact with the charged residues near the catalytic cleft and part of the SH2 domain is inserted into the catalytic cleft. In this state there is a blockade of the active site to its substrates (Figure 1.3). Truncation of the SH2

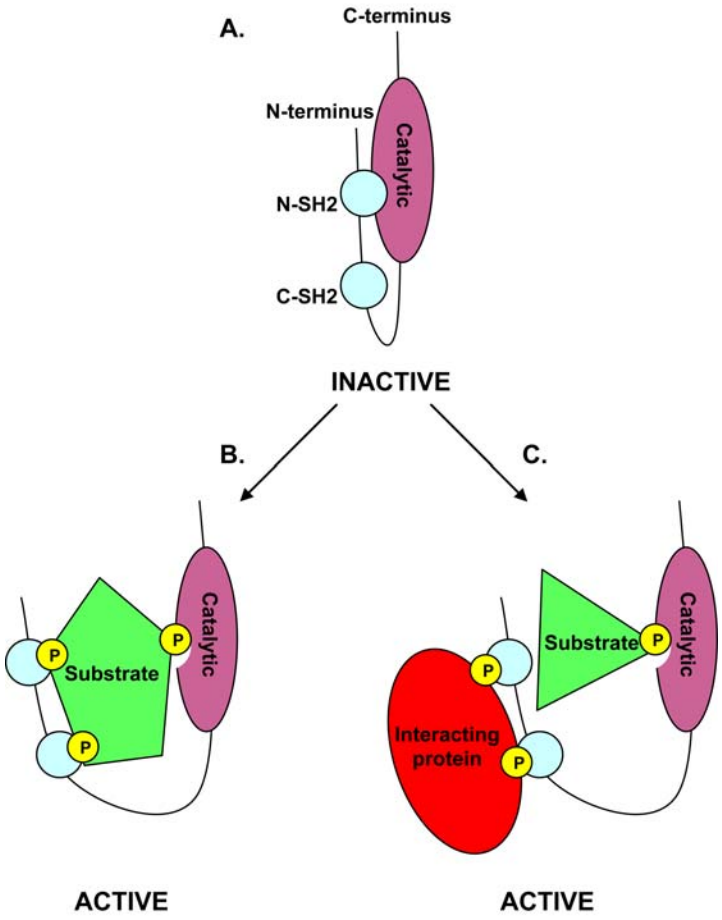

Figure 1.3: Regulation of SHP-1 phosphatase activity. (**A**) Inactive state of SHP-1, in which the N-terminal SH2 domain sits within the catalytic domain of the molecule restrict access of substrates to the phosphatase. The C-terminal SH2 domain is exposed and searches for potential binding partners which contain the pYXX(V/IL) consensus motif. Upon interaction with a phosphorylated substrate (**B**) or phosphorylated interacting protein (*i.e.* a receptor tyrosine kinase) (**C**) the phosphatase is activated.

domain(s) results in activation of SHP-1 (Pei et al., 1994; Townley et al., 1993). Truncation of the two SH2 domains of SHP-1 (amino acids 1-204) or the last 35 amino acids of the C-terminal SH2 increases SHP-1 activity by 30-fold and 20-34 fold relative to the wildtype, respectively (Pei et al., 1994) as well as its affinity towards synthetic phosphotyrosyl peptides (Townley et al., 1993). Conventionally, binding of the N-terminal SH2 domain to a phosphopeptide causes a conformation change in the N-terminal domain so that it "opens" (now an activated form), thereby disrupting the interaction between the SH2 domain and the catalytic domain. When in the activated form, the catalytic domain of SHP-1 is accessible to its substrates for dephosphorylation (Poole and Jones, 2005) (Figure 1.3).

The active site of the catalytic domain of SHP-1 contains three important amino acid residues: Cys455 acts as a nucleophil to attack the substrate; Arg459 stabilizes the negative charge of the phosphotyrosine substrate; and Asp421, the proton donor and proton acceptor involved in product release. Both biochemical and crystallographic data have shown that the catalytic domain of SHP-1 prefers substrates containing the consensus sequence (D/E)X(L/I/V)X_{1-2}pYXX(L/I/V) (Yang et al., 2000; Yang et al., 1998). This consensus sequence is often referred to in the literature as an immunoreceptor tyrosine-based inhibitory motif (ITIM).

1.2.2 Regulation of signalling through ITIMs and ITAMs.

ITIMs and immunoreceptor tyrosine-based activation motifs (ITAMs), as their names suggest, were first characterized in the immune system. The activation of many immune cell types occurs through ITAMs, which contains the consensus sequence YXXL/I(X_{6-8})YXXL/I (Cambier, 1995; Reth, 1989). ITAMs are encoded by the cytoplasmic tail of the ligand binding transmembrane receptor, *e.g.* FcγRIIA. Upon ligand binding and receptor clustering, tyrosine residues are phosphorylated by Src family protein tyrosine kinases. ITAMs serve as docking sites for the tandem SH2 domains of cytoplasmic protein tyrosine kinases, ZAP-70 or Syk (Samelson, 2002; Weiss, 1993).

The immune system negatively regulates ITAM-containing receptors (Daeron et al., 1995) through co-ligation with ITIM-containing co-receptors (Vivier and Daeron,

1997), which can result in a reduced or absent cellular response. ITIMs share a consensus amino acid sequence in their cytoplasmic tail, (I/V/L/S)XYXX(L/V) (Ravetch and Lanier, 2000). Upon co-ligation with an activating ITAM-containing receptor or ligand engagement, Src family protein tyrosine kinases phosphorylate the tyrosine residue in the ITIM sequence (Binstadt et al., 1996; Burshtyn et al., 1996; Ravetch and Lanier, 2000). The tyrosine phosphorylated ITIM-containing receptor can then recruit SH2 domain–containing negative regulators: the inositol phosphatase SHIP (Src homology 2–containing inositol polyphosphate 5'-phosphatase) (Ravetch and Lanier, 2000), the tyrosine phosphatase SHP-1 (Burshtyn et al., 1996) or the tyrosine phosphatase SHP-2 (Barrow and Trowsdale, 2006). Negative regulation by ITIM-bearing molecules is correlated with the recruitment of SH2 domain-bearing phosphatases SHIP and SHP-1 by phosphorylated ITIMs (Scharenberg and Kinet, 1996). However, the role of SHP-2 in ITIM-mediated inhibition is less clear than for the other mediators (Barrow and Trowsdale, 2006).

1.2.3 Function of the SHP-1 C-terminus.

While much of the characterization of SHP-1 is centered on its function as a PTP, there is evidence to suggest that its C-terminus has a role in regulating its function and activity. The C-terminus of SHP-1 consists of approximately 60 amino acids. Truncation of the last 35 amino acids of SHP-1 greatly enhances its phosphatase activity and mutations within these 60 amino acids of SHP-1 increases its phosphatase activity (Pei et al., 1994). The C-terminal tail is also important for association of SHP-1 with the insulin receptor *in vitro* (Uchida et al., 1994). However, this is not a generalized mechanism as it does not mediate association with the EGF receptor (Tenev et al., 1997). Although the structure and function of the C-terminal tail remains unknown, primarily because the full-length SHP-1 structure is not solved, there are several functions that have been demonstrated for this region of SHP-1. These include (i) tyrosine and serine phosphorylation (Jones et al., 2004; Lorenz et al., 1994; Uchida et al., 1994), (ii) phospholipid binding (Frank et al., 1999), (iii) lipid raft localization (Fawcett and Lorenz, 2005) and (iv) nuclear localization (Craggs and Kellie, 2001).

1.2.4 Tyrosine phosphorylation of SHP-1.

Phosphorylation of SHP-1 at the C-terminus is critical for its activity. The major phosphorylation sites have been mapped to Tyr536 and Tyr564. Initially, SHP-1 was demonstrated to undergo tyrosine phosphorylation in a T cell hybridoma cell line and in primary thymocytes upon stimulation of surface receptors, including cluster of differentiation 4 (CD4) or CD8 (Lorenz et al., 1994). These authors identified Tyr536 and Tyr564 as major phosphorylation sites and provided evidence for phosphorylation of Tyr564 by the tyrosine kinase p56Lck. Upon RTK stimulation with insulin (Uchida et al., 1994) or PDGF (Bouchard et al., 1994), SHP-1 undergoes phosphorylation on Tyr538 (*note*: Tyr538 in the epithelial variant of SHP-1 is equivalent to Tyr536), which results in an increase in phosphatase activity. Bouchard *et al.* (1994) showed phosphorylation on Tyr538 may be regulated through autodephosphorylation. Normally, upon stimulation of HEK293 cells with PDGF, SHP-1 undergoes a rapid autodephosphorylation; however, phosphorylation at this site is maintained by the addition of pervanadate (a general PTP inhibitor) or mutation of the catalytic Cys residue of SHP-1 (Bouchard et al., 1994). Another tyrosine kinase, Src kinase, phosphorylates SHP-1. Cells transformed with v-*src* have high levels of tyrosine phosphorylated SHP-1, whereas non-transformed cells do not (Matozaki et al., 1994). Src also phosphorylates SHP-1 *in vitro* leading to an increase in phosphatase activity and again Tyr538 and Tyr538/566 are implicated as substitution of these Tyr for Phe decreases the ability of SHP-1 to dephosphorylate phosphoproteins (Frank et al., 2004).

1.2.5 SHP-1 phospholipid binding.

SHP-1 is activated through direct interaction with acidic phospholipids, including phosphatidic acid (PA), phosphatidylserine (PS), cardiolipin (CL), phosphatidylglycerol (PG) and phosphatidylinositol (PI) (Zhao et al., 1993). PA also increases the association of SHP-1 with the EGFR and dephosphorylation of the EGFR is enhanced in the presence of PA (Tomic et al., 1995). It is now known that the last 41 C-terminal amino acids of SHP-1 bind directly to acidic phospholipids (Frank et al., 1999). This region has a preference for PA, phosphatidylinositol-3,4,5-*tris*phosphate ($PI3,4,5P_3$) and dipalmitoylphosphatidic acid over other phospholipids and has less

affinity for dipalmitoylphosphatidylglycerol, phosphatidylinositol-3,4-*bis*phosphate ($PI3,4P_2$) and phosphatidylserine (Frank et al., 1999). These authors speculated that lipid binding to SHP-1 may confer structural changes similar to that following phosphopeptide binding, thus facilitating substrate access to the active site. C-terminal truncation abrogates activation by the C-terminal lipid binding site (Frank et al., 1999). In addition to the contribution of lipid binding to SHP-1 has to its phosphatase activity, it is not unreasonable to consider that its lipid binding capacity could also mediate the cellular localization of SHP-1.

1.3 Regulation of signal transduction pathways by SHP-1.

1.3.1 Motheaten mice.

The importance of SHP-1 in regulating cell function is highlighted by the motheaten mouse (Shultz and Green, 1976). In motheaten mice homozygous (*me/me*) there is a naturally occurring mutation in the SHP-1 locus that results in no expression of SHP-1 and in the allelic viable (me^v/me^v) mice a functionally inactive form of SHP-1 is expressed (Shultz et al., 1993; Tsui et al., 1993). Mice with this deficiency are characterized by widespread autoimmune phenomena, caused by an inability to negatively regulate immune responses. These mice display severe hematopoietic disruption with chronic inflammation, systemic autoimmunity and hemorrhagic pneumonitis (inflamed and bleeding lungs) that results in death at about 2-3 (*me/me*) or 9-12 (me^v/me^v) weeks (Bignon and Siminovitch, 1994; Green and Shultz, 1975). The functional loss of SHP-1 in motheaten mouse brain is associated with a reduced infarct volume, less caspase-3-positive cells and increased neuronal survival (Beamer et al., 2006). However, this same loss of SHP-1 is also associated with a reduction in the number of glia (Wishcamper et al., 2001). These effects on brain function and structure will be discussed later.

Parenthetically, mutations in SHP-2, the orthologue for SHP-1, also affect cell function. For example, loss of SHP-2 expression is embryonic lethal (Saxton et al., 1997) and the D61G gain-of-function mutation results in cardiomyopathies associated with Noonan syndrome (Tartaglia et al., 2003).

1.3.2 SHP-1 regulation of immunity.

The characterization of SHP-1 function focuses on the numerous immune defects displayed in mouthеaten mice and strongly implicates SHP-1 as a negative regulator of immune responses. It is now well established that SHP-1 is a central component in the negative regulation of signalling pathways of several immune cells including B lymphocytes (B cells), T lymphocytes (T cells), natural killer cells and myeloid cell function and that most of this occurs through modulation of immune cell receptors and effector molecules (Zhang et al., 2000). A brief review of the associated literature is included so as to demonstrate the diverse effects of SHP-1 during immune responses.

Stimulation of the antigen receptors on B and T cells evokes a complex cascade of signal transduction events, which provide guidance for proliferation, differentiation and other biological outcomes (Alberola-Ila et al., 1997; Healy and Goodnow, 1998). For example, the B and T cell antigen receptors (BCR and TCR, respectively) represent multimeric complexes containing either a peptide/major histocompatibility complex (MHC) or antigen recognition module associated with signalling molecules composed of various transmembrane proteins such as CD3γ, δ, ε and TCRζ chains in T cells and Ig-α and Ig-β chains in B cells. Antigen receptor engagement is rapidly followed by the activation of Src family protein tyrosine kinases, such as Lyn, Fyn and/or Blk in B cells and Lck and Fyn in T-cells, with the consequent tyrosine phosphorylation of ITAMs present in the cytosolic region of the antigen receptor signalling subunits. ITAMs allow for membrane recruitment of other signalling molecules, providing docking sites for the SH2-domain containing kinases Syk and ZAP-70 in B and T cells, respectively (Campbell, 1999; Chan and Shaw, 1996; van Oers, 1999). In contrast to Src kinases, SHP-1 is identified as a negative regulator of antigen receptor signalling and lymphocyte activation.

Through modulation of the BCR, SHP-1 affects proliferation and BCR-evoked apoptosis (Ono et al., 1997; Pani et al., 1995; Wu et al., 1998; Wu et al., 1995). While the mechanism involved in the effects of SHP-1 in BCR signalling is not fully understood, data suggest that direct protein-protein interaction is occurring. For example, SHP-1 constitutively associates with the BCR complex to dephosphorylate Ig-α and Ig-β chains in resting B cells (Dustin et al., 1999). This association forces the

BCR complex into an inactive state, enabling stimuli to initiate BCR signalling. In contrast to resting cells, B cells activated by BCR engagement do not contain SHP-1 within the BCR complex (Dustin et al., 1999). Instead, SHP-1 is recruited to other sites within the cell, such as the co-modulatory receptors implicated in the inhibition of BCR signalling (Bolland and Ravetch, 1999; Cambier, 1997). Unlike the ITAM-containing receptors and receptor subunits which promote BCR signalling, the BCR inhibitory receptors contain ITIMs, which upon phosphorylation, enable SHP-1 binding and termination of BCR signalling (Bolland and Ravetch, 1999; Cambier, 1997). SHP-1 is found to interact with other ITIM-containing BCR modulators upon BCR ligation including CD22 (Doody et al., 1995), FcγRIIB (D'Ambrosio et al., 1995), paired immunoglobulin-like receptor B (PIR-B/p91A) (Blery et al., 1998) and CD72 (Adachi et al., 1998). In addition to its effect on the BCR and its co-receptors, SHP-1 is also linked to the modulation of several cytosolic molecules involved in BCR signalling. Some of these BCR effectors include Lyn (Somani et al., 2001), Syk (Dustin et al., 1999) and BLNK/SLP-65, an adaptor protein which is recruited into the BCR signalling cascade followed by activation of Syk (Fu et al., 1998).

Natural killer (NK) cells have a significant role in infection control and tumor surveillance. One important activation pathway implicated in natural killing is initiated by Fc receptor (FcR) engagement and the consequent activation of kinases such as Lck, ZAP-70 and Syk (Brumbaugh et al., 1998; Leibson, 1997) (Figure 1.4). This activation pathway has been shown to be negatively regulated by a number of inhibitory receptors including the killer inhibitory receptor (KIR), gp49 and leukocyte Ig-like receptor 1/Ig-like transcript 2 (LTR-1/ILT-2) receptors (Brumbaugh et al., 1998; Leibson, 1997; Yokoyama, 1998). Although these receptors are structurally diverse, most contain ITIMs in their cytosolic domain and interact with MHC class I molecules and transduce an inhibitory signal following their interaction with MHC class I protein on target cells (Long, 1999; Yokoyama, 1998). Upon engagement of the KIR by target cell MHC class I molecules there is an induction of tyrosine phosphorylation of the KIR ITIMs, recruitment of SHP-1, and consequently inhibition of target cell cytolysis (Binstadt et al., 1996; Burshtyn et al., 1996) (Figure 1.4). To further support this finding, catalytically inactive SHP-1 overexpression abrogates KIR-mediated inhibitory effects

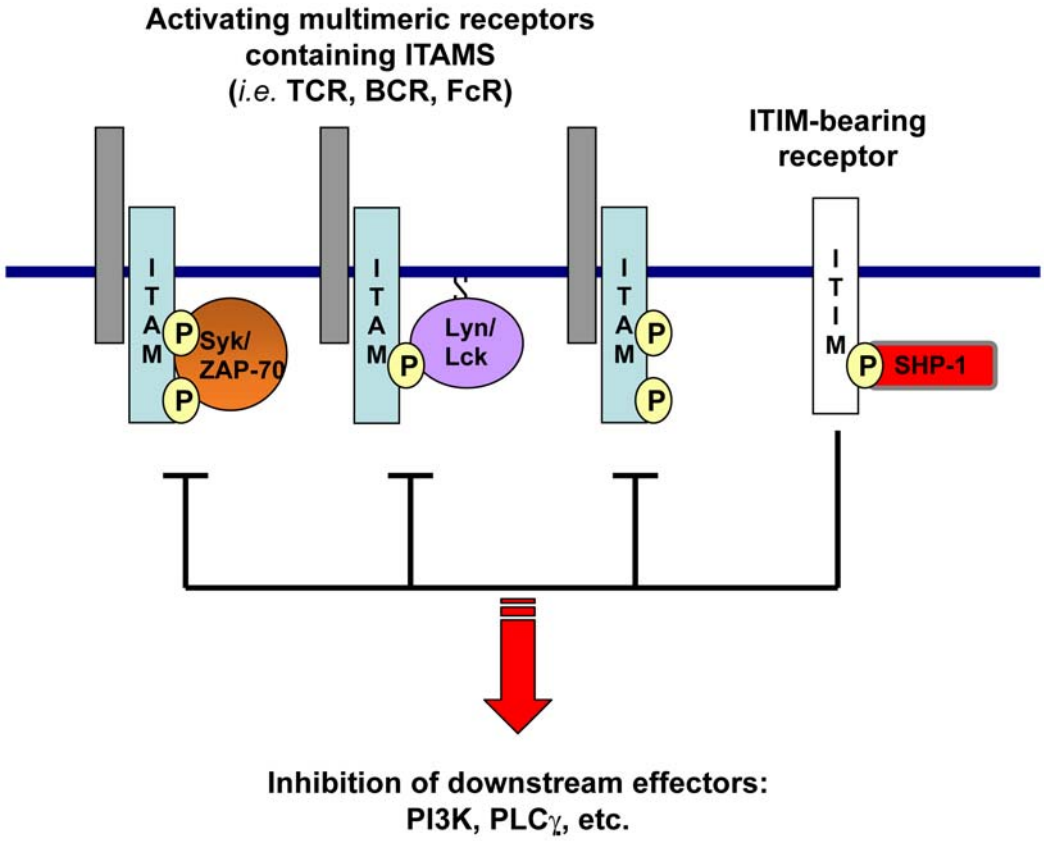

Figure 1.4: Inhibition of NK cell ITAM-bearing receptors by SHP-1 associated-ITIM-bearing receptors. Upon engagement of ITIM bearing receptors, ITIMs are tyrosine phosphorylated and recruit protein tyrosine phosphatases, such as SHP-1. Substrates of SHP-1 include a variety of tyrosine phosphorylated proteins, such as ITAM-bearing receptors, *i.e.* B cell receptors (BCR) or T cell receptors (TCR) or Fc receptors (FcR), Src-family protein tyrosine kinases (Lyn and Lck) or Syk-family protein tyrosine kinases (ZAP-70 and Syk). Dephosphorylation of such signalling molecules leads to inhibition of the NK activating signalling pathways (Adapted from: Tomasello et al., 2000).

on NK cell cytolytic activity (Binstadt et al., 1996; Burshtyn et al., 1996). Other ITIM-containing inhibitory receptors, which SHP-1 interacts with and is responsible for attenuating NK-cell mediated killing, include Ly-49A (Nakamura et al., 1997), gp48B (also present in mast cells) (Rojo et al., 1997) and p75/AIRMI (Falco et al., 1999). These data reveal the ability of SHP-1 to inhibit NK-cell activating receptor signalling.

Cells of myeloid lineage, including macrophages, neutrophils, monocytes and mast cells can be activated through interactions with chemotactic peptides, cytokines and other ligands (Downey et al., 1995). The role for SHP-1 in these signalling pathways is demonstrated by the severe inflammation found in *motheaten* mice. SHP-1 suppresses signalling pathways that promote myeloid cell growth, survival and activation (Dong et al., 1999) and regulates adhesive properties of myeloid cells as well as the chemotactic responses of both immature and mature myeloid cells (Koo et al., 1993; Roach et al., 1998). The inhibitory effects on myeloid cell physiology can again be attributed in part to association with a number of ITIM-containing inhibitory receptors on these cells. These include PIR-B (Timms et al., 1998), which is expressed on macrophages and mast cells, gp49B (Lu-Kuo et al., 1999), LIR-1 and LIR-2 (Wang et al., 1999), two MHC class-I binding receptors present on monocytes. The latter receptors associate with SHP-1 upon tyrosine phosphorylation and, when co-ligated with FcγRI, inhibit tyrosine phosphorylation of FcγRI and Syk. These effects result in the inhibition of FcγRI-mediated monocyte activation. SHPS-1, a plasma membrane-associated glycoprotein binds PTPs *via* its ITIMs and is involved in growth-factor-cell adhesion-induced signalling. For example, upon tyrosine phosphorylation of the two ITIMs in SHPS-1, SHP-1 is recruited and downregulates integrin-mediated signalling (Timms et al., 1999; Veillette et al., 1998). Finally, the myloid cell paired immunoglobulin-like receptor (PILR-α) contains several ITIMs and associates with SHP-1 upon tyrosine phosphorylation (Mousseau et al., 2000).

1.4 Phosphatidylinositol 3'-kinase (PI3K) pathway.

As described above, SHP-1 is clearly a negative regulatory of a number of signalling pathways in hematopoietic cells. However, its function in non-hematopoietic cells is not as clear. SHP-1 negatively regulates phosphatidylinositol 3'-kinase (PI3K)

signalling, but is a positive regulator of mitogen-activated protein kinase (MAPK) and Janus kinases/signal transducers and activators of transcription (JAK/STAT) signalling. One objective of this thesis was to determine the role of SHP-1 in modulating PI3K in the CNS.

1.4.1 RTK activation of the PI3K pathway.

The PI3K pathway is activated by a number of RTKs with ligands as diverse as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) (Dolcet et al., 1999; Encinas et al., 1999; Hetman et al., 1999; Yao and Cooper, 1995), as well as PDGF, EGF, angiotensin II, insulin and insulin-like growth factor-1 (IGF-1) (Borgatti et al., 2000; Isenovic et al., 2002; Zheng et al., 2000).

Regardless of how PI3K is activated, it ultimately initiates signalling cascades that regulate cellular activities such as the promotion of cell survival, proliferation, differentiation, apoptosis, cytoskeletal rearrangement and vacuolar trafficking (Datta et al., 1999). The factors that determine cellular function are complex and are determined based on the type of stimulus, the isoform of PI3K and the nature of the second messenger lipid.

1.4.2 PI3K classification and subunits.

There are multiple isoforms of PI3K in mammalian cells that are divided into three classes, denoted as I, II and III, according to their substrate preference (Domin and Waterfield, 1997). All class I PI3Ks form a heterodimeric complex and are responsive to ligand stimulation and can be subdivided into class IA and IB depending on their activation by RTKs and G-protein coupled receptors, respectively (Domin and Waterfield, 1997). The PI3Ks from class IA are diverse in mammals. These form a heterodimeric complex consisting of a 110-120-kDa catalytic subunit and a regulatory protein often called p85 proteins, based on the molecular weight of the first two isoforms to be identified. The mammalian class IA catalytic subunits include three isoforms, p110α, p110β and p110δ (Hiles et al., 1992; Hu et al., 1993; Vanhaesebroeck et al., 1997). The catalytic subunits all contain a C-terminal kinase domain and a nearby N-

terminal binding site for the regulatory subunit, p85 (Holt et al., 1994) and a region for binding with p21ras-GTP (Rodriguez-Viciana et al., 1996).

There are five regulatory subunits for class IA that have been identified to associate with the catalytic subunit. The regulatory subunits are generated by expression and splicing of three different genes, p85α, p85β and p55γ (Vanhaesebroeck and Alessi, 2000) (Figure 1.5). The two homologous p85 regulatory subunits, p85α and p85β, contain an N-terminal SH3 domain followed by a proline-rich domain, a breakpoint cluster region homology domain, a second proline-rich domain and two SH2 domains. Shorter forms (p55) lack the SH3 and BCR homology domains (Antonetti et al., 1996; Pons et al., 1995) (Figure 1.5). All of the p85/p55 proteins contain putative coiled-coil domains that mediate the stable dimerization with the p110 catalytic subunits (p110α, p110β and p110δ) (Dhand et al., 1994a). Class IB PI3Ks are not as diverse as the class IA and are present in platelets and neutrophils, acting downstream of receptors which signal through G-proteins (Kucera and Rittenhouse, 1990; Stephens et al., 1993). This class of PI3Ks is found only in mammals and contains a 110 kDa catalytic subunit that is associated with a 101 kDa regulatory subunit.

1.4.3 Activation of PI3K.

Activation of class IA PI3Ks, which is focused on in this thesis, is mediated through binding of the p85 regulatory subunit either directly to RTKs or through adaptor molecules, *i.e.* Grb2-associated binder-1 (Gab1) (Ong et al., 2001; Rodrigues et al., 2000) (Figure 1.6). Upon activation of a RTK and autophosphorylation of its cytoplasmic domain, PI3K is recruited directly to the receptor through binding of the p85 SH2 domains. Nearly all p85 adapter subunits and splice variants contain two class III SH2 domains, which enable p85 to bind phosphotyrosine residues. In all known p85 proteins both the N-terminal and the C-terminal SH2 domains bind preferentially to a specific amino acid consensus sequence, pYXXM (Songyang et al., 1994). Under resting conditions, p85 stabilizes the p110 protein and inhibits PI3K lipid kinase activity. PI3K, in its inactive state, is localized to the cytoplasm (Klippel et al., 1996). The inhibitory effect of p85 on p110 is relieved by binding of the p85 SH2 domains, particularly the N-terminal SH2 domain, to tyrosine-phosphorylated peptides or receptors such as the

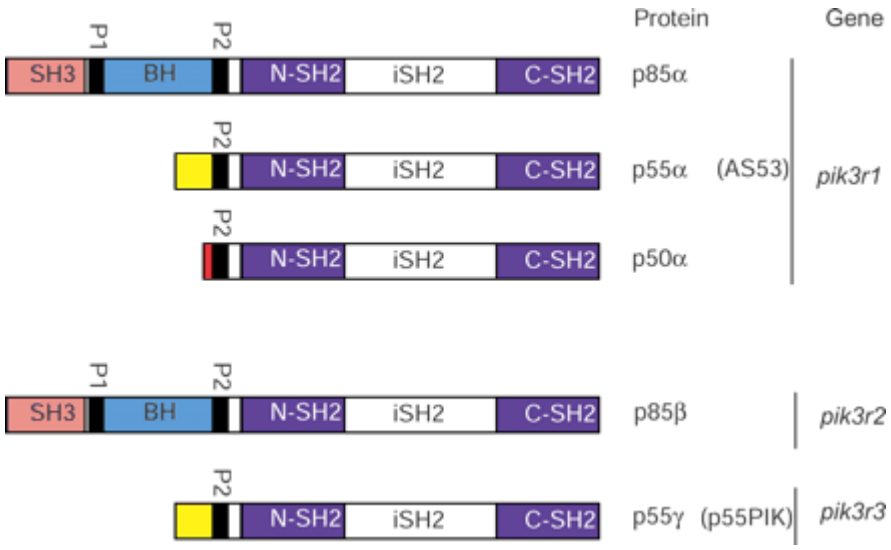

Figure 1.5: The main isoforms of the class IA PI3K regulatory subunits. P1 and P2, proline-rich regions 1 and 2, respectively; iSH2, the inter-SH2 domain; N-SH2, the NH_2-terminal SH2 domain; C-SH2, the COOH-terminal SH2 domain. The names in parenthesis represent other names by which the proteins are known. The p55α and p50α N-termini of 34 and 6 amino acids are shown in yellow and red, respectively. (From: K. Okkenhaug and B. Vanhaesebroeck (2001) New responsibilities for the PI3K regulatory subunit p85 alpha. Sci STKE *2001*, PE1. Reprinted with permission from AAAS)

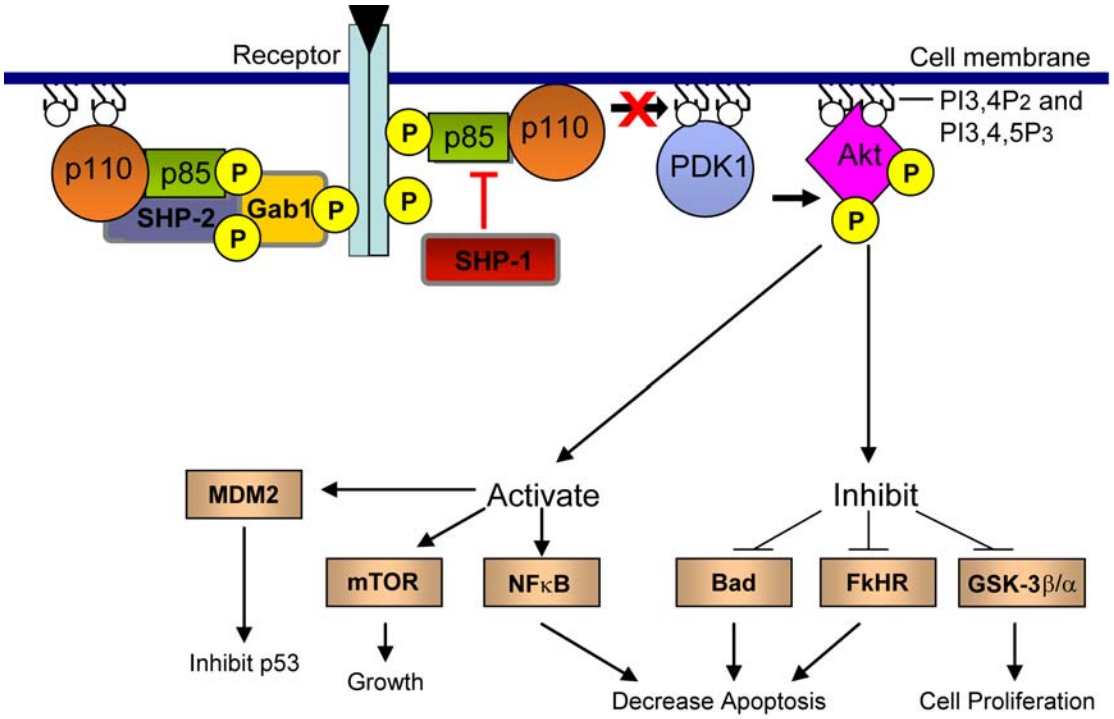

Figure 1.6: The PI3K/PDK1/Akt signalling cascade. Ligand stimulation of a receptor initiates the activation of the PI3K/PDK1/Akt pathway. PI3K is activated by directed recruitment by the SH2 domain of the p85 regulatory subunit to the tyrosine phosphorylated receptor or through recruitment by the Gab1 adaptor molecule. When PI3K is activated it phosphorylates 3'-OH position of the inositol ring of membrane-localized phosphatidylinositols generating phosphatidylinositol-3,4-*bis*phosphate ($PI3,4P_2$) and phosphatidylinositol-3,4,5-*tris*phosphate ($PI3,4,5P_3$). Akt and PDK1 bind $PI3,4P_2$ and $PI3,4,5P_3$ by their individual PH-domains and are localized to the plasma membrane where PDK1 phosphorylates Akt on Thr308 which leads to phosphorylation on Ser473. Once Akt is activated it phosphorylates its downstream targets which results in promotion of cell survival and proliferation. SHP-1 inhibits PI3K/PDK1/Akt signalling through dephosphorylation of p85.

PDGFR or to molecules containing the pYXXM motif (Yu et al., 1998b). Site-directed mutagenesis of the PDGFR at sites containing the consensus sequence, *e.g.* Y^{740}MDM and Y^{751}VPM, disrupt binding of p85 (PI3K) to the PDGFR (Yu et al., 1998b). Binding of the N-terminal SH2 domain of p85 to its tyrosine phosphorylated substrates and the resulting relief of the inhibitory effect of p85 on p110 is critical for PI3K localization to the plasma membrane and activation of the PI3K pathway (Klippel et al., 1996; McGlade et al., 1992).

The Grb2-associated binder (Gab) family of adaptor molecules mediates PI3K localization to RTKs that lack p85 binding sites. For example, Gab1 binding to p85 is a route for PI3K activation downstream of fibroblast growth factor receptors (FGFRs) (Ong et al., 2001) and the EGFR (Rodrigues et al., 2000). Gab proteins also recruit PI3K in response to stimulation of receptors that have p85 binding sites, such as the TrkA (tropomyosin receptor kinase A)-receptor (Holgado-Madruga et al., 1997) and Ret (rearranged during transfection)-receptor (Besset et al., 2000) or receptor systems in which co-receptors also recruit PI3K, such as the BCR (Ingham et al., 2001) and TCR (Pratt et al., 2000; Yamasaki et al., 2001). All Gab family proteins share a common architecture consisting of a highly conserved N-terminal pleckstrin homology (PH) domain, a central proline-rich domain and multiple tyrosines that are binding motifs for various SH2 domain-containing proteins. Most Gab protein-receptor interactions occur indirectly *via* Grb2. Gab proteins contain several proline-rich motifs, two of which can mediate binding of Gab1 or Gab2 to Grb2 SH3 domains (Lock et al., 2000; Schaeper et al., 2000). Grb2 contains an SH2 domain, which targets the constitutive Grb2-Gab complex to receptors containing Grb2 SH2 domain binding sites (pYXNX, Note: this is an example of a class II SH2 domain) (den Hertog et al., 1994). In some signalling pathways, Grb2-Gab complexes are recruited to receptors indirectly by other tyrosyl-phosphorylated adaptor proteins. For example, recruitment of Gab1 and Gab2 to the thrombopoietin receptor (Bouscary et al., 2001) and the Ret-receptor (Besset et al., 2000) involves a Shc-Grb2-Gab2 complex. In the FGFR pathway, Gab1 phosphorylation involves an additional scaffolding adaptor, fibroblast growth factor receptor substrate (FRS2). Upon receptor activation, FRS2 becomes tyrosine phosphorylated and binds to Grb2 that, in turn, recruits Gab1 (Hadari et al., 2001; Ong et al., 2001). The Trk

receptors, TrkA and neurotrophin (NT), also evoke FRS2 and Gab1 phosphorylation and recruitment to the receptor (Meakin et al., 1999).

The interaction between p85(PI3K) and Gab proteins occurs through three YXXM motifs, which are consensus binding site for the SH2 domain of p85, present in all mammalian Gab proteins. Mutations at the p85-binding sites of mammalian Gab1 and Gab2 result in inhibition of several signalling pathways (Gu et al., 2000; Holgado-Madruga et al., 1997; Laffargue et al., 1999; Yart et al., 2001). The physical association between p85 and Gab1 or Gab2 is crucial in mediating the PI3K/Akt signalling pathway induced by a variety of stimuli including cytokines IL-2 and IL-3 (Gu et al., 2000; Gu et al., 2001), NGF (Holgado-Madruga et al., 1997), EGF (Rodrigues et al., 2000) and hepatocyte growth factor (Maroun et al., 1999). Overexpression of Gab1 potentiates FGF-induced Akt activity, whereas overexpression of the p85-binding mutant of Gab1 decreases Akt activation (Ong et al., 2001) and is unable to provide anti-apoptotic signals in response to NGF stimulation (Holgado-Madruga et al., 1997). Similarly, mutations at the p85-binding sites of Gab2 were found to impair the ability of IL-3 to activate Akt and to induce cell growth (Gu et al., 2000). Moreover, the activation of PI3K leads to the production of phosphatidylinositol-3,4-*bis*phosphate ($PI3,4P_2$) and phosphatidylinositol-3,4,5-*tris*phosphate ($PI3,4,5P_3$) also recruits the PH domain of Gab proteins and presumably promotes further activation of PI3K, a positive feedback loop could be formed to amplify the signals through the Gab proteins (Rodrigues et al., 2000).

Recruitment to the RTK either directly or indirectly is an important step in relieving the inhibitory effect the regulatory p85 subunit exerts on the p110 catalytic subunit. Recruitment of p85 to RTKs allows for tyrosine phosphorylation on p85 which changes the conformation of p85 enough to relieve its inhibitory effect on p110. Supporting evidence is provided by the increased tyrosine phosphorylation state of p85 in response to a variety of stimuli including PDGF simulation and other tyrosine kinases, such as Abl and Lck (von Willebrand et al., 1994; von Willebrand et al., 1998; Yu et al., 1998c). Elevated levels of tyrosine phosphorylation of p85 also correlate with proliferation in Jurkat cells (Martinez-Lorenzo et al., 2000) and altered SH2 domain binding properties of p85 (von Willebrand et al., 1998). PI3K activity is higher in tyrosine phosphorylated p85 immunoprecipitates when compared to those with depleted

p85 tyrosine phosphorylation (Cuevas et al., 2001). A critical tyrosine residue, Tyr688, mapped within the C-terminal SH2 domain on p85, may directly regulate PI3K activity. Tyr688 on p85 is a target for Src kinase family members Lck and Abl (von Willebrand et al., 1998) and coexpression of Lck with PI3K in COS-7 cells results in an increase in PI3K activity (Cuevas et al., 1999). The importance of phosphorylation of Tyr688 for the regulation of PI3K activity was further demonstrated through mutating Tyr688 to Ala, which results in inhibition of PI3K activity (Cuevas et al., 2001). Substituting this same residue with an Asp, which would mimic phosphorylation, results in the activation of PI3K and its downstream targets Akt and nuclear factor-kappa B (NF-κB) in COS-7 cells (Cuevas et al., 2001). These authors suggest that phosphorylation of p85 on Tyr688 could allow for an intramolecular association of the phosphorylated Tyr688 residue within the p85 C-terminal tail to the p85 N-terminal SH2 domain, which would relieve its inhibitory effect on p110. The authors also propose that Tyr688 phosphorylation triggers an intermolecular interaction between individual p85 proteins or other substrates of p85, again inducing a disruption of the inhibitory effect of p85 on p110.

The regulation of p85 is not limited to its phosphorylation on Tyr688. For example, the p85 is phosphorylated on Y508 by the PDGFR activation (Kavanaugh et al., 1994) and phosphorylation of Ser608 by p110 acts as a negative feedback regulatory mechanism (Dhand et al., 1994b)

While the role of PI3K during growth factor stimulation is apparently quite clear, there is evidence that this model may not apply to all cases. This is particularly evident following the removal of essential growth factors from cell cultures. Removing serum from cell cultures is often used as a model of growth factor withdrawal (Liang et al., 2007; Lieberthal et al., 1998; Wei et al., 2004) and often inhibits PI3K activity. This initiates apoptotic events in neuronal cell lines (Jin et al., 2000; Poser et al., 2003; Zhong et al., 1993) and can affect proliferation of glial cells (Fan, 1983; Michler-Stuke and Bottenstein, 1982). Absence or withdrawal of growth factors induces caspase activity and leads to cells death of PC12 cells (Batistatou and Greene, 1991). Hypoxia, which can initiate specific adaptive responses, can activate PI3K and, thus, protect against apoptosis during serum withdrawal (Alvarez-Tejado et al., 2001). In cortical neurons

cAMP antagonizes neurotrophin-mediated protection during serum withdrawal by inhibiting the PI3K pathway (Poser et al., 2003).

Serum withdrawal does not always readily induce apoptosis as it can also, for example, reduce cell growth and promote differentiation of glial cells (Chou and Langan, 2003; Fan, 1983). The effect of removing serum on signalling pathways is unclear as removal of serum from U937 (human leukemic monocyte lymphoma) cell cultures increases PI3K activity (Lee et al., 2005), but induces the loss of Akt phosphorylation and the induction of the proapoptotic protein Bax in HeLa cultures (Tsuruta et al., 2002).

1.4.4 PI3K/PDK1/Akt signalling.

When PI3Ks are activated, the p110 catalytic subunit catalyzes the transfer of a phosphate from ATP to the 3'-OH position of the inositol ring of membrane-localized phosphatidylinositols (PI), generating PI3,4P_2 and PI3,4,5P_3 (Figure 1.7). These phosphorylated lipids exist in the inner portion of the plasma membrane and once generated, they serve as docking sites for signalling molecules that contain pleckstrin homology (PH) domains. A major downstream target of PI3,4P_2 and PI3,4,5P_3 is Akt, also known as protein kinase B (PKB) (Franke et al., 1997; Franke et al., 1995; Kulik and Weber, 1998). Akt is a 57 kDa serine/threoine kinase and belongs to the subfamily of the mammalian AGC family of kinases. Akt exists as one of three isoforms, PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (Coffer et al., 1998).

The binding to PI3,4P_2 and PI3,4,5P_3 *via* its PH domain localizes Akt to the plasma membrane (Franke et al., 1997; Frech et al., 1997; James et al., 1996; Kulik and Weber, 1998) (Figure 1.6). At the plasma membrane, Akt is activated by phosphorylation on two critical residues, Thr308 and Ser473 (Bellacosa et al., 1998). The phosphorylation on Thr308 is mediated by 3'-phosphoinositide-dependent protein kinase-1 (PDK1), which is also localized to the plasma membrane through binding of its

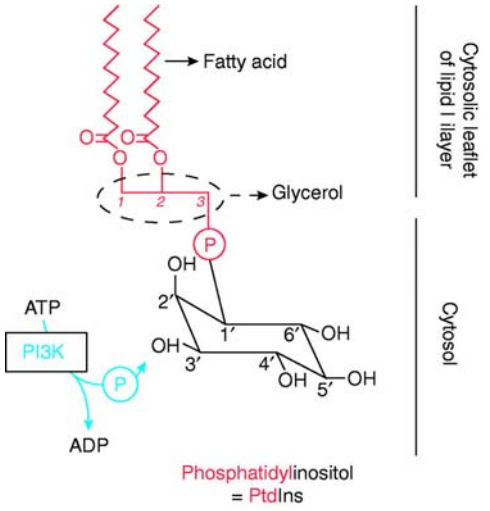

Figure 1.7: Generation of 3'-phosphatidylinositide. Inositol-containing lipids consist of a glycerol backbone with fatty acids attached at positions 1 and 2, and an inositol 1-phosphate group at position 3. If the inositol ring has no additional phosphates, it is called **p**hospha**t**i**d**yl**in**o**s**itol (PtdIns/PI). When PI3Ks is activated the p110 catalytic subunit catalyzes the transfer of a phosphate from ATP to the 3-OH position of the inositol ring of membrane-localized phosphatidylinositols (PtdIns/PI), generating PI3,4P_2 and PI3,4,5P_3. (Reproduced with permission, from B. Vanhaesebroeck and D.R. Alessi, 2000, Biochem J, 346, 561-576. © the Biochemical Society)

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own PH domain to $PI3,4P_2$ and $PI3,4,5P_3$ (Anderson et al., 1998; Stephens et al., 1998) (Figure 1.6). Interestingly, the PH domain of PDK1 has a higher affinity for $PI3,4,5P_3$ than the Akt PH domain (Stokoe et al., 1997), undoubtedly accounting for its constitutive association with the plasma membrane. It is not clear how Ser473 is phosphorylated, but it may undergo autophosphorylation (Toker and Newton, 2000a) or be targeted by the rictor-mTor complex (Sarbassov and Sabatini, 2005). There are several other kinases that phosphorylate Ser473 *in vitro*, including integrin-linked kinase (ILK), MAPK-activated protein kinase (MAPKAPK-2), p90 ribosomal S6 kinase (RSK) (Delcommenne et al., 1998), as well as NEK6 (Belham et al., 2001).

Once activated, Akt detaches from the membrane and targets cytosolic, mitochondrial and nuclear substrates such as the proapoptotic proteins, glycogen synthase kinase-3 beta (GSK-3β) (Shaw et al., 1997), BCL2 antagonist of cell death (BAD) (Datta et al., 1997), caspase-9 (Cardone et al., 1998), forkhead transcription factors (Brunet et al., 1999) and nuclear receptor subfamily 4, group A, member 1 (NR4A1) (Masuyama et al., 2001). Also targeted are the antiapoptotic proteins, NF-κB (Romashkova and Makarov, 1999) and cAMP-response element-binding protein (CREB) (Pugazhenthi et al., 2000) (Figure 1.6).

Most research regarding PI3K/PDK1/Akt signalling is based on phosphoinositide generation and signalling from the plasma membrane, yet phosphoinositides and their biosynthetic machinery are also present in the nucleus (D'Santos et al., 1998). Regulation of these two inositol pools is independent, suggesting a different functional importance for nuclear phosphoinositides. Not surprisingly, nuclear PI3K is activated in response to different cellular responses. For example, translocation of PI3K to the nucleus occurs under conditions of differentiation in PC12 cells by NGF (Neri et al., 1994), in Saos-2 (human osteosarcoma) cells with IL-1 stimulation (Bavelloni et al., 1999), in MC-3T3-E1 (murine osteoblastic-like) cells in response to mitogenic factors such as insulin-like growth factor-1 (IGF-1) or PDGF (Martelli et al., 2000), and in C6 cells grown in serum-free medium (Sephton and Mousseau, 2007, manuscript submitted). There is evidence that nuclear PI3K may play a role in RNA metabolism (Bunney et al., 2000), regulation of transcription (Yu et al., 1998a) and activation of

protein kinase C zeta (PKC-ζ) (Toker et al., 1994). The nuclear function of PI3K is still unclear and nuclear targets are still being defined.

1.5 3'-phosphoinositide-dependent protein kinase-1 (PDK1).

1.5.1 Regulation of the PI3K/Akt pathway by PDK1.

PDK1 is a central activator and the next "player" in the PI3K/PDK1/Akt pathway. As described in Section 1.4.4, a model has been proposed for PI3K/Akt activation by hormone or growth factor stimulation of receptors. In summary, activation of cell surface receptors increases the levels of PI3Ks' lipid products, PI3,4P_2 and PI3,4,5P_3, which leads to the translocalization of PDK1 and Akt to the plasma membrane where PDK1 phosphorylates and activates Akt (Figure 1.6).

The PDK1 gene maps to chromosome 16p13.3 and the translated protein is a 63 kDa monomer with serine/threonine kinase activity. PDK1 is ubiquitously expressed in human tissues and cells (Alessi et al., 1997a; Stephens et al., 1998). The kinase domain of PDK1 is situated within the N-terminal domain, followed by a linker region and a C-terminal PH domain. PDK1 was the first kinase identified to phosphorylate Akt on Thr308 and to initiate Akt activation in a PI3,4,5P_3-dependent manner (Alessi et al., 1997b). The interaction with PI3,4P_2 and PI3,4,5P_3 is mediated by the individual PH domains found in Akt and PDK1. In the presence of phospholipids, PI3,4P_2 or PI3,4,5P_3 (but no other PIs), Akt phosphorylation is enhanced over 1000-fold (Alessi et al., 1997b). The major effect of PI3,4P_2/PI3,4,5P_3 binding of PDK1, in addition to directing the localization of PDK1 to the plasma membrane to phosphorylate Akt, is the effect PI3,4,5P_3 binding has on PDK1 substrates (Alessi et al., 1997b). Akt binding to PI3,4P_2/PI3,4,5P_3 induces a conformational change that increases the accessibility of PDK1 to the Thr308 residue on Akt (Vanhaesebroeck and Waterfield, 1999; Yang et al., 2002a) (Figure 1.8). This enables other kinases to access and phosphorylate Ser473 leading to full activation of Akt (Alessi et al., 1996). In the absence of PI3,4P_2/PI3,4,5P_3, PDK1 is unable to phosphorylate wildtype Akt (Alessi et al., 1997a; Stephens et al., 1998). Point mutations in the PH domain of Akt, which block its interaction with PI3,4,5P_3, prevent PDK1 phosphorylation in the presence of PI3,4,5P_3

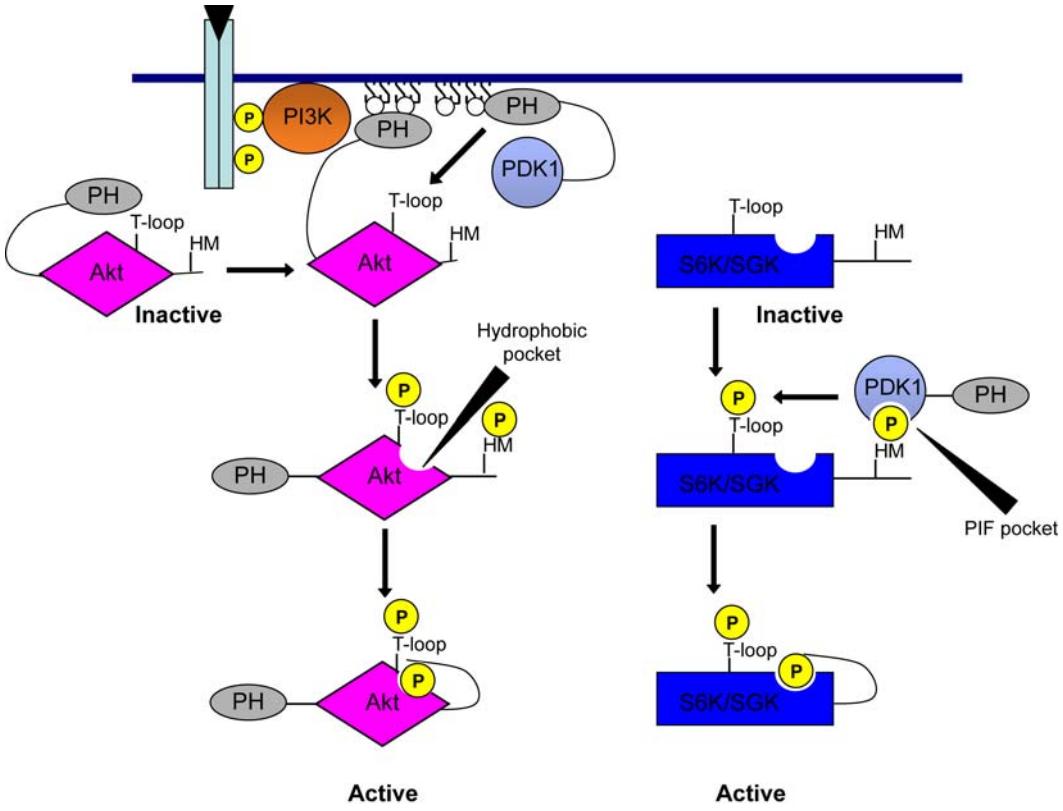


Figure 1.8: Mechanism of activation of Akt, S6K and SGK by PDK1. Akt is activated following its recruitment to the plasma membrane through binding of its PH domain to $PI3,4,5P_3$, where it is phosphorylated at its T-loop by PDK1 and its hydrophobic motif (HM) by an unknown kinase. Once Akt is phosphorylated at its T-loop, intramolecular binding occurs through the phosphorylated HM the hydrophobic pocket resulting in maximal Akt activation. In contrast, phosphorylation of the HM of S6K and SGK enables PDK1 to interact with these enzymes through its PIF-pocket, and permits the T-loop phosphorylation of these substrates (Adapted from: Mora et al., 2004).

(Stokoe et al., 1997) and Akt with a deleted PH domain is not activated in the presence of $PI3,4,5P_3$ (Alessi et al., 1997a). However, PDK1 is still able to phosphorylate Akt that lacks its PH domain (ΔPH-Akt) (Alessi et al., 1997a; Stephens et al., 1998). Moreover, artificially promoting the interaction of PDK1 with wildtype Akt and ΔPH-Akt, by the attachment of a high-affinity PDK1 interaction motif to these enzymes, induces phosphorylation of the T-loop in ΔPH-Akt, but not in wildtype Akt unstimulated cells (Biondi et al., 2001), which further supports that $PI3,4,5P_3$ induces a conformational change in Akt which allows for phosphorylation by PDK1. In contrast, removing the PH domain on PDK1 does not obstruct Akt phosphorylation and activation, but the rate of Akt activation by this PDK1 mutant was reduced around 30-fold compared to wildtype PDK1 (Alessi et al., 1997a).

There are reported cases where phosphorylation on Thr308 and Ser473 does not conform to the expected paradigm. For example, in PDK1-deficient cells, Akt is largely inactive, yet phosphorylation on Ser473 remains responsive to insulin stimulation (Williams et al., 2000). In other cases, staurosporine, an inhibitor of PDK1 activity, prevents Thr308 phosphorylation, but does not inhibit Ser473 phosphorylation (Hill et al., 2001; Scheid et al., 2002). A new model for Akt phosphorylation has been proposed by Scheid *et al.* (2002), who suggest that Ser473 regulates the phosphorylation of Thr308. This model describes PI3K-induced Akt translocation to the plasma membrane where Ser473 is first phosphorylated, allowing for subsequent PDK1-mediated phosphorylation of Thr308. Scheid *et al.* (2002) show that substitution of Ser473 to alanine reduced the degree of Thr308 phosphorylation, an effect that was not observed when Ser473 was mutated to aspartic acid. A point mutation in the substrate-binding region (PIF-pocket) of PDK1 (L155E) resulted in a reduced level of phosphorylation on Thr308 and did not affect the level of phosphorylation on Ser473 at the plasma membrane. However, this mutant completely abolished Akt phosphorylation in the cytosol (Scheid et al., 2002). These data suggest two distinct mechanisms of Akt phosphorylation in the cytoplasm *versus* at the plasma membrane. [Note: others have shown that the PIF pocket is not required for binding or activation of Akt: Biondi *et al.* (2000)].

1.5.2 Activation of AGC kinases by PDK1.

The diverse metabolic, proliferative and survival effects initiated by PI3K activation and the ensuing generation of $PI3,4,5P_3$ are mediated by the activation of a subgroup of AGC family of protein kinases. These include isoforms of Akt (Brazil and Hemmings, 2001), p70 ribosomal S6 kinase (S6K) (Avruch et al., 2001), serum- and glucocorticoid-induced protein kinase (SGK) (Lang and Cohen, 2001) and atypical isoforms of protein kinase C (PKC) (Newton, 2003). These enzymes are activated within minutes of insulin or growth factor stimulation of PI3K. Akt, as previously discussed, regulates apoptosis, cell division and glucose metabolism (Lawlor and Alessi, 2001). S6K controls protein synthesis required for cell growth and amino acid storage (Volarevic and Thomas, 2001) and SGK regulates cell growth and ion transport (Lang and Cohen, 2001). The roles of atypical PKC isoforms (ζ, λ/ι) downstream of PI3K are less well defined.

All AGC kinases have residues equivalent to Thr308 of Akt, known as the T-loop or activation loop which is located in the core of their kinase catalytic domain, and display a high degree of primary sequence conservation within their respective kinase domains (Niederberger and Schweingruber, 1999; Toker and Newton, 2000b). Another common feature of AGC kinases is the presence of a C-terminal hydrophobic motif (HM) FXXF/Y[S/T]F/Y (the S/T represents the site of phosphorylation) which is involved in stabilization of the active conformation of these kinases (Johnson et al., 2001; Knighton et al., 1991) (Figure 1.8). Outside of these catalytic domains, the AGC kinases generally show little similarity.

Activation of AGC kinases, including those activated by PI3K (Akt, SGK, S6K and atypical PKC isoforms), is dependent on their phosphorylation by PDK1. PDK1 targets members of this family in a different manner than established for Akt, *e.g.* phosphoinositide-dependence. All recognized PDK1 substrates, other than Akt, are phosphorylated efficiently in a phosphoinositide-independent manner *in vitro* (Vanhaesebroeck and Alessi, 2000). With substrates other than Akt, PDK1 interacts directly through the C-terminal HM. In some AGC kinases, the HM contains a phosphorylation site (FXXF/Y[S/T]F/Y) that, when phosphorylated, triggers the interaction with PDK1. HM phosphorylation is required for the interaction of PDK1

with substrates such as S6K, SGK and RSK (Balendran et al., 1999; Biondi et al., 2001; Frodin et al., 2002; Frodin et al., 2000) (Figure 1.8).

Although an AGC kinase itself, PDK1 differs from other members in that it does not possess an equivalent HM, instead having a homologous pocket, with no intramolecular HM-binding partner. In PDK1, this pocket interacts with the C-terminal HM of protein substrates, and is termed PIF (PDK1-interacting fragment)-binding pocket (Biondi et al., 2000). This interaction results in PDK1 activation (Biondi et al., 2000; Frodin et al., 2000). Evidence for a role for the PIF pocket in the regulation of PDK1 intrinsic activity is further supported by the generation of PIF-pocket mutants, which can stabilize active conformations of PDK1 (Biondi et al., 2000). Peptides that encompass the HM of S6K (Biondi et al., 2002) and RSK (Frodin et al., 2000) induced a four- to six-fold activation of PDK1, indicating that peptide binding of the PIF pocket on PDK1 is important for its enhanced activity.

S6K and SGK, which do not contain a PH domain, become activated upon phosphorylation at the two highly conserved Ser/Thr residues. One is located in the T-loop (activation-loop), and the other is located C-terminal to the catalytic domain in the hydrophobic motif (Kobayashi and Cohen, 1999; Park et al., 1999; Pearson et al., 1995). PDK1 efficiently catalyzes the phosphorylation of the activation-loop residues of isoforms of S6K (Pullen et al., 1998) and SGK (Williams et al., 2000) (Figure 1.8). Phosphorylation of both residues is required for maximal activation of these enzymes. Substitution of the Ser/Thr residue in the HM of either S6K1 (Pullen et al., 1998) or SGK1 (Kobayashi and Cohen, 1999; Park et al., 1999) to an acidic residue to mimic phosphorylation greatly enhances the phosphorylation of these enzymes by PDK1. Hydrophobic pocket mutants, such as PDK1L155E, were incapable of binding or phosphorylating S6K and SGK (Biondi et al., 2001), which supports the importance of the PIF-pocket of PDK1 as a substrate-docking site. Mutations of S6K and SGK that make them constitutively active do not affect their activity or T-loop phosphorylation by inhibitors of PI3K (Kobayashi and Cohen, 1999; Park et al., 1999; Pullen et al., 1998). These results suggest that PI3K promotes the activation of S6K and SGK by controlling hydrophobic motif phosphorylation of these enzymes.

Other AGC kinases are constitutively phosphorylated, as is the case for the classical members of the PKC family (Newton, 2001; Parekh et al., 2000). Similar to Akt, PKC has a related pattern of activation, in which PKCδ can be phosphorylated by PDK1 on its activation loop site, Thr505 (Le Good et al., 1998). In contrast with Akt, PKCδ is fully activated through co-recruitment to membranes/liposomes by its own allosteric activator diacylglycerol (DAG), in combination with $PI3,4,5P_3$ (Le Good et al., 1998). Similarly, modification of the HM of the atypical PKC isoform, PKCζ, reduces the ability of PDK1 to interact with and phosphorylate PKCζ at its T-loop residue (Balendran et al., 2000).

1.5.3 PDK1 function in development.

The role for PDK1 activation of certain AGC kinase members was established by the finding that mouse embryonic stem (ES) cells lacking PDK1 failed to activate Akt, S6K and RSK in response to stimuli that trigger the activation of these enzymes in wildtype ES cells (Williams et al., 2000). It was unexpected that ES cells lacking PDK1 were viable, morphologically indistinguishable from wildtype cells and proliferated at the same rate, because Akt and RSK have often been reported to play important roles in regulating survival and proliferation in other cell types (Williams et al., 2000). Thus, in ES cells, PDK1 is not intrinsically required for survival and proliferation. However, knocking out PDK1 homologues in *Saccharomyces cerevisiae* (Niederberger and Schweingruber, 1999), in *Caenorhabditis elegans* (Paradis et al., 1999), in *Drosophila* (Cho et al., 2001; Rintelen et al., 2001) and in mice (Lawlor et al., 2002) has revealed that PDK1 is required for the normal development and viability of these organisms. Specifically, PDK1-deficient *Drosophila* embryos die during early larval stages (Cho et al., 2001; Rintelen et al., 2001). Similarly, PDK1 is important for the normal development of mice. The PDK1 knockout in mice ($PDK1^{-/-}$) is embryonic lethal (day 9.5) and the $PDK1^{-/-}$ embryos display multiple abnormalities including lack of somites, forebrain and neural crest-derived tissues (Lawlor et al., 2002). In addition to the normal development of mice, PDK1 plays a role in regulating cell size independently of cell number or proliferation and mediates the effects of insulin on activating Akt, S6K and RSK (Lawlor et al., 2002).

1.5.4 Regulation of PDK1 activity.

1.5.4.1 PDK1 response to growth factors.

In unstimulated cells PDK1 is mainly cytosolic, with some localization at the plasma membrane (Anderson et al., 1998; Deak et al., 1999). PDK1 was once thought to be constitutively active in resting cells and its activity did not change by growth factor stimulation (Casamayor et al., 1999). A pool of PDK1 is constitutive active in cells in the absence of growth factor stimulation which may be partly due to its constitutive association with the plasma membrane. Although, PDK1's PH domain binds $PI3,4,5P_3$ with higher affinity than other PIs such as $PI3,4P_2$ (Stokoe et al., 1997), the constitutive localization of PDK1 to the plasma membrane is attributed to its association with endogenous phosphatidylinositol-4,5-*bis*phosphate (Vanhaesebroeck and Alessi, 2000).

It is uncertain whether PDK1 is directly activated or inhibited by any extracellular signals. Some reports show that PDK1 is growth factor-insensitive and others show PDK1 is growth factor-dependent and translocates to the plasma membrane. For example, PDGF stimulation of endothelial cells (Anderson et al., 1998) and insulin stimulation of HeLa cells (Filippa et al., 2000) and HEK293 cells (Park et al., 2001) cause a redistribution of PDK1 to the plasma membrane in a PI3K-dependent manner. However, others are unable to induce membrane localization of PDK1 in response to IGF-1 stimulation of HEK293 cells, although an increase in PDK1 kinase activity was demonstrated (Alessi et al., 1997a; Currie et al., 1999). Similarly, using Porcine Aortic Endothelial (PAE) cells overexpressing the PDGFR, translocation of PDK1 to the plasma membrane does not occur after stimulation with either IGF-1 or PDGF, but Akt activity is increased 6- and 8-fold respectively, suggesting PDK1 is activated (Currie et al., 1999). Similarly, insulin stimulation of adipocytes does not induce membrane translocation of PDK1, yet pervanadate, which is used to mimic the effect of insulin, potently induces PDK1 localization to the plasma membrane (Grillo et al., 2000).

It is possible that $PI3,4P_2$ and $PI3,4,5P_3$ regulate the cellular localization of PDK1. However, the binding and localization of PDK1 to the plasma membrane may not significantly contribute to its intrinsic protein kinase activity. The PH domain of PDK1, in addition to its role in mediating membrane association, acts to auto-inhibit the

kinase activity of PDK1, and PI3,4,5P_3 binding relieves this inhibition. Thus, the total effect of PI3,4,5P_3 binding may also increase the catalytic activity of PDK1 towards its substrates (Filippa et al., 2000).

1.5.4.2 PDK1 regulation by serine phosphorylation

After the discovery of PDK1, there was much attention surrounding the regulation of PDK1 kinase activity by serine phosphorylation. The original PDK1 studies identified several serine (Ser) residues, including Ser25, Ser241, Ser393, Ser396 and Ser410, that were phosphorylated on PDK1 in unstimulated HEK293 cells, and the phosphorylation on these sites was unaffected by stimulation with IGF-1 (Casamayor et al., 1999). Substitutions of the individual serine sites with alanine, designed to mimic dephosphorylation, did not affect PDK1 activity. However, Ser241, unlike the other serine phosphorylation sites, contributes positively to PDK1 catalytic activity (Casamayor et al., 1999). Of these serine residues, Ser241 is also the only one that is highly conserved across different species and is located on the activation loop of PDK1 kinase domain in an equivalent position to the site that PDK1 phosphorylates on its AGC kinase substrates (Mora et al., 2004). While phosphorylation on the activation loop is shown in certain instances to be required for PDK1 activity, it is not clear how the phosphorylation of this site is regulated. PDK1 purified from rabbit skeletal muscle could not be dephosphorylated on Ser241 or inactivated following the incubation with high concentrations of serine/threonine-specific protein phosphatase 2A (PP2A) or protein phosphatase-1 (PP-1) (Alessi et al., 1997a). It is possible that Ser241 is resistant to dephosphorylation by protein phosphatases because it is buried inside the PDK1 protein (Cheng et al., 1998; Steinberg et al., 1993). PDK1 expressed in bacteria is active and phosphorylated on Ser241, demonstrating PDK1 can autophosphorylate at this residue (Casamayor et al., 1999). However, it cannot be ruled out that in mammalian cells other kinases besides PDK1 may phosphorylate Ser241 of PDK1.

Contrary to the reported importance of Ser241 phosphorylation on PDK1, others have reported only a modest increase in Ser241 and Ser25 in response to stimulation of cells with insulin (Chen et al., 2001) or pervanadate (Park et al., 2001). Recently, a study showed that upon insulin stimulation PDK1 is phosphorylated on Ser164 and that its

activity is independent of Ser241 (Riojas et al., 2006). This study indicates that Ser164 phosphorylation on PDK1 is necessary and contributes to PDK1 activity (Riojas et al., 2006).

1.5.4.3 PDK1 regulation by tyrosine phosphorylation.

Tyrosine phosphorylation has recently emerged as an important component to PDK1 activation and function. Treatments including insulin, NGF, hydrogen peroxide (H_2O_2), pervanadate (an inhibitor of PTPs) and Src kinases increase tyrosine phosphorylation on PDK1 (Fiory et al., 2005; Grillo et al., 2000; Park et al., 2001; Prasad et al., 2000). Pervanadate significantly increases the tyrosine phosphorylation of PDK1 with only a modest increase in serine phosphorylation. Both H_2O_2 and pervanadate activate PI3K and increase PDK1 activity towards its substrates SGK and Akt (Prasad et al., 2000). Pervanadate stimulation induces a 1.5-3 fold increase in PDK1 activity and specifically increases tyrosine phosphorylation on Tyr9, Tyr373 and Tyr376 (Park et al., 2001). Tyr9 facilitates Tyr373/376 phosphorylation and Tyr373/376 phosphorylation is important for PDK1 activity and membrane localization (Park et al., 2001).

Specific tyrosine kinases, *i.e.* c-Src and c-Abl, also phosphorylate PDK1 *in vitro*. Tyrosine phosphorylation of PDK1 by Abl kinase results in increased activity of PDK1 toward SGK and Akt (Prasad et al., 2000) and Src is capable of phosphorylating PDK1 on Tyr9, Tyr373 and Tyr485 (Park et al., 2001). Both Tyr373/376 are phosphorylated by Src kinase *in vitro*, and expression of Src leads to tyrosine phosphorylation and activation of PDK1 in HEK293 cells (Grillo et al., 2000; Park et al., 2001). Due to the regulation of PDK1 by insulin and Src kinases, PDK1 is implicated in insulin signalling (Grillo et al., 2000). PDK1 is recruited to the insulin receptor in response to insulin stimulation and binds directly to the receptor (a kinase) resulting in PDK1 tyrosine phosphorylation by the insulin receptor, an event required for activation of glucose uptake and glycogen synthesis (Fiory et al., 2005).

The tyrosine phosphorylation state of PDK1 is not always dependent on PI3K. For example, effects of pervanadate may be dependent on PI3K, whereas those of Src and H_2O_2 may be independently of PI3K. The increase in tyrosine phosphorylation on

PDK1 by Src kinase and H_2O_2 (Prasad et al., 2000) is reported to be wortmannin (PI3K inhibitor)-insensitive, suggesting they act independent of PI3K, whereas, pervanadate- and insulin-induced tyrosine phosphorylation is dependent on PI3K activity (Fiory et al., 2005; Park et al., 2001). Both serine and tyrosine phosphorylation have emerged as important mechanisms of PDK1 regulation, though it is unclear what the exact contribution of each has in controlling PDK1 activity and its influence on PI3K/Akt signalling. It is very interesting, however, that the Tyr residues in PDK1 targeted by Src reside in YXX(V/I/L) motifs similar to the ITIMs targeted by SHP-1 (Yang et al., 2000; Yang et al., 1998).

1.6 Inactivation of PI3K signalling.

PI3K signalling is terminated in different ways. One is by the dephosphorylation of $PI3,4P_2$ and $PI3,4,5P_3$ by lipid phosphatases. PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a $PI3,4P_2$ and $PI3,4,5P_3$ phosphatase and functions as a tumor suppressor (Cantley and Neel, 1999). It converts $PI3,4P_2$ to phosphatidylinositol-4-*mono*phosphate (PI4P) and $PI3,4,5P_3$ to phosphatidylinositol-4,5-*bis*phosphate ($PI4,5P_2$) (Cantley and Neel, 1999), blocking the recruitment of Akt to the plasma membrane and inhibiting Akt activation. In several types of human cancers PTEN is mutated and/or inactivated such that the PI3K/PDK1/Akt signalling pathway is constitutively activated due to the high levels of $PI3,4,5P_3$ (Li et al., 1997). Mutations, frameshifts, or deletions in PTEN contribute to the phenotype in several glioblastoma cells lines (Li et al., 1997).

Other phosphatases which downregulate PI3K signalling include SHIP-1 and SHIP-2 (for Src Homology domain-containing Inositol Phosphatases). These lipid phosphatases are capable of removing the 5'-phosphate from $PI3,4,5P_3$ to yield $PI3,4P_2$ (Damen et al., 1996; Lioubin et al., 1996). PI3K can also be inactivated by the direct dephosphorylation of the regulatory subunit, p85 by protein tyrosine phosphatases such as SHP-1 (Cuevas et al., 1999; Yu et al., 1998c).

1.6.1 Regulation of PI3K signalling by SHP-1.

As discussed above, SHP-1 is a regulator of a variety of signalling cascades, including the PI3K/Akt pathway. Most studies support SHP-1 as a negative regulator of this signalling cascade. The mode of regulation of PI3K signalling can occur either through direct regulation of the receptor or through binding to p85(PI3K) (Cuevas et al., 1999; Yu et al., 1998c). It is the N-terminal SH2 domain of SHP-1 that mediates the interaction between its substrates *i.e.* tyrosine phosphorylated receptors or p85 (Cuevas et al., 1999; Yu et al., 1998c). SHP-1 dephosphorylates p85 upon stimulation with IL-4 (Imani et al., 1997) or PDGF (Yu et al., 1998c), and upon ligation of the TCR (Cuevas et al., 1999). SHP-1 interacts with and dephosphorylates receptors for IGF-1, PDGF, EGF (Tonks and Neel, 2001) and Ros (Keilhack et al., 2001). In response to PDGF stimulation of MCF-7 cells (human breast adenocarcinoma cell line) and TRMP cells (canine kidney epithelial cell line), SHP-1 associates with both p85 and the overexpressed PDGFR, resulting in the negative regulation of this signalling cascade (Yu et al., 1998c). SHP-1 specifically dephosphorylates p85 on Tyr688, which is required for PI3K activity, resulting in the inactivation of the PI3K pathway (Cuevas et al., 2001). Insulin stimulates the activation of SHP-1 and its association with the insulin receptor (Bousquet et al., 1998; Uchida et al., 1994). Viable motheaten mice (me^v/me^v), which bear a functionally deficient SHP-1 protein, display enhanced glucose tolerance and insulin sensitivity compared to wildtype littermates. me^v/me^v mice are found to have enhanced insulin receptor signalling as measured by an increase in PI3K activity and associated Akt phosphorylation in liver and muscle (Dubois et al., 2006). Other evidence for the negative modulation of PI3K activity by SHP-1 is demonstrated in T cell antigen receptor-stimulated thymocytes from SHP-1-deficient motheaten mice. In the motheaten thymocytes, PI3K activity and the level of Akt phosphorylation on Ser473 is markedly higher compared to wildtype cells (Cuevas et al., 2001).

There is evidence that SHP-1 may also regulate PI3K through regulation of p85 protein expression. Treatment of MCF-7 cells stably expressing SHP-1 with trichostatin-A (TSA), a histone deacetylase (HDAC) inhibitor, results in a loss of p85 expression and Akt phosphorylation, and increases sensitivity of cells to TSA (Xu et al., 2003). Interestingly, different HDAC inhibitors affect the total cellular phosphotyrosine status

of cells by means of different mechanisms; butyrate represses the mRNA (and protein expression) of the c-*src* tyrosine kinase (Kostyniuk et al., 2002), wherease TSA induces SHP-1 expression specifically by increasing the activity of the tissue-specific P1 promotor, as demonstrated in MCF-7 cells (Xu et al., 2001).

In contrast, SHP-2, the orthologue to SHP-1, has been implicated in the positive regulation of PI3K in response to growth factor and cytokine signal transduction pathways. Like SHP-1, SHP-2 regulates signalling through association with the receptor or through binding to p85(PI3K). SHP-2 associates with and dephosphorylates the PDGFR (DeMali et al., 1999) and IGF-1R (Maile and Clemmons, 2002). SHP-2 also associates with IRS-1 to modulate the ability of EGF, insulin or IGF-1 to activate PI3K (Hayashi et al., 2004; Ugi et al., 1996). A more direct role for SHP-2 in regulating PI3K is through its association with p85(PI3K). EGF stimulation of mouse fibroblast cells induces p85 co-immunoprecipitation with SHP-2 (Wu et al., 2001). Expression of a SHP-2 mutant with an N-terminal SH2 domain deletion results in impaired stimulation of PI3K and Akt phosphorylation in response to EGF, PDGF (Wu et al., 2001) and IGF-1 (Ling et al., 2003). Similar to SHP-1, the SH2 domain of SHP-2 mediates its binding to target proteins and binds to the tyrosine motif, YxxV/I/L (Fujioka et al., 1996; Maile and Clemmons, 2002; Myers et al., 1998). SHP-2 was shown to associate with p85 in response to IL-2 in hematopoietic cells (Gesbert 1998, Craddock Welham 1997) and in U87MG glioblastoma cells in response to EGF stimulation (Wu et al., 2001). Wu *et al.* (2001) showed that EGF induced an association of p85 with SHP-2 and that deletion of the N-terminal SH2 domain of SHP-2 impaired PDGF- and IGF-induced Akt phosphorylation. Furthermore, they demonstrated that SHP-2 association with p85 was correlated with PI3K lipid kinase activity and Akt phosphorylation. More recently, in smooth muscle cells, IGF-1 induced SHP-2 binding to p85, an association which is disrupted with the substitution of tyrosines 528 and 556 to phenylalanine on p85 (Kwon et al., 2006). The loss of interaction under these conditions impaired PI3K activity and the cell migration associated with IGF-1 stimulation of smooth muscle cells (Kwon et al., 2006).

1.7 SHP-1 as a positive regulator of signalling pathways.

Most research has been focused on the function of SHP-1 as a negative regulator of hematopoiesis and the immune system. However, there is evidence SHP-1 may be a positive regulator in development and differentiation of the CNS. SHP-1 is implicated as a positive regulator of neurotransmission and is reported to be localized at synaptic vesicles and interacts with vesicle-associated protein, synaptophysin (Jena et al., 1997). In the brains of motheaten mice, there is a decrease in the number of astrocytes and microglia and reduced myelination in the CNS, also implicating SHP-1 as a positive regulator of differentiation and proliferation of glia (Massa et al., 2004; Wishcamper et al., 2001).

There is strong support for a positive role for SHP-1 modulation of the mitogen-activated protein kinase (MAPK) pathway, which can regulate gene expression, mitosis, differentiation and cell survival (Pearson et al., 2001). In epithelial cells, SHP-1 activity was shown to be required for MAPK activation in response to EGF stimulation. When the catalytically inactive mutant of SHP-1, SHP-1(C455S), is overexpressed in HEK293 cells the stimulatory effects of EGF or serum on cell proliferation, early gene transcription and DNA synthesis are strongly repressed (Su et al., 1996). Similarly, the phosphorylation of MAPK and of the mitogen and extracellular signal-activated protein kinase kinase (MEK) is markedly inhibited by overexpression of SHP-1(C455S) (Su et al., 1996). Knockdown of the SHP-1 gene using siRNA causes a reduction of MAPK and Akt activation in response to EGF stimulation (Wang et al., 2006).

Positive modulation of the MAPK pathway by SHP-1 has largely been characterized in non-hematopoietic cells. However, SHP-1 activity is also required for Ras-dependent activation of the MAPK pathway in hematopoietic cells (Krautwald et al., 1996). Using macrophages from viable motheaten mice the authors demonstrated a decreased activation of MAPK in response to colony-stimulating factor 1 (CSF-1), which is a growth factor that stimulates macrophage survival, growth and differentiation. Using a CSF-1-dependent macrophage cell line (BAC-1.2F5), they demonstrated that expression of a dominant-negative Ras mutant strongly reduced CSF-1-mediated stimulation of MEK and MAPK. In these cells SHP-1 was activated in the course of

mitogenic signal transduction in a Ras-dependent manner and the phosphatase activity was necessary for the activation of the MAPK pathway (Krautwald et al., 1996).

Another pathway that has been identified as being positively modulated by SHP-1 is the Janus kinases/signal transducers and activators of transcription (JAK/STAT) signalling pathway. The JAK/STAT pathway regulates cellular responses to cytokines and growth factors (Rawlings et al., 2004). This pathway plays a central role in cell fate decisions, regulating cell proliferation, differentiation and apoptosis (Rawlings et al., 2004). SHP-1 also functions as a positive regulator of EGF- and interferon (INF)γ-induced STAT activation in HeLa cells (You and Zhao, 1997).

1.7.1 SHP-1 involvement in differentiation.

SHP-1 has been identified as a central regulator in differentiation of both neurons and glia in the CNS. In *me/me* brains, there is a decrease in the number of astrocytes and microglia (Wishcamper et al., 2001) as well as a reduction in myelination in the CNS (Massa et al., 2004). These reports suggest SHP-1 may play a role in astrocyte differentiation and proliferation as well as for oligodendrocytes differentiation, maturation and survival. However, a positive role for SHP-1 during glial development may be specific to development as SHP-1 functions as a negative regulator in mature activated glia and microglia (Sorbel et al., 2002).

SHP-1 may contribute to differentiation of PC12 cells. NGF results in differentiation of PC12 cells to a neuronal phenotype (Greene and Tischler, 1976; Huff et al., 1981) and results in the tyrosine phosphorylation of SHP-1 (Vambutas et al., 1995). In contrast, the activated EGFR, which is also able to phosphorylate and activate SHP-1 (You and Zhao, 1997), does not induce differentiation in PC12 cells, but induces proliferation (Huff et al., 1981). Obviously, the role of SHP-1 is greatly dependent on the growth factor receptor that is stimulated. A direct role for SHP-1 in NGF-mediated events is suggested by the fact that SHP-1 can bind to the TrkA-receptor and that anti-TrkA immunoprecipitates have protein tyrosine phosphatase activity (Vambutas et al., 1995). This same group later demonstrated that SHP-1 negatively regulates TrkA through direct dephosphorylation of the receptor and that mice lacking SHP-1 had increased numbers of sympathetic neurons during the period of naturally occurring cell

death (Marsh et al., 2003). Although the role for SHP-1 in NGF-mediated differentiation remains unclear, these authors suggested that although SHP-1 in their study appeared as a negative regulator of TrkA, it may function in this manner in order to sustain TrkA sensitivity to NGF during the time of differentiation (Marsh et al., 2003). Src kinase, which phosphorylates SHP-1 *in vitro*, is also activated during NGF-induced PC12 differentiation (Kremer et al., 1991), yet activation of the Src kinase member Lyn (often viewed as an anti-apoptotic event) is inhibited, most likely by SHP-1, during NGF-induced PC12 differentiation (Daigle et al., 2002).

This review of the literature has provided information on PI3K/PDK1/Akt signalling and how this can influence cell function; it has touched upon the role of tyrosine phosphorylation and the role of SHP-1 in modulating these events. It has also provided evidence that PDK1 is a kinase central to PI3K function. Not surprisingly, the tyrosine residues on PDK1 that may contribute to its function are targets for Src kinase and reside in YXX(V/I/L) motifs, putative targets for SHP-1. The function of Tyr-phosphorylated PDK1, however, remains unclear.

Given that both SHP-1 and PDK1 have been implicated in differentiation and that differentiation invariably involves a nuclear event, the last section of this review of the literature will provide information on the nucleus and on its potential as a subcellular target for SHP-1 and PDK1 localization.

1.8 Nuclear shuttling of proteins.

Over the last several years, there has been mounting evidence for the presence of a nuclear-specific PI3K signalling pathway. In addition, key signalling components including an independent pool of $PI3,4,5P_3$ and downstream effectors such as PDK1, Akt and PKCζ, are all present and activated in the nucleus (Neri et al., 2002; Neri et al., 1999). In addition, modulators of PI3K signalling such as SHP-1 (Craggs and Kellie, 2001; Yang et al., 2002b), PTEN (Lachyankar et al., 2000) and SHIP-2 (Deleris et al., 2003) are also present in the nucleus.

1.8.1 Nuclear pore complex (NPC).

The transport of proteins between the nucleus and the cytoplasm is necessary for the exchange of information within the cell. The bidirectional movement of molecules across the nuclear envelope is mediated by channels within the nuclear pore complex (NPC). The NPC is a huge macromolecular assembly with a mass of ~120 MDa in vertebrates and is composed of approximately 30 proteins, called nucleoportins (Fried and Kutay, 2003). The overall structure can be divided into basic parts: the nuclear basket, the central core and the cytoplasmic fibrils (Figure 1.9). Passive diffusion across the NPC occurs with ions and molecules smaller than 25-40 kDa (Fried and Kutay, 2003). In contrast, proteins and RNA molecules larger than 40 kDa do not diffuse across the NPC (Fried and Kutay, 2003). Rather, macromolecules are carried through the central channel of the NPC by specific transport receptor proteins. These carriers are collectively referred to as karyopherins (Radu et al., 1995), with those involved in import (Gorlich et al., 1994) and export (Stade et al., 1997) termed importins and exportins, respectively. Many transport receptors are members of the importinβ superfamily. In some instances cargo proteins (*i.e.* proteins that are cotransported in and out of the nucleus) can bind directly to importinβ. However, most often, the interaction between importinβ and the cargo is mediated by the adaptor molecule importinα. Transport receptors identify specific signals, such as a nuclear localization signal (NLS) present within the cargo molecules which allows for the interaction and transport of that molecule into the nucleus. This type of transport requires energy, usually derived from GTP hydrolysis (Fried and Kutay, 2003).

The energy for nuclear transport is provided by the small Ras family of GTPase, Ran (Quimby and Dasso, 2003). Like other GTPases, Ran cycles between a GTP- and a GDP-bound state (Bourne et al., 1990). The different forms of Ran are not evenly distributed in the cell, with RanGTP being found predominantly in the nucleus and RanGDP in the cytoplasm (Kalab et al., 2002; Smith et al., 2002). Therefore, import receptors bind cargo in the cytoplasm in the absence of RanGTP and release cargo proteins in the nucleus upon RanGTP binding to the complex. The importin-RanGTP complex is then recycled to the cytoplasm, where RanGTP is displaced from the

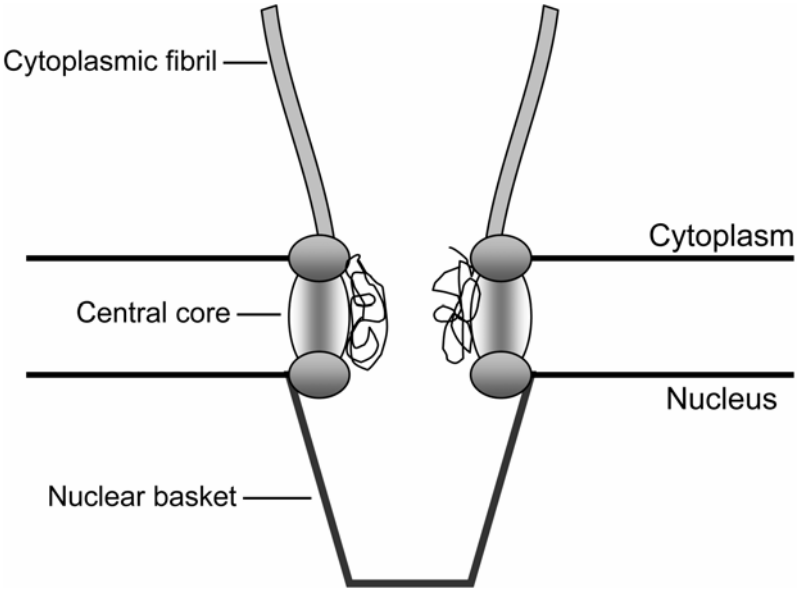


Figure 1.9: The main structure of the nuclear pore complex (NPC). The NPCs are composed of approximately 30 proteins, collectively called nucleoportins. This huge macromolecular assembly is a calculated mass of 120 MDa in vertebrates. The basic architecture of the NPC can be divided into three basic elements: the nuclear basket, the central core and the cytoplasmic fibrils (Adapted from: Fried and Kutay, 2003).

importin by RanBP1 and/or RanBP2 and subsequently hydrolyzed by the RanGTPase in the presence of RanGAP (Figure 1.10). In contrast, export receptors bind cargo proteins in the nucleus in a complex with RanGTP. The trimeric complex is then translocated through the NPC to the cytoplasm, where RanGTP is removed from the complex by GTP hydrolysis to GDP and this triggers the dissociation of the exportin from the cargo protein (Figure 1.10).

Proteins containing NLS are transported into the nucleus *via* NPC recognition. A NLS is a short peptide sequence with high content of basic amino acids residues (Jans et al., 2000; Moroianu, 1999). Three classes of NLS have been identified: (i) the SV40-like NLS is composed of a single peptide region containing basic residues, (ii) the bipartite NLS is composed of two regions of basic residues separated by a spacer, (iii) the third class NLS is uncommon and defined by the N-terminal signal of the yeast protein, Mat α2, K-I-P-I-K (Hall et al., 1984). There are some rare examples that do not conform to these three classes (Boehm et al., 1995; Dingwall and Laskey, 1991; Siomi and Dreyfuss, 1995). The NLS are specifically recognized by distinct importin proteins that help transport the targets into the nucleus (Moroianu, 1999). Binding of these proteins can be influenced by phosphorylation, which may increase the identification and interaction or hide the NLS of the protein.

Exportins facilitate the nuclear export of several different proteins, RNA molecules and enzymes. The export receptor chromosomal region maintenance protein 1 (CRM1) is the most versatile of all export factors, as it is involved in export of many different classes of proteins including cell cycle regulators, transcription factors and RNA binding proteins. Most commonly, CRM1's export substrates that contain a short, leucine-rich nuclear export signal (NES) and the export complex formed with NES-containing substrates requires RanGTP binding to CRM1 (Fornerod et al., 1997).

1.8.2 Nuclear shuttling of SHP-1.

Expression of SHP-1 is found in a variety of cells including hematopoietic and non-hematopoietic cells. In most hematopoietic cells, SHP-1 expression is high and localized predominantly in the cytoplasm. In contrast, SHP-1 in non-hematopoietic cells is found in both the cytoplasm and the nucleus under resting conditions

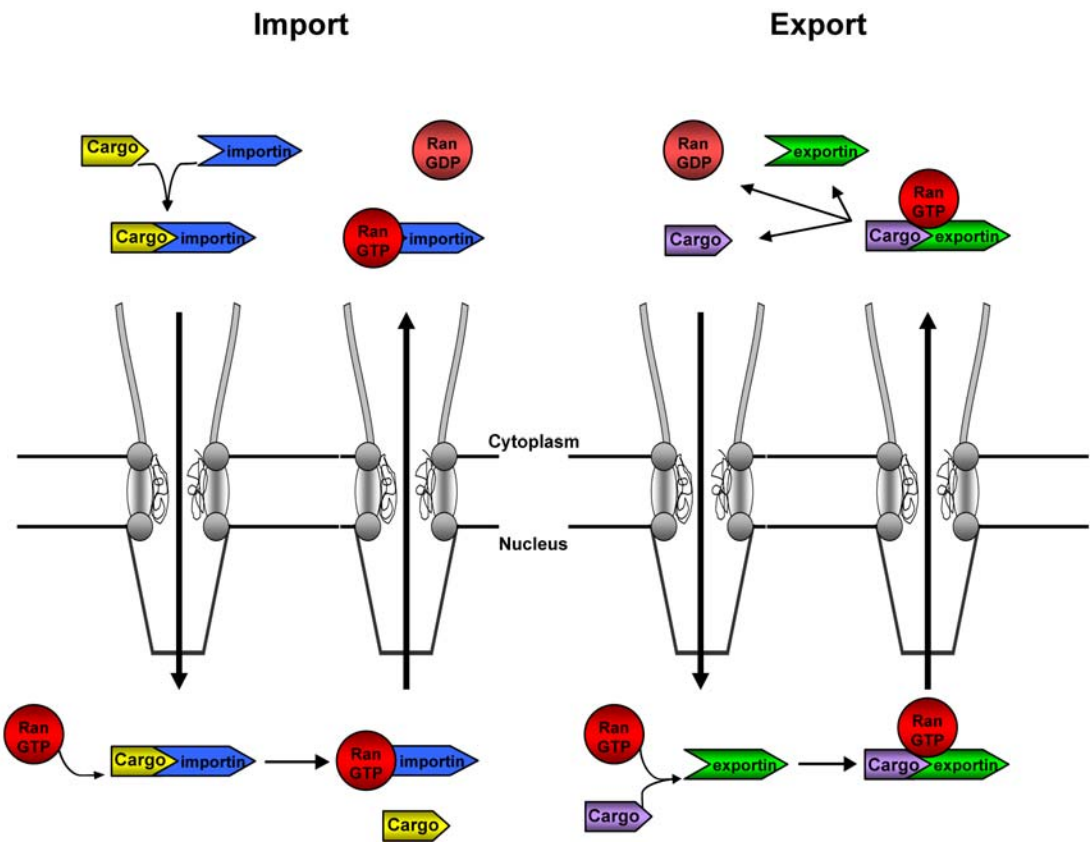


Figure 1.10: Nuclear shuttling of cargo proteins by importins and exportins. (Left) In the cytoplasm an importin binds to cargo proteins containing a nuclear localization signal and mediates interactions with the nuclear pore complex (NPC) to translocate the complex into the nucleus. Nuclear RanGTP binds to the importin and induces the release of the cargo protein from the complex. The importin–RanGTP complex is then recycled to the cytoplasm, where RanGTP is displaced from the importin by RanBP1 and/or RanBP2 and subsequently hydrolyzed by RanGTPase in presence of RanGAP. (Right) In the case of export, cargo protein with a nuclear export signal binds to an exportin induced by RanGTP in the nucleus and the trimeric complex is then translocated through the NPC to the cytoplasm, where RanGTP is removed from the complex by GTP hydrolysis and the exportin dissociates from the cargo. The exportin recycles back to the nucleus and is ready to translocate more cargo proteins to the cytoplasm (Adapted from: Fried and Kutay, 2003).

(Brumell et al., 1997; Ganesan et al., 2003; He et al., 2005). In both hematopoietic and non-hematopoietic cells, growth factor stimulation is shown to induce SHP-1 nuclear localization. For example, SHP-1 is constitutively present in the both cytosol and nucleus of liver cells extracted from Fischer rats, with the amount of SHP-1 present in the nucleus increasing in response to growth hormone (GH) (Ram and Waxman, 1997). Nuclear localization of SHP-1 is reported in non-hematopoietic cells A431 (skin cancer), COS-7 (monkey kidney), Swiss 3T3 (mouse fibroblast), A549 (human alveolar basal epithelial cells), MCF-7 and HeLa and in hematopoietic cells U937 (human lymphoma) (Craggs and Kellie, 2001; Tenev et al., 2000). EGF stimulation of HEK293 and COS-7 as well as A431 and HT29 cells increases the translocation of SHP-1 to the nucleus (Craggs and Kellie, 2001; He et al., 2005). Other examples of ligand-induced nuclear localization of SHP-1 include; IL-4 stimulation of NIH3T3 cells and IL-4 and IL-7 stimulation of PBLC-1, a hematopoietic cell line (Yang et al., 2002b). The subcellular localization and/or its potential to relocalize to the nucleus in different cell types could explain how SHP-1 can act as a negative regulator as well as a positive regulator of cell signalling.

1.8.2.1 Mechanism of SHP-1 nuclear import.

The mechanism of SHP-1 nuclear import has not been extensively studied. When SHP-1 was first cloned, it was reported to contain three nuclear localization-like motifs at the C-terminus, TTKKKLE (aa 518-524), KVKKQRSAD (aa 576-584) and KNKGSLKRK (aa 587-595) (Yi et al., 1992). The latter basic sequences fall into a typical bipartite nuclear localization signal (NLS). Craggs and Kellie (2001) reported that SHP-1 is localized to the nucleus of several non-hematopoietic cell lines including, HEK293, HeLa, MCF-7, A549, primary rat embryo fibroblast and CHO (Chinese hamster ovary) cells, and excluded from the nucleus in several other hematopoietic cell lines including, HL-60, U937 macrophage and Jurkat T-cell leukemia. These authors mutated the various NLS on SHP-1 and identified the C-terminal cluster of basic amino acids (KRK) as functioning independently and required for nuclear import (Craggs and Kellie, 2001). This finding was unexpected as the accepted paradigm for bipartite NLS is that neither basic region can function independently to target proteins to the nucleus.

However, this finding was not unprecedented; T cell protein tyrosine phosphatase (TCPTP) is another example of a PTP where the basic regions of the bipartite NLS function independently to a limited degree (Tiganis et al., 1997).

There remains some controversy regarding the requirement for the bipartite NLS for nuclear localization of SHP-1. Yang *et al.* (2002) demonstrate that both basic regions of the SHP-1 NLS are required for nuclear localization in NIH3T3 cells. Using EGFP-fusion proteins containing three different fragments of the C-terminal region of the SHP-1 NLS, (amino acid (aa) 551-595; aa 576-595; aa 588-595) the subcellular localization of the fusion proteins was determined. Fusion proteins containing both C-terminal bipartite regions completely localized to the nucleus. This finding was also reproduced in a macrophage cell line, Bac1.2f5 cells, using the fusion protein, EGFP(551-595). In these cells expression of the EGFP(551-595) protein fragment plasmid is expressed exclusively in the nucleus of all transfected cells (Yang et al., 2002b).

Nuclear import of SHP-1 is likely mediated through recognition of its NLS by the NPC. This has not been extensively researched, but there is evidence that SHP-1 nuclear import occurs through a GTP-independent mode of import (Tenev et al., 2000). GTP-independent import, unlike GTP-dependent import, is activated by increased cytoplasmic calcium (Ca^{2+}) levels and occurs in a calmodulin-dependent manner. Treatment of A431 and HaCaT (human epidermal keratinocytes) cells with the calcium ionophore, A23187, increases the localization of SHP-1 to the nucleus. This localization is blocked with a calmodulin antagonist, calmidazolium chloride, suggesting SHP-1 nuclear import is mediated by a Ca^{2+}-calmodulin-dependent mechanism (Tenev et al., 2000). GTP-independent and Ca^{2+}-calmodulin dependent nuclear transport has been reported to occur (Sweitzer and Hanover, 1996). Although GTP-dependent nuclear import of SHP-1 has not been demonstrated, it can not be excluded as a mechanism of SHP-1 nuclear import.

1.8.2.2 Role for nuclear SHP-1.

Functions for SHP-1 in the nucleus have not been fully characterized. It is possible that SHP-1 localization to the nucleus might be a way to prevent interactions

with cytoplasmic signalling molecules or that it has specific nuclear phosphoprotein targets and/or that it modulates nuclear signalling cascades.

SHP-1 localizes to the nucleus of cultured liver cells and in rat liver *in vivo* in response to stimulation with GH (Ram and Waxman, 1997). GH regulates the transcription of a variety of genes that mediate growth and metabolism (Thomas, 1998). The actions of GH are mediated through activation of STAT transcription factors which are involved in regulating cell proliferation, differentiation and apoptosis (Thomas, 1998). GH also induces nuclear translocation of SHP-1 and binding to tyrosine-phosphorylated STAT5b, (but not to STAT3) (Ram and Waxman, 1997). These authors suggest that the nuclear inactivation of STAT5b by SHP-1 may contribute to the sustained sensitivity of STAT5b to GH, unlike STAT3 (which does not bind SHP-1), which becomes desensitized to GH over time (Ram and Waxman, 1997). An indirect link between SHP-1 and STATs is suggested by the observation that overexpression of SHP-1 in A431 cells leads to the attenuation of the EGF-dependent STAT signalling (Tenev et al., 2000). In inducible SHP-1-expressing A431 cells, SHP-1 mediates a reduction in STAT1/3 DNA binding activity when stimulated with EGF (Tenev et al., 2000).

1.8.3 Other PTPs that contain a NLS.

There are several PTPs besides SHP-1 that contain a NLS and shuttle between the cytoplasm and the nucleus including PTEN, PEST-enriched phosphatase (PEP) (Flores et al., 1994), non-receptor protein tyrosine phosphatase-ε (NRPTPε) (Kraut et al., 2002) and PP-1 (Lesage et al., 2004). For example, PTEN accumulates in the nucleus during cell differentiation (Lachyankar et al., 2000) or cell cycle (Gil et al., 2006; Ginn-Pease and Eng, 2003). Localization of PTEN to the nucleus is mediated by its own NLS-like motif which is located in the N-terminal region of PTEN (Chung and Eng, 2005; Chung et al., 2005). The role of importins in PTEN nuclear entry is not clear, but it is known that nuclear accumulation of PTEN occurs in a RanGTP-dependent manner (Gil et al., 2006). Several reports have demonstrated that the function of PTEN in the nucleus is independent of its catalytic activity and depends on the direct interaction of PTEN with nuclear target proteins, such as the tumor suppressor p53

(Freeman et al., 2003) or the oncoprotein MSP58/MCRS1 (Okumura et al., 2005). However, the phosphatase activity of PTEN is required for other functions mediated by nuclear PTEN. For example, the catalytic activity of PTEN mediates the decrease in cyclin D1 levels in the nucleus leading to cell cycle arrest (Chung and Eng, 2005; Radu et al., 2003; Weng et al., 2001). Interestingly, the catalytic activity of nuclear PTEN may mediate growth suppression independent of Akt activity (Liu et al., 2005). In addition, several findings indicate a proapoptotic function for nuclear PTEN. Active PTEN purified from vascular smooth muscle cell nuclei dephosphorylated $PI3,4,5P_3$ (Deleris et al., 2003), and nuclei from NGF-treated PC12 cells incubated with recombinant PTEN showed increased apoptotic DNA fragmentation (Ahn et al., 2004).

A role for nuclear TCPTP is implicated in regulating cell proliferation. Alternative splicing of the TCPTP transcript generates a 45-kDa form that lacks the hydrophobic segment at the C-terminial (Champion-Arnaud et al., 1991; Tillmann et al., 1994). This 45-kDa form of TCPTP is found in the nucleus, and a NLS has been identified in the C-terminal segment (residues 550-381) (Lorenzen et al., 1995; Tillmann et al., 1994). The nuclear localization of TCPTP suggests a potential role for this phosphatase in cell cycle regulation. TCPTP lacking the NLS can revert transformation of Rat2 cells, as well as inhibit the potential of these cells to induce tumor growth in nude mice (Zander et al., 1993). Furthermore, overexpression of TCPTP in U87MG glioblastoma cells inhibits proliferation and anchorage-independent growth *in vitro* and suppresses the tumorigenicity of U87MG cells implanted in mice (Klingler-Hoffmann et al., 2001). There is a decreased proliferation rate of TCPTP null fibroblasts compared to wildtype fibroblasts (Ibarra-Sanchez et al., 2001). Further investigation of the cell cycle revealed a reduced progression through the G1 phase and delayed induction of cyclin D1 in TCPTP null cells. These cells presented delayed activation of NFκB and decreased IκB kinase (IKKβ) activity, suggesting that TCPTP might exert a positive regulatory effect on the cell cycle by enhancing signalling through the NFκB pathway (Ibarra-Sanchez et al., 2001). The ability of NFκB to stimulate cell proliferation and to prevent apoptosis can undoubtedly favor cancer development, and activation of NFκB has been linked with many human cancers (Ghosh and Karin, 2002). In other types of cancer,

such as colon cancer, decreased activity of NFκB is associated with tumorigenesis (Deng et al., 2002).

1.8.4 Nuclear shuttling of PDK1.

In unstimulated cells, endogenous PDK1 resides mostly in the cytoplasm with a small pool being localized at the plasma membrane. In most cells constitutive nuclear PDK1 is not often observed (Grillo et al., 2000; Kim et al., 2001; Lim et al., 2003; Scheid et al., 2005). However, it can localize to the nucleus upon growth factor (i.e. insulin, IGF-1, NGF and PDGF) stimulation (Lim et al., 2003; Salinas et al., 2000; Scheid et al., 2005). This suggests PDK1 shuttles between the nucleus and cytoplasm, with the rate of nuclear export occurring at a higher rate than the import rate (Kikani et al., 2005). The above mentioned growth factors all have been associated with activation of the PI3K pathway. Lim *et al.* (2003) demonstrate an increase in the nuclear accumulation of PDK1 in PTEN-/- embryonic fibroblasts which suggest that PDK1 nuclear shuttling is regulated by the PI3K pathway and the availability of $PI3,4,5P_3$.

1.8.4.1 PDK1 mechanism of nuclear export.

The nuclear export of PDK1 is mediated through its own nuclear export signal (NES). Deletion mapping and mutagenesis of mouse PDK1 identified a functional NES located between the kinase domain and the PH domain (amino acid residues 382-391) (Lim et al., 2003). It is most likely that the NES of PDK1 associates with the nuclear export protein CRM1 which allows for its cytoplasmic relocalization through the NPC (Kutay and Guttinger, 2005). Evidence for this is demonstrated by treatment of cells with leptomycin-B (LMB), a compound that dissociates CRM1 from RanGTP and substrates (Kutay and Guttinger, 2005). LMB treatment of CHO/IR (Chinese hamster ovary cells overexpressing the insulin receptor) and MCF-7 cells causes nuclear accumulation of PDK1 (Lim et al., 2003; Scheid et al., 2005).

1.8.4.2 Mechanism of PDK1 nuclear import.

The mechanism used for the import of PDK1 into the nucleus is unknown. It has been suggested by Scheid *et al.* (2005) that phosphorylation on Ser396 located close to

the NES is important for shuttling of PDK1 to and from the nucleus. Indeed, mutating Ser396 to Ala reduces the import of PDK1 into the nucleus of PTEN-/- MEF (mouse embryonic fibroblast) cells stimulated with PDGF and in MCF-7 cells treated with IGF-1, although some localization of PDK1Ser396Ala to the nucleus still occurs. These same authors offer two explanations for their observation; first, phosphorylation on Ser396 may occur at the plasma membrane which subsequently allows for its import into the nucleus. Second, phosphorylation on Ser396 may occur in the nucleus where it could influence the adjacent NES resulting in the disruption of the CRM1-PDK1 complex that forms prior to nuclear export. Interestingly, these same authors observed that overexpression of PDK1 did not result in a proportional increase in nuclear PDK1 upon growth factor stimulation, suggesting that a component of the nuclear import mechanism may be saturated and, thus, be unable to contribute to additional PDK1 nuclear accumulation. Others have tried to explain nuclear entry of PDK1 by suggesting PDK1 contains a NLS bipartite-like motif which could influence nuclear localization (Kikani et al., 2005). *Drosophila* PDK1, Dstpk61, has a putative bipartite nuclear localization signal (Clyde and Bownes, 2000), which could influence nuclear localization; however, this has never been adequately demonstrated.

1.8.4.3 Roles of nuclear PDK1.

Similar to SHP-1, the function of nuclear PDK1 and its nuclear targets are currently being characterized. There is evidence that shuttling of PDK1 to the nucleus is required for growth and proliferation. Stable expression of PDK1 in Comma-1D (mammary epithelial) cells induces anchorage-independent growth whereas stable expression of PDK1ΔNES, which is unable to localize to the cytoplasm because of a deleted NES (ΔNES), inhibits anchorage-independent growth (Lim et al., 2003). Similarly, NMuMg (mouse mammary epithelial) cells stably expressing PDK1 survived better than cells expressing PDK1ΔNES when exposed to UV induced cell death (Lim et al., 2003).

Nuclear shuttling of PDK1 may also mediate signalling of the nuclear PI3K pathway. The presence of nuclear PI3,4,5P_3 and key signalling components of the PI3K pathway, including PI3K, Akt, PKCζ, p70S6KβI and p70S6KβII, are all found present

and activated in the nucleus (Neri et al., 2002). PDK1 could directly phosphorylate its substrates in the nucleus. For example, insulin, which stimulates PI3K activity and Akt phosphorylation, also induces PDK1 localization to the nucleus in cells (Lim et al., 2003). Scheid *et al.* (2005) demonstrated that overexpressed PDK1mNES (with serine substitution at L380 and F384 causing it to accumulate in the nucleus) colocalized with Akt and repressed transcriptional activity of FOXO3a. Upon phosphorylation Akt and other AGC kinases phosphorylate FOXO3a causing it to redistribute to the cytoplasm where it docks with the 14-3-3 sequestering proteins (Brunet et al., 1999). This reduces FOXO3a transcriptional activity and, ultimately, inhibition of apoptosis (Brunet et al., 1999). These findings suggest a functional role for PDK1 in the nucleus; however, they do not support the finding by Lim *et al.* (2003) who demonstrate that PDK1 localization to the nucleus does not alter Ser241 phosphorylation or kinase activity of PDK1, but simply negatively regulates its function (*i.e.* activation of Akt) by virtue of removal from the cytoplasm.

Nuclear PDK1 has been implicated in the effects of NGF in PC12 cells (Martelli et al., 2003) and COS-7 cells (Salinas et al., 2000). In the latter case, NGF stimulation induces nuclear and perinuclear localization of PDK1 (Salinas et al., 2000). Nuclear-specific PI3K signalling has been suggested to be mediated by PDK1 (Neri et al., 2002). PDK1 has also been shown to translocate to the nucleus with peroxisome proliferator activated-receptor-γ (PPARγ) in response to PPARγ agonists and to induce transcription of genes required for adipocyte differentiation (Yin et al., 2006).

1.8.5 Nuclear import by "piggy-back."

There are numerous proteins that do not contain recognition signals for the NPC (i.e. NLS or NES), but are still able to undergo nuclear shuttling. The nuclear shuttling of such proteins can occur *via* a "piggy-back" mechanism. For example, IL-5 affects nuclear transport of its α- and β-receptor subunits apparently through a "piggy-back" mechanism. IL-5 is central in regulating eosinophilia in allergic disease and parasitic infections (Sanderson, 1990). The receptor for IL-5 is comprised of an α-subunit which recognizes IL-5 specifically (Takaki et al., 1990) and a β-subunit which lacks ligand-binding activity, but is essential for signal transduction (Devos et al., 1991). Human

(h)IL-5 possesses a functional NLS signal and localizes to the nucleus of intact receptor-expressing cells (Jans et al., 1997b). Only in the presence of both the hIL-5 binding α-subunit and hIL-5 was the β-subunit (which lacks IL-5 binding activity) able to accumulate in the nucleus (Jans et al., 1997a). Similiary, the nuclear accumulation of the IL-5-binding α-subunit was dependent on the presence of IL-5 (Jans et al., 1997a).

The serine/threonine phosphatase PP-1 is expressed in all eukaryotic cells and controls numerous cellular processes including metabolism, cell division, apoptosis and protein synthesis (Bollen, 2001; Cohen, 2002). Although PP-1 is very abundant in the nucleus, the mechanism underlying its nuclear translocation is unknown. PP-1 (36-38 kDa) is small enough to enter the nucleus passively; however, this is not the most likely mechanism because PP-1 does not appear to exist in the cell as a free monomer (Bollen and Stalmans, 1992). PP-1 isoforms contain classical bipartite nuclear NLS that mediate transport by importins (Okano et al., 1997), but the functionality of the NLS of many of these isoforms is unclear. Another possible mechanism for nuclear translocation is "piggy-back" transport involving the cotransport with an interactor that contains an NLS (Mizuno et al., 1996; Turpin et al., 1999; Zhao and Padmanabhan, 1988). At least four nuclear interactors of PP-1 have either putative NLS as is the case for PP-1 nuclear targeting subunit (PNUTS) and Sds22 or established NLS found in the nuclear inhibitor of PP-1 (NIPP) and SIPP1, making them candidate cotransporters of PP-1 (Allen et al., 1998; Jagiello et al., 2000; Llorian et al., 2004; Stone et al., 1993). Also, strains of *Saccharomyces cerevisiae* that carried temperature-sensitive *sds22* alleles showed a rapid loss of nuclear PP-1 under restrictive conditions, providing additional evidence for a role of Sds22 in maintaining the normal nuclear localization of PP-1 (Peggie et al., 2002). One isoform, PP-1γ_1, does not contain a functional NLS and its nuclear accumulation and nuclear targeting depends on the interaction with NIPP1 and PNUTS that contain both a NLS and a binding motif (RVXF) for PP-1γ_1. PP-1γ_1 mutants with a cytoplasmic accumulation are retargeted to the nucleus by the overexpression of NIPP1 and PNUTS with a functional RVXF motif (Lesage et al., 2004).

The mechanism of "piggy-backing" also occurs for the export of proteins from the nucleus. For example, the dual-specificity MKP-3 is found to regulate the nuclear localization of ERK2. MKP-3 is localized in the cytoplasm and contains a NES within

its N-terminal non-catalytic domain (Karlsson et al., 2004). This NES mediates the CRM1-dependent nuclear export of MKP-3 and functions independently of a conserved KIM (kinase interaction motif), which mediates ERK binding and is also located in this domain of the protein. The observation that either mutation of the NES or exposure of cells to the export inhibitor LMB, causes MKP-3 to accumulate in the cell nucleus, suggests that MKP-3 is capable of nucleo–cytoplasmic shuttling (Karlsson et al., 2004). In addition to dephosphorylation and inactivation of ERK2, MKP-3 is able to sequester ERK2 in the cytoplasm. Furthermore, mutation of either the NES or the KIM abolished cytoplasmic anchoring, indicating that both motifs within MKP-3 are required for this function. These findings suggest that MKP-3 regulates the activity as well as the subcellular localization of ERK2 (Karlsson et al., 2004).

Similarly, the oncoprotein v-ErbA, a virally-derived, dominant negative variant of thyroid hormone receptor (TR), contains a NES and is actively exported to the cytoplasm by the CRM1 export receptor (Bunn et al., 2001; DeLong et al., 2004). *In situ* protein-protein interaction assays, and colocalization studies in transiently transfected cells indicate that v-ErbA dimerizes with TRα and the retinoid X receptor (RXR) and sequesters a significant fraction of the two nuclear receptors in the cytoplasm (Bonamy et al., 2005). Bonamy *et al.*, (2005) suggest that the mislocalization is most likely due to the CRM1-mediated nuclear export of TRα and RXR, which are "piggy-backed" to the cytoplasm by v-ErbA after formation of the dimers in the nucleus (Bonamy and Allison, 2006). The mislocalization of TR, RXR and possibly other coactivators by v-ErbA blocks these transcription factors from their normal function in the nucleus which may contribute to cellular transformation by v-ErbA, resulting in cancer (Bonamy and Allison, 2006).

1.8.6 Can SHP-1 regulate PDK1 function and localization?

Both SHP-1 and PDK1 function at the plasma membrane; however, independent reports also provide evidence of their being localized to the nucleus. SHP-1 interacts with proteins that contain ITIMS, with the consensus sequence pYXX(V/I/L). SHP-1 is capable of dephosphorylating Src kinase substrates (Frank et al., 2004). As PDK1 is a

Src kinase substrate (Park et al., 2001) and as it contains putative ITIM-like motifs, it is not unreasonable to consider a regulatory role for SHP-1 in PDK-1 function.

Furthermore, it is obvious that not all proteins that translocate to the nucleus have a NLS, thus suggesting that the notion of a "piggy-back"-like mechanism may be prevalent. The same holds true for proteins that do not contain a NES but appear to cycle through the nuclear compartment. Perhaps a similar mechanism accounts for the ability of SHP-1 (with its NLS) and PDK1 (with its NES) to shuttle into and out of the nucleus under a variety of conditions?

1.9 Objectives.

This review of the literature reflects the linear PI3K signalling model, initiated by membrane-bound receptor tyrosine kinases and PI3K-mediated phospholipid production at the plasma membrane, as a means for regulating Akt function. While this paradigm is championed repeatedly in the growth factor receptor field, several issues must be considered; first, PI3K function/activity is more often than not measured indirectly as it is simply more convenient to infer/assess its activity by the phosphorylation status of its primary effector Akt. However, Akt phosphorylation need not always directly reflect PI3K function, second; our laboratory has recently shown that activation of the sigma2 receptor system by the antipsychotic drug haloperidol induces PI3K activity and a concurrent loss of Akt phosphorylation (Dai *et al.*, 2007, in press). Furthermore, differentiation of C6 glioma cell cultures using dibutyryl-cAMP (in combination with serum withdrawal) can induce PI3K activity (Roymans et al., 2001), while inhibiting the phosphorylation of Akt (Van Kolen and Slegers, 2004). A similar uncoupling is observed in differentiated 3T3-L1 adipocytes chronically treated with GH (Takano et al., 2001). It is the contention of this laboratory that this "uncoupling" of PI3K activity and Akt phosphorylation has been observed before, but is not published.

With this in mind, this thesis was originally undertaken to determine why PI3K signalling does not concord with Akt phosphorylation during growth of C6 cells in serum-free medium and to determine how this compares/contrasts with "normal" linear PI3K/PDK1/Akt signalling. During the course of this work, a unique means of regulation of PI3K function by SHP-1 was observed in these cells. The regulation of PDK1 phosphorylation/localization/function by SHP-1 became the focus of this thesis.

The specific objectives of this thesis are:

(1) To identify the function of SHP-1 in CNS cell lines.

(2) To determine how SHP-1 modulates the PI3K/PDK1/Akt pathway in C6 glioblastoma cells using serum withdrawal as a model of cell stress.

(3) To determine if SHP-1 can interact with PDK1 in a phosphotyrosine-dependent manner.

(4) To determine the significance of PDK1 and SHP-1 nucleo-cytoplasmic shuttling.

2. MATERIALS AND METHODS

2.1 Reagents.

All reagents used were of molecular biological or analytical grade. The names of the reagents and their suppliers are listed in Table 2.1. The addresses of the individual suppliers from which all supplies were obtained are given in Table 2.4.

2.2 Culture of mammalian cell lines.

C6 rat glioblastoma (ATCC #CCL-107: *Rattus norvegicus*), human embryonic kidney (HEK293) (ATCC #CRL-1573: *Homo sapiens*) and pheochromocytoma (PC12) (#CRL-1721: *Rattus norvegicus*) cell lines were utilized throughout the course of this work. C6 and HEK293A cells were propagated in Dulbecco's modified Eagle's Medium (DMEM): Nutrient Mixture 4.0 mM/L L-glutamine, 1000 mg/L glucose, 110 mg/L sodium pyruvate, supplemented with 10% fetal bovine serum (FBS) in tissue culture plates and grown at 37°C in a 5% CO_2 environment.

C6 and HEK293A cells were passaged bi-weekly by aspirating the medium from the culture plates, washing with PBS and adding Trypsin-EDTA. The detached cells were resuspended in DMEM containing 10% FBS, and seeded to a ratio of 1:4.

PC12 cells were cultured on cell cultured plates coated with rat tail collagen type I (200 μg/mL) using medium containing Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 82.5%; horse serum, 10%; FBS, 5%. They were propagated bi-weekly by removing the adhered cells with 5 mL of fresh medium and cell suspensions were passed through a syringe 5 times to break up the cell clusters. Cells were then seeded to a ratio of 1:3.

Stocks were prepared by detaching cells from their plates with trypsin (C6 and HEK293A) or by medium (PC12), as detailed above. The cells were then suspended in Gibco recovery cell culture freezing medium. The cells were placed in a NalgeneCryo 1°C Freezing Container and placed at -70°C for 24 hrs, then stored in liquid nitrogen.

Table 2.1: Lists of Reagents and Suppliers.

Reagents	Supplier
Absolute Ethanol	BDH
Acrylamide	Bio-Rad
Agarose	Invitrogen
Ammonium Persulfate	EMD
Bovine Serum Albumin	EMD
Bromophenol Blue	Sigma
Calcium Chloride	BDH
Chloroform	BDH
Coomassie Brilliant Blue R-250	Sigma
1,2-Diacyl-*sn*-glycero-3-phospho-L-serine	Sigma
Diethylpyrocarbonate (DEPC)	BDH
Dimethylsulfoxide (DMSO)	Sigma
N,N-Dimethylformamide	BDH
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT)	Calbiochem
Ethidium Bromide	Sigma
Ethylene-Diamine Tetraacetic Acid Disodium Salt (EDTA)	Sigma
Ethylene Glycol Bis (2-Aminoethyl Ether)-N,N,N'N'-Tetraacetic Acid Disodium Salt (EGTA)	Sigma
Goat Serum	Sigma
Glacial Acetic Acid	EMD
D-Glucose	BDH
Glycerol	MP Biomedicals
β-Glycerophosphate	BDH
L-Glycine	MP Biomedicals
N-2-Hydroethylpiperazine-N'-2-Ethane Sulfonic Acid (HEPES)	USB
Hydrochloric Acid (HCl)	EMD
Isobutanol	BDH
Isopropanol	EMD
LiCl	EMD
L-α-Phosphatidylinositol 4,5-diphosphate sodium salt	Sigma
Magnesium Chloride ($MgCl_2$)	EMD
Magnesium Sulfate ($MgSO_4$)	EMD
β-Mercaptoethanol	EMD
Methanol	BDH
Non-fat Dry Milk Carnation	Nestle
NZ amine (casein hydrolysate)	Sigma
Okadaic Acid	Sigma
Paraformadehyde	EMD
Phenol	Sigma
Phenylmethylsulfonyl Fluoride (PMSF)	Sigma
1,4-Piperazine Diethane Sulfonic Acid, Sodium Salt (PIPES)	EMD
Polyethylene Glycol (PEG)	Sigma
Potassium Chloride (KCl)	BDH

Table 2.1: Lists of Reagents and Suppliers. (continued)

Reagents	Supplier
Protease Inhibitor Cocktail	Sigma
Sodium Acetate	BDH
Sodium Chloride (NaCl)	VWR
Sodium Bicarbonate (Na_2CO_3)	EMD
Sodium Dodecyl Sulfate (SDS)	ICN
Sodium Fluoride (NaF)	Sigma
Sodium Hydroxide (NaOH)	EMD
Sodium Orthovanadate	Sigma
Sodium Pyrophosphate	Sigma
Sucrose	BDH
N,N'- methylene-bis-acrylamide	Bio-Rad
N,N,N',N'-Tetramethylenediamine (TEMED)	Bio-Rad
Tris-Acetate	EMD
Tris-HCl	INC
Triton-X100	Sigma
Trizol™	GIBCO-BRL
Trypsin-EDTA	GIBCO-BRL
Tween-20	EMD
Cell Growth Reagents	**Supplier**
Ampicillin	EMD
Bacto-Agar	BD
Bacto-Tryptone	BD
Bacto-Yeast Extract	BD
Calf Serum	GIBCO-BRL
Collagen Type I (rat tail)	BD
Dulbecco's Modified Eagle's Medium: DME/Low	Hyclone
Fetal Bovine Serum	GIBCO-BRL
Kanamycin	Sigma
Opti-MEM Reduced Serum Medium	Invitrogen
Recovery Cell Culture Freezing Medium	GIBCO-BRL
Pharmacological Agents	**Supplier**
A23187	Sigma
BAPTA-AM	Sigma
Leptomycin-B	Cedarlane
LY294002	Cell Signaling
Transfection Reagents	**Supplier**
ExGen500	Fermentas
Lipofectamine2000	Invitrogen
SiLentFect Lipid Reagent	BioRad
Radioactive-Substrates	**Supplier**
^{32}P (250 μCi/μl)	Perkin-Elmer

Table 2.2: List of Antibodies and the Dilutions used for Western Blotting.

Primary Antibody	Dilution	Supplier
β-Actin (A5441)	1 : 3000	Sigma-Aldrich Inc
Akt (total)	1 : 750	Cell Signaling Technology (CST)
ERK1 and ERK2 MAP Kinase (M5670)	1 : 2000	Sigma-Aldrich Inc
FOXO3a	1 : 1000	CST
Gab-1 (C-Terminal)	1 : 1000	Upstate
GSK-3β	1 : 1000	Santa Cruz Biotechnology (SCB)
HA-tag	1 : 750	Biovision
PDK1 Monoclonal	1 : 1000	CST
PDK1 Polyclonal	1 : 1000	SCB
PDK1 (Ser241)	1 : 1000	CST
Phosphatidylinositol-3,4,5-triphosphate-Biotin conjugated	1 : 5000	Echelon
Phospho-p44/42 MAP Kinase (Thr202/Tyr204) (E10) Monoclonal (#9106)	1 : 1000	CST
Phospho-Akt (Ser473)	1 : 750	CST
Phospho-Akt (Thr308)	1 : 500	CST
Phospho-GSK-3β (Ser9) 4G10	1 : 1000	SCB
Phosphotyrosine 4G10	1 : 3000	Upstate
Phosphoserine	1 : 1000	Sigma-Aldrich Inc
Phospho-S6 Ribosomal protein Ser235/236	1 : 1000	CST
PtdIns (3,4,5)P_3 IgM Biotin	1 : 100	Echelon
PTEN-HPR (milk only)	1 : 1000	SCB
p85 (SH3) Monoclonal	1 : 1200	Upstate
p85 (SH2) Polyclonal	1: 1000	Upstate
SHP-1/PTP1C Monoclonal	1 : 750	BD Biosciences
SHP-1/SHPTP1 (C19) Polyclonal	1 : 1000	SCB
SHP-2/PTP1D Monoclonal	1 : 1500	BD Biosciences
Secondary Antibody	**Dilution**	**Supplier**
Alexa Fluor 488 Goat Anti-Rabbit IgG	1 : 1000	Invitrogen
Alexa Fluor 594 Goat Anti-Rabbit IgG	1 : 700	Invitrogen
Alexa Fluor 488 Streptavidin conjugate	1 : 2000	Invitrogen
Donkey Anti-Goat IgG, HRP-conjugate	1 : 3000	SCB
Goat Anti-Rabbit IgG, HRP-conjugate	1 : 3000	Cedarlane
Goat Anti-Mouse IgG, HRP-conjugate	1 : 3000	Cedarlane
Rabbit Anti-Sheep IgG, HRP-conjugate (#12-342)	1 : 6000	Upstate

Table 2.3: List of Commercial Kits.

Commercial Kit	Supplier
BCA™ Protein Assay Kit	Pierce
SuperScript™ III First-strand Synthesis System for RT-PCR	Invitrogen
Qiaex II Gel Extraction Kit 500	Qiagen
Quantum Prep® Plasmid Midiprep Kit	BioRad
QuikChange® Site-Directed Mutagenesis Kit	Stratagene

Table 2.4: List of Oligonucleotides (Invitrogen) for Site-directed Mutagenesis, Subcloning and siRNA S=Sense A.S.= Anti-Sense.

Gene Name and mutation	Primer Sequence and Orientation for Mutagenesis
p85 Y688D	5'-CCCTATAACTTGGATAGCTCTCTGAAAG-3' (S)
p85 Y688D	5'-CTTTCAGAGAGCTATCCAAGTTATAGG-3' (A.S.)
PDK1 Y9F	5'-CCAGCCAGCTGTTTGACGCCGTGCC -3' (S)
PDK1 Y9F	5'- GGCACGGCGTCAAACAGCTGGCTGG-3' (A.S.)
PDK1 Y333F	5'-TGAGGAAATGGAAGGATTCGGACCTCTTAAAGCAC -3' (S)
PDK1 Y333F	5'- GTGCTTTAAGAGGTCCGAATCCTTCCATTTCCTCA-3' (A.S.)
PDK1 Y373F	5'-ACGACGAGGACTGCTTTGGCAATTATGACAATCTC -3' (S)
PDK1 Y373F	5'-GAGATTGTCATAATTGCCAAAGCAGTCCTCGTCGT -3' (A.S.)
PDK1 Y376F	5'-GGACTGCTATGGCAATTTTGACAATCTCCTGAGCC -3' (S)
PDK1 Y376F	5'-GGCTCAGGAGATTGTCAAAATTGCCATAGCAGTCC -3' (A.S.)
PDK1 S241E	5'-CAAGCCAGGGCCAACGAATTCGTGGGAACAGC-3'(S)
PDK1 S241E	5'-GCTGTTCCCACGAATTCGTTGGCCCTGGCTTG-3' (A.S.)
Primer Name	**Primer Sequence and Orientation for Subcloning**
SHP-1 FXhoI	5'-ATCTCGAGTGCTGTCCCGTGGGTGG -3' (S)
SHP-1 REcoRI	5'-GGAATTCTCACTTCCTCTTGAGGGAACCC -3' (A.S.)
PDK1 FSalI	5'-TTAGTCGACTTGCCAGGACCACCAGCC-3' (S)
PDK1 RBglII	5'-ATAAGATCTCTGCACAGCGGCGTCC-3' (A.S.)
siRNA Oligomers	**Primer Sequence and Orientation**
SHP-1 5	5'-GCUCUACUCUGUGACCAACUGUAAA-3' (S)
SHP-1 5	5'-UUUACAGUUGGUCACAGAGUAGAGC-3' (A.S.)
SHP-1 6	5'-GCGAGAGGUAUCAUGGUCACAU-3' (S)
SHP-1 6	5'-AUGUGACCAUGAUACCACCUCUCGC-3' (A.S.)
SHP-1 7	5'-CCACUCGGGUAAAUGCAGCAGACAU-3' (S)
SHP-1 7	5'-AUGUCUGCUGCAUUUACCCGAGUGG-3' (A.S)

Table 2.5: Names and Addresses of Suppliers.

Supplier	Address
BD (Becton Dickinson)	2771 Bristol Circle, Oakville, ON., Canada
BDH	501-45th Street West, Saskatoon, SK., Canada
Bio-Rad	5671 McAdam Road, Mississauga, ON., Canada
Cedarlane Laboratories	5516 8th Line, Hornby, ON., Canada
Cell Signaling Technology Inc.	159J Cummings Center Beverly, MA 01915, USA
Echelon Biosciences Inc.	Box 58537, Salt Lake City, UT 84158-0537, USA
EMD Biosciences Inc.	10394 Pacific Center Court, San Diego, CA 92121, USA
Fermentas Life Sciences	830 Harrington Crt., Burlington, ON., Canada
GIBCO-BRL	Box 9418, Gaithersburg, MD 20898, USA
ICN Biomedicals Inc	15 Morgan, Irvine, CA 92618-2005, USA
Invitrogen	1600 Faraday Avenue, Carlsbad, CA 92008, USA
Molecular Probes	
MP Biomedicals	29525 Fountain Pkwy., Solon, OH 44139, USA
Perkin-Elmer	501 Rowntree Dairy Road, Woodbridge, ON., Canada
Promega	2360 Argentia Road, Mississauga, ON., Canada
Santa Cruz Biotechnology	2161 Delaware Ave., Santa Cruz, CA, USA
Sigma	2149 Winston Park Drive, Oakville, ON., Canada
Upstate USA Inc.	4588 Collections Center Drive Chicago, IL 60693, USA
USB	300 Laurier Blvd., Brockville, ON., Canada
Qiagen	2800 Argentia Road, Unit 7 Mississauga, ON., Canada

2.3 Western blotting.

2.3.1 Isolation of protein lysates from mammalian cell lines.

Medium was aspirated from cells and the cells were rinsed in 10 mL of PBS. Lysis buffer consisting of 1% TritonX-100, 1% NP40, 10% glycerol, 1 mM EDTA, 20 mM Tris pH 7.4, 1 mM Na orthovanadate, and 1X of mammalian anti-protease cocktail, was added to each 100 mm culture dish. Cells were scraped into microcentrifuge tubes, lysed for 30 min on ice and centrifuged at 12000×*g* at 4°C for 30 min. Protein concentration of lysates was determined using a BCA Protein Assay Kit and concentrations of proteins were analyzed using a Spectra Max Plus384 microplate reader from Molecular Devices, at a wavelength of 562 nm. Protein lysates were diluted to 1 µg/µL with lysis buffer and 1X loading buffer and boiled for 5 min at 95°C.

2.3.2 Protein immunoprecipitation.

300-500 μg of cell protein lysates were incubated with 3-5 μg of antibody and incubated overnight at 4°C with rocking. 30 μL Sepharose A for polyclonal antibodies or Sepharose G for monoclonal antibodies was added to the lysates and incubated for 1 hour at 4°C with rocking. Samples were centrifuged at 8000×*g* for 5 min and washed with ice-cold lysis buffer three times. 25 μL of 1X loading buffer was added to the Sepharose pellet and boiled for 5 min at 95°C.

2.3.3 Subcellular fractionation.

Cells were grown in a 100 mm cell culture plate. Following treatment or transfection, cells were washed with ice-cold PBS and removed from plate using Trypsin-EDTA. Total cell lysates were obtained as described above. Subcellular fractions were obtained by centrifuging cell suspension at 2500×*g*, for 5 min and 320 μL suspension buffer containing 20 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EGTA, 1 mM DTT, 320 mM sucrose and 1X protease inhibitor cocktail, was added to pellet and the cell suspension was homogenized. Cells were disrupted with 20 strokes using a Dounce Homogenizer. Homogenates were centrifuged at 900×*g* at 4°C for 10 min to remove nuclei and cellular debris. The supernatant was then centrifuged at 18000×*g* for 40 min at 4°C to separate the heavy membrane compartment from the soluble fraction. The 18000×*g* pellets were resuspended in cold lysis buffer and represented the mitochondria-rich fraction. The 18000×*g* supernatant was used as the cytosolic fraction.

To isolate nuclear extracts, cells were resuspended in 1 mL Hypotonic buffer containing 20 mM HEPES, 1 mM $MgCl_2$, 10 mM KCl, 2 mM PMSF, 0.5 μM DTT, 5 μg/mL Aprotinin, 5 μg/mL Leupeptin, 0.1 % Triton X-100 and 20 % Glycerol and the cell suspension was homogenized. Cells were disrupted with 20 strokes using a Dounce Homogenizer. Homogenates were centrifuged at 900 rcf at 4°C for 5 min to separate the soluble fraction from the nuclei. The nuclei were resuspended in 100 μL of cold Extraction buffer containing 20 mM HEPES, 1 mM $MgCl_2$, 10 mM KCl, 2 mM PMSF, 0.5 μM DTT, 5 μg/mL Aprotinin, 5 μg/mL Leupeptin, 0.1 % Triton X-100, 20 % Glycerol and 420 mM NaCl, and rocked for 20 min at 4°C. Samples were centrifuged

for 10 min at 18000×*g*, to pellet nuclear matrix. The remaining supernatant contained the nuclear extract.

2.3.4 Resolution of proteins by SDS-PAGE.

Proteins were resolved by SDS-PAGE. The resolving gel was composed of acrylamide (X1 for 8%; X2 for 10%): N,N'-methylene-bis-acrylamide (37.3:1-acryl:bis), 375 mM Tris-HCl at pH 8.8, 0.1% SDS. The stacking gel was composed of acrylamide: N,N'-methylene-bis-acrylamide (37.3:1-acryl:bis), 130 mM Tris-HCl at pH 6.8 and 0.1% SDS. The resolving gel and stacking gel were polymerized with 0.1 % APS and 0.04% TEMED. Quantified protein lysates were added onto the gel and proteins were electrophoresed at 100 volts (Bio-Rad PowerPac200) in running buffer composed of 25 mM Tris-HCl pH 7.4, 250 mM glycine and 0.1% SDS.

The resolved samples were then transferred onto a nitrocellulose membrane using a Bio-Rad Transblot apparatus at 0.23 amps for 55 min at 4°C in a buffer composed of 39 mM glycine, 48 mM Tris-HCl and 20% methanol. The membrane was incubated with a 5% milk solution (Carnation non-fat dry milk dissolved in TBS) and blocked for 1 hr at RT. The membrane was then incubated with primary antibody in TBS containing 0.5% Tween-20 (TBST) with 5% milk solution for 2 hr at RT or overnight at 4°C, and washed three times with the TBST-5% milk solution. The membrane was incubated with secondary antibody conjugated to horseradish peroxidase (HPR) in TBST-5% milk for 1 hr. This was followed by three 10 min washes with TBST-5% milk. The Amersham Biosciences ECL™ Western Blotting Detection Reagent was used to detect conjugated HPR. The signal protein-antibody complex was detected using Amersham Biosciences Hyperflim™ or Kodak T-Mat L/RA film.

2.4 Bacterial strains and medium preparation.

The *Escherichia coli* (*E. coli*) strains DH5α (methylating) and BW58 (non-methylating) were used for replication of plasmid DNA. Details of the strain can be found elsewhere (Hanahan 1983; Bethesda Research Laboratories 1986).

Double yeast and tryptone (dYT) plates were used to propagate the bacterial cells following transformation with plasmid. Selection was dependent on the ampicillin

(Amp) or the kanamycin (Kan) resistance gene expressed by the plasmid. The plates were prepared by combining 16 g of bacto-tryptone, 10 g of bacto-yeast extract, 5 g of NaCl and 15 g of agar in 1 L of double distilled water (ddH_2O). This solution was autoclaved for 20 min at 15 lb/sq. After the temperature of the medium had cooled sufficiently, ampicillin or kanamycin was added to a final concentration of 50 μg/mL and the medium was poured into Petri dishes.

Terrific Broth, as described by Sambrook *et al.* (1989), was used to propagate bacterial cells in a liquid medium overnight at 37ºC with agitation. This medium was prepared by combining 12 g of bacto-tryptone, 24 g of bacto-yeast extract and 4 mL of glycerol in 900 mL of ddH_2O. This solution was autoclaved as above. The medium was complete by adding 100 mL sterile solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 and the appropriate antibiotic.

2.5 General biochemical techniques.

Protocols in this section are based on those described in (Sambrook et al., 1989).

2.5.1 Plasmid DNA.

SHP-1 and SHP-2 cDNA cloned into the pcDNA3 mammalian expression vector as well as their catalytic (SHP-1C455S and SHP-2C453S) and substrate binding (SHP-1D421A and SHP-2D419A) mutants were obtained from Dr. S.H. Shen (Molecular Pharmaceutical Sector, National Research Council of Canada). The PTENC124S phosphate-dead plasmid was a kind gift from Dr. D.H. Anderson (Saskatchewan Cancer Agency, Canada). PH-mRFP1 was provided by Dr. E.R. Prossnitz (Department of Cell Biology and Physiology, University of New Mexico) with permission for transfer of an mRFP1-based plasmid obtained from Dr. R.Y. Tsien (Howard Hughes Medical Institue, UCSD). The constituively active, *N*-myristoylated full-length membrane-directed mouse Akt (myr-Akt) cDNA was a gift from Dr. A.B. Vojtek (University of Michigan, Ann Arbor, MI). The *N*-myristoylated full-length membrane-directed PDK1 (myr-PDK1) and pEGFP (green fluorescent protein)-PDK1 expression vectors were obtained from Drs. S. Kim and J. Chung (Korea Advanced Institute of Science and Technology, Republic of Korea). Any mutagenesis was done according to section 2.6.8.

2.5.2 Preparation of competent cells.

A single colony of *E. coli* was used to inoculate 5 mL of LB broth in a 50 mL sterile conical tube and incubated overnight at 37°C with agitation. A 2 mL aliquot of this culture was used to inoculate 200 mL SOB medium (2% Bacto-trypone, 0.5% yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$), and a sample was removed and used to determine the optical density (O.D.) of the culture at time zero. The culture was grown at 37°C with agitation until an O.D. of 0.45 was reached. The culture was aliquoted into 50 mL pre-chilled sterile conical tubes and incubated on ice for 10 min before pelleting at 2500 rpm for 10 min at 4°C in a mid-range Alegra 25R centrifuge from Beckman Coulter. The supernatant was removed and the cell pellets were resuspended in 32 mL of an ice-cold transformation buffer containing 15 mM $CaCl_2$, 250 mM KCl, 10 mM PIPES and 55 mM $MnCl_2$ at pH 6.7 and left on ice for 10 min. The cells were pelleted and then resuspended in 8 mL of fresh transformation buffer containing 7% DMSO and incubated on ice for 10 min. Cells were aliquoted into pre-chilled sterile tubes and stored at -80°C.

2.5.3 Large scale isolation of plasmid DNA.

2.5.3.1 Transformation of competent cells with plasmid DNA.

A 50 µL aliquot of competent cells was incubated with 1 µg of plasmid DNA in a 13 mL polypropylene tube and incubated for 30 min on ice. The cells were subjected to heat-shock at 42°C for 45 sec to allow transformation. The cells were incubated on ice for 2 min, SOC medium (SOB medium containing 20 mM glucose) was added and incubated for 30 min at 37°C with shaking, 250 rpm, after which they were plated on an dYT-selection agar plate and incubated overnight at 37 °C. Single colonies of bacteria were selected and propagated for plasmid isolation.

A 500 mL culture of Terrific Broth containing 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 and the appropriate antibiotic was inoculated with a 5 mL culture grown from a single bacterial colony and grown overnight at 37°C with agitation (250 rpm).

2.5.3.2 DNA extraction.

Bacterial cells were centrifuged at 5000×*g* for 10 minutes. The pellet was washed with 100 mL ice cold 1X PBS and centrifuged at 5000×*g* for 10 minutes. The pellet was resuspended in 20 mL of solution I (50 mM Glucose, 25 mM Tris-Cl, 10 mM EDTA, pH 8) by vortexing. 40 mL of NaOH/SDS lysis solution II (0.2 M NaOH, 1% SDS) was then added and the tube was gently inverted 8 times. Solution III (20 mL) was added and the tube was gently inverted eight times. Solution III neutralizes the NaOH in solution II and precipitates the genomic DNA and SDS. The solution was then centrifuged at 5000×*g* for 15 min. The supernatant was filtered through cheesecloth in a centrifuge bottle and 48 mL of isopropanol was added and mixed followed by centrifugation 5000×*g*, 15 min at RT. The supernatant was removed and the DNA pellet was rinsed with 85% ethanol (EtOH) and allowed to evaporate. The pellet was then dissolved in 3 mL TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) and poured into a 15 mL Cortex tube.

2.5.3.3 Removal of RNA.

4.8 mL of ice cold 5M LiCl was added to the above solution and vortexed. The solution was centrifuged at 9000×*g* for 10min to pellet the precipitated RNA. The supernatant containing the plasmid DNA was transferred to a 30 mL cortex tube and 7.8 mL of isopropanol was added and mixed to precipitate the plasmid DNA. The solution was centrifuged at 9000×*g* for 10 min (RT) and the supernatant was removed. The pellet was rinsed with 85% EtOH. The EtOH was allowed to evaporate and the pellet was dissolved in 500 µL TE. The remaining RNA was digested with 0.1 mg/mL RNase at RT for 30 min.

2.5.3.4 Protein extraction.

The plasmid DNA was precipitated with 400µl PEG solution (1.6 M NaCl and 13% (w/v) PEG800) and pelleted with centrifugation at 12000×*g* for 2min, 4°C. The DNA pellet was dissolved in 500 µL TE and proteins were extracted twice with 500 µL phenol. After each extraction, samples were centrifuged at 14000×*g* for 5 min and the top (aqueous) layer was transferred to a fresh tube. The phenol was removed using 500

μL chloroform, then centrifuged at 14000×*g* for 5 min and the top (aqueous) layer was transferred to a fresh tube.

2.5.3.5 Precipitation of pure DNA.

NaCl was added to the sample to a final concentration of 125 mM and DNA was precipitated by adding two volumes of absolute EtOH (400 μL each time) and centrifuged at 12000×*g* for 5min (RT). 85% EtOH was added to samples and vortexed briefly before being centrifuged at 12000×*g* for 2 min (RT, 4°C). The supernatant was removed and the EtOH was allowed to precipitate before dissolving the DNA in sterile ddH_2O. The plasmid DNA pellet was resuspended in sterile ddH_2O to a concentration of 1 μg/μL as determined by O.D. at 260 nm where 1 O.D. corresponds to 50 μg plasmid DNA.

2.5.4 Medium scale isolation of plasmid DNA.

For medium scale isolation of plasmid DNA the BioRad Quantum Prep® Plasmid midiprep Kit is based on the alkaline lysis method described in Section 2.5.3 Large scale isolation of plasmid DNA. The kit was used as per the manufacturer's instructions.

2.5.5 Mini scale isolation of plasmid DNA.

A 2 mL culture of Terrific Broth with the appropriate antibiotic was inoculated with a single bacterial colony and grown overnight at 37°C. Isolation of the plasmid was based on the alkaline lysis method described by Sambrook *et al.* (1989). The plasmid DNA pellet was resuspended in sterile ddH_2O to a concentration of 1 μg/μL as determined by O.D. at 260 nm where 1 O.D. corresponds to 50 μg plasmid DNA.

2.6 DNA manipulation.

2.6.1 Isolation of total RNA from mammalian cell lines.

The cell lines were grown to 70% cell confluency on 100 mm cell culture plates. Medium was removed and the cells were washed with PBS. A 0.75 mL volume of

TRIzol™ reagent (GibcoBRL) was added to each plate. Cells were scraped into a microcentrifuge tube and incubated for 5 min. A 200 µL volume of chloroform was added to the tube, gently agitated, and incubated at RT for 15 min. The emulsion was separated by centrifugation at 12000×*g* for 15 min at 4°C. The supernatant was removed and transferred into 500 µL of isopropanol, followed by incubating 10 min at RT. RNA was pelleted by centrifugation at 12000×*g* for 10 min at 4°C. Supernatant was removed and the pellet washed with 70% ethanol/DEPC. The ethanol was allowed to evaporate and the pellet suspended in 50 µL DEPC-treated ddH_2O. The RNA was heated at 65°C for 5 min to dissolve. RNA concentration was determined using a Du640 spectrophotometer from Beckman Coulter. An O.D. ratio (*e.g.* 260/280 nm) between 1.6-1.9 indicated that the isolated RNA is free of DNA and protein.

2.6.2 Reverse transcription of mRNA.

Reverse transcription of mRNA was performed using Invitrogen's Superscript™ III First-strand Synthesis System for RT-PCR. The RNA/primer mixture was prepared by combining 5 µg of RNA with 1 µL of 50 ng/µL random hexamers, 1 µL 10 mM dNTP mix to 10 µL DEPC-treated water. The mix was incubated for 5 min at 65°C and placed on ice for 1 min. The cDNA synthesis mix was prepared in a solution containing 1X RT buffer, 5 mM $MgCl_2$, 10 mM DTT, 40 U RNaseOUT (40 U/µL) and 200 U Superscript III RT (200 U/ µL), and 10 µL was added to each RNA/primer mixture and incubated for 10 min at 25°C followed by 50 min at 50°C. Reactions were terminated by heating at 85°C for 5 min. Cooled reactions were then incubated with 1 µL RNase H, which was added to each tube and incubated for 20 min at 37°C. cDNA synthesis reactions were stored at -20°C or used for PCR.

2.6.3 Polymerase chain reaction (PCR).

PCR is an *in vitro* technique used for exponentially amplifying DNA, *via* enzymatic replication. This technique is based on polymerases, *i.e.* Taq-polymerase is used to amplify DNA for screening purposes and Pfu-polymerase, which contains a proofreading function, is used to amplify DNA for the purpose of subcloning or sequencing.

Taq-based PCR began with a 1 min, 95°C denaturing step, followed by 25 cycles of denaturing (30 sec, 94°C), annealing (30 sec, 50°C), and extension/elongation (1 min/kb, 72°C). The PCR program included a final elongation time of 5 min at 72°C.

Pfu-based PCR began with a 1 min, 95°C denaturing step, followed by 30 cycles of denaturing (30 sec, 94°C), annealing (30 sec, 52°C), and extension/elongation (1 min/kb, 72°C). The PCR program included a final elongation time of 5 min at 72°C.

2.6.4 Agarose gel electrophoresis.

DNA was subjected to agarose gel electrophoresis on a 1% agarose gel in a 40 mM Tris-Acetate and 1 mM EDTA at pH 8.0 (TAE) buffer and 0.5 μg/mL ethidium bromide in a TAE running buffer. DNA samples were mixed with an appropriate volume of 5X agarose gel sample buffer, consisting of 50% glycerol, 50 mM EDTA, a trace of bromophenol blue and xylene cyanol FF at pH 8.0, before being loaded onto the gel. Electrophoresis was typically carried out between 80 and 100 Volts, until the necessary resolution was achieved.

2.6.5 DNA extraction.

DNA extractions from agarose were performed using Qiaex II Gel Extraction Kit 500 as per the manufacturer's instructions.

2.6.6 Restriction digest of DNA.

Digestion of DNA with restriction enzymes was carried out using 1 μg DNA in a solution containing 1X Reaction buffer (selected by manufacturer's recommendations), and 1-2 U of individual restriction endonucleases in a final volume of 20 μL with ddH_2O. Typically, samples were incubated at 37°C for 1-2 hr.

2.6.7 Subcloning.

After DNA extraction from agarose gel, digested insert and plasmid were combined with >3 fold excess of insert to plasmid in 2 μL of 10X ligation buffer and 5U of T4 DNA ligase (5U/μL) in a final volume of 20 μL with ddH_2O. The ligation mixture was incubated overnight at 21°C. Following the ligation, 5 μL of the ligation mixture

was transformed into the appropriate bacterial strain and plated on dYT plates containing the appropriate antibiotic selection and grown overnight at 37°C. Colonies were selected and grown in dYT medium with antibiotic selection and screened for insertion of the ligated DNA insert by restriction digestion of islolated plasmid DNA.

2.6.8 Site-directed mutagenesis.

Mutagenesis of DNA was performed using QuikChange® Site-Directed Mutagenesis Kit as per the manufacture's instructions. Briefly, 25 ng of double-stranded (ds)DNA was combined with a solution containing 1X reaction buffer, 125 ng forward primer and reverse primer and 1 μL of dNTP mix. The mixture was then subjected to PCR for incorporation of the mutation. Following PCR, the methylated parent dsDNA was digested using 10 U of Dpn I (10 U/ μL), which selectively cleaves methylated DNA, for 1h at 37°C. The remaining mutated dsDNA was then transformed into XL1-Blue supercompetent cells and 0.5 mL NZY^+ broth (10 g NZ amine casein hydrolysate, 5 g yeast extract, 85.5 mM NaCl, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$, 20 mM glucose, pH 7.5) was added to the transformed bacteria. The transformed cells were then incubated at 37°C with agitation for 1 h and plated on dYT plates containing the appropriate antibiotic selection.

2.7 Treatment of mammalian cell lines.

2.7.1 Transfection of mammalian cell lines.

Cells were plated the day before transfection experiments onto 100 mm cell culture plates, and grown to 70-80% confluency. The transfection mixture was made by combining 20 μg DNA in 914 μL 150 mM NaCl solution incubated with 66 μL ExGen500. The combined mixture was incubated for 10 min at room temperature (RT) before adding it to the cultured cells. Transfected cells were then incubated at 37°C in 5% CO_2 for 4 h. The medium was aspirated and replaced with DMEM with 10% FBS. Transfection efficiency ranged from 20% in C6 cells to 50% in PC12 cells and 80% in HEK293A cells.

When conducting the MTT reduction assay, cells were transfected with Lipofectamine2000. Cells were passaged the day before onto 96-well culture plates and grown to 70-80% confluency. The transfection mixture was made by combining 0.2 μg DNA in 25 μL DMEM serum-free medium and mixed with 0.5 μL Lipofectamine2000 in 25 μL DMEM serum free medium which had been pre-incubated for 5 min at RT. The transfection mixture was incubated at RT for 20 min before adding it to the cultured cells, transfected cells were then incubated at 37°C in 5% CO_2 for 4 h. The medium was then aspirated and replaced with DMEM with 10% FBS.

2.7.2 Pharmacological treatment of cell lines.

2.7.2.1 PI3K signalling inhibitor.

Cells were passaged the day before treatment and grown to 70-80% confluency in 100 mm cell culture plates. The PI3K signalling pathway was inhibited using the chemical inhibitor LY294002 (25 μM, 30 min). After treatment of cells, protein lysates were subject to Western blot (see Section 2.3).

2.7.2.2 Nuclear pore complex (NPC) inhibitors.

Leptomycin B (LMB) is a specific inhibitor of CRM-1, which is responsible for the export of proteins out of the nucleus (Kutay and Guttinger, 2005; Lim et al., 2003; Scheid et al., 2005). BAPTA-AM is a calcium chelator used to inhibit nuclear pore complex (NPC) formation (Macaulay and Forbes, 1996). The calcium ionophore, A23187 is used to activate Ca^{2+}-calmodulin dependent nuclear transport of proteins by the NPC (Tenev et al., 2000). Cells were treated with LMB (50 nM, 3 h), BAPTA-AM (100 μM, 30 min), or A23187 (5 μM, 10 min) before fixing cells for confocal microscopy (see Section 2.8) or collecting protein lysates for Western blot (see Section 2.3).

2.7.2.3 Protein tyrosine phosphate (PTP) inhibitor.

Pervanadate is a general PTP inhibitor. Before treating cells, cell culture medium was preincubated with vanadate (100 μM) and H_2O_2 (100 μM) at RT for 15 min to allow

formation of pervanadate. Cell culture medium was aspirated and replaced with the pervanadate solution and incubated for 30 min, 37°C.

2.8 Confocal microscopy.

2.8.1 Fluorescence.

Cells were grown in chambered cell culture slides and transfected with DNA designed to express fluorophores, *e.g.* GFP or RFP. After 24 h of transfection, cells were rinsed once with PBS, warmed to 37°C and removed. Cells were fixed using 4% paraformaldehyde and incubated for 30 min at RT or over night (O/N) in a humidity box at 4°C. After fixing with paraformaldehyde, cells were washed with PBS three times. For nuclear staining, cells were first permeabilized with 0.5% Triton-100/PBS for 15 min at RT and washed with PBS three times followed by staining with DAPI (1 μg/mL) for 5 min. Cells were washed three times with PBS. Mounting medium, ProLong Gold antifade (Molecular Probes), was then added to the slides and a 22 X 50 mm (1 once) micro coverslip was used to cover cells. The slide was allowed to cure on a flat surface and analyzed using an Olympus FV300 confocal microscope**.**

2.8.2 Immunofluorescence.

Cells were grown in chambered cell culture slide and fixed with 4% paraformaldehyde for 30 min at RT or O/N in a humidity box at 4°C. Cells were washed with PBS three times and then permeabilized with 0.5% Triton-100/PBS for 15 min at RT. Cells were washed three times with PBS. The slide was blocked with 3% BSA/TBS and incubated for 30 min followed by three PBS washes. Primary antibody (monoclonal 1:100 and polyclonal 1:500) diluted in 3% BSA/TBST was added and incubated at RT for 90 min and washed with PBS three times. Secondary antibody diluted in H_2O was added and incubated for 90 min and washed three times with PBS. Nuclear staining was done using DAPI as described above in section 2.8.1. The slide was dried and mounting medium, ProLong Gold antifade reagent, was added. A coverslip was added and allowed to cure on a flat surface in the dark. Samples were analyzed using an Olympus FV300 confocal microscope**.**

2.8.2.1 PI3,4,5P_3 immunofluorescence.

Cells were grown in chambered cell culture slide and fixed with an equal volume of 4% paraformaldehyde in cell medium for 20 min at RT. Cells were washed three times with TBS then permeabilized with 0.5% Triton-100/TBS for 15 min at RT. Cells were washed with TBS three times and blocked with 10% goat serum (GS)/TBS 30 min at 37°C or overnight at 4°C. Anti- PI3,4,5P_3 antibody (1:100) diluted in TBST-GS was added to cells and allowed to incubate for 60 min followed by three washes with TBST-GS. Streptavidin-AlexaFluor 488 (1:2000) diluted in TBS was added to cells and incubated for 30 min at 37°C. Following incubation, cells were rinsed with H_2O, dried and sealed with mounting medium, ProLong Gold antifade reagent. The slide was allowed to cure on a flat surface in the dark. Samples were analyzed using an Olympus FV300 confocal microscope**.**

2.9 PI3K activity assay.

Cells were grown on 100 mm culture plates. After treatment, cells were washed with PBS and PLC lysis buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM NaPPi (pyrophosphate), 100 mM NaF with freshly added 1x protease cocktail and 1 mM Na orthovanadate, was added to each 100 mm culture dish. Cells were scraped into microcentrifuge tubes, lysed for 30 min and centrifuged at 12000×*g* at 4°C for 30 min. Protein concentration of lysates was determined using a BCA Protein Assay Kit.

Samples were immunoprecipitated using the p85 (SH3) antibody O/N at 4°C. Following incubation, 30 µL of protein Sepharose A was added to lysates and incubated for 1h at 4ºC for 30-60 min. Samples were centrifuged at 5000×*g* for 30 sec. Liquid was aspirated and the Sepharose pellet was washed with 500 µl HNTG wash buffer containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 1.0% Triton X-100, 10% glycerol and 1 mM Na orthovanadate and centrifuged. The washes and centrifugation were repeated three times. Samples were kept at 4ºC until ready to use for [γ-^{32}P]ATP incorporation.

The immunoprecipitates were washed with each of the following: wash 1 (phosphate-buffered saline), wash 2 (100 mM Tris-HCl, pH 7.4, 50 mM LiCl), and wash 3 (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA). Excess liquid was removed

from the immunoprecipitates. Lipid micelles were generated by sonicating phosphatidylserine and phosphatidylinositol in PI3K assay buffer (25 mM Hepes, pH 7.4, 10 mM $MgCl_2$) in a sonicating water bath for 20 min. Each sample was incubated with lipid micelles (5 µg of phosphatidylserine + 2.5 µg of phosphatidylinositol) in PI3K assay buffer and 10 µCi of [γ-^{32}P]ATP in a total volume of 50 µl for 15 min at 20°C while gently rocking. The reaction was stopped by the addition of HCl (to 1.7 M). Lipids were extracted into chloroform:methanol (1:1) and further washed with methanol, 1 N HCl (1:1). Reaction products were dried down, resuspended in chloroform:methanol (1:1), and spotted onto a thin layer chromatography plate (Silica Gel 60; VWR Canlab). Samples were developed in 1-propanol, water, acetic acid (17.4:7.9:1) in a chromatography chamber for 4 h, dried, and exposed to a PhosphorImager screen. Results were visualized and quantified using Quantity One software (Bio-Rad), and statistical analysis was performed using Prism software (GraphPad Software, Inc., San Diego, CA).

2.10 Cell viability/growth assays.

2.10.1 MTT conversion/reduction assay.

Cells were grown in a 96-well plate. After treatment or transfection, the growth medium was gently aspirated. To each well 50µl 0.5 mg/mL 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) mix (0.5 mg/mL MTT diluted in DMEM containing 1% FBS) was added and incubated for 3-4 hrs at 37°C. After incubation, 100 µl DMSO was added to each well to dissolve the generated purple formazan crystals. Formazan crystal dissolution was then allowed to occur at room temperature for 5 min. The O.D was determined with a Spectra Max Plus384 microplate reader from Molecular Devices, at a wavelength of 570 nm.

2.10.2 Flow cytometry.

Cells were grown in a 100 mm plate. Following treatment or transfection, cells were washed with PBS and removed from the plate using Trypsin-EDTA. Cells were washed, counted, and diluted to a concentration of 10^6 cells/mL. Cells were pelleted and

resuspended in an ice-cold solution containing 30% PBS and 70% absolute ethanol. Ethanol was added slowly. Fixed cells were kept on ice for at least 2 hours or overnight at 4°C. Cells were transfered to an Eppendorff tube and pelleted at 300×*g* for 5 min. The pellet was resuspended with PBS containing 100 μg/mL RNase and 50 μg/mL propidium iodide (PI) and incubated for 20 min. Samples were filtered with a nylon filter and placed on ice for DNA analysis. DNA content was measured by exciting propidium iodide at 488 nm and measuring the emission at 580 nm. Samples were analyzed by a Coulter® EPICS® XL flow cytometer using EXPO32 ADC software.

2.11 Statistical analysis.

Significance (set at $P<0.05$) was assessed by unpaired Student's *t*-test (two groups) or by analysis of variance (three groups or more) with *post-hoc* analysis relying on Newman-Keuls Multiple Comparison Test (GraphPad Software, Inc., San Diego, CA). Data are represented as mean ± standard deviation (S.D.).

3. RESULTS

3.1 Characterization of C6 glioma cells.

Cell culture is a useful model for examining aspects of cellular biology. Primary cell cultures, which are extracted directly from the host organism, are ideal models given that they better represent the host; however, they generally lack the ability to proliferate. Immortalized cell lines grow and divide indefinitely, which allows for a limitless source of that cell type. These cells can be naturally generated in the case of cancer or generated by chemical means. For example, C6 glioma cells were generated from a rat glial tumor induced by N-nitrosomethylurea (Benda et al., 1968). In this case, N-nitrosomethylurea was injected into rats and the resulting glial tumors were propagated in culture (Druckrey et al., 1965). In the case of the human embryonic kidney (HEK293) cell line, often used for overexpression and characterization studies, the cells have been immortalized following transformation with the adenovirus early region 1B (E1B) 19K protein (a prosurvival Bcl-2-like protein) (Han et al., 1996).

3.1.1 C6 cell morphology.

The glioblastoma C6 cell line displayed a fibroblast-like morphology (Figure 3.1) as previously described (Benda et al., 1968). In comparison, the pheochromocytoma PC12 cell line displayed a round, polygonal morphology. The HEK293A cell line displayed an epithelial-like morphology (Figure 3.1).

3.1.2 Protein expression in selected cell lines.

The endogenous expression of proteins of interest, specifically SHP-1, SHP-2, p85 and ERK1/2, were examined in C6 cells and compared with other cell lines, *e.g.* HEK293A, N2a and SHSY5Y. SHP-1 expression in C6 and HEK293A cells was comparable and higher than in the neuronal cell lines, N2a and SHSY5Y (Figure 3.2).

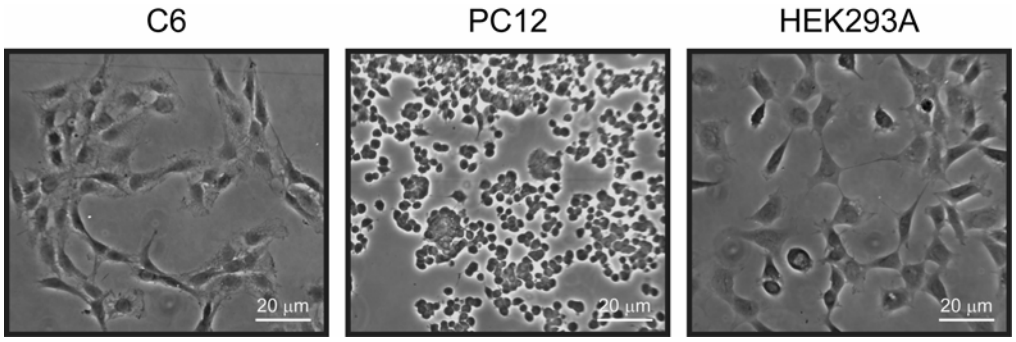


Figure 3.1: Morphology of C6, PC12 and HEK293A cell lines. C6 and HEK293A cells were grown on a glass-coverslip and PC12 cells were grown on a glass-coverslip coated with rat tail collagen type I (200 μg/mL) and fixed for 30 min with 4% paraformaldehyde. Cell morphology was assessed using phase contrast on an IMT-2 Olympus microscope.

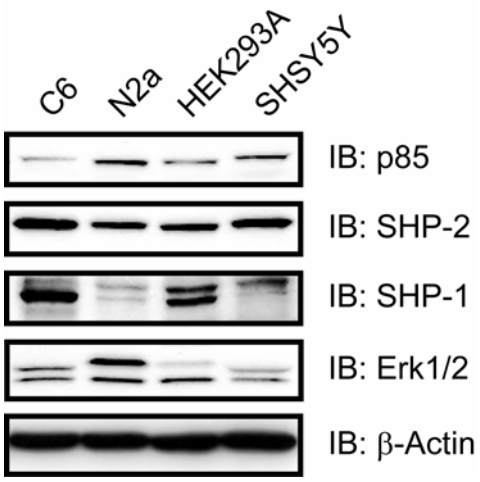


Figure 3.2: Expression of SHP-1 and selected proteins in C6 cells and other cell lines. Cells were harvested at 80% confluence and total cellular proteins were extracted. Proteins (30 μg per lane) were resolved by SDS-PAGE and immunoblotted (IB) for p85, SHP-1, SHP-2, Erk1/2 and β-actin using the appropriate antibodies.

The expression of p85 in C6 and HEK293A cells was lower than in the neuronal cell lines (Figure 3.2).

3.2 Characterization of C6 cells cultured in serum-free medium.

Removing serum from cell cultures is routinely used to induce cell cycle arrest at G1 (Chou and Langan, 2003) and to reduce a cell's ability to grow and multiply (Heldin et al., 1980). Removal of serum does not readily induce apoptosis in glial cells, although it can promote differentiation (Chou and Langan, 2003; Fan, 1983) and promote morphological changes from a fibroblast-like flattened shape to a rounded astrocyte-like morphology (Baranska et al., 2004).

3.2.1 Serum withdrawal affects growth rate and cell cycle.

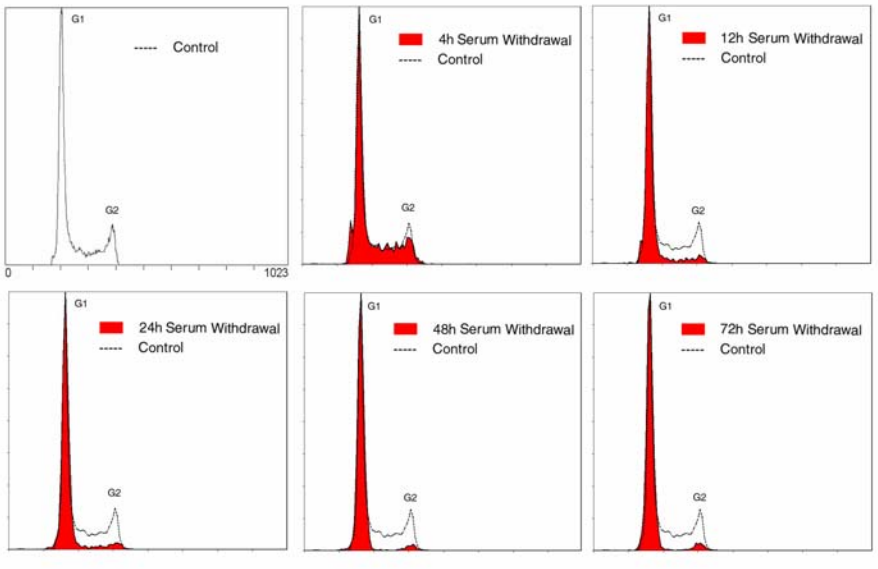
Phases of the cell cycle of C6 cells cultured in serum-free medium were assessed using flow cytometry. Twelve hours of growth in serum-free conditions resulted in an increase in the number of cells in G1/S phase and a reduction in the number of cells progressing into G2/M phase (Figure 3.3). G1/S arrest extended over the 72-hour test interval (Figure 3.3).

The MTT conversion assay, which measures cell number/proliferation, was used to assess growth of C6 cells in response to serum withdrawal (SWD). A decreased MTT conversion (indicating diminished cell number) was evident as early as 12 hours following SWD (Figure 3.4).

3.2.2 C6 cell morphology changes in response to SWD.

C6 cell morphology was examined at several time points following SWD. After 30 minutes of SWD, cells displayed a heterogenous cell morphology including some that were unchanged, flat without polarity, whereas others were less flat and more elongated

A.



B.

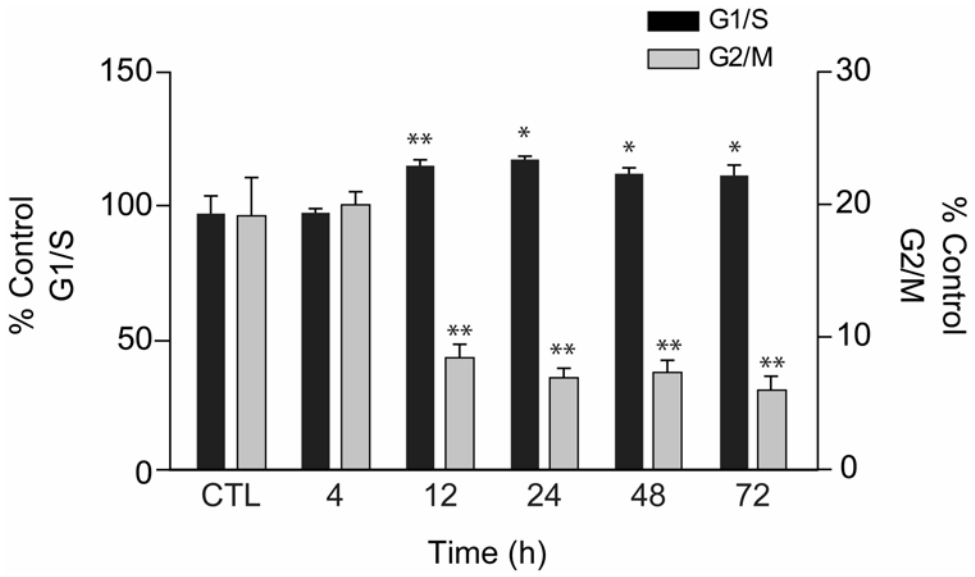


Figure 3.3: Cell cycle arrest of C6 cells cultured in serum-free medium. C6 cells were grown in serum-free medium for the indicated times (h) or maintained in growth medium (DMEM, 10% FBS) (CTL). Cells were suspended at a concentration of 10^6/ml and stained with 50 µg/ml propidium iodide. DNA content was analyzed by a Coulter® EPICS® XL flow cytometer. (**A**) Flow cytometry analysis overlays of control cells and cells grown in serum-free medium. (**B**) Statistical representation of flow cytometry analysis. SWD: serum withdrawal; * & **: P<0.05 and 0.01, respectively, *versus* control (CTL). Data are represented as mean ±SD, n=4.

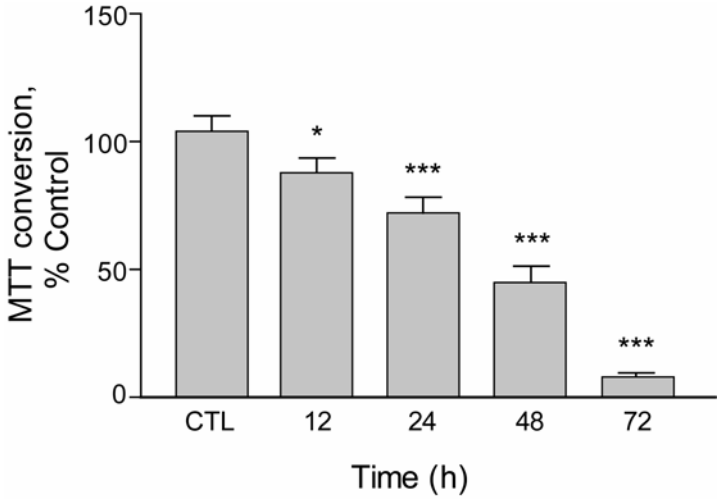


Figure 3.4: Loss of C6 cell proliferation in response to serum withdrawal (SWD). Cells were grown in a 96 well plate and cultured in serum-free medium for the indicated times (h). Medium was removed and MTT was added to a concentration of 0.5 mg/ml. The reaction (4 h at 37°C) was terminated by addition of 100 µl of DMSO. The O.D. was determined using a SpectraMax Plus384microplate reader at 570 nm.* & ***: $P<0.01$ and $P<0.001$ *versus* control (CTL). Range for control was 0.3-0.9, O.D. 570 nm and data are represented as mean ± SD, n=3.

(Figure 3.5). By 24 hours, all cells were more elongated and these changes were maintained over the time intervals studied (Figure 3.5).

3.3 Effect of SWD on the PI3K/Akt pathway.

There are reports that C6 glioma cell cultured in serum-free medium in combination with dibutyryl-cAMP can induce PI3K activity (Roymans et al., 2001), while inhibiting the phosphorylation of Akt (Van Kolen and Slegers, 2004). The mechanism behind this observation is unclear.

The commonly accepted paradigm suggests that Akt phosphorylation is dependent on PI3K activation (Figure 3.6A). This depends on the recruitment of PI3K to RTKs at the plasma membrane either directly or by scaffolding molecules such as Gab1 (Ingham et al., 2001). The tyrosine phosphorylation status of p85, which contributes to p110/PI3K activation (Cuevas et al., 2001), can be modified by its interaction with protein tyrosine phosphatases such as SHP-1 (Cuevas et al., 2001) and SHP-2 (Wu et al., 2001). Similarly, Akt recruitment to the plasma membrane where it is phosphorylated and activated is dependent on the generation of PI3K lipid products $PI3,4P_2$ and $PI3,4,5P_3$ (Bellacosa et al., 1998; Franke et al., 1997; Franke et al., 1995; Klippel et al., 1997). The balance between PI3K and the phosphoinositide phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) frequently determines $PI3,4,5P_3$ levels and Akt function (Gericke et al., 2006). The effect of SWD on components involved in PI3K activation was further examined.

3.3.1 SWD induces PI3K activity, but inhibits Akt phosphorylation.

Protein lysates of C6 cells cultures (SWD for 4, 24 and 48 hours) were immunoprecipitated with the p85 antibody and used for the p110/PI3K lipid kinase assay, based on [γ-^{32}P]ATP incorporation into PI. p85-associated PI3K activity was found to be increased at 4 hours (Figure 3.6B,C). The PI3K activity at 4 hours did not correspond with the decreased levels of Akt phosphorylation in corresponding lysates (Figure 3.7).

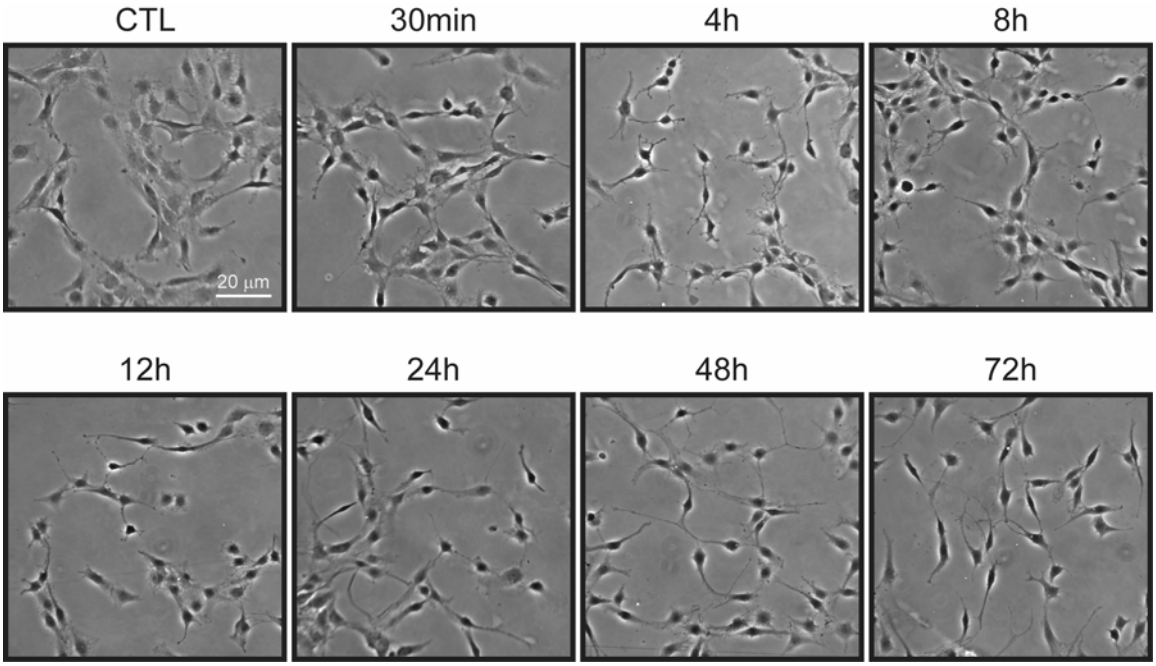
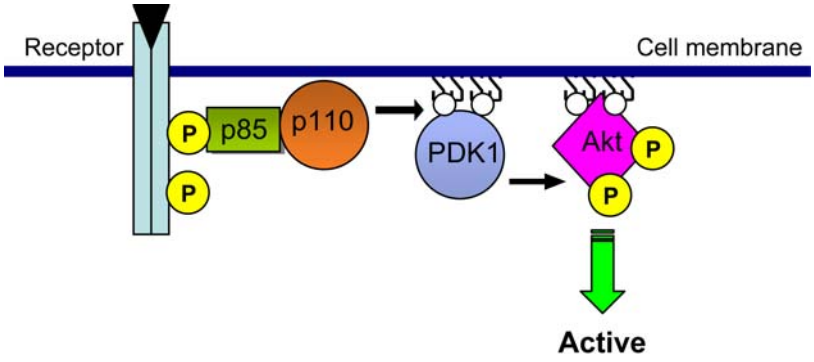


Figure 3.5: Morphology of C6 cells cultured in serum-free medium. Cells were grown on a glass-coverslip and cultured in serum-free medium for the indicated times (h) then fixed for 30 min with 4% paraformaldehyde. Cell morphology was assessed using phase contrast on an IMT-2 Olympus microscope. Data are representative of three independent experiments.

A.



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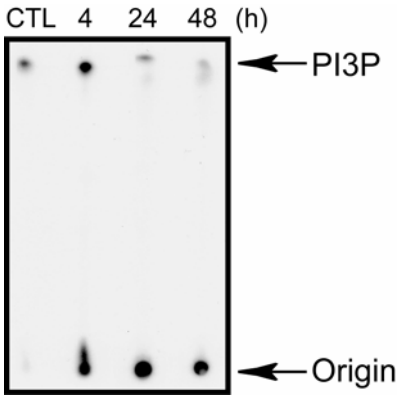


C.

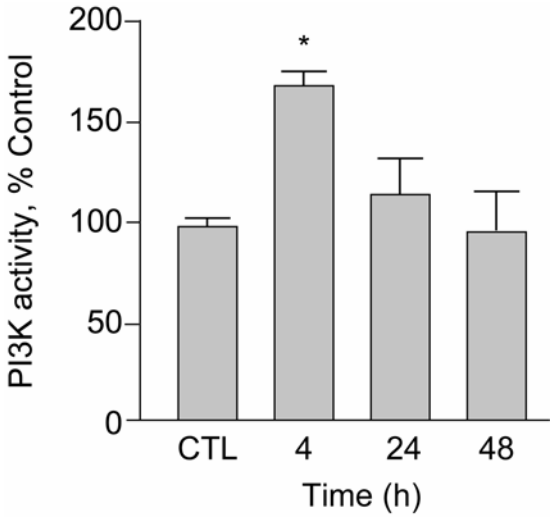


Figure 3.6: Increase in PI3K activity in C6 cells grown in serum-free medium. (**A**) The schematic of PI3K signalling through the PDK1/Akt pathway (originally shown in Figure 1.4) is included to remind the reader of "normal" growth factor-dependent PI3K signalling. (**B,C**) Cells were cultured in serum-free medium for the indicated times. (**B**) p85 was immunoprecipitated from 300 µg of total protein and used to assay for associated PI3K activity. [γ^{32}P]ATP-labeled PI3K lipid products were resolved by thin layer chromatography. (**C**) Results were quantified using Quantity One software (Bio-Rad). PI3P: phosphatidylinositol-3-*mono*phosphate. *:P<0.05 (ANOVA); data are represented as mean ± SD, n=3.

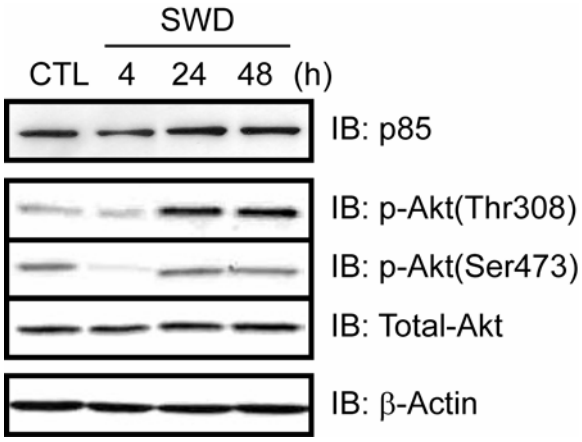


Figure 3.7: C6 cells cultured in serum-free medium have a transient loss of Akt phosphorylation. Cells were cultured in serum-free medium for the indicated times (h, SWD: serum withdrawal). Proteins (25 μg per lane) were resolved by SDS-PAGE and immunoblotted (IB) for phosphorylated Akt(Thr308) and Akt(Ser473) and for total Akt, as well as for p85 and β-actin. Data are representative of three independent experiments.

3.3.2 Membrane-bound Akt does not protect against the effects of SWD.

The constitutively active, myristoylated (myr)-Akt protein contains a N-terminal myristoylation sequence that localizes Akt to the plasma membrane (Goswami et al., 1999; Kohn et al., 1996).

C6 cells overexpressing myr-Akt were cultured in serum-free medium for 4 hours. There was no loss of Akt phosphorylation on Thr308 and Ser473 in these cells under these conditions (Figure 3.8A). Overexpression of myr-Akt did not protect against the inhibition of cell proliferation induced by SWD (Figure 3.8B), which was expected as this protein is bound to the plasma membrane and may not be capable of targeting some of its downstream substrates. (*note*, 24 h of SWD was used as 4h of SWD does not induce a significant inhibition of cell number).

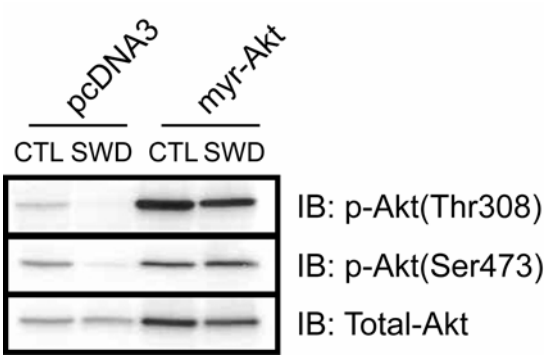
3.3.3 Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) does not affect Akt phosphorylation during SWD.

The loss of Akt phosphorylation observed during SWD could result from the activation of the lipid phosphatase PTEN and the subsequent dephosphorylation of plasma membrane-bound $PI3,4,5P_3$ necessary for recruitment of Akt. Loss-of-function mutations in PTEN can contribute to tumorigenicity in cell lines.

In order to compare and contrast PTEN expression in a variety of cell lines, including the C6 cell line which had previously been shown to be PTEN null (Kubiatowski et al., 2001), several cell lines were screened for PTEN expression. These included PC12, N2a, T98G (human glioma), HT22 (mouse hippocampal cells) and HEK293A cells. PTEN was expressed in all cell lines (Figure 3.9A). To determine if a mutation was contributing to C6 cell phenotype, the PTEN gene was amplified by RT-PCR (Figure 3.9B) and sequenced. There was no evidence of any deletions, insertions, frame-shifts, or other mutations to the PTEN gene in our C6 cells (data not shown).

Next, the influence of PTEN on the loss of Akt phosphorylation in C6 cells cultured in serum-free medium was assessed using the PTENC124S catalytically inactive mutant of PTEN (Maehama and Dixon, 1998; Ono et al., 2001). C6 cells overexpressing PTENC124S were grown in serum-free medium for 4 hours. PTEN overexpression was difficult to assess by Western blot (Figure 3.10).

A.



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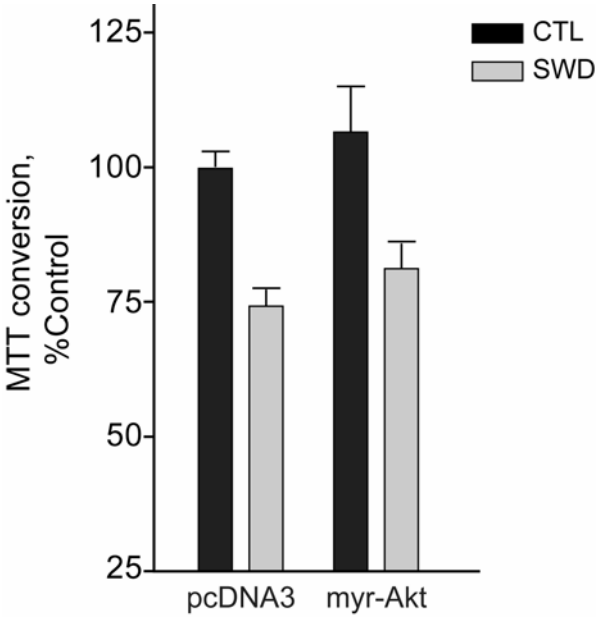
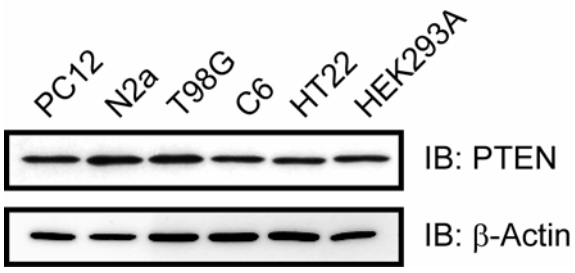


Figure 3.8: Effect of activated Akt (myr-Akt) on C6 cell proliferation in response to SWD. C6 cells were transfected with pcDNA3 or myristoylated (myr) pCS2+-Akt (myr-Akt). (**A**) 24 h post-transfection cells were cultured in serum-free medium (4 h, SWD: serum withdrawal) and proteins (25 µg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) for phosphorylated Akt(Thr308) and Akt(Ser473) and for total Akt. (**B**) Similar cells were grown in serum-free medium (24 h). Medium was then removed and MTT was added to a concentration of 0.5 mg/ml. The reaction (4 h at 37°C) was terminated by addition of 100 µl of DMSO. Range for control was 0.3-0.9, O.D. 570 nm and data are represented as mean ± SD, n=3.

A.



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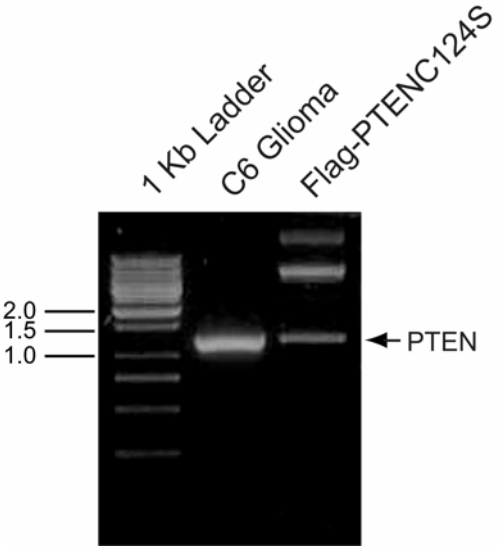


Figure 3.9: PTEN expression in C6 cells. (**A**) Total cell protein (25 μg of protein per lane) from six cell lines was resolved by SDS-PAGE and immunoblotted (IB) for PTEN and β-actin. (**B**) RNA was extracted from C6 cells and transcribed to DNA using RT-PCR. Using specific primers, PTEN was amplified from C6 cells and Flag-PTENC124S using PCR. Products were resolved on a 5% agarose gel.

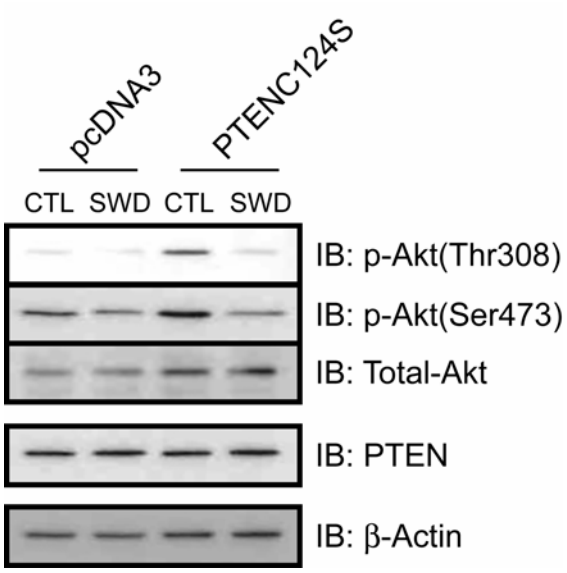


Figure 3.10: Catalytically inactive PTEN (PTENC124S) does not protect against the loss of Akt phosphorylation in response to SWD. C6 cells were transfected with pcDNA3 or Flag-PTENC124S and 24 h post-transfection cells were cultured in serum-free medium (4 h). Proteins (25 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) for phosphorylated Akt(Thr308) and Akt(Ser473) and for total Akt, as well as for PTEN. Levels of β-actin were used to monitor protein loading. Data are representative of two independent experiments. SWD: serum withdrawal.

It is not clear at this point why it was difficult to detect, but PTENC124S expression was implied indirectly by the increase in Akt phosphorylation under control conditions. PTENC124S was ineffective in protecting against the loss of Akt phosphorylation during SWD (Figure 3.10).

3.3.4 SWD does not affect the association between p85 and Gab1

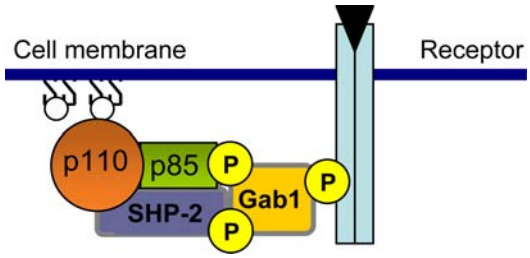
Gab1 is a scaffolding/docking protein that helps to recruit p85/PI3K to RTKs for activation (Ingham et al., 2001). It is generally accepted that the interaction between the phosphatase SHP-2 and Gabl positively regulates PI3K activity (Kong et al., 2000; Maeda et al., 2004) (Figure 3.11A). However, there are a few exceptions (Zhang et al., 2002).

The interaction between p85 and Gab1 was examined in C6 cells cultured in serum-free medium. Following SWD (4 h), the cells were treated with pervanadate (PV), a general tyrosine phosphatase inhibitor that allows for the accumulation of phosphotyrosine-dependent associations between proteins. Protein lysates were immunoprecipitated for p85. The association between p85 and Gab1 during SWD was maintained and was increased by PV, indicating the association is phosphotyrosine-dependent (Figure 3.11B). The decreased mobility of Gab1 in PV-treated groups confirmed a post-translational modification, presumably tyrosine phosphorylation (Figure 3.11B). The association between p85 and SHP-2 was decreased in response to SWD, but enhanced by the combination of SWD and PV (Figure 3.11B).

Protein lysates were also immunoprecipitated for SHP-2. The association between SHP-2 and Gab1 was enhanced in response to SWD (Figure 3.11C) and further enhanced by treatment with PV. The association between SHP-2 and p85 was decreased in response to SWD and, again, enhanced by the combination of SWD and PV (Figure 3.11C), confirming the corresponding observations using p85 immunoprecipitates (Figure 3.11B). Similarly to Gab1, the decreased mobility of SHP-2 in PV-treated groups confirmed a post-translational modification, again presumably tyrosine phosphorylation (Figure 3.11C).

The fact that the association between p85 and SHP-2 is disrupted by SWD is counter-intuitive given the demonstrated increase in PI3K lipid kinase activity. As SWD

A.



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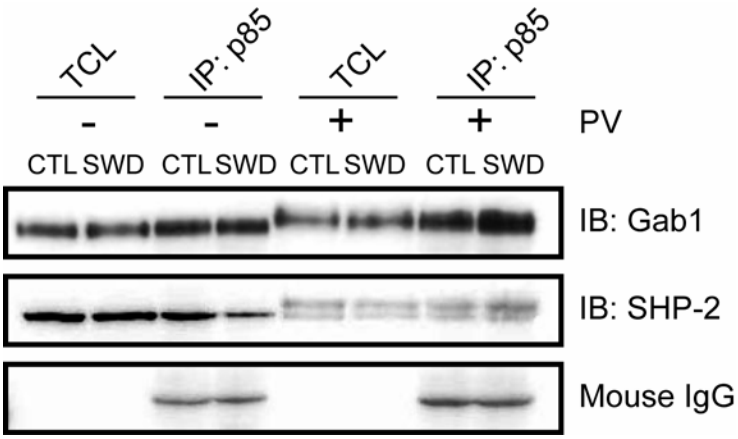


C.

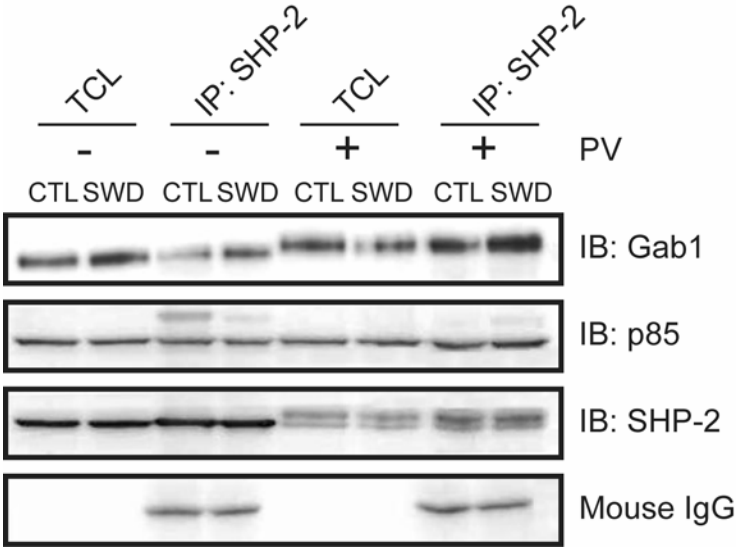


Figure 3.11: Effect of SWD on p85 association with Gab1 and SHP-2. (**A**) Reminder of aspects of growth factor-dependent PI3K signalling. C6 cells were cultured in serum-free medium (4 h, SWD: serum withdrawal) and treated with pervanadate (PV) for 30 min. (**B**) p85 and (**C**) SHP-2 were immunoprecipitated (IP: 300 µg of total protein) and immune complexes were resolved by SDS-PAGE and immunoblotted (IB) with anti-Gab1, anti-p85 and anti-SHP-2 antibodies. Data are representative of two independent experiments.(TCL: total cell lystate)

decreases the association between p85 and SHP-2, and PV enhances the association, it is possible that the phosphorylation of p85 is affected by SWD.

3.3.5 Tyrosine phosphorylation of p85 is lost in response to SWD.

There was a sustained loss of p85 tyrosine phosphorylation over the SWD intervals studied (Figure 3.12), which is unexpected as PI3K at 4h of SWD was increased (recall Figure 3.6). A tyrosine-to-aspartate (Y-to-D) substitution mutant [*i.e.* a phosphorylation mimic] of p85 was generated (Figure 3.13A) based on the observation that the phosphorylation of p85Tyr688 (p85Y688) increases Akt phosphorylation (Cuevas et al., 2001) by affecting PI3K activity independent of ligand stimulation. Wild type p85 was not used as it can act as a dominant-negative protein in a variety of cases (Ueki et al., 2000). The p85Y688D substitution mutant was overexpressed in C6 cells and did not affect Akt phosphorylation (Figure 3.13B). The inhibition of cell proliferation by SWD (24 h) was not affected by overexpression of p85Y688D (Figure 3.13C) (*note*, 24 h of SWD was used as 4h of SWD does not induce a significant inhibition of cell number).

3.3.6 SWD induces p85 and PI3,4,5P_3 redistribution to the nucleus.

Recruitment of p85 to ligand-stimulated RTKs allows for tyrosine phosphorylation on p85 leading to the conformation change that relieves its inhibitory effect on p110/PI3K (Yu et al., 1998b; Yu et al., 1998c). However, SWD increased PI3K activity did not correspond with an increase in p85 tyrosine phosphorylation. Therefore, the follow experiments were conducted in order to determine the localization of p85 and cellular production of PI3K-associated lipid products in reponse to SWD.

C6 cells were cultured in serum-free medium for 4 hours and then fixed for confocal microscopic visualization of the localization of p85. In response to SWD, p85 (Figure 3.14; upper panel) was redistributed to the vicinity of the nucleus and a perinuclear distribution, although present in control C6 cells, was much more evident in cells grown in sum-free medium. PI3,4,5P_3, used to indirectly monitor PI3K activity, was also detected in this same region in cells cultured in serum-free medium (Figure 3.14; lower panel).

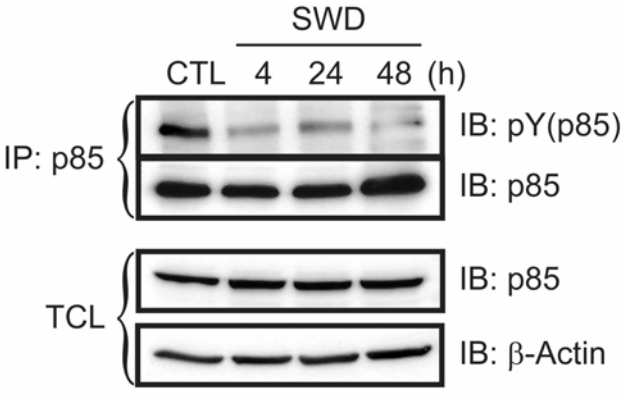


Figure 3.12: SWD induces a loss of p85 tyrosine phosphorylation. C6 cells were cultured in serum-free medium for the indicated times (h, SWD: serum withdrawal). Proteins (300 μg of total protein) were immunoprecipitated (IP) for p85 and resolved by SDS-PAGE. Membranes were probed with anti-phosphotyrosine and anti-p85 antibodies. Corresponding protein lysates (25 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) p85 and β-actin. Data are representative of three independent experiments

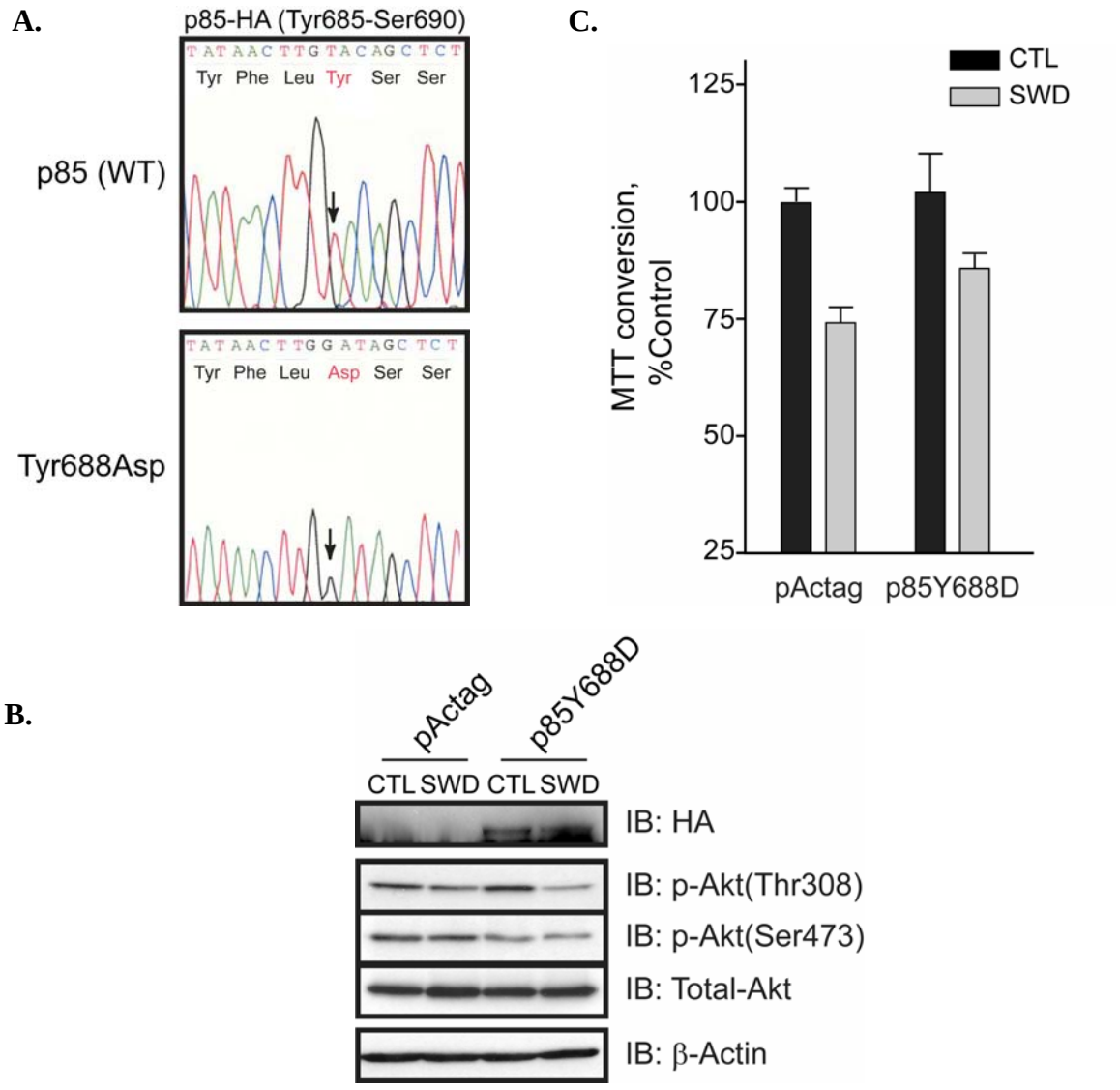


Figure 3.13: Activated p85 (p85Y688D) does not affect C6 cell proliferation in response to SWD. p85 tyrosine (Tyr/Y) 688 was substituted with an aspartic acid (Asp/D). Plasmid DNA was extracted using the alkaline lysis method and sequenced. (**A**) Chromatogram of p85 (WT) and p85Tyr688Asp. Cells were transfected with pActag-HA-tagged or pActag-HA-tagged-p85 (p85), or pActag-HA-tagged-p85Y688D (p85Y688D). (**B**) 24 h post-transfection protein lysates (30 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) for phosphorylated Akt(Thr308) and p-Akt(Ser473) and for total Akt, HA and β-actin using antibodies. (**C**) Similar cells were grown in serum-free medium for 24 h and tested for MTT conversion. Data are represented as mean ± SD, n=3.

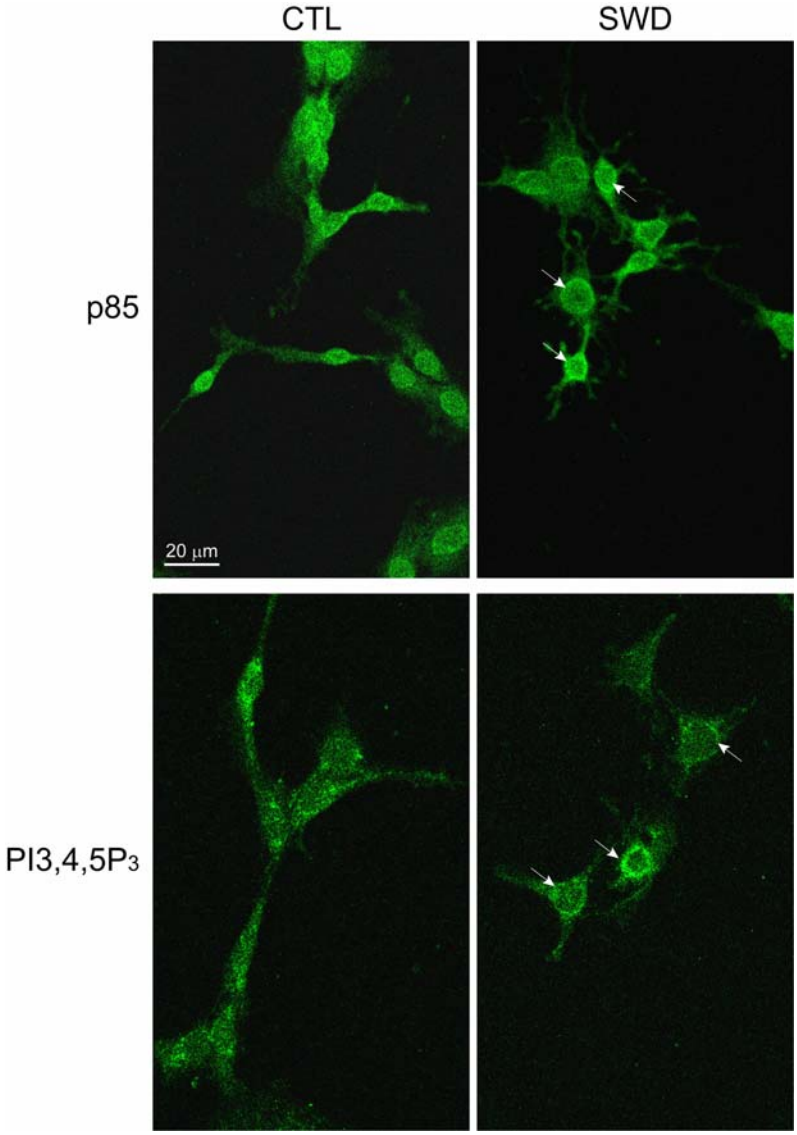


Figure 3.14: Perinuclear distribution of p85 and PI3,4,5P$_3$ in response to SWD. C6 cells were cultured in the presence or absence of serum (4 h) in chambered culture slides. Cells were then fixed with 4% paraformaldehyde and incubated with either anti-p85 or anti-phosphatidylinositol-3,4,5-*tris*phosphate (PI3,4,5P$_3$) IgM-Biotin. FITC-conjugated secondary antibody (green) or streptavidin-AlexaFluor 488 (green), respectively were used for visualization by confocal microscopy. SWD: serum withdrawal. Excitation/emission wavelengths for both secondary antibody conjugates are 495/520 nm. Data are representative of two independent experiments.

3.3.7 Growth factor stimulation with PDGF reverses the effects of SWD in C6 cells.

A number of receptor tyrosine kinases, with ligands as diverse as the platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF) and angiotensin II (Kazlauskas and Cooper, 1990; Okamoto et al., 1993; Ram and Ethier, 1996; Saward and Zahradka, 1997) are known to stimulate PI3K activity. C6 cells have been well characterized and contain both the EGFR and the PDGFR (Grobben et al., 2002). Supplementing medium with both EGF or PDGF is reported to maintain cell proliferation of C6 cells cultured in serum-free medium (Heldin et al., 1980). The following experiments were conducted to determine the role, if any, of PDGF and EGF on the effects observed in the present model.

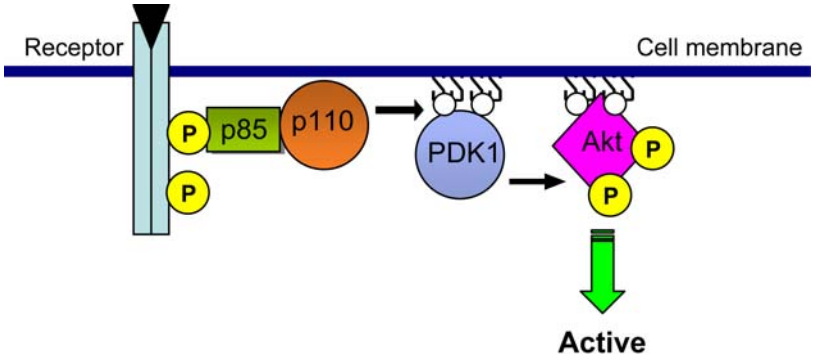
Stimulation with PDGF, but not EGF, maintained Akt phosphorylation in serum-free conditions (Figure 3.15B). In addition, PDGF stimulation increased the tyrosine phosphorylation of the PDGF receptor and its association with p85 (Figure 3.15B). EGF stimulation increased basal Akt phosphorylation (indicating the presence of an active EGF system in C6 cells), but did not protect Akt from dephosphorylation in response to SWD (Figure 3.15B).

3.3.8 The nuclear redistribution of PI3,4,5P_3 and the loss of Akt phosphorylation occur as early as 30 min following SWD.

To determine if the loss of Akt phosphorylation was the direct result of a signalling event, shorter intervals of SWD were used. The dephosphorylation of Akt was observed as early as 30 minutes following the removal of serum (Figure 3.16).

C6 cells overexpressing a fluorophore (red fluorescent protein: RFP)-tagged pleckstrin homology protein, PH-mRFP1, which makes use of the pleckstrin homology (PH) from *Dictyostelium* to detect PI3,4,5P_3 distribution (Revankar et al., 2005), were cultured in serum-free medium for 30 min and 4 hours. PH-mRFP1 expression was diffusely distributed in control cells. The PH-mRFP signal was redistributed to the nucleus upon SWD (Figure 3.17). It was also noted that after 4 hours of removing serum from C6 cell cultures there were morphological changes (cell shrinkage), which was

A.



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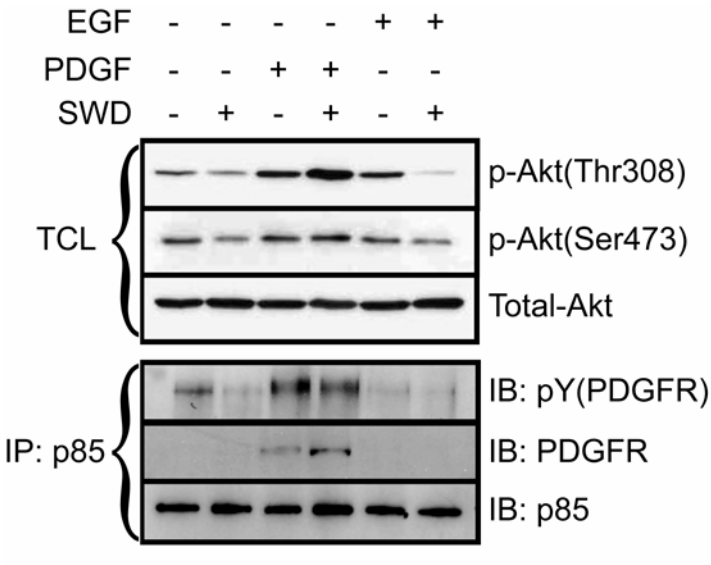
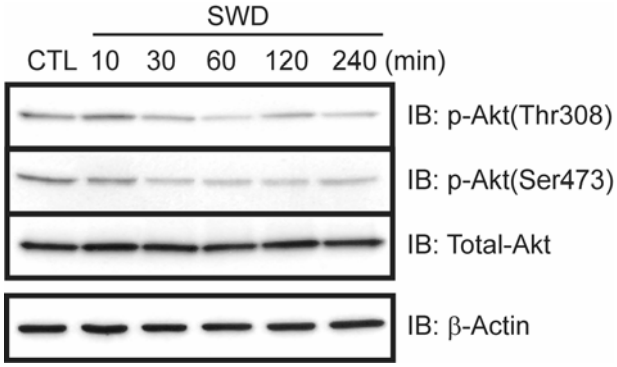


Figure 3.15: The loss of Akt phosphorylation in C6 cells cultured in serum-free medium is sensitive to PDGF stimulation. (**A**) Reminder of growth factor-dependent PI3K signalling. (**B**) C6 cells cultured in serum-free medium (4 h) were stimulated with either 50 ng/ml of PDGF (platelet-derived growth factor) or EGF (epidermal growth factor) for 10 min. Protein lysates (25 µg of protein per lane) were resolved by SDS-PAGE and probed for phosphorylated Akt(Thr308) and Akt(Ser473) and for total Akt. Proteins (300 µg) were also immunoprecipitated (IP) with anti-p85 and resolved by SDS-PAGE. Membranes were probed with anti-phosphotyrosine (pY), anti-PDGF receptor (PDGFR) and anti-p85 antibodies. Data are representative of two independent experiments. SWD: serum withdrawal.

A.



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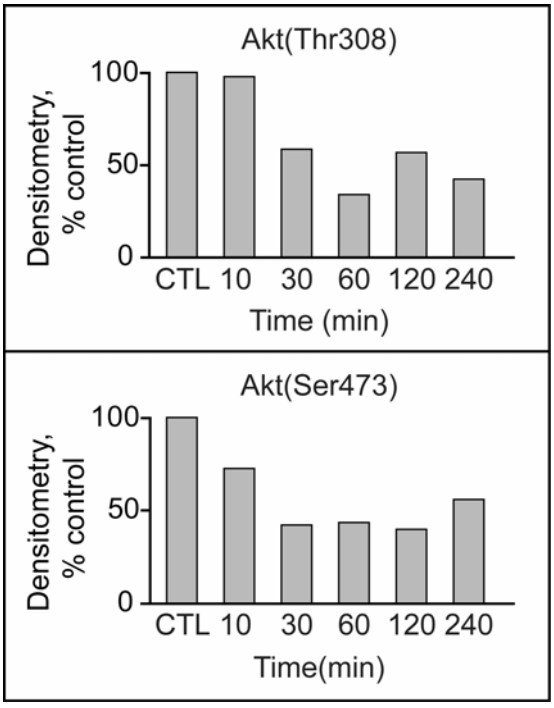


Figure 3.16: Akt phosphorylation is decreased within 30 minutes of removing serum from C6 cell cultures. (**A**) Cells were cultured in serum-free medium for the indicated times (min). Proteins (25 μg of protein per lane) were subjected to SDS-PAGE and immunoblotted (IB) for phosphorylated Akt(Thr308) and Akt(Ser473) and for total Akt. The levels of β-actin were used to monitor protein loading. (**B**) Densitometric analysis of resolved proteins in (**A**); expressed as percent control. Data are representative of two independent experiments.

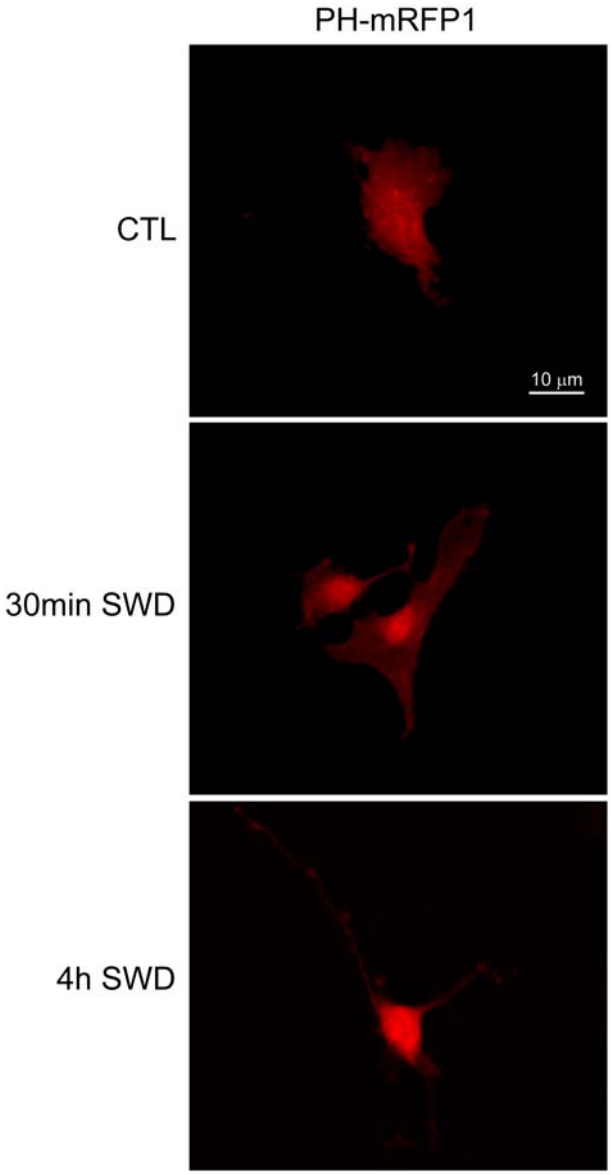


Figure 3.17: Distribution of PI3,4,5P$_3$ at 30 min compared to 4 h of SWD. C6 cells were transfected with PH-mRFP1 (red) and post-transfection (24 h) were cultured in serum-free medium (30 min and 4 h). Cells were fixed with 4% paraformaldehyde, and analyzed using an Olympus FV300 confocal microscope. SWD: serum withdrawal. Excitation/emission wavelengths (nm) are 584/607. Data are representative of three independent experiments.

identified as a potential confounding factor when studying localization using confocal microscopy. There was virtually no shrinkage of the cells over the 30-minute interval.

3.3.9 PDGF reverses the effects of SWD and stimulates membrane-associated $PI3,4,5P_3$ production.

The PH-mRFP1 protein, which binds to $PI3,4,5P_3$, was again used to monitor $PI3,4,5P_3$ distribution in the cell. C6 cells overexpressing PH-mRFP1 were grown in serum-free medium (30 min) and stimulated with PDGF (10 min). In response to SWD, nuclear PH-mRFP1 was confirmed (Figure 3.18). PDGF stimulation of cells grown in serum-free medium induced a detectable increase in plasma-membrane associated PH-mRFP1 (arrows in Figure 3.18).

3.3.10 C6 cells grown in serum-free medium are sensitive to PDGF.

The effect of supplementing serum-free cultures of C6 cells with PDGF or EGF on cell cycle was determined. PDGF had modest effects on preventing G1/S arrest. PDGF decreased the number of cells in G1/S arrest and increased the number of cells in G2/M (Figure 3.19). There was no effect of EGF on cell cycle during SWD (Figure 3.19).

3.4 Effect of protein tyrosine phosphatases on PI3K signalling induced by SWD.

PI3K function and activity can be modulated by the protein tyrosine phosphatases SHP-1 and SHP-2 (Cuevas et al., 2001; Wu et al., 2001). While both phosphatases are known to dephosphorylate p85 on tyrosine residues, SHP-2 is considered as a positive regulator of p85 and PI3K activity (Wu et al., 2001), while SHP-1 is traditionally considered as a negative regulator of p85/PI3K function (Cuevas et al., 1999; Imani et al., 1997).

The interaction between p85 and either SHP-1 or SHP-2 was examined in C6 cells after 4 hours of SWD. Protein lysates were collected and immunoprecipitated for p85. The association between SHP-2 and p85 was decreased (Figure 3.20, upper panel) and the association of SHP-1 and p85 was maintained (Figure 3.20, lower panel) during SWD. It was noted that the tyrosine phosphorylation state of p85 associated with SHP-1

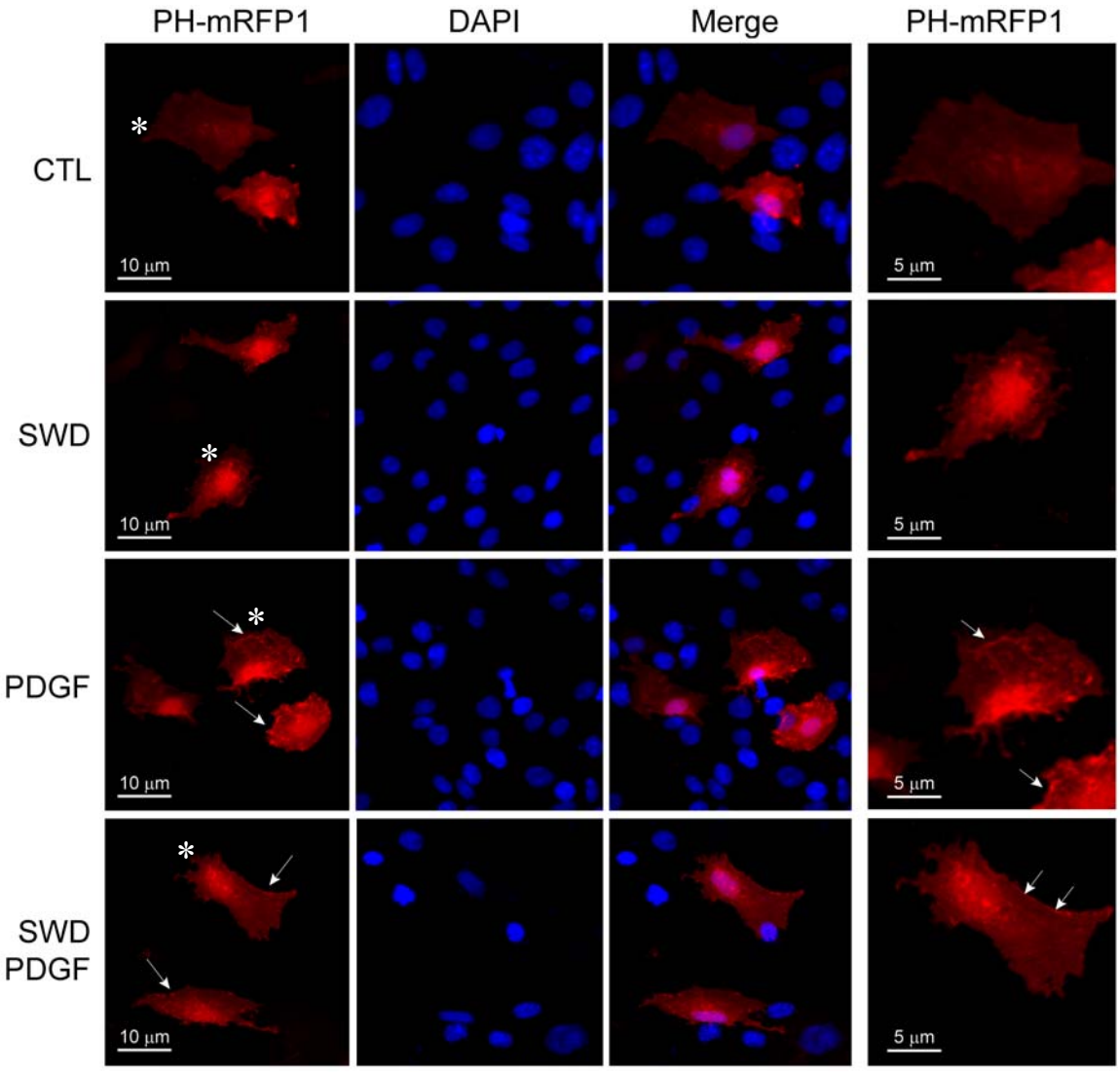


Figure 3.18: The distribution of PI3,4,5P$_3$ in C6 cells cultured in serum-free medium is sensitive to PDGF stimulation. Cells were transfected with PH-mRFP1 expression vector. 24 h post-transfection, serum was removed from cultures (30 min) followed by stimulation with PDGF (50 ng/ml, 10 min) prior to preparation for confocal microscopy. Nuclei were counterstained with DAPI (blue, 1 µg/µl, 5 min). Samples were analyzed using a Zeiss LSM 510 META confocal microscope. Excitation/emission wavelengths (nm) are mRFP1 (red): 584/607 and DAPI (blue): 358/461. (arrows: indicate increased RFP-PH signal at the plasma-membrane following PDGF treatment; asterisk: represent cells that were magnified; shown in far right column).

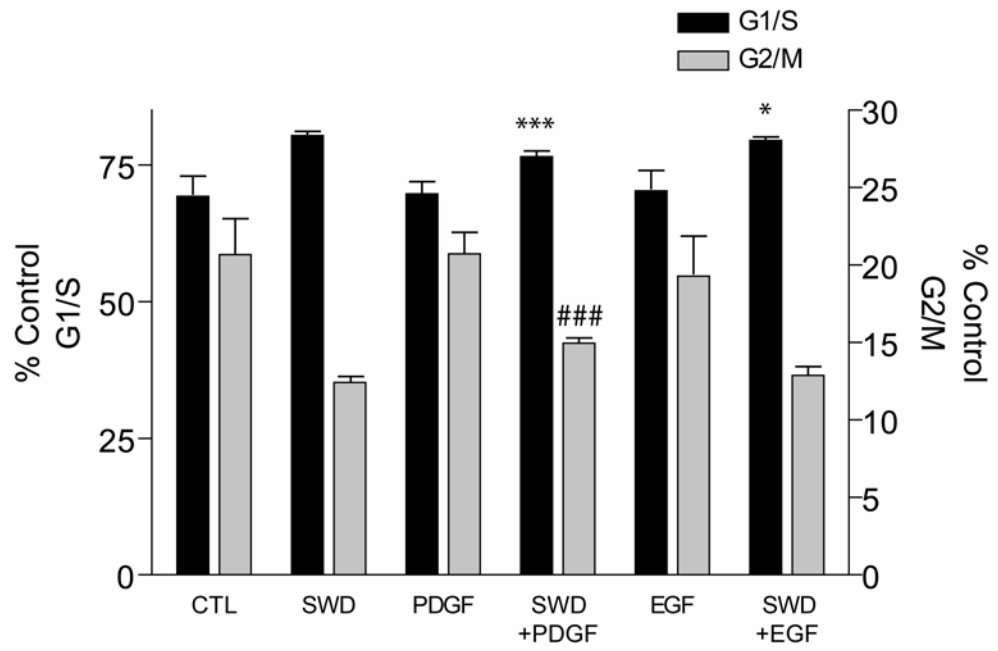


Figure 3.19: C6 cell cycle arrest induced by removal of serum is sensitive to PDGF. Cells cultured in serum-free medium (12 h) were supplemented with 50 ng/ml of PDGF or EGF. Cells were suspended at a concentration of 10^6/ml and stained with propidium iodide (50 µg/ml). DNA content was analyzed by a Coulter® EPICS® XL flow cytometer. SWD: serum withdrawal; * and ***: $P<0.05$ and 0.001, respectively, *versus* SWD-G1/S; ###: $P<0.001$, *versus* SWD-G2/M alone. Data are represented as mean ± SD, n=3.

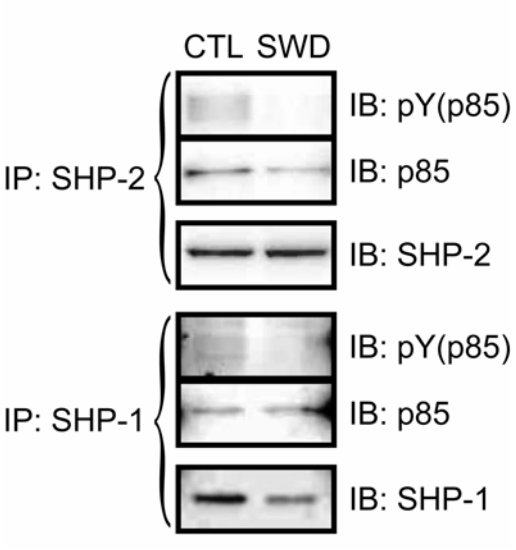


Figure 3.20: The interaction between p85 and SHP-1 is maintained in response to SWD. C6 cells were cultured in serum-free medium for 4 h. Endogenous SHP-1 or SHP-2 were immunoprecipitated (IP) from 300 μg of total protein and resolved by SDS-PAGE and immunoblotted (IB) with anti-phosphotyrosine, anti-p85, anti-SHP-1, or anti-SHP-2 antibodies. Data are representative of three independent experiments.

and SHP-2 was decreased with SWD (Figure 3.20). As the interaction between p85 and SHP-1 was maintained, the effects of SHP-1 on p85 were assumed to be predominant. However, it was not clear whether SHP-1 exerts a positive or negative effect on p85 under these conditions.

3.4.1 Effect of SHP-1 catalytic activity on Akt phosphorylation.

Substitution mutations in the protein tyrosine phosphatases, SHP-1 and SHP-2, can be used to study their catalytic function. The substitution of Cys/C455 to Ser/S generates a catalytically inactivate SHP-1, SHP-1C455S (Yu et al., 1998c). Similarly, the SHP-2C453S substitution mutant is catalytically inactive.

The pcDNA3-SHP-1 and pcDNA3-SHP-2 plasmids were confirmed by DNA sequencing (Figure 3.21). Peak expression of SHP-1 in C6 cells (Figure 3.22) was determined to occur at about 24 h (Figure 3.22). SHP-1 and SHP-2 proteins were overexpressed in C6 cells for 24 h. Overexpressed SHP-1 (WT) and SHP-1C455S (Figure 3.23A), but not SHP-2 (WT) or SHP-2C453S (Figure 3.23B), increased Akt phosphorylation on Ser473.

C6 cells transiently overexpressing SHP-1 (WT) and SHP-1C455S were grown in serum-free medium for 4 hours. SHP-1, but not SHP-1C455S, protected against the loss of Akt phosphorylation in response to SWD (Figure 3.24).

The overexpression of SHP-1 (WT) protected against the inhibition of proliferation in cells grown in serum-free medium (Figure 3.25). The overexpression of SHP-1C455S did not protect against the inhibition of proliferation induced by SWD (Figure 3.25)

3.5 Regulation of PDK1 in C6 cells.

Akt and PDK1 are recruited to the plasma membrane by $PI3,4P_2$ and $PI3,4,5P_3$ generated by PI3K (Anderson et al., 1998; Bellacosa et al., 1998; Stephens et al., 1998). At the plasma membrane, PDK1 phosphorylates Akt on Thr308 allowing for the subsequent activation of Akt (Bellacosa et al., 1998). The phosphorylation of PDK1 on Ser241 is thought to be required for its activation (Casamayor et al., 1999). The effect of SWD on PDK1 was determined.

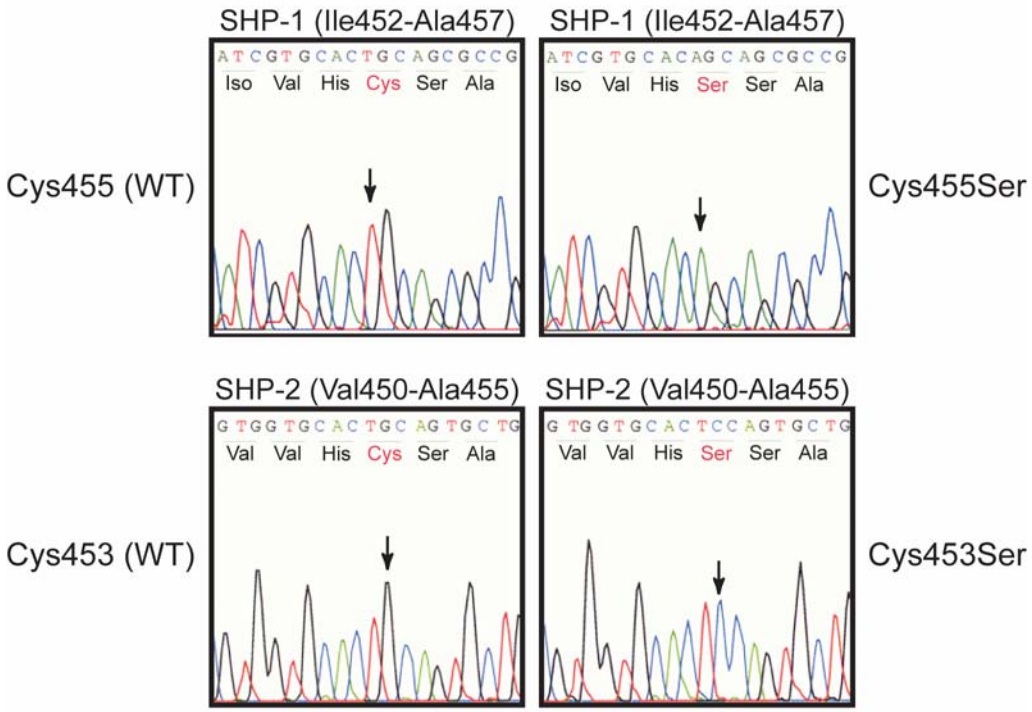


Figure 3.21: Chromatograms of SHP-1 and SHP-2 wildtype (WT) and catalytically inactive mutants. SHP-1 and SHP-2 cDNA plasmids were sequenced and mutations determined. SHP-1 WT and catalytic Cys455Ser mutants (upper panel) and the SHP-2 WT and catalytic Cys453Ser mutants (lower panel) were confirmed.

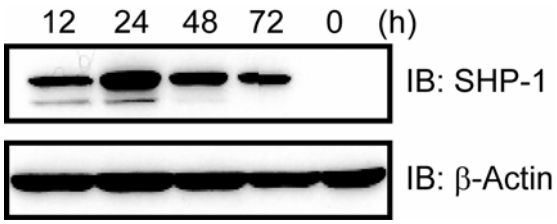
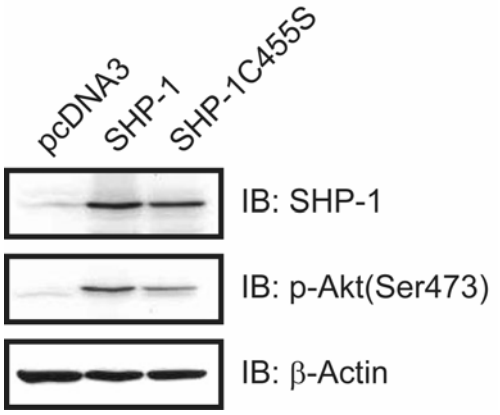


Figure 3.22: Transient expression of SHP-1 in C6 cells. Cells were transfected with pcDNA3-SHP-1 and SHP-1 was overexpressed for the indicated times (h). Proteins (10 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) for SHP-1 and β-actin. Data are representative of two independent experiments.

A.



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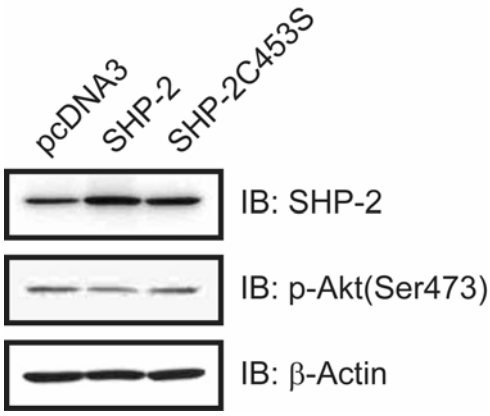


Figure 3.23: Effect of SHP-1 and SHP-2 on Akt phosphorylation. (**A**) C6 cells were transfected with pcDNA3 or pcDNA3-SHP-1 (SHP-1), or pcDNA3-SHP-1C455S (SHP-1C455S). (**B**) C6 cells were transfected with pcDNA3 or pcDNA3-SHP-2 (SHP-2), or pcDNA3-SHP-2C453S (SHP-2C453S). Post-transfection (24 h) proteins (25 μg of protein per lane) were resolved by SDS-PAGE and probed with anti-Akt(Ser473) and anti-SHP-1 or anti-SHP-2 antibodies. The levels of β-actin were used to monitor protein loading. Data are representative of two independent experiments.

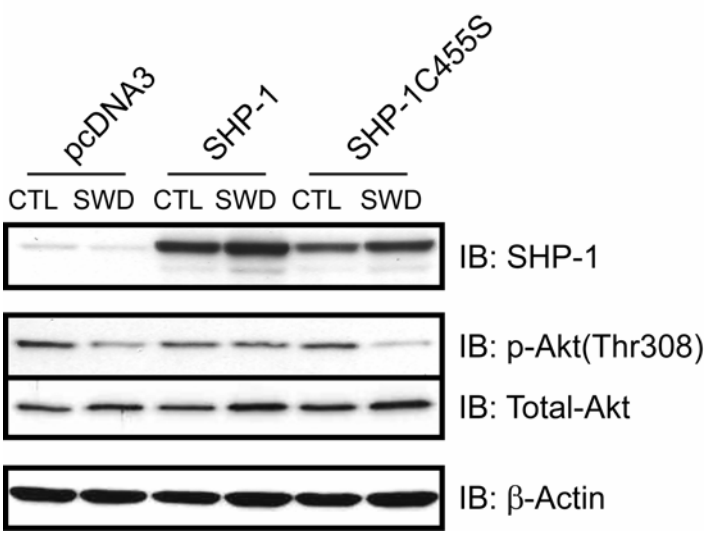


Figure 3.24: SHP-1 attenuates Akt dephosphorylation in response to SWD. C6 cells were transfected with pcDNA3 or pcDNA3-SHP-1 (SHP-1) or pcDNA3-SHP-1C455S (SHP-1C455S) and 24 h post-transfection cultured in serum-free medium for 4 h. Proteins (25 μg of protein per lane) were resolved by SDS-PAGE and probed for phosphorylated Akt(Thr308) and for total Akt and SHP-1. The levels of β-actin were used to monitor protein loading. Data are representative of two independent experiments.

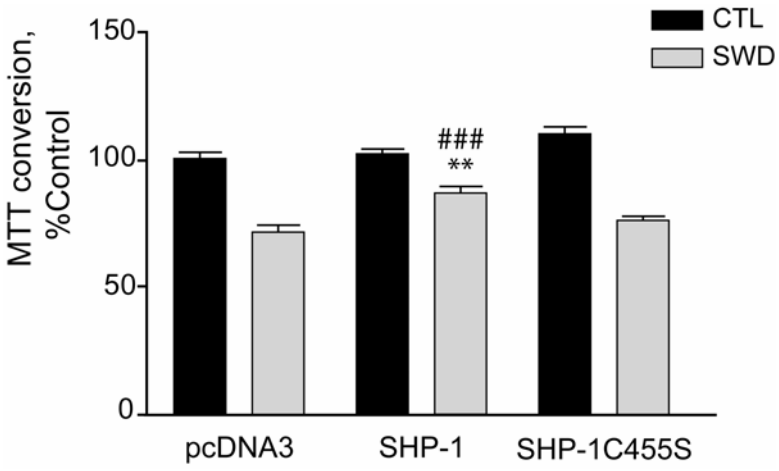


Figure 3.25: SHP-1 attenuates the loss of C6 cell proliferation induced by SWD. C6 cells were transfected with pcDNA3 or pcDNA3-SHP-1 (SHP-1), or pcDNA3-SHP-1C455S (SHP-1C455S), and 24 h post-transfection were cultured in serum-free medium for a further 24 h. MTT conversion was assessed. SWD: serum withdrawal. ###: $P<0.001$ *versus* SWD-pcDNA3; and **: $P<0.01$ *versus* SWD-SHP-1C455S. Data are represented as mean ± SD, n=4.

3.5.1 Expression of PDK1 in different cell lines.

PDK1 is a monomeric ~63 kDa serine/threonine kinase ubiquitously expressed in human tissues and cells (Alessi et al., 1997a; Stephens et al., 1998). Several cell lines including, C6, PC12, HEK293A and T98G, were screened for PDK1 expression. PDK1 was expressed in all cell lines, but expression levels and molecular weights varied between cell lines (Figure 3.26).

3.5.2 The response time of C6 cells to SWD on Akt phosphorylation.

The phosphorylation of PDK1 on Ser241 was not affected by SWD; however, a decrease in PDK1 mobility was observed (Figure 3.27), which suggests PDK1 may be modified (*i.e.* phosphorylated) at a site other than Ser241 under these conditions.

3.5.3 The PDK1 Ser241 phosphorylation mimic (PDK1S241E) does not protect against the loss of Akt phosphorylation in reponse to SWD.

To confirm the finding that phosphorylation of Ser241 does not affect Akt phosphorylation in reponse to SWD, a phosphorylation mimic was generated by substituting Ser241 to glutamic acid (Glu/E). The pEGFP-PDK1S241E cDNA was sequenced to confirm the substitution (Figure 3.28A). Overexpression of EGFP-PDK1S241E did not block the loss of Akt phosphorylation induced by SWD (4 h) in C6 cells (Figure 3.28B).

3.5.4 Membrane-bound PDK1 does not affect Akt phosphorylation in response to SWD.

The constitutively membrane-bound PDK1 mutant (myr-PDK1) containing a N-terminal myristoylation signal is able to constitutively activate co-expressed Akt protein *in vivo* (Anderson et al., 1998). C6 cells overexpressing myr-PDK1 were cultured in serum-free medium for 4 hours. Myr-PDK1 did not protect against the loss of Thr308 phosphorylation (Figure 3.29).

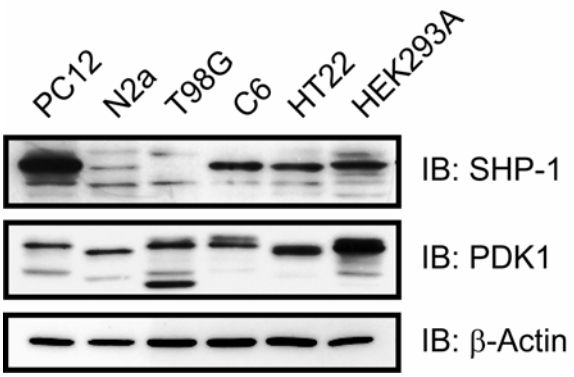


Figure 3.26: PDK1 expression in cell lines. Proteins (25 μg of protein per lane) from the indicated cell lines were resolved by SDS-PAGE and immunoblotted (IB) for PDK1, SHP-1 and β-actin.

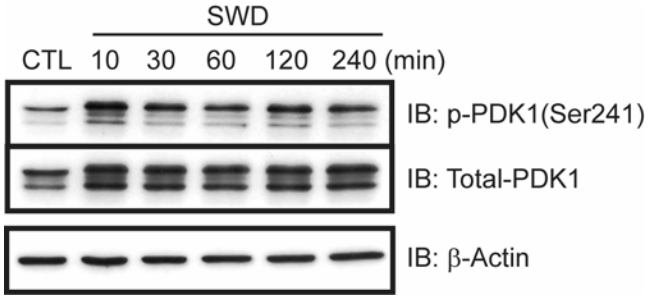
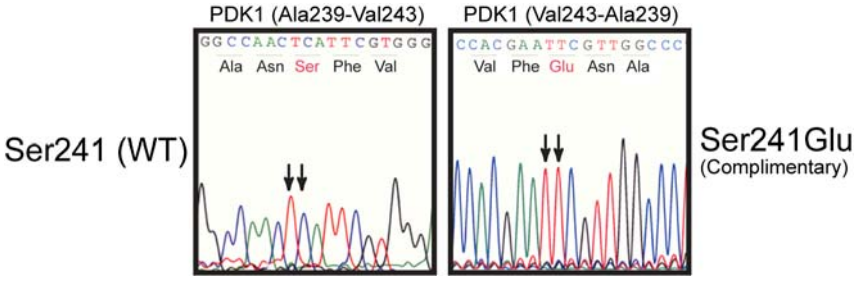


Figure 3.27: SWD induces a decrease in PDK1 mobility in C6 cells. Cells were cultured in serum-free medium for the indicated times (min). Proteins (25 μg of protein per lane) were resolved to SDS-PAGE and immunoblotted (IB) for phosphorylated PDK1(Ser241) and for total PDK1. The levels of β-actin were used to monitor protein loading. Data are representative of three independent experiments.

A.



B.

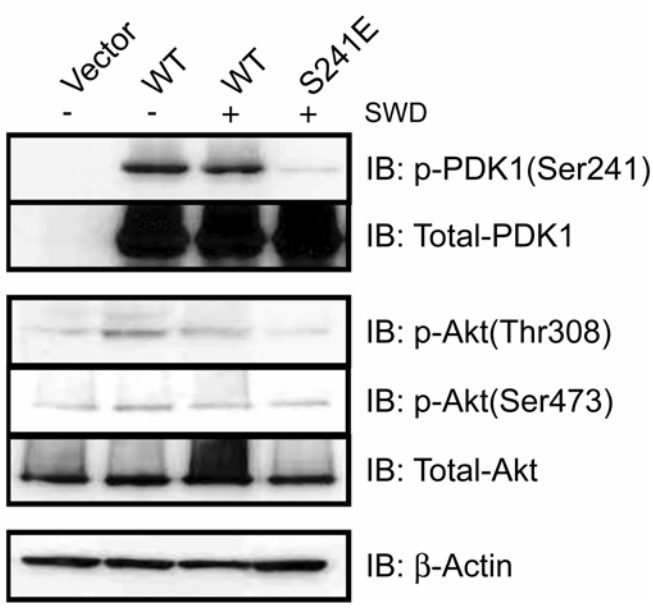


Figure 3.28: "Activated" PDK1 (PDK1S241E) does not attenuate Akt dephosphorylation induced by SWD. pEGFP-PDK1 (Ser/S) 241 was substituted with a glutamic acid (Glu/E). Plasmid DNA was extracted using the alkaline lysis method and sequenced. (**A**) Chromatogram of PDK1 wildtype (WT) and PDK1Ser241Glu. (**B**) C6 cells were transfected with pEGFP (vector), pEGFP-PDK1 (WT), or pEGFP-PDK1S241E (S241E). 24 h post-transfection cells were cultured in serum-free medium for 4 h. Proteins (25 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) for phosphorylated Akt(Thr308) and Akt(Ser473) and for total Akt, and for phosphorylated PDK1(Ser241) and for total PDK1. The levels of β-actin were used to monitor protein loading. Data are representative of two independent experiments. SWD: serum withdrawal.

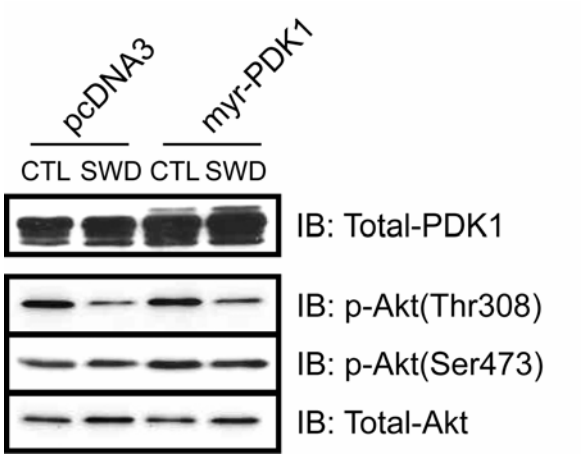


Figure 3.29: Membrane-bound PDK1 (myr-PDK1) does not attenuate Akt dephosphorylation induced by SWD. C6 cells were transfected with pcDNA3 or myristoylated (myr) pBJ5-myr-PDK1-FLAG (myr-PDK1) and 24 h post-transfection cells were cultured in serum-free medium for 4 h. Resolved proteins (25 μg of protein per lane) were immunoblotted (IB) for phosphorylated Akt(Thr308) and Akt(Ser473) and for total Akt and PDK1. Data are representative of two independent experiments.

3.5.5 SWD-induced decreased PDK1 mobility is PI3K-independent.

C6 cells overexpressing myr-Akt were cultured in serum-free medium for 4 hours. Akt phosphorylation in these cells was maintained during SWD. It did not affect the change in PDK1 mobility induced by serum-free conditions, thus suggesting that Akt does not appear to phosphorylate PDK1 as a possible feedback mechanism contributing to PDK1 mobility (Figure 3.30)

The decreased mobility of PDK1 in response to removing serum from C6 cell cultures may be induced by the increased PI3K activity demonstrated in Figure 3.6. C6 cells were treated with the PI3K inhibitor, LY260004, followed by the removal of serum from cultures. Inhibition of PI3K did not affect the mobility of PDK1 in response to SWD (Figure 3.31).

3.6 Tyrosine phosphorylation of PDK1.

There is evidence that tyrosine phosphorylation may also contribute to the regulation of PDK1 activity and function (Park et al., 2001; Prasad et al., 2000). The influence of SWD on tyrosine phosphorylation was investigated and compared with known inducers of PDK1 tyrosine phosphorylation, *e.g.* PV and Src.

3.6.1 SWD increases the tyrosine phosphorylation of PDK1.

C6 cells were grown in serum-free medium, treated with PV, or transfected with activated chicken SrcY527F (Src). Protein lysates were immunoprecipitated for PDK1 and analyzed for tyrosine phosphorylation using the phosphotyrosine-specific antibody 4G10. The tyrosine phosphorylation of PDK1 was slightly increased in response to SWD but to a greater exent with PV and activated Src (Figure 3.32). All treatments increased the total cellular tyrosine phosphorylation state (Figure 3.32), though SWD showed the smallest increase.

Cells were grown in serum-free medium followed by treatment with PV. The effect on PDK1 mobility was then assessed. The combination of SWD and PV resulted in a greater decrease in PDK1 mobility than with either SWD or PV alone (Figure 3.33), suggesting PV and SWD could be inducing PDK1 phosphorylation on different residues.

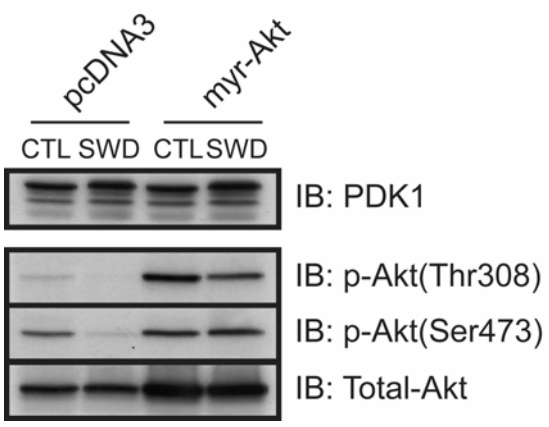


Figure 3.30: Myr-Akt does not affect the decrease in PDK1 mobility induced by SWD. C6 cells were transfected with pcDNA3 or myristoylated (myr) pCS2+-Akt (myr-Akt) and 24 h post-transfection cells were cultured in serum-free medium for 4 h. Resolved proteins (25 μg of protein per lane) and immunoblotted (IB) for phosphorylated Akt(Thr308) and Akt(Ser473) and for total Akt as well as for PDK1.

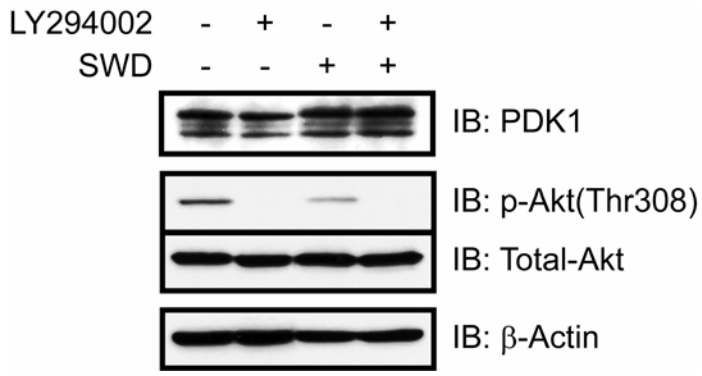
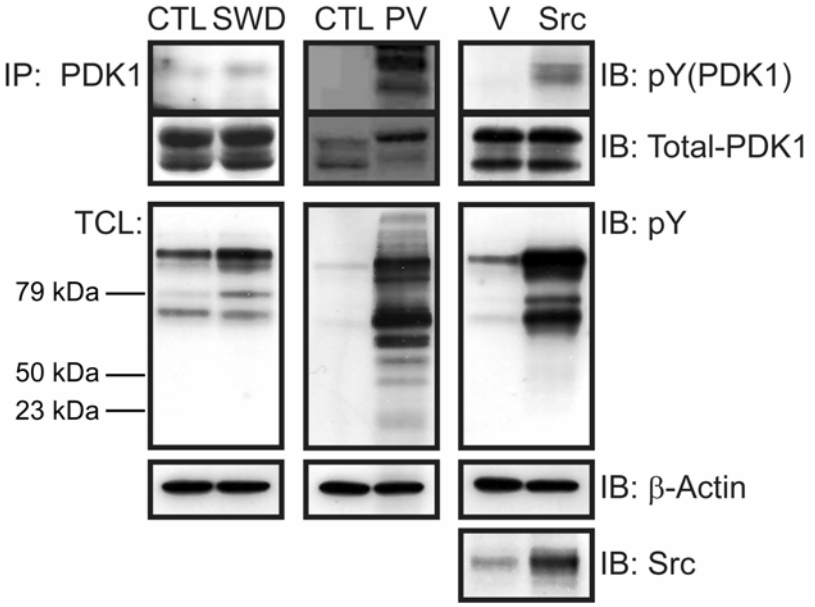


Figure 3.31: The SWD-induced decrease in PDK1 mobility is independent of PI3K. C6 cells were cultured in serum-free medium (4 h) and treated with 25 μM LY294002 for 30 min. Resolved proteins (25 μg of protein per lane) were immunoblotted (IB) for phosphorylated Akt(Thr308) and for total Akt and PDK1. The levels of β-actin were used to monitor protein loading. Data are representative of two independent experiments.

A.



B.

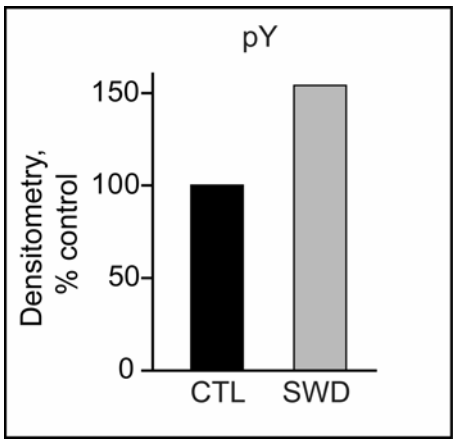


Figure 3.32: SWD, pervanadate and Src kinase induce an increase in PDK1 tyrosine phosphorylation. (**A**) C6 cells were grown in serum-free medium (SWD: serum withdrawal, 4 h), or treated with pervanadate (PV, 30 min), or transfected with pcDNA3 (V) or activated chicken SrcY527F kinase (Src). 300 μg protein lysates were used to immunoprecipitate (IP) PDK1. 25 μg of protein from corresponding total cell lysates (TCL) and immunoprecipitants were resolved by SDS-PAGE and immunoblotted (IB) for phosphotyrosine (pY), total PDK1, Src and β-actin. (**B**) Densitometric analysis of tyrosine phosphorylated PDK1 in response to SWD in (**A**); expressed as percent control. Data are representative of two independent experiments.

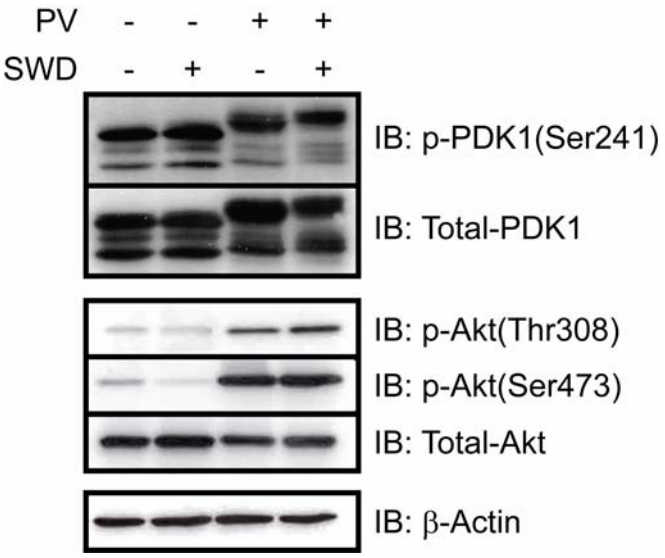


Figure 3.33: Effect of SWD and pervanadate on PDK1 mobility. C6 cells were grown in serum-free medium for 4 h and treated with pervanadate (PV) for 30 min. Proteins (25 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) for phosphorylated Akt(Thr308) and Akt(Ser473) and for total Akt, as well as for phosphorylated PDK1(Ser241) and for total PDK1. β-Actin were used to monitor protein loading. Data are representative of two independent experiments.

It was noted that treatment with PV increased the phosphorylation of Akt on Thr308 and Ser473 and blocked the loss of Akt phosphorylation induced by SWD (Figure 3.33).

3.7 Regulation of PDK1 by SHP-1.

The PDK1 sequence contains several tyrosine residues, several of which are found in immunoreceptor tyrosine-based inhibitory motifs (ITIMs). ITIMs with the consensus sequence pYXX(V/I/L), are recognized by the SH2 domains of SHP-1 (Doody et al., 1995). PDK1 Tyr, 9, 156, 248, 273, 299, 333, 376 and 486 fall into putative ITIMs for SHP-1 (Figure 3.34). Interestingly, two of them are also targeted by Src and PV, *e.g.* Tyr9 and Tyr376 (Park et al., 2001) (Figure 3.34). A recent study indicated that the SHP-1 is capable of dephosphorylating Src substrates (Frank et al., 2004). The potential regulation of PDK1 by SHP-1 was investigated.

3.7.1 SHP-1 associates with PDK1 in a phosphotyrosine-dependent manner.

The association between PDK1 and SHP-1 was examined using SWD, PV and activated Src, all of which are inducers of PDK1 tyrosine phosphorylation, SWD having only a modest effect on PDK1 tyrosine phosphorylation (recall Figure 3.32). SWD increased the association between PDK1 and SHP-1 (Figure 3.35A). It was noted that the basal tyrosine phosphorylation levels of PDK1 allowed for an association with SHP-1 (Figure 3.35A). In cells treated with PV the association with PDK1 was decreased, however, the PDK1 that was associated with SHP-1 had a decreased mobility compared with the control group (Figure 3.35B). Overexpressing activated Src induced an increased association between SHP-1 and PDK1 (Figure 3.35C).

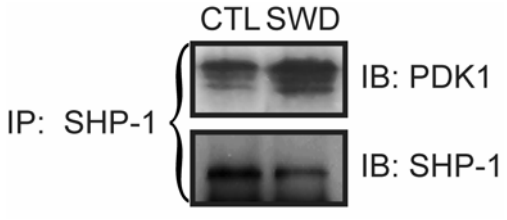
To determine if the association between SHP-1 and PDK1 was affected by PI3K activity, cells were treated with the PI3K inhibitor, LY294002. PI3K inhibition with LY294002 treatment was confirmed indirectly by the reduction in Akt phosphorylation in LY-treated groups (Figure 3.36). Inhibiting PI3K did not affect the association between SHP-1 and PDK1 (Figure 3.36).

SHP-1 contains three important amino acid residues that mediate dephosphorylation of tyrosine phosphorylated proteins. Cys455 acts as a nucleophile to attack the substrate; Arg459 stabilizes the negative charge of the phosphotyrosine

MARTTSQL**Y**[9]**DAV**PIQSSVVLCSCPSPSMVRTQTESSTPPGIPGGSRQGPAMD
GTAAEPRPGAGSLQHAQPPPQPRKKRPEDFKFGKILGEGSFSTVVLARELATS
REYAIKILEKRHIIKENKVPYVTRERDVMSRLDHPFFVKLYFTFQDDEKL**Y**[156]
FGLSYAKNGELLKYIRKIGSFDETCTRFYTAEIVSALEYLHGKGIIHRDLKPE
NILLNEDMHIQITDFGTAKVLSPESKQARAN**S**[241]FVGTAQ**Y**[248]**VSP**ELLTEKSA
CKSSDLWALGCII**Y**[273]**QLV**AGLPPFRAGNEYLIFQKIIKLE**Y**[299]**DFP**EKFFPKAR
DLVEKLLVLDATKRLGCEEMEG**Y**[333]**GPL**KAHPFFESVTWENLHQQTPPKLT
AYLPAMSEDDEDCY[373]GN**Y**[376]**DNL**LSQFGCMQVSSSSSSHSLSASDTGLPQRS
GSNIEQYIHDLDSNSFELDLQFSEDEKRLLLEKQAGGNPWHQFVENNLILKM
GPVDKRKGLFARRRQLLLTEGPHLY[485]**Y**[486]**VDP**VNKVLKGEIPWSQELRPEA
KNFKTFFVHTPNRTYYLMDPSGNAHKWCRKIQEVWRQRYQSHPDAAVQ

Figure 3.34: PDK1 protein sequence. The human PDK1 protein sequence was obtained from the National Center for Biotechnology Information (accession number NP_002604). The putative immunoreceptor tyrosine-based inhibitory motif (ITIMs) recognized by the SH2 domains of SHP-1 are highlighted in bold: Tyrosine (Y)9, Y156, Y248, Y273, Y299, Y333, Y376 and Y486. The Src kinase-targeted tyrosines are underlined: Y9, Y373, Y376 and Y485.

A.



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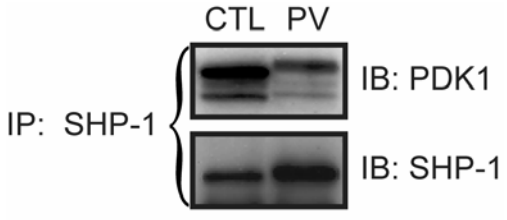


C.

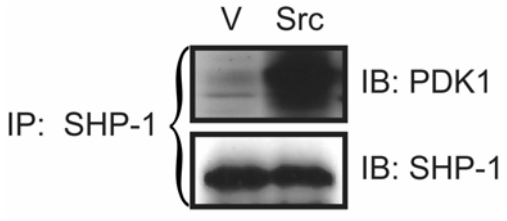


Figure 3.35: SHP-1 associates with PDK1. C6 cells were (**A**) cultured in serum-free medium (SWD: serum withdrawal, 4 h) or (**B**) treated for 30 min with pervanadate (PV) or (**C**) transfected with vector control (V) or SrcY527F (Src). Proteins (300 μg) were also immunoprecipitated (IP) with anti-SHP-1 and resolved by SDS-PAGE. Membranes were probed with anti-PDK1 and anti-SHP-1 antibodies. Data are representative of three independent experiments.

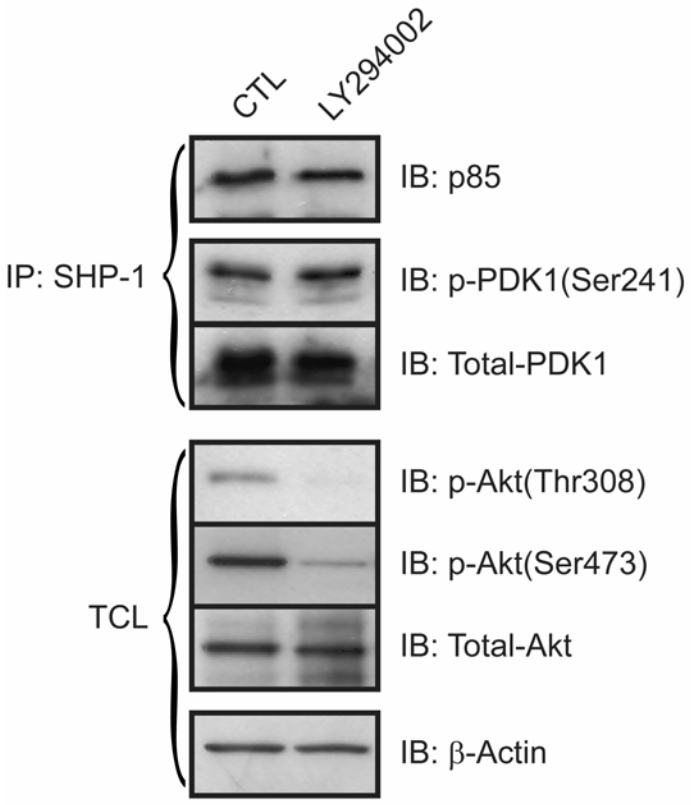


Figure 3.36: Inhibition of PI3K does not affect the association between SHP-1 and PDK1. C6 cells were treated with LY294002 (25 μM, 30 min). Resolved proteins (300 μg) were immunoprecipitated (IP) with anti-SHP-1 and immunoblotted (IB) for phosphorylated PDK1(Ser241) and for total PDK1 and p85. Corresponding proteins (25 μg of protein per lane) were resolved by SDS-PAGE and probed for phosphorylated Akt(Thr308), Akt(Ser473) and for total Akt. The levels of β-actin were used to monitor protein loading. Data are representative of two independent experiments.

substrate; and Asp421, the proton donor and proton acceptor controls substrate release (Yang et al., 2000; Yang et al., 1998). Dephosphoryation of tyrosine phosphorylated proteins by SHP-2 also follows this paradigm (Barford, 1999). Mutagenesis of the Asp that is responsible for substrate release (*i.e.* Asp421 for SHP-1 and Asp419 for SHP-2) can be used to study the interaction of these phosphatases with substrate proteins. Substitution of Asp421 to Ala in SHP-1 (and the analogous Asp419 to Ala in SHP-2) generates substrate-trapping mutants, SHP-1D421A and SHP-2D419A (Flint et al., 1997; Yu et al., 1998c). These mutants were used to identify a phosphotyrosine-dependent interaction with PDK1.

The pcDNA3-SHP-1 and pcDNA3-SHP-2 DNA plasmids were purified and sequenced (Figure 3.37). These were then coexpressed with activated SrcY527F (Src). The respective phosphatases were immunoprecipitated with specific antibodies. There was an increase in detectable PDK1 in SHP-1D241A immunoprecipitates suggesting that PDK1 is constitutively tyrosine phosphorylated. This was modestly increased by co-overexpressing activated Src (Figure 3.38A). In contrast, the association between SHP-2D419A and PDK1 was decreased in cells coexpressing activated Src (Figure 3.38B).

3.7.2 SHP-1 catalytic activity affects the tyrosine phosphorylation of PDK1.

A strategy for determining the function of a phosphatase is to use a catalytically inactive mutant protein to act as a dominant negative and block the function of the normal endogenous protein. C6 cells overexpressing SHP-1 (WT) and the inactive mutant SHP-1C455S were cultured in serum-free medium for 4 hours. Expression of SHP-1C455S blocked the decreased mobility of PDK1 in response to SWD (Figure 3.39). The change in PDK1 mobility induced by SWD was unaffected by overexpression of the pcDNA3 plasmid vector and SHP-1 (Figure 3.39).

To determine whether SHP-1 regulates the tyrosine phosphorylation on PDK1, C6 cells were cotransfected with activated Src and either SHP-1 or SHP-1C455S. Protein lysates were immunoprecipitated for PDK1. There was a significant decrease in the tyrosine phosphorylation of immunoprecipitated PDK1 in cells overexpressing SHP-1 and activated Src as compared to Src alone (Figure 3.40). Whereas a significant increase in PDK1 tyrosine phosphorylation was observed in immunoprecipitates from

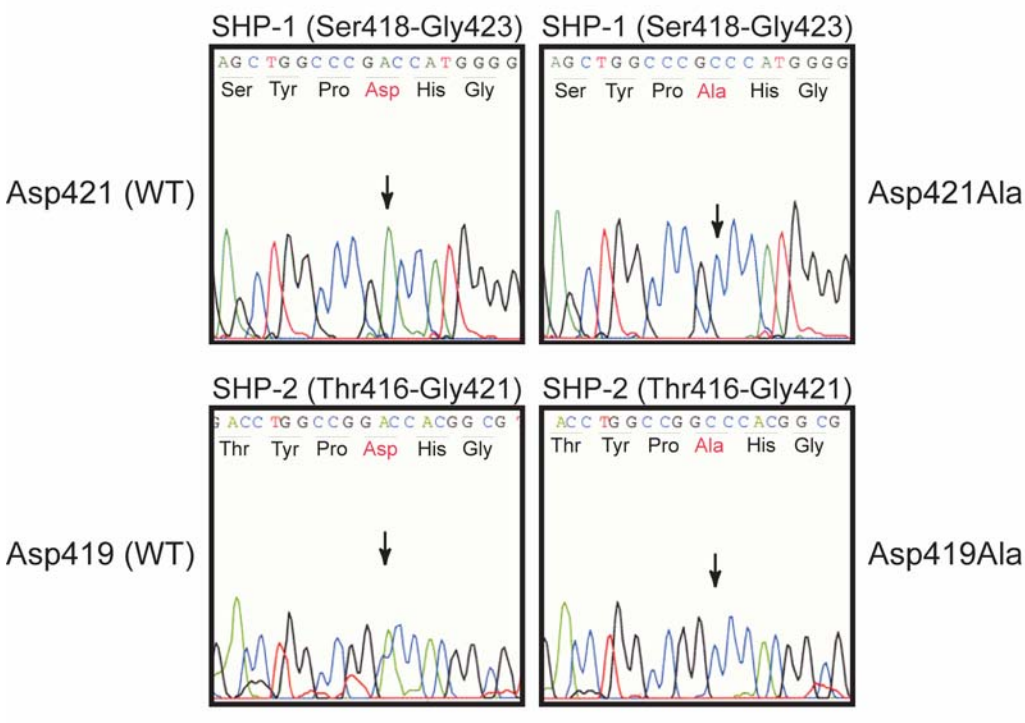
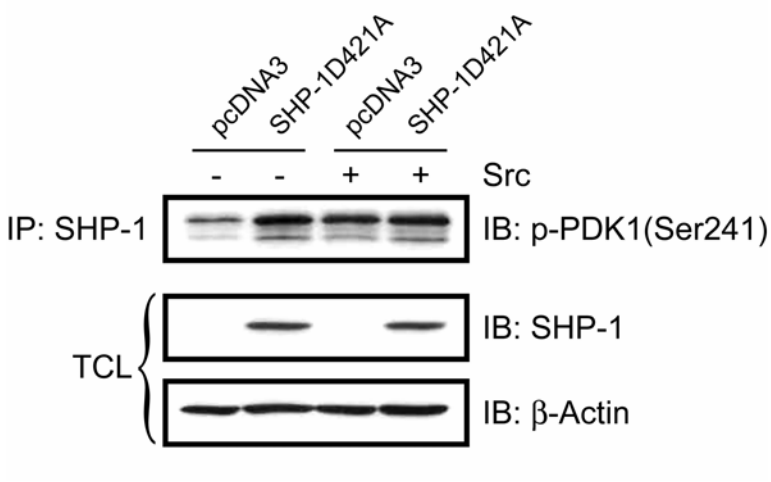


Figure 3.37: Chromatograms of SHP-1 and SHP-2 wildtype (WT) and substrate trapping mutants. SHP-1 and SHP-2 cDNA plasmids were sequenced and mutations were determined. (upper panel) SHP-1 WT and substrate trapping mutant Asp241Ala and the (lower panel) SHP-2 WT and substrate trapping mutant Asp419Ala were confirmed.

A.



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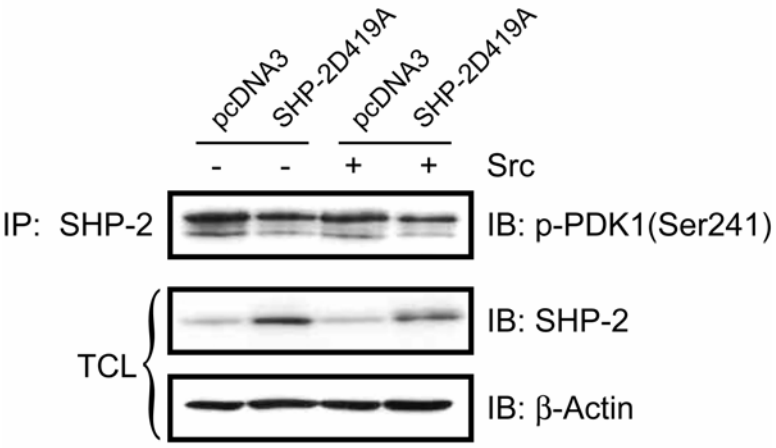


Figure 3.38: PDK1 associates with SHP-1 or SHP-2 substrate trapping mutants. (**A**) C6 cells were cotransfected with activated SrcY527F (Src) or pcDNA3-SHP-1D421A (SHP-1D421A) and 24 h post-transfection, proteins (300 μg) were immunoprecipitated with anti-SHP-1. (**B**) C6 cells were cotransfected with activated SrcY527F (Src) or pcDNA3-SHP-2D419A (SHP-2D419A) and proteins were immunoprecipitated with anti-SHP-2. Immunoprecipitants and corresponding protein lysates (25 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) with anti-PDK1(Ser241), anti-SHP-1 or anti-SHP-2 antibodies. The levels of β-actin were used to monitor protein loading. Data are representative of two independent experiments.

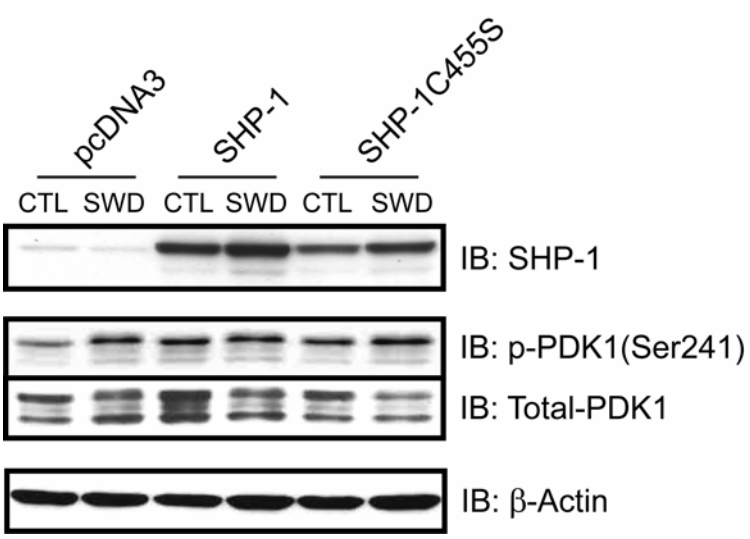


Figure 3.39: SHP-1 catalytic activity contributes to the decrease in PDK1 mobility induced by SWD. C6 cells were transfected with pcDNA3 or pcDNA3-SHP-1 (SHP-1) or pcDNA3-SHP-1C455S (SHP-1C455S) and 24 h post-transfection, cells were grown in serum-free medium for 4 h. Resolved proteins (25 μg of protein per lane) were probed for phosphorylated PDK1(Ser241) and for total PDK1 and SHP-1. The levels of β-actin were used to monitor protein loading. Data are representative of two independent experiments.

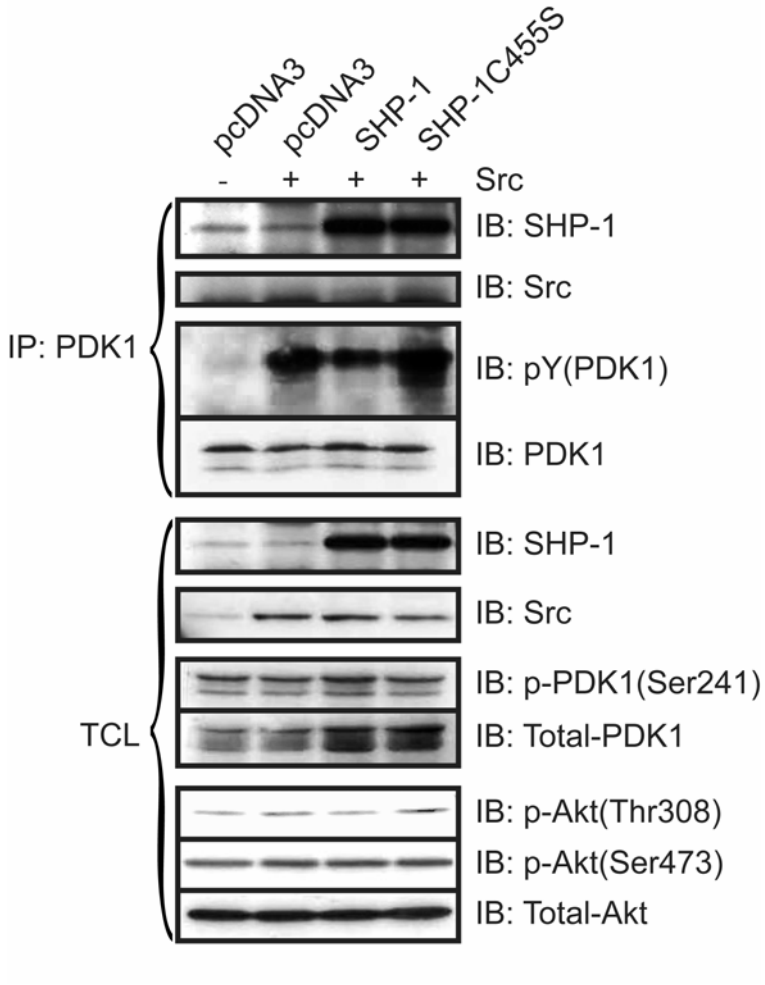


Figure 3.40: The catalytic activity of SHP-1 affects PDK1 tyrosine phosphorylation. C6 cells were cotransfected with activated SrcY527F (Src) and pcDNA3 or pcDNA3-SHP-1 (SHP-1) or pcDNA3-SHP-1C455S (SHP-1C455S). Proteins (300 μg) were immunoprecipitated (IP) from cell lysates with a PDK1 antibody. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted (IB) with anti-phosphotyrosine (pY) and anti-PDK1 antibodies. Corresponding proteins were resolved by SDS-PAGE and immunoblotted for phosphorylated Akt(Thr308) and Akt(Ser473) and for total Akt, as well as for phosphorylated PDK1(Ser241) and for total PDK1. Overexpression of proteins was determined using anti-SHP-1 and anti-Src antibodies. Data are representative of three independent experiments.

cells overexpressing the catalytic mutant as compared to Src alone and cells coexpressing Src and SHP-1 (Figure 3.40).

3.7.3 Mutagenesis of PDK1 on tyrosine residues.

Substituting tyrosine (Tyr/Y) residues with phenylalanine (Phe/F) [*i.e.* dephosphorylation mimic] is a strategy for examining the importance of specific tyrosine residues on the association between proteins. In order to distinguish endogenous PDK1 from PDK1 substitution mutants when employing Western blot/immunoprecipitations (and planning ahead for immunofluorescence studies), PDK1 subcloned into the pEGFP expression vector was used. pEGFP-PDK1 single as well as double Y-to-F substitution mutants were generated (Y9F, Y333F, Y373F, Y376F, Y9/376F and Y333/373F). The single mutations were chosen based on ITIMS for SHP-1 binding and Src phosphorylation sites (recall Figure 3.34).

Primers for mutagenesis of PDK1 were designed based on the human PDK1 nucleotide sequence, accession number NM002613 (Figure 3.41). The generated pEGFP-PDK1 plasmids were purified and sequenced (Figure 3.42A shows an example chromatogram and Figure 3.42B shows the deduced amino acid sequence). The Y-to-F substitution mutants were generated by site-directed mutagenesis and confirmed by sequencing (Figure 3.43).

3.7.4 Effect of PDK1 tyrosine substitution mutants on the association with SHP-1 and on Akt phosphorylation.

The EGFP-PDK1 mutants were overexpressed in C6 cells and migrate at a molecular weight of ~90 kDa (which corresponds to PDK1: ~63 kDa plus GFP: ~30 kDa) (Figure 3.44). The interaction between endogenous SHP-1 and the EGFP-PDK1 mutants was not consistent in that some experiments showed a decreased association between SHP-1 and EGFP-Y9F and -Y376F mutants and other experiments showed the association was maintained (Figure 3.44).

The overexpression of the EGFP-PDK1 tyrosine mutants exerted effects on Akt phosphorylation that also were not very clear/consistent. The experiment was repeated several times and each experiment generated different results regarding the

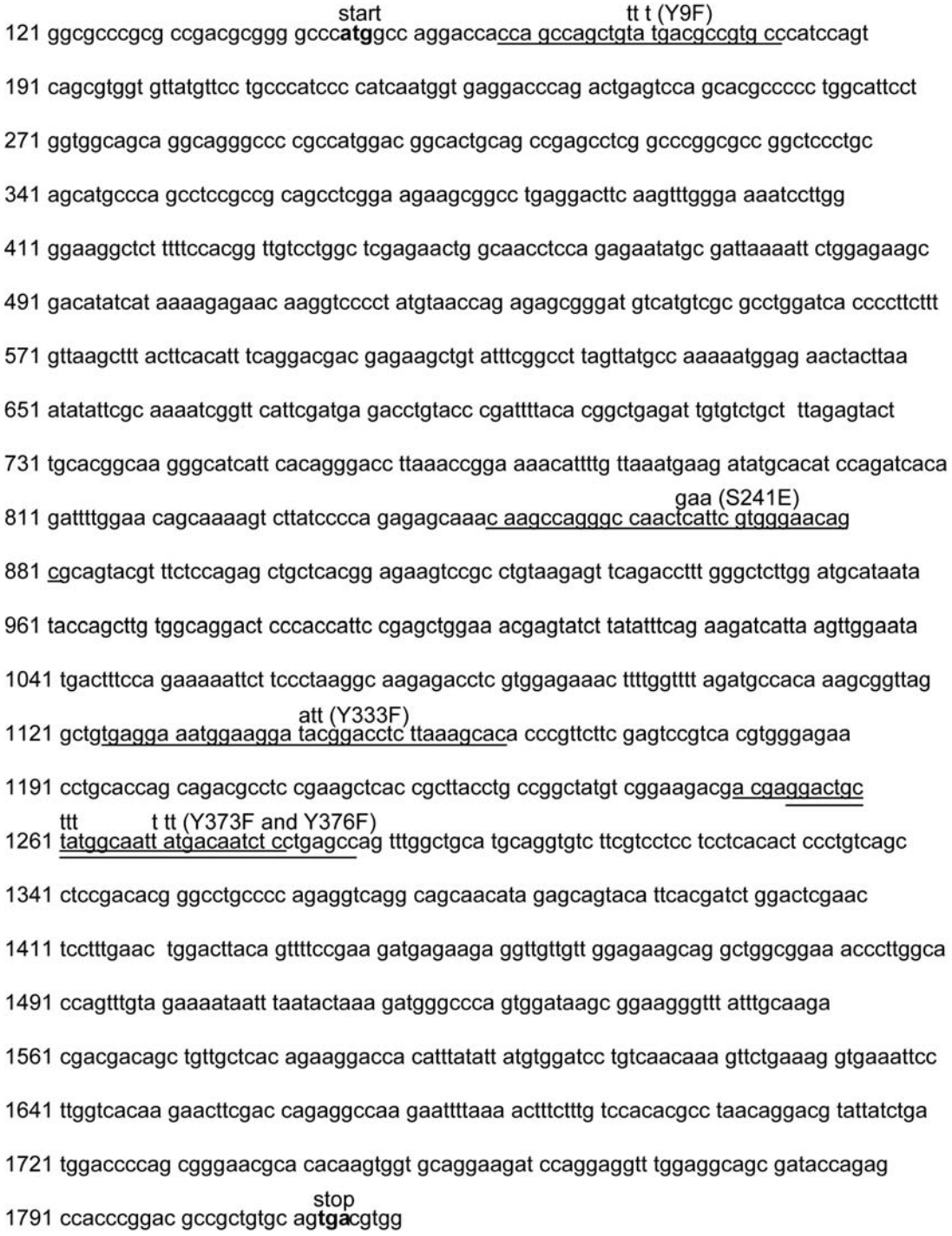
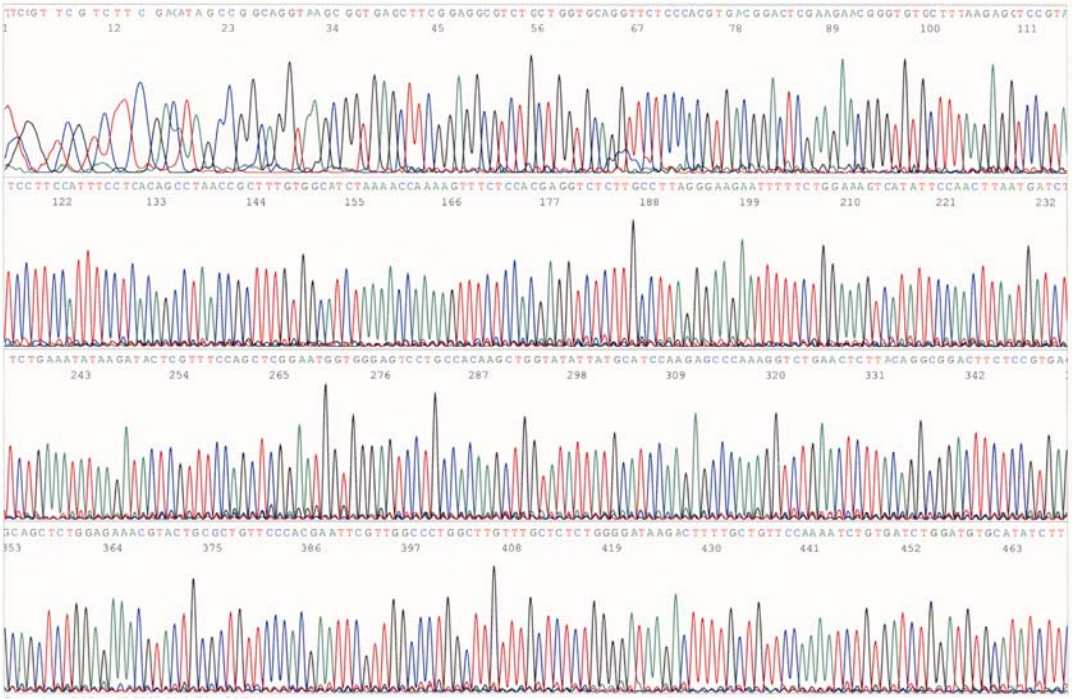


Figure 3.41: PDK1 DNA sequence. Primers were designed based on the human PDK1 nucleotide sequence obtained from the National Center for Biotechnology Information (accession number NM_002613). The underlined portion of the sequence indicates where the primers for mutagenesis were designed. The text above the underlined portion of the DNA indicates the generated mutation and in brackets the generated amino acid change. Start codon (atg) and stop codon (tgc) are as indicated.

A.



B.

MARTTSQL**Y**[9]DAVPIQSSVVLCSCPSPSMVRTQTESSTPPGIPGGSRQGPAMDGTAA
EPRPGTGSLQHAQPPPQPRKKRPEDFKFGKILGEGSFSTVVLARELATSREYAIKILEK
RHIIKENKVPYVTRERDVMSRLDHPFFVKLYFTFQDDEKLYFGLSYAKNGELLKYIRKIG
SFDETCTRFYTAEIVSALEYLHGKGIIHRDLKPENILLNEDMHIQITDFGTAKVLSPESKQ
ARAN**S**[241]FVGTAQYVSPELLTEKSACKSSDLWALGCIIYQLVAGLPPFRAGNEYLIFQKII
KLEYDFPEKFFPKARDLVEKLLVLDATKRLGCEEMEG**Y**[333]GPLKAHPFFESVTWENLH
QQTPPKLTAYLPAMSEDDEDC**Y**[373]GN**Y**[376]DNLLSQFGCMQVSSSSSSHSLSASDTGL
PQRSGSNIEQYIHDLDSNSFELDLQFSEDEKRLLLEKQAGGNPWHQFVENNLILKMGP
VDKRKGLFARRRQLLLTEGPHLYYVDPVNKVLKGEIPWSQELRPEAKNFKTFFVHTPN
RTYYLMDPSGNAHKWCRKIQEVWRQRYQSHPDAAVQ

Figure 3.42: PDK1 chromatogram and deduced amino acid sequence. PDK1 plasmid DNA was extracted using the alkaline lysis method and sequenced. (**A**) Chromatogram of wildtype PDK1. (**B**) PDK1 amino acid (single-letter code) sequence.

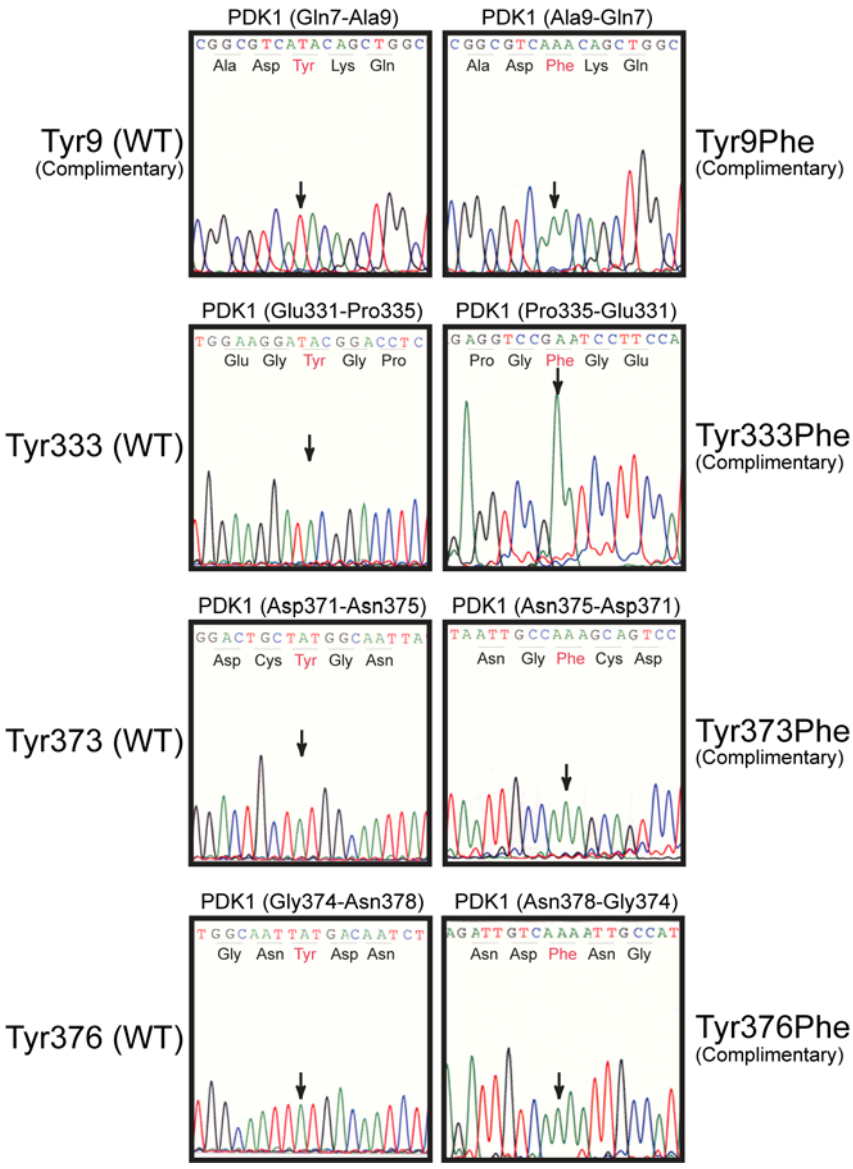


Figure 3.43: Chromatograms showing PDK1 mutations. PDK1 tyrosines (Tyr)9, 333, 373 and 376 were substituted with a phenylalanine (Phe) using the Quikchange® site-directed mutagenesis kit. PDK1 plasmid DNA was extracted using the alkaline lysis method and sequenced.

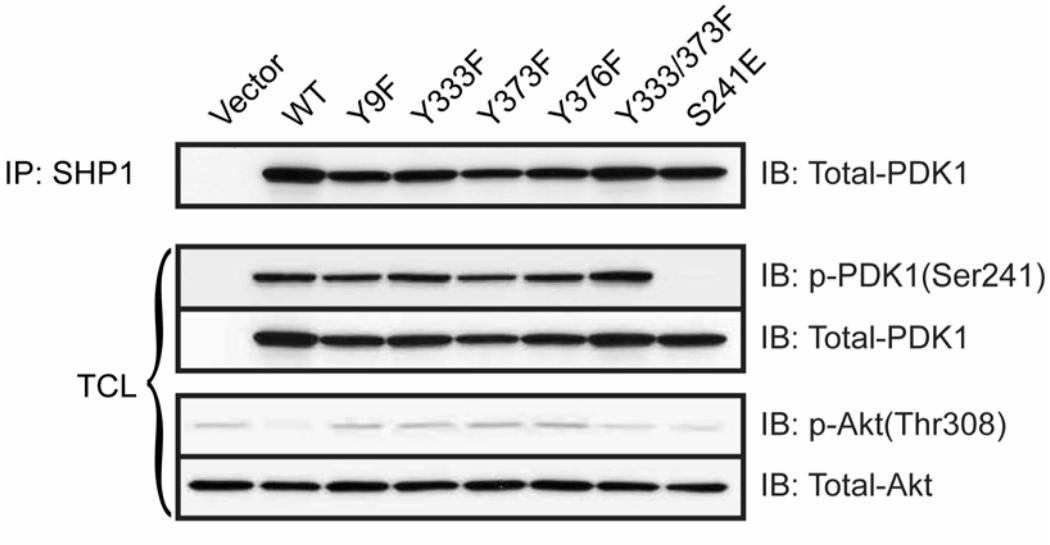


Figure 3.44: PDK1 substitution mutants, Y9F, Y333F, Y373F, Y376F, Y333/373F and S241E associate with SHP-1. C6 cells were transfected with pEGFP (Vector) or pEGFP-PDK1 wildtype (WT) or pEGFP-PDK1 substitution mutants, Y9F, Y333F, Y373F, Y376F, Y333/373F, or S241E. 24 h post-transfection, proteins (300 μg) were immunoprecipitated (IP) from cell lysates with the SHP-1 antibody and resolved by SDS-PAGE and immunoblotted (IB) with for PDK1 (the GFP-fusion protein migrates at ~90 kDa). Corresponding proteins (25 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) for phosphorylated Akt(Thr308) and for total Akt, and for phosphorylated PDK1(Ser241) and for total PDK1. Data are representative of three independent experiments.

phosphorylation of Akt (Figure 3.44). The expression of the activated EGFP-PDK1 mutant, S241E, did not increase the phosphorylation of Akt (Figure 3.44).

3.7.5 PDK1 Y-to-F mutants do not generally affect proliferation of C6 cells.

The effect of the EGFP-PDK1 substitution mutants on cell proliferation was assessed using the MTT reduction assay. Cells overexpressing EGFP-Y373F or EGFP-S241E had a modest reduction in proliferation compared to cells overexpressing EGFP-PDK1 (WT) or any of the other substitution mutants (Figure 3.45).

3.7.6 PDK1Y9F is insensitive to PV treatment.

C6 cells overexpressing the EGFP-PDK1 substitution mutants were treated with PV for 30 minutes. All EGFP-PDK1 substitution mutants, except for EGFP-Y9F responded to pervanadate with a decrease in mobility (Figure 3.46), as expected (recall Figure 3.33). Akt phosphorylation was also examined in these lysates and was increased by PV treatment in cells overexpressing EGFP-PDK1, -Y9F and -Y376F (Figure 3.46). The increase was lower in cells overexpressing EGFP-Y333F, -Y373F and -S241E (Figure 3.46). This suggests that the change in PDK1 mobility upon PV-treatment may be due to phosphorylation at Tyr9.

3.7.7 Effect of SWD on the interaction between SHP-1 and PDK1 mutants.

C6 cells overexpressing the EGFP-PDK1 substitution mutants were grown in serum-free medium for 4 hours. There was an increased association between SHP-1 and the EGFP-PDK1 substitution mutants EGFP-Y333F and -Y373F compared to EGFP-PDK1 (WT) (Figure 3.47). Akt phosphorylation was modestly decreased in groups expressing EGFP-PDK1 (WT), -Y9F and -Y376F (Figure 3.47), but maintained in groups overexpressing the EGFP-Y333F and -Y373F substitution mutants (Figure 3.47).

3.7.8 Effect of PDK1 double Y-to-F mutants on the interaction with SHP-1.

The results in Sections 3.7.6 and 3.7.7 indicate that in response to pervanadate or SWD, the PDK1 substitution mutants EGFP-Y9F and -Y376F exerted different effects than did the EGFP-Y333F and -Y373F mutants on Akt phosphorylation.

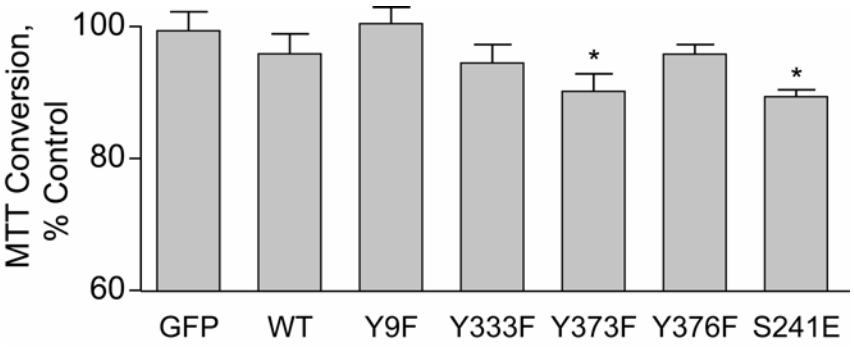


Figure 3.45: PDK1 Y-toF substitution mutants do not generally affect C6 cell proliferation. Cells were grown in a 96 well plate and transfected with pEGFP (GFP) or pEGFP-PDK1 wildtype (WT) or pEGFP-PDK1 substitution mutants, Y9F, Y333F, Y373F, Y376F, or S241E. 24 h post-transfection, MTT conversion (proliferation) was assessed. Y: tyrosine, F: phenylalanine, S: serine, E: glutamic acid. *: $P<0.05$ *versus* vector control (GFP). Data are represented as mean ± SD, n=3.

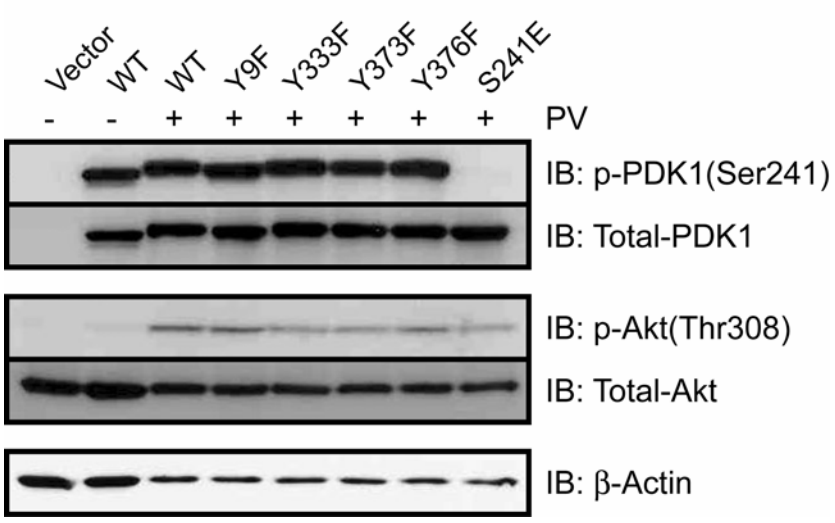
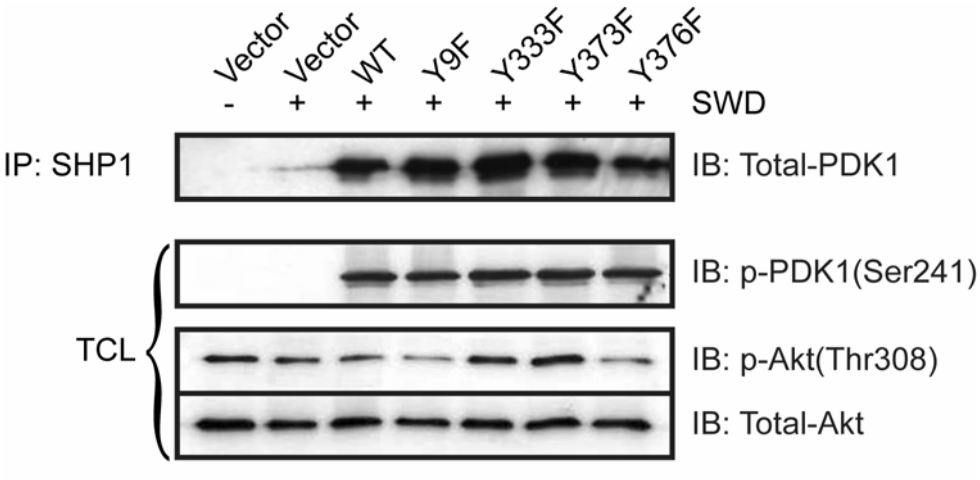


Figure 3.46: PDK1 substitution mutant, Y9F does not respond to pervanadate (PV) treatment. C6 cells were transfected with pEGFP (Vector) or pEGFP-PDK1 wildtype (WT), or pEGFP-PDK1 substitution mutants, Y9F, Y333F, Y373F, Y376F, Y333/373F, or S241E and 24 h post-transfection treated with pervanadate (PV) for 30 min. Proteins (25 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) for phosphorylated Akt(Thr308) and for total Akt, as well as for phosphorylated PDK1(Ser241) and for total PDK1. The levels of β-actin were used to monitor protein loading. Y: tyrosine, F: phenylalanine, S: serine, E: glutamic acid. Data are representative of one experiment.

A.



B.

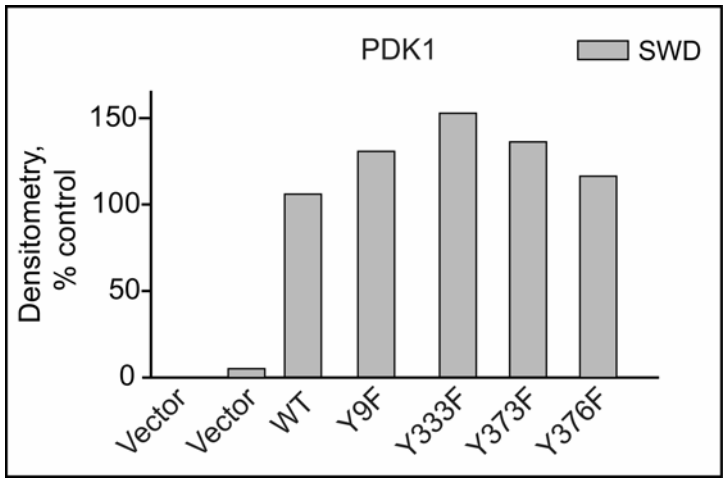


Figure 3.47: SWD induces an increased association between SHP-1 and PDK1 substitution mutants, Y333F and Y373F. (**A**) C6 cells were transfected with pEGFP (Vector) or pEGFP-PDK1 wildtype (WT), or pEGFP-PDK1 substitution mutants, Y9F, Y333F, Y373F, Y376F, or S241E. 24 h post-transfection, cells were grown in serum-free medium (SWD) for 4h. Proteins (300 μg) were immunoprecipitated (IP) from cell lysates with the SHP-1 antibody and resolved by SDS-PAGE and immunoblotted (IB) with for PDK1. Corresponding proteins (25 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) for phosphorylated Akt(Thr308) and for total Akt, as well as for phosphorylated PDK1(Ser241) and for total PDK1. (**B**) Densitometry, % control for PDK1 associated with immunoprecipitated SHP-1 in (**A**). Data are representative of two independent experiments. Y: tyrosine, F: phenylalanine.

Double substitution mutations of these tyrosine pairs were generated and sequenced (Figure 3.43). C6 cell overexpressing the double substitution mutants were grown in serum-free medium for 30 min. The association between EGFP-Y9/376F and SHP-1 was decreased both under control and SWD (Figure 3.48). The association between EGFP-Y333/373F and SHP-1 was unchanged (Figure 3.48).

Overexpression of EGFP-Y9/376F decreased the phosphorylation of Akt (Thr308) and did not have any additional effects on Akt phosphorylation when serum was removed (Figure 3.48). EGFP-Y333/373F had no effect on Akt phosphorylation during SWD (Figure 3.48). This suggests that unlike EGFP-Y333/373F, EGFP-Y9/376F is functioning like a dominant negative towards Akt phosphorylation/function.

3.7.9 Src decreases the association between SHP-1 and PDK1Y9/376F.

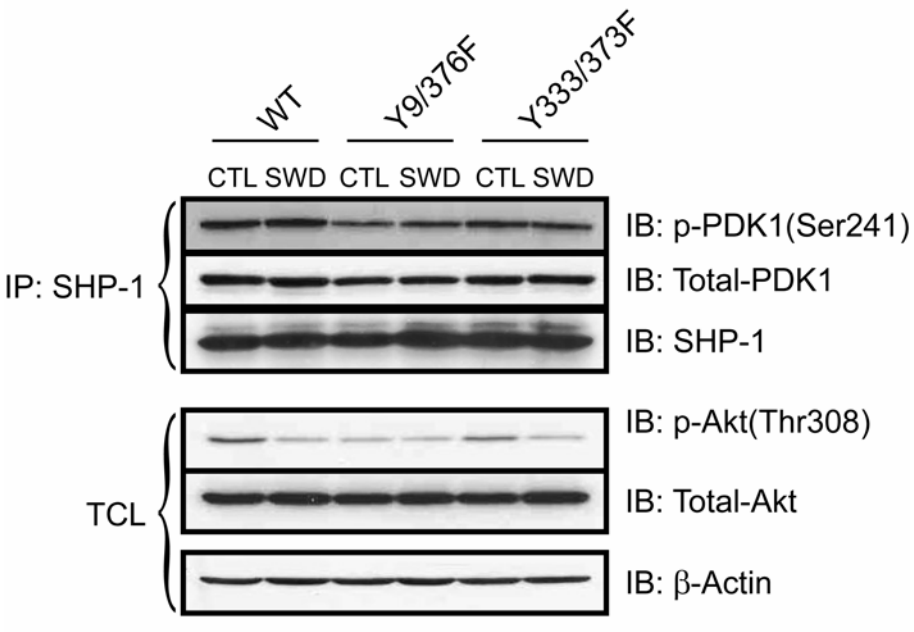
Src was used in the following experiment to determine the importance of tyrosine residues Y9 and Y376 for the recognition and interaction with SHP-1.

C6 cells were cotransfected with activated Src, and either EGFP-Y9/376F or EGFP-Y333/373F. There was a significant loss of association between endogenous SHP-1 and EGFP-Y9/376F when co-expressed with Src (Figure 3.49). The association between endogenous SHP-1 and EGFP-Y333/373F was unchanged from control groups (Figure 3.49). These data suggest Src phosphorylation of PDK1 on Y9 and Y376 contributes to the interaction between SHP-1 and PDK1, but does not address the possibility that Src could be indirectly affecting PDK1 Y9 and Y376 *via* some other cellular intermediate.

3.8 Cellular localization of SHP-1 and PDK1.

Both SHP-1 and PDK1 are shown to function at the plasma membrane (Anderson et al., 1998; Cuevas et al., 1999; Stephens et al., 1998; Yu et al., 1998c); however, there are reports that they can also localize to the nucleus (Craggs and Kellie, 2001; Lim et al., 2003; Yang et al., 2002b). SHP-1 localization to the nucleus depends on its nuclear localization sequence (NLS) (Craggs and Kellie, 2001; Yang et al., 2002b), however, the role of nuclear SHP-1 is unclear as is its means of export from the nucleus.

A.



B.

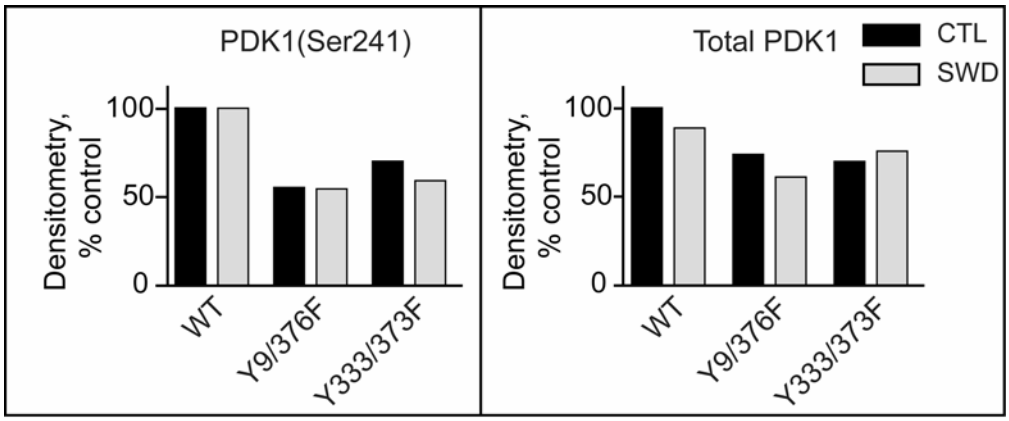
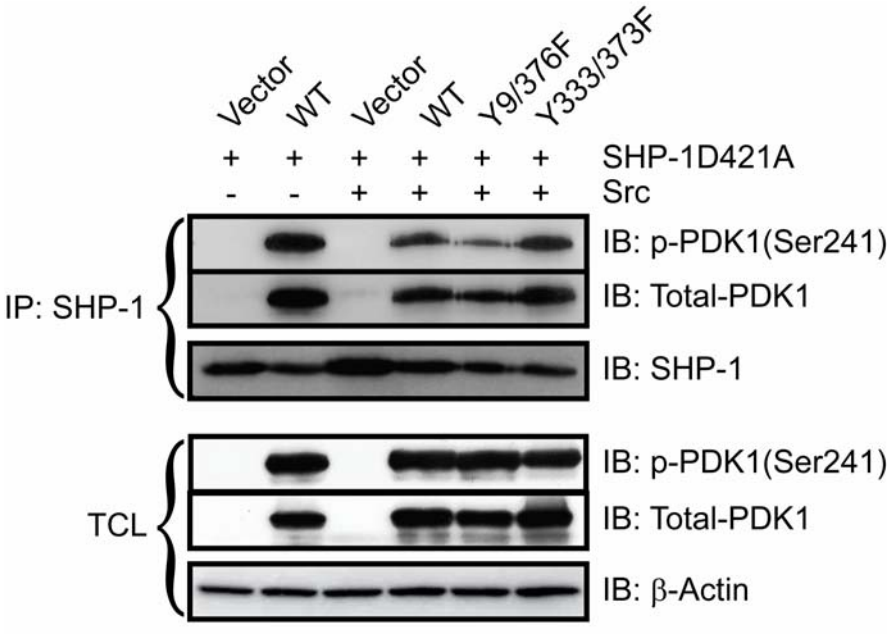


Figure 3.48: There is a decreased association between SHP-1 and the PDK1 double substitution mutant Y9/376F. C6 cells were transfected with pEGFP-PDK1 wildtype (WT), or pEGFP-PDK1 double substitution mutants, Y9/376F or Y333/373F. 24 h post-transfection, cells were grown in serum-free medium (SWD, 30 min). Proteins (300 μg) were immunoprecipitated (IP) from cell lysates with the SHP-1 antibody and resolved by SDS-PAGE and immunoblotted (IB) with for PDK1. Corresponding proteins (25 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) for phosphorylated Akt(Thr308) and for total Akt as well as for phosphorylated PDK1(Ser241) and for total PDK1 and β-actin. (**B**) Densitometric analysis of SHP-1-immunoprecipitated PDK1 in (**A**); expressed as percent control of WT PDK1. Data are representative of two independent experiments. Y: tyrosine, F: phenylalanine.

A.



B.

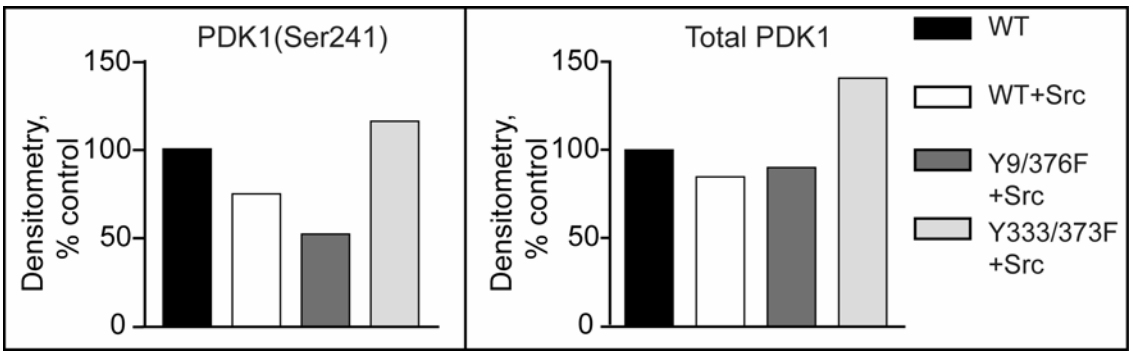


Figure 3.49: There is a decreased association between SHP-1 and the PDK1 double substitution mutant Y9/376F when cotransfected with activated Src. C6 cells were cotransfected with SHP-1D421A and SrcY527F (Src) and pEGFP-PDK1 wildtype (WT), or pEGFP-Y9/376F (Y9/376F), or pEGFP-Y333/373F (Y333/373F). Proteins (300 μg) were immunoprecipitated (IP) with the SHP-1 antibody and resolved by SDS-PAGE. Membranes were immunoblotted (IB) for phosphorylated PDK1(Ser241) and for total PDK1 and for SHP-1. Corresponding total cell lysates (TCL) were immunoblotted for the same proteins. The levels of β-actin were used to monitor protein loading. (**B**) Densitometric analysis of SHP-1-immunoprecipitated PDK1 in (**A**); expressed as percent control of WT PDK1/no Src. Data are representative of two independent experiments. Y: tyrosine, F: phenylalanine.

Export of PDK1 from the nucleus has been demonstrated to be mediated through its own nuclear export sequence (NES) (Lim et al., 2003). However, the mechanism used for nuclear import by PDK1 is also unknown.

3.8.1 SHP-1 and PDK1 are redistributed to the nucleus in response to SWD in C6 cells.

To determine if SHP-1 interacts with PDK1 at the plasma membrane during SWD, membrane-directed myr-PDK1 (myr-PDK1) was expressed in C6 cells. Its association with SHP-1 was then determined. Under control conditions there was a significant association between endogenous SHP-1 and myr-PDK1. During SWD the association with membrane-directed PDK1 was significantly decreased (Figure 3.50).

The association between endogenous SHP-1 and PDK1 was examined in subcellular fractions. The association between SHP-1 and PDK1 was maintained in the in the nuclear extracts in response to SWD (Figure 3.51). In the cytoplasmic fraction PDK1-associated with SHP-1 has a modest decreased mobility as compared with PDK1-assciated with SHP-1 in the nuclear extract (Figure 3.51).

Confocal microscopy was used to monitor the localization of these proteins in C6 cells during SWD. It was demonstrated that endogenous SHP-1 and EGFP1-PDK1 colocalized to the nucleus in response to SWD (Figure 3.52). This corresponded with a loss of cytoplasmic Akt (Ser473 and Thr308) phosphorylation in response to SWD (Figure 3.53). This loss of phosphorylation was not due to a loss of total Akt protein during SWD (Figure 3.53, upper panel), thus confirming our results obtained using Western blot (recall Figure 3.7). Subcellular fractionation was conducted to confirm theses findings (Figure 3.54).

Scheid *et al.* (2005) demonstrated that nuclear localization of PDK1, and the ensuing increase in nuclear Akt, blocked the translocation of FOXO3a to the nucleus. Localization of FOXO3a was examined in C6 cells and was distributed throughout the cell. The signal began to localize more to the nucleus after 30 min of SWD (Figure 3.55).

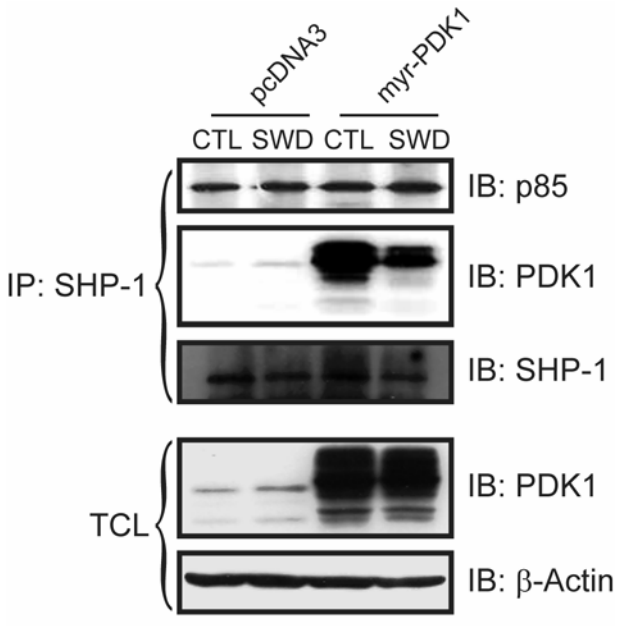


Figure 3.50: The association between SHP-1 and PDK1 is reduced at the plasma membrane in response to SWD. C6 cells were transfected with pcDNA3 and myristoylated (myr) pBJ5-myr-PDK1-FLAG (myr-PDK1) and 24 h post-transfection grown in serum-free medium for 4 h. Proteins (300 μg) were immunoprecipitated (IP) with anti-SHP-1 antibody and the immunoprecipitates were resolved by SDS-PAGE and probed for p85 and PDK1. Corresponding proteins were resolved by SDS-PAGE and immunoblotted (IB) with anti-PDK1 and anti-β-actin. SWD: serum withdrawal. Data are representative of two independent experiments.

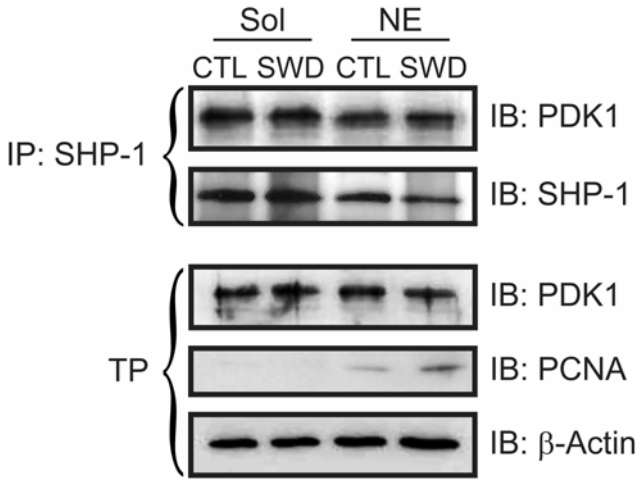


Figure 3.51: SHP-1 associates with PDK1 in the nucleus of C6 cells. C6 cells were grown in serum-free medium (SWD: serum withdrawal, 4 h) and nuclear extracts (NE) were obtained. Proteins (300 μg) were immunoprecipitated (IP) with anti-SHP-1 antibody and the immunoprecipitates were resolved by SDS-PAGE and probed for phosphorylated PDK1(Ser241) and for total PDK1. Corresponding total protein (TP, 30 μg of protein per lane) were resolved by SDS-PAGE and immunoblotted (IB) with anti-PDK1(Ser241), anti-total PDK1 and anti-β-actin. Fraction purity was determined using anti-PCNA. Data are representative of two independent experiments.

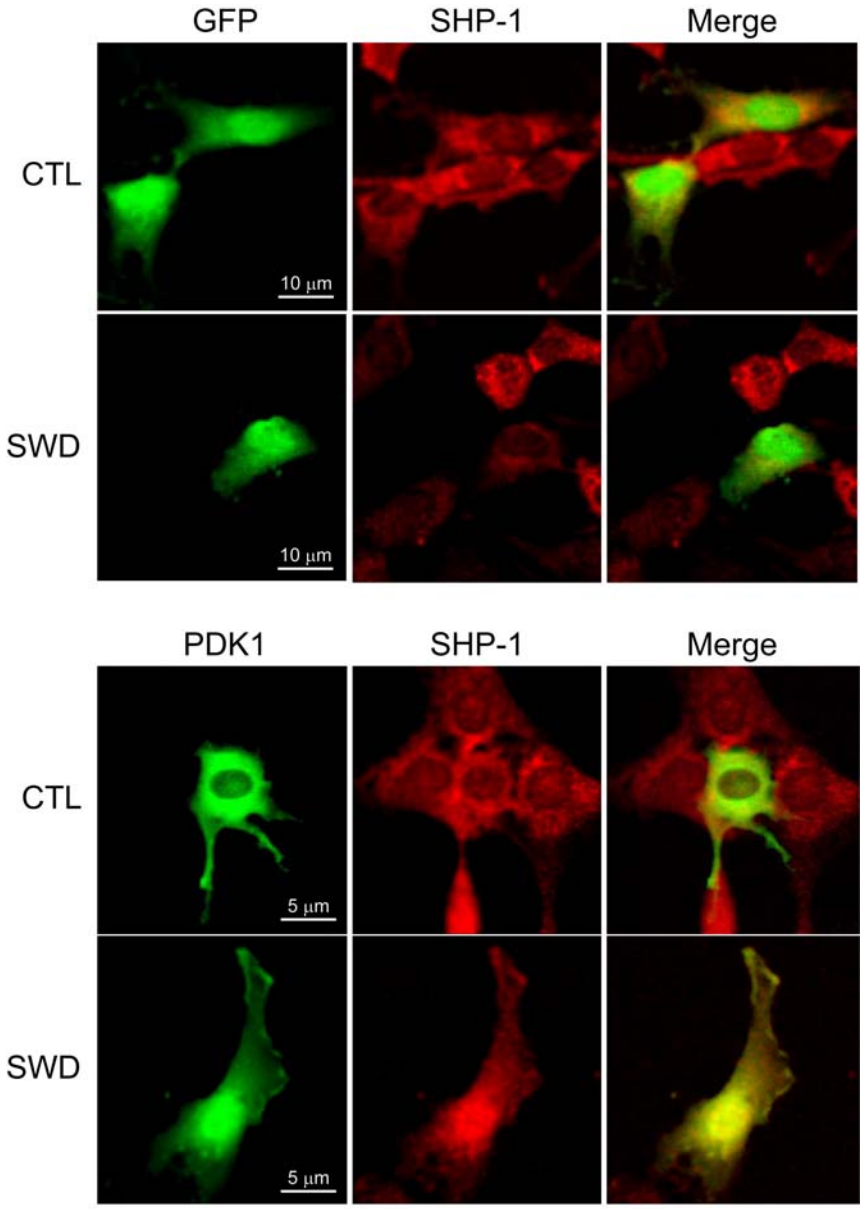


Figure 3.52: SHP-1 and PDK1 localize to the nucleus in response to SWD. C6 cells were transfected with pEGFP (EGFP) or pEGFP-PDK1 (PDK1) and 24 h post-transfection grown in serum-free medium (30 min). Cells were fixed, permeabilized, and incubated with primary antibody SHP-1 (1:500), followed by incubation with the secondary antibody AlexaFluor 594 (1:1000). Samples were analyzed using an Olympus FV300 confocal microscope. SWD: serum withdrawal. Excitation/emission wavelengths (nm) are AlexaFluor 594 (red): 590/617 and EGFP (green): 488/507. Data are representative of three independent experiments.

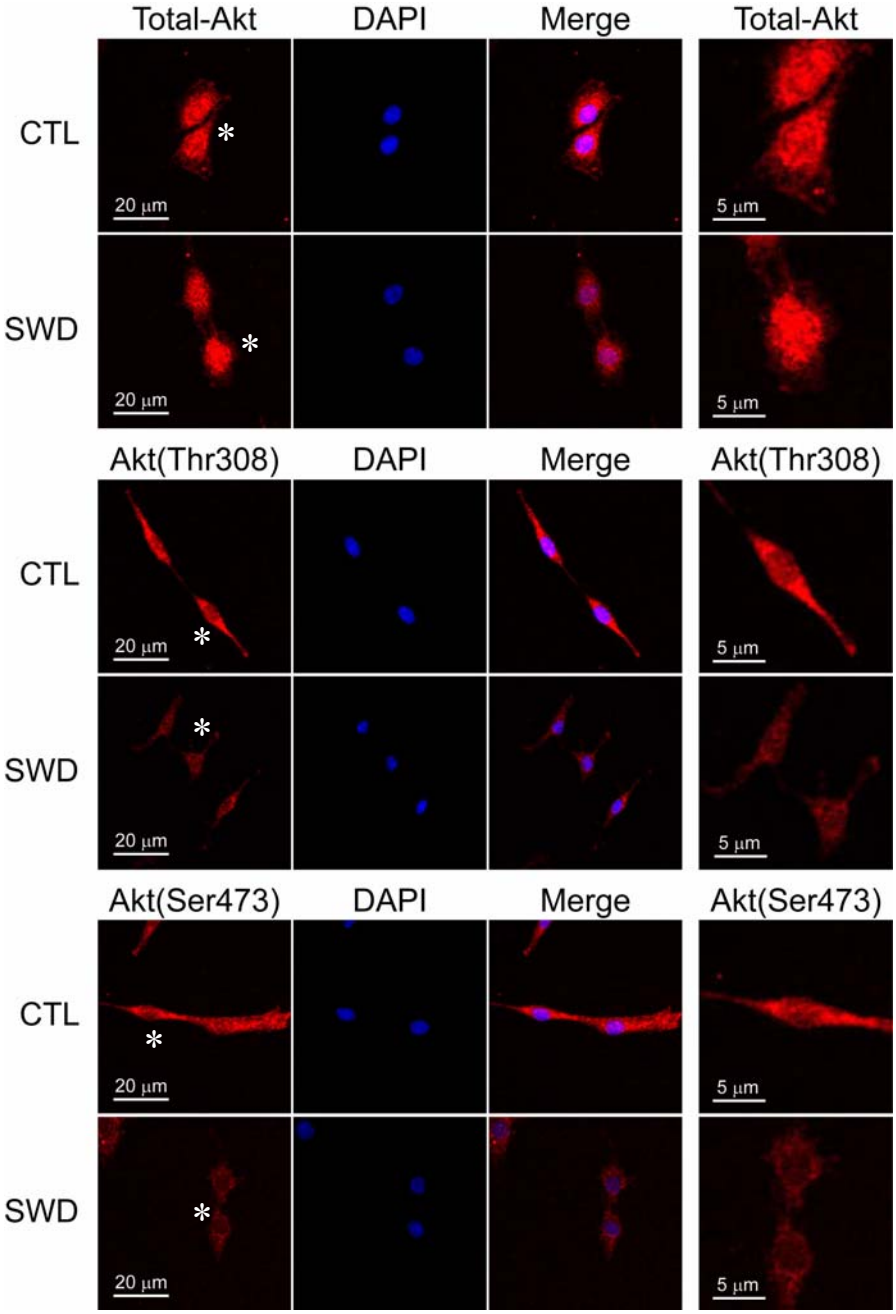
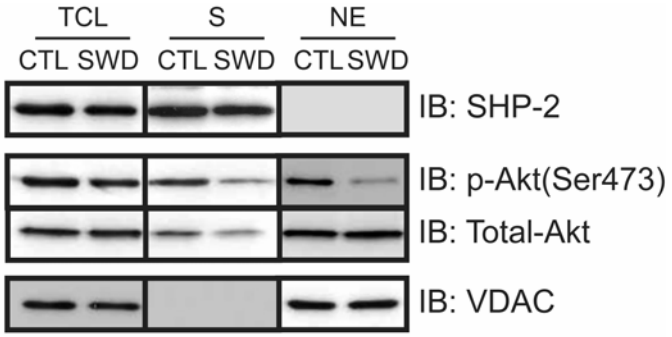


Figure 3.53: Cellular distribution of Akt phosphorylation in response to SWD. C6 cells were grown in serum-free medium (30 min). Cells were fixed, permeabilized, and incubated with primary antibodies for total Akt, Akt(Thr308), or Akt(Ser473), followed by incubation with the secondary antibody AlexaFluor 594 (1:1000). Samples were then stained with DAPI (1 µg/ml, 5 min) and analyzed using a Zeiss LSM 510 META confocal microscope. SWD: serum withdrawal. Excitation/emission wavelengths (nm) are AlexaFluor 594 (red): 590/617 and DAPI (blue): 358/461. Data are representative of one experiment. (asterisk: represent cells that were magnified; shown in far right column).

A.



B.

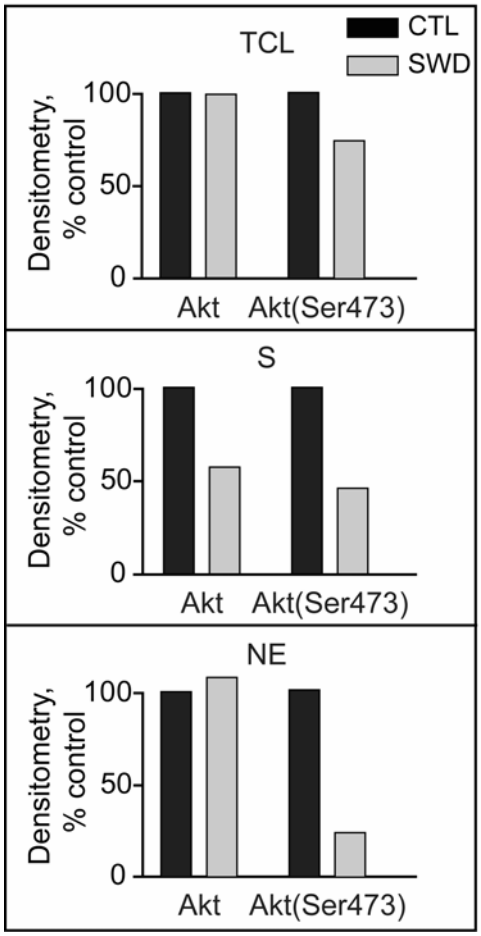


Figure 3.54: Cellular distribution of Akt phosphorylation in response to SWD. (**A**) C6 cells were grown in serum-free medium and proteins from cytosol (S) and nuclear extracts (NE) were obtained. Proteins (30 μg of protein per lane) were resolved by SDS-PAGE and membranes were probed using antibodies for Akt(Ser473), total Akt, SHP-2 and VDAC. (**B**) Densitometry, % control for resolved proteins in (**A**). Data are representative of three independent experiments.

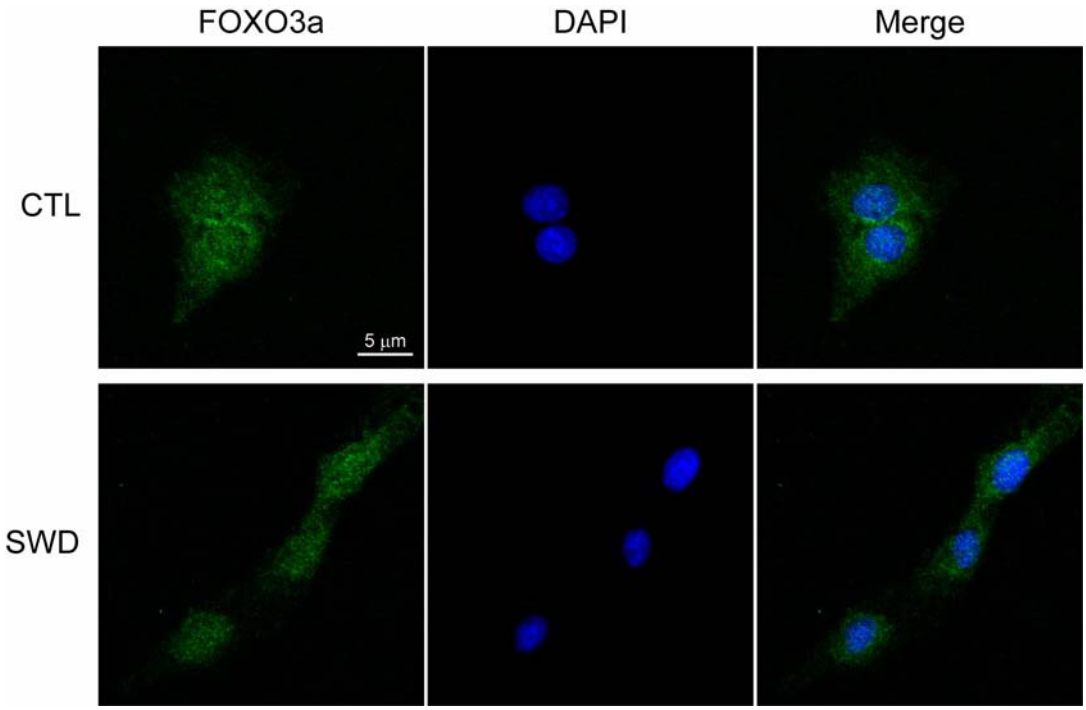


Figure 3.55: Distribution of FOXO3a in C6 cells grown in serum-free medium. C6 cells were grown in serum-free medium (30 min). Cells were fixed, permeabilized, and incubated with primary antibodies for FOXO3a (1:500), followed by incubation with the secondary antibody AlexaFluor 488 (1:1000). Samples were stained with DAPI (1 µg/ml, 5 min) and analyzed using a Zeiss LSM 510 META confocal microscope. SWD: serum withdrawal. Excitation/emission wavelengths (nm) are AlexaFluor 488 (green): 495/520 and DAPI (blue): 358/461. Data are representative of one experiment.

3.8.2 SHP-1 and PDK1 are not redistributed to the nucleus in response to SWD in HEK293A cells.

The effects of SWD on the subcellular localization of PDK1, SHP-1 and Akt were determined in a non-neuronly derived cell line, HEK293A. The cellular distribution of SHP-1 and PDK1 were determined in HEK239A cells and grown in serum-free medium for 30 min. Endogenous SHP-1 was expressed throughout the cells and EGFP-PDK1 was predominantly expressed in the cytoplasm (Figure 3.56). In response to SWD, there was no observable redistribution of either protein throughout the cell (Figure 3.56). SWD did not change the cellular distribution of total Akt and there was only a slight decrease in phosphorylation of Akt(Ser473) in the cytoplasm and nuclear region upon SWD, and perhaps a modest decrease in phosphorylation of Akt(Thr308) in the cytosol upon SWD (Figure 3.57).

3.8.3 Endogenous SHP-1 and the PDK1Y9/376F mutant are not co-localized in the cell when coexpressed with activated Src.

In Figure 3.48 the interaction between endogenous SHP-1 the EGFP-Y9/376F mutant was decreased in control conditions and during SWD. The subcellular distribution of endogenous SHP-1 and EGFP-PDK1 substitution mutants, Y9F, Y376F and Y9/376F were examined using confocal microscopy. In C6 cells cultured in serum-free medium the EGFP-Y9F, -Y376F and -Y9/376F substitution mutants did not localize to the nucleus of cells (Figure 3.58). In cells expressing these substitution mutants there was some redistribution of SHP-1 to the nucleus in response to SWD (Figure 3.58).

The subsequent confocal microscopy experiments were ultimately going to lead to mutagenesis of SHP-1. To monitor the mutated protein a fluorophore-tagged fusion protein was needed. The SHP-1 gene was subcloned into the pRFP-momomer-C1 plasmid, which is a mammalian expression vector that encodes pRFP-Monomer (DsRed.M1). The SHP-1 gene was subcloned into DsRed.M1 (Figure 3.59A). The expression of the red fluorescent protein-tagged-SHP-1 (RFP-SHP-1) fusion protein was confirmed by Western blot (Figure 3.59C).

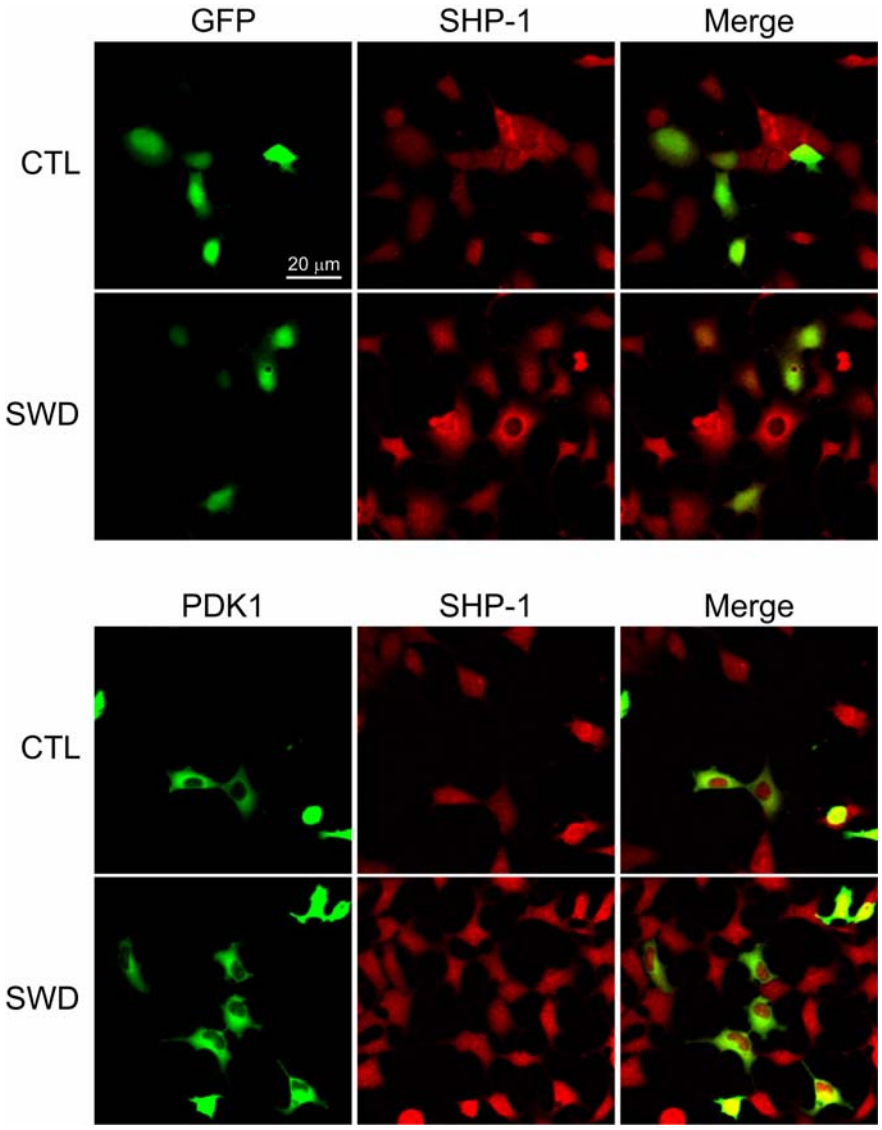


Figure 3.56: SHP-1 and PDK1 do not redistribute to the nucleus of HEK293A cells in response to SWD. Cells were transfected with pEGFP (EGFP) or pEGFP-PDK1 (PDK1) and 24 h post-transfection grown in serum-free medium (30 min). Cells were prepared and analyzed as described in Figure 3.54. SWD: serum withdrawal. Excitation/emission wavelengths (nm) are AlexaFluor 594 (red): 590/617 and EGFP (green): 488/507. Data are representative of two independent experiments.

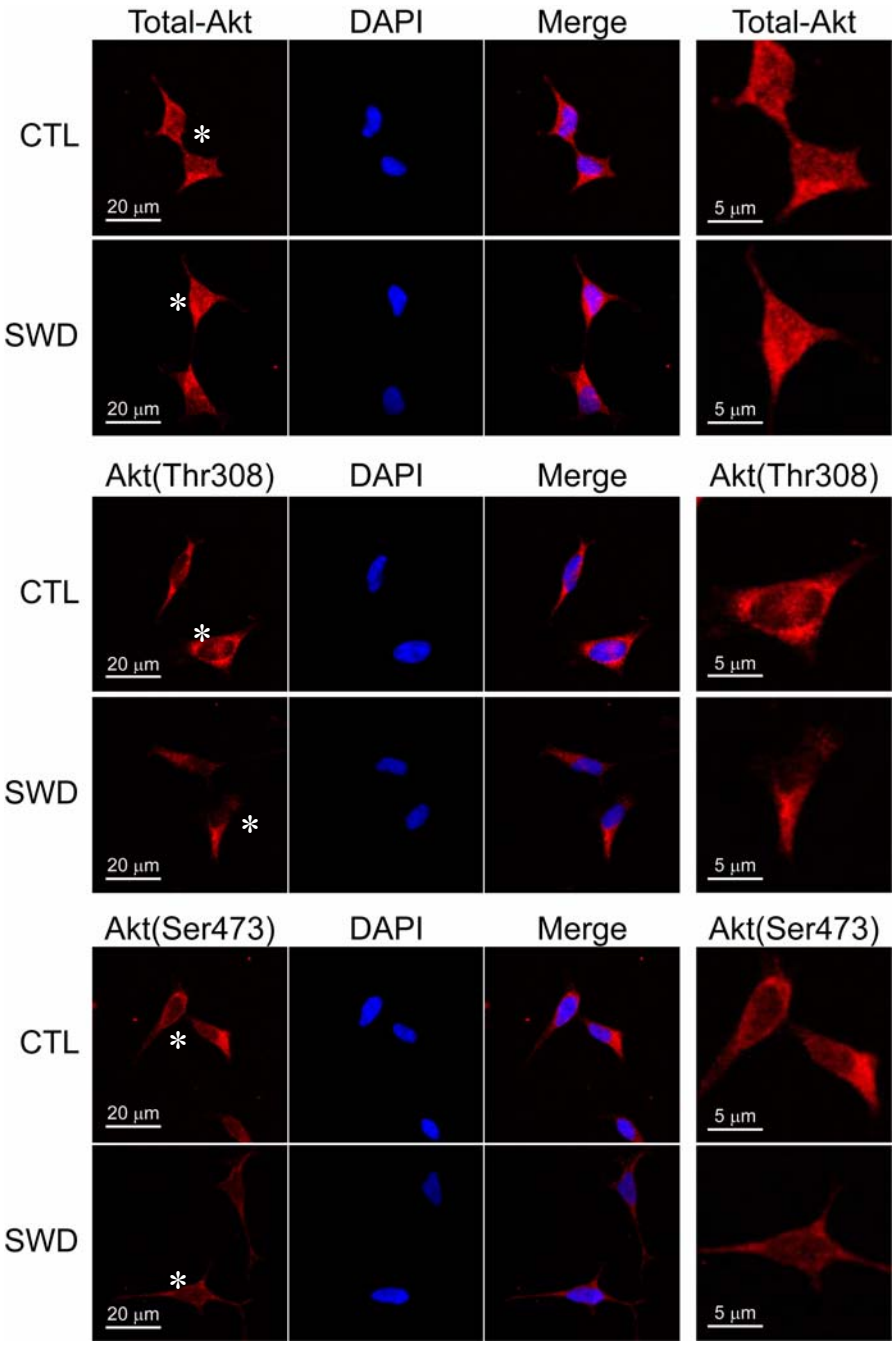


Figure 3.57: Cellular distribution of Akt phosphorylation in HEK293A cells in response to SWD. C6 cells were grown in serum-free medium (30 min). Cells were prepared and analyzed as described in Figure 3.55. SWD: serum withdrawal. Excitation/emission wavelengths (nm) are AlexaFluor 594 (red): 590/617 and DAPI (blue): 358/461. Data are representative of one experiment. (asterisk: represent cells that were magnified; shown in far right column).

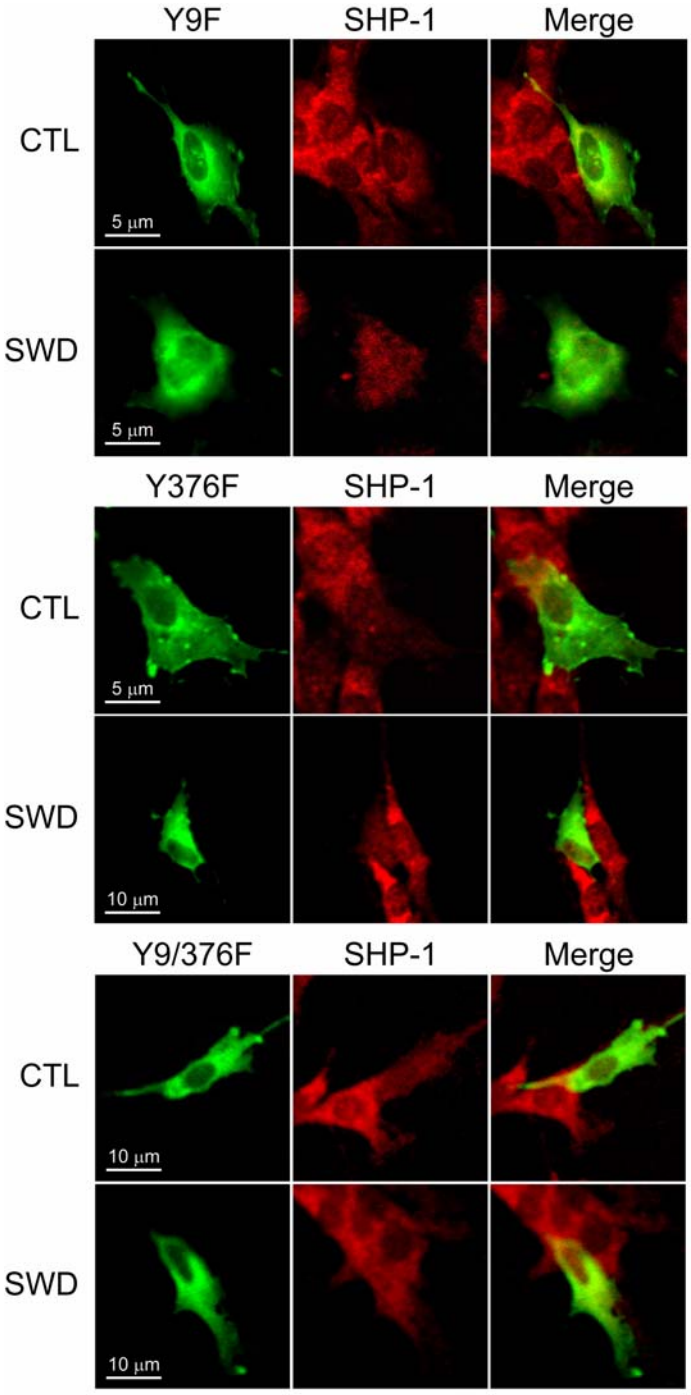


Figure 3.58: PDK1 tyrosine residues: Y9 and Y376F affect the distribution of PDK1 to the nucleus in C6 cells in response to SWD. Cells were transfected with pEGFP-Y9F (Y9F) or pEGFP-Y376F (Y376F), or pEGFP-Y9/376F (Y9/376F) and 24 h post-transfection grown in serum-free medium (30 min). Cells were prepared and analyzed as described in Figure 3.54. SWD: serum withdrawal. Excitation/emission wavelengths (nm) are AlexaFluor 594 (red): 590/617and EGFP (green): 488/507. Data are representative of three independent experiments.

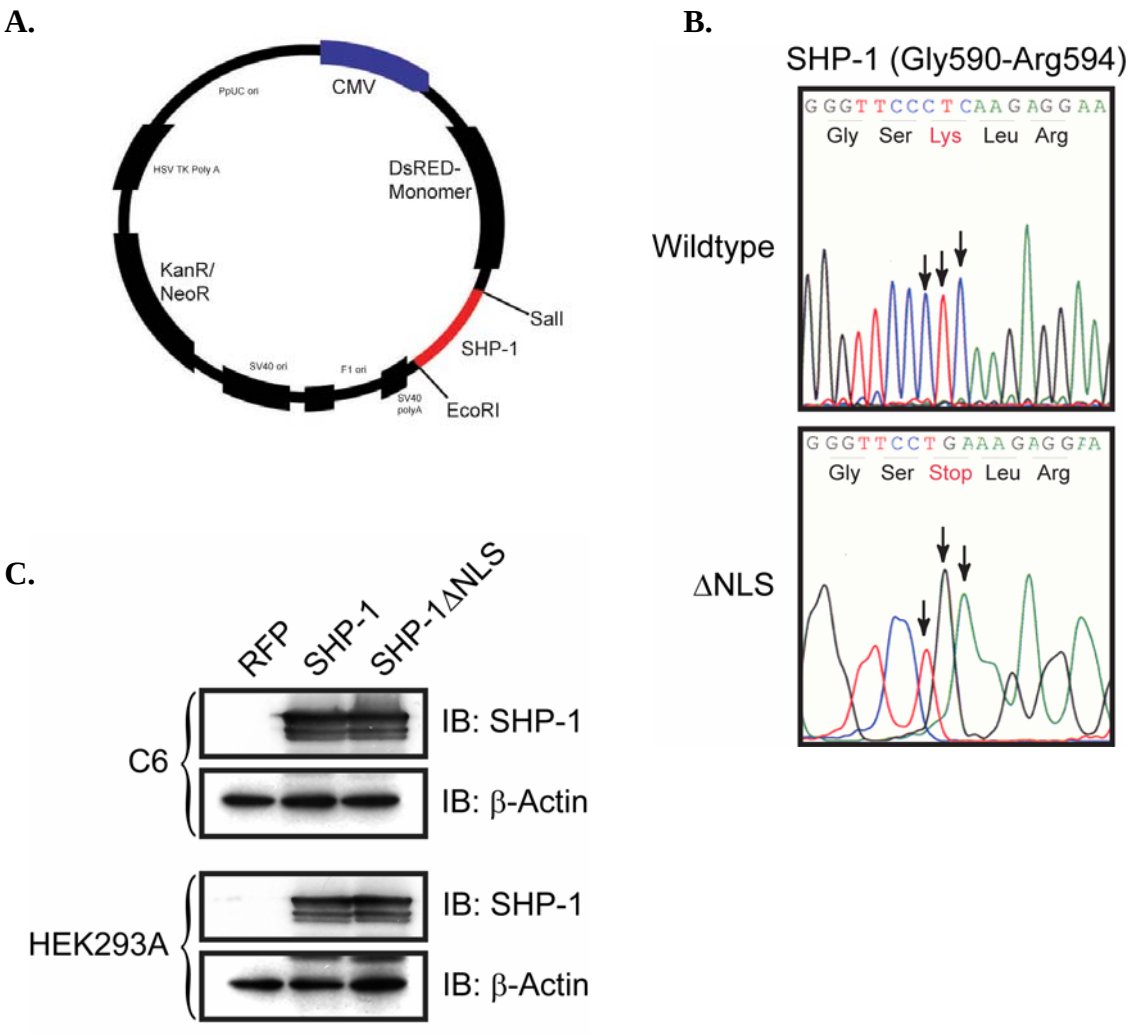


Figure 3.59: Red fluorescent protein-tagged-SHP-1 (pRFP-SHP-1). (**A**) The SHP-1 gene (human) was subcloned into pRFP-monomer-C1 using *EcoR*I and *Sal*I restriction sites. KanR/NeoR: kanamycin/neomycin resistant genes, SV40 ori: mammalian origin of replication, SV40 polyA: polyadenylation signal, PpUC ori: origin of replication for *E. coli*: F1 ori: origin for ssDNA production, Herpes simplex virus thymidine kinase (HSV TK polyA): polyadenylation signal allows stably transfected eukaryotic cells using G418. (**B**) Deletion of the SHP-1 nuclear localization sequence (NLS), was accomplished by inserting a STOP codon at Lysine (Lys) 592 using Quikchange® site-directed mutagenesis kit. (**C**) Expression of pRFP-SHP-1 constructs in C6 and HEK293A cells using Western blot. Membranes were probed using antibodies for SHP-1 and equal loading was monitored using β-actin

In Figure 3.49, the interaction between endogenous SHP-1 and EGFP-Y9/376F was decreased in the presence of activated Src. C6 cells were cotransfected with pRFP-SHP-1 and either pEGFP1-Y9/376F or pEGFP1-Y333/373F, in the absence or presence of activated Src, and their subcellular localization was examined using confocal microscopy. There was a distinct colocalization of RFP-SHP-1 and EGFP1-Y9/376F throughout the cells, however, in the presence of Src, RFP-SHP-1 was localized more to the nucleus and EGFP1-Y9/376F was localized more in the cytoplasm. The colocalization of RFP-SHP-1 and EGFP-Y333/373F was not affected by Src overexpression (Figure 3.60).

3.8.4 SHP-1 and PDK1 localization to the nucleus occurs *via* the nuclear pore complex.

Shuttling of proteins in and out of the nucleus occurs through the nuclear pore complex (NPC). Nuclear import of SHP-1 has been demonstrated to be mediated by its NLS (Craggs and Kellie, 2001). The calcium inonophore, A23187, has been demonstrated to induce Ca^{2+}-calmodulin dependent activation of the NPC resulting in SHP-1 nuclear accumulation (Tenev et al., 2000). PDK1 nuclear export has been demonstrated to occur *via* its NES and leptomycin-B (LMB), which is an inhibitor of CRM-1 of the NPC-export machinery, has been demonstrated to cause nuclear accumulation of PDK1 (Lim et al., 2003; Scheid et al., 2005).

Blocking NPC-mediated nuclear export with LMB or enhancing NPC-mediated import with A23187 both resulted in the nuclear accumulation of both EGFP1-PDK1 and RFP-SHP-1 (Figure 3.61). Treatment with BAPTA-AM, which blocks NPC-mediated import as a result of inhibition of the assembly of the NPC (Macaulay and Forbes, 1996), resulted in a slightly stronger cytoplasmic signal for both RFP-SHP-1 and EGFP1-PDK1 (Figure 3.61).

3.8.5 SHP-1 associates with the nuclear matrix upon dissociation from PDK1.

C6 cells co-expressing SHP-1 (WT) and the double mutant PDK1Y6/376F were treated with LMB. The nuclei were isolated and the nuclear matrix was separated from

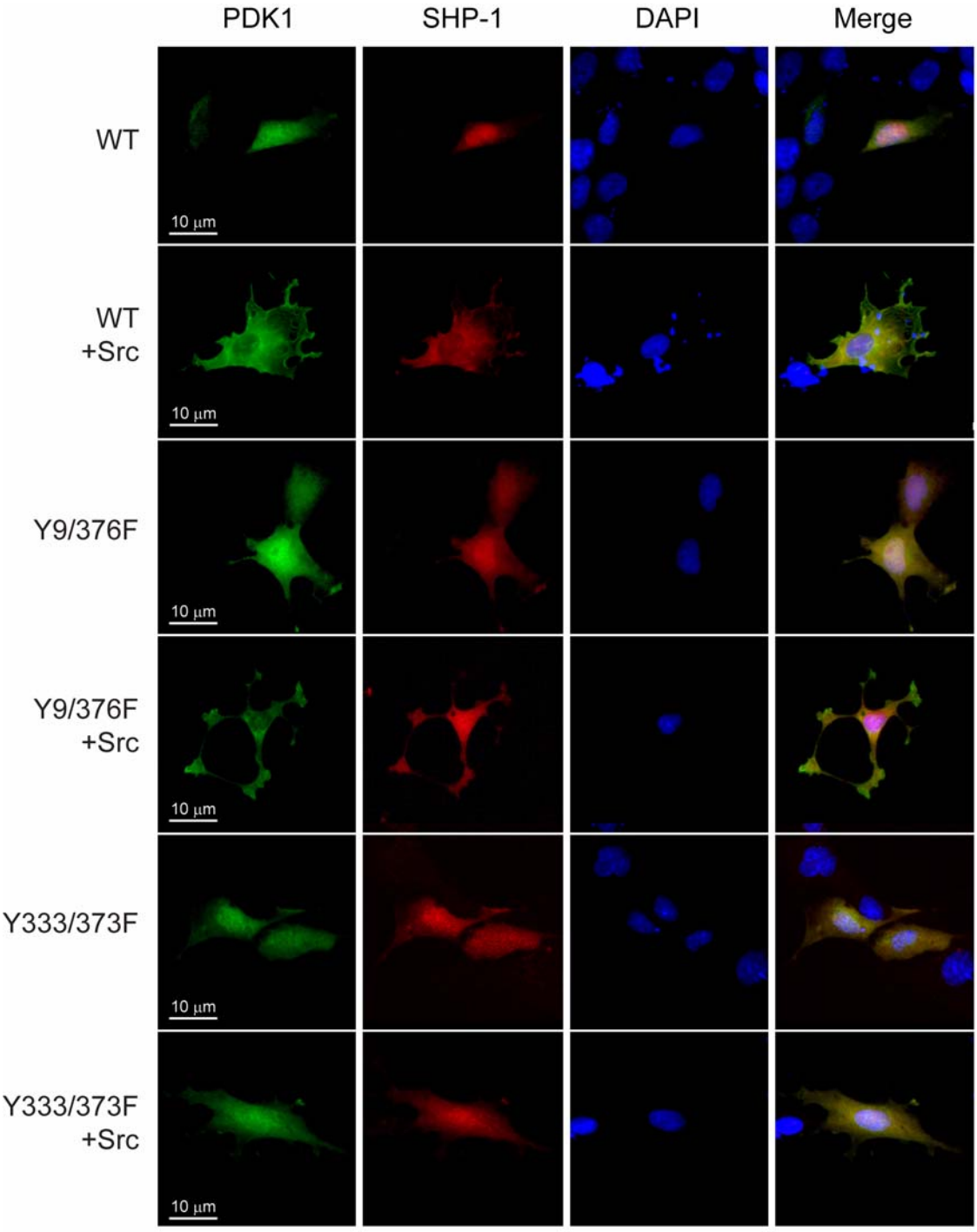


Figure 3.60: The SHP-1 and PDK1 double substitution mutant Y9/376F do not colocalize in cells cotransfected with Src. C6 cells were cotransfected with pRFP-SHP-1 (SHP-1) or pEGFP-PDK1 (WT) or pEGFP-Y9/376F (Y9/376F) or pEGFP-Y333/373F (Y333/373F) and activated chicken SrcY527F (Src). Samples were fixed, permeabilize, stained with DAPI (1 μg/ml, 5 min), and analyzed using a Zeiss LSM 510 META confocal microscopy. Data are representative of two independent experiments.

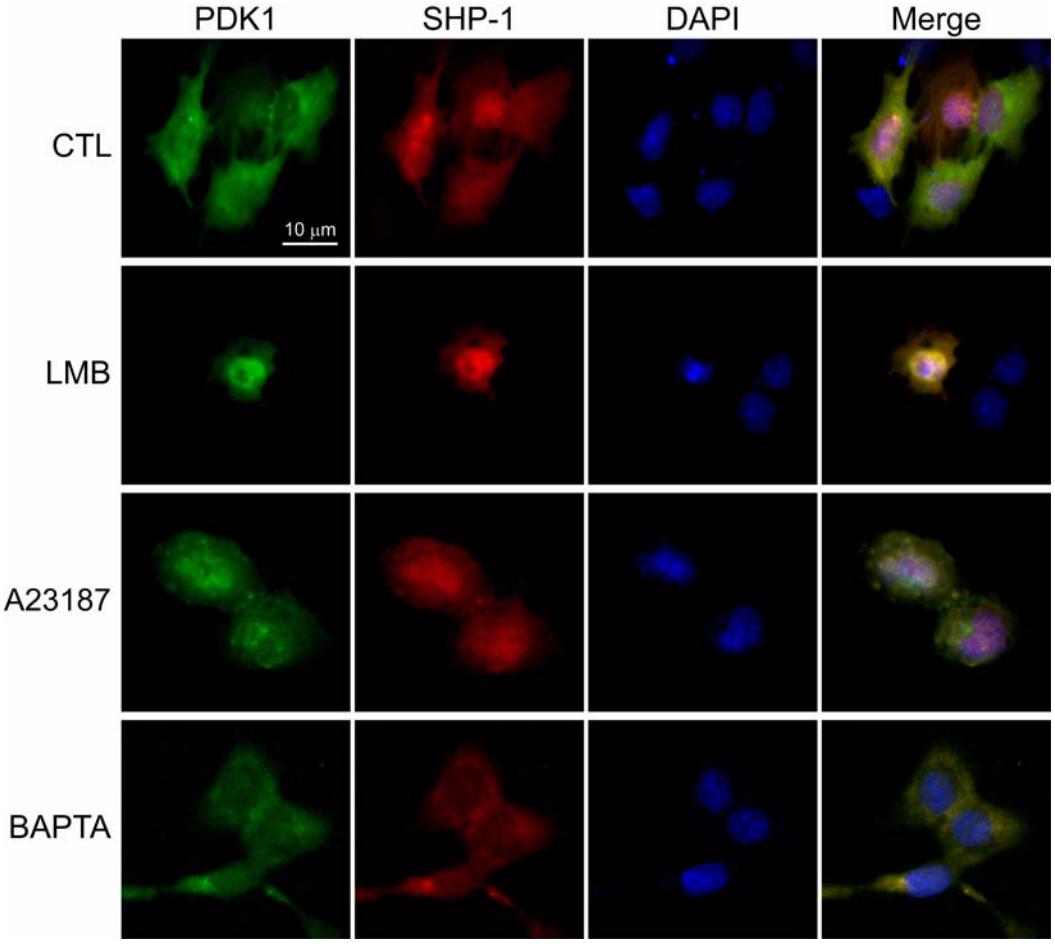


Figure 3.61: The nuclear pore complex mediates SHP-1 and PDK1 nuclear shuttling. C6 cells were cotransfected with pEGFP-PDK1 (PDK1) and pRFP-SHP-1 (SHP-1) and treated with leptomycin-B (LMB; 50 nM, 3 h) or A23187 (5 μM, 10 min), or BAPTA-AM (100 μM, 30 min). Cells were prepared and analyzed as described in Figure 3.61. Excitation/emission wavelengths (nm) are RFP (red): 557/585, EGFP (green): 488/507 and DAPI (blue): 358/461. Data are representative of three independent experiments.

the soluble fraction. Proteins were resolved by SDS-PAGE and examined for SHP-1 and PDK1 expression (Figure 3.62).

3.8.6 The SHP-1 NLS contributes to the nuclear localization of PDK1.

The following experiments were conducted to determine if the nuclear localization signal (NLS) of SHP-1 influences the import of RFP-SHP-1 (and EGFP1-PDK1) to the nucleus. The last 3 amino acids of the C-terminus of SHP-1 constitute a NLS (Craggs and Kellie, 2001). This NLS was deleted by insertion of a STOP codon at the Lysine (Lys) 592 immediately preceding the NLS (Figure 3.59B).

C6 cells were treated with LMB to determine if the SHP-1 mutant lacking the NLS (RFP-SHP-1ΔNLS) could block the localization and accumulation of EGFP1-PDK1 in the nucleus. RFP-SHP-1 and EGFP1-PDK1 both accumulated in the nucleus of LMB-treated cells (Figure 3.63). The RFP-SHP-1ΔNLS mutant was not completely excluded from the nucleus (Figure 3.63). EGFP1-PDK1 was also excluded from the nucleus of these cells, even following treatment with LMB (Figure 3.63). The RFP-SHP-1ΔNLS mutant was not completely excluded from the nucleus, perhaps due to a contribution to nuclear import by the N-terminal NLS on SHP-1.

The effect of RFP-SHP-1ΔNLS on the nuclear accumulation of EGFP1-PDK1 in response to SWD was examined. RFP-SHP-1ΔNLS was effective in reducing the nuclear accumulation of EGFP1-PDK1 in response to SWD (Figure 3.64). Once again the RFP-SHP-1ΔNLS was not completely excluded from the nucleus in C6 cells. Yang *et al.*, (2002) reported that the nuclear localization of SHP-1 can be dependent on both the C and N-terminal localization signals of SHP-1. However, Craiggs and Kellie (2001), reported that the C-terminal NLS and not the N-terminal NLS regulates SHP-1 nuclear localization in HEK293 cells. Therefore, HEK293A cells were used to test the influence of the RFP-SHP-1ΔNLS on EGFP1-PDK1 localization.

HEK293A cells overexpressing the RFP-SHP-1ΔNLS clearly had reduced nuclear SHP-1 signal intensity and a reduced nuclear accumulation of EGFP-PDK1, even when treated with LMB (Figure 3.65).

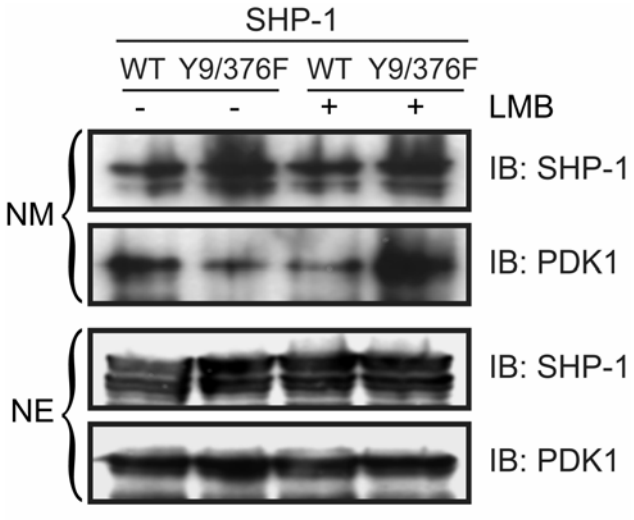


Figure 3.62: SHP-1 associates with the nuclear matrix upon dissociation from PDK1. C6 cells were transfected with pRFP-SHP-1 and pEGFP-PDK1 (WT) or pEGFP-Y9/376F and 24 h post-transfection treated with leptomycin-B (LMB; 50 nM, 3 h). Proteins from the nuclear matrix (NM) and nuclear extracts (NE) were obtained. Proteins (30 μg of protein per lane) were resolved by SDS-PAGE and membranes were probed using antibodies for SHP-1 and PDK1.

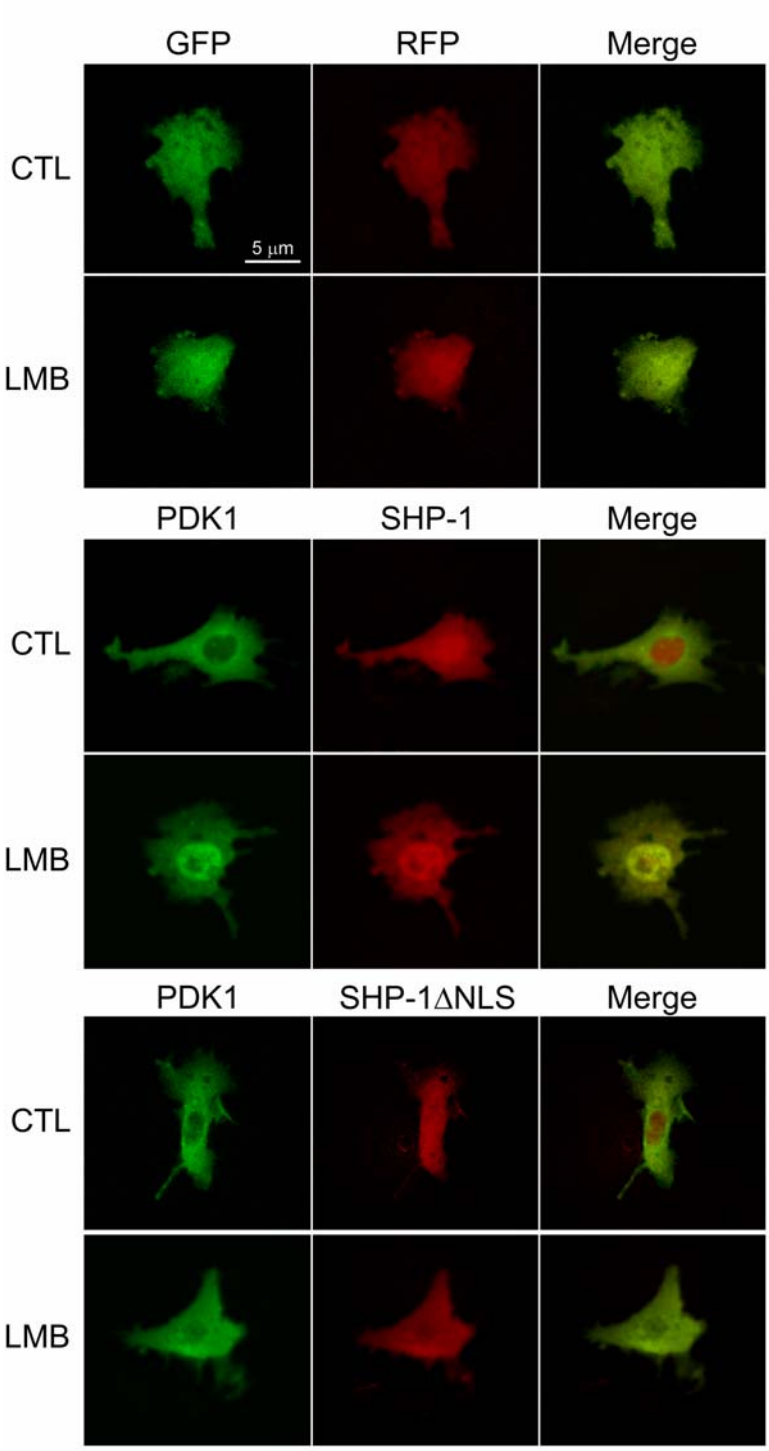


Figure 3.63: Deletion of the SHP-1 nuclear localization signal (NLS) inhibits the nuclear accumulation of SHP-1 and PDK1 in C6 cells. Cells were cotransfected with pEGFP (EGFP) and pRFP (RFP), or pEGFP-PDK1 (PDK1) and pRFP-SHP-1 (SHP-1) or pRFP-SHP-1ΔNLS (SHP-1ΔNLS) and treated with leptomycin-B (LMB; 50 nM, 4 h). Cells were fixed, permeabilized and analyzed by confocal microscopy. Excitation/emission wavelengths (nm) are RFP (red): 557/585 and EGFP (green): 488/507. Data are representative of three independent experiments.

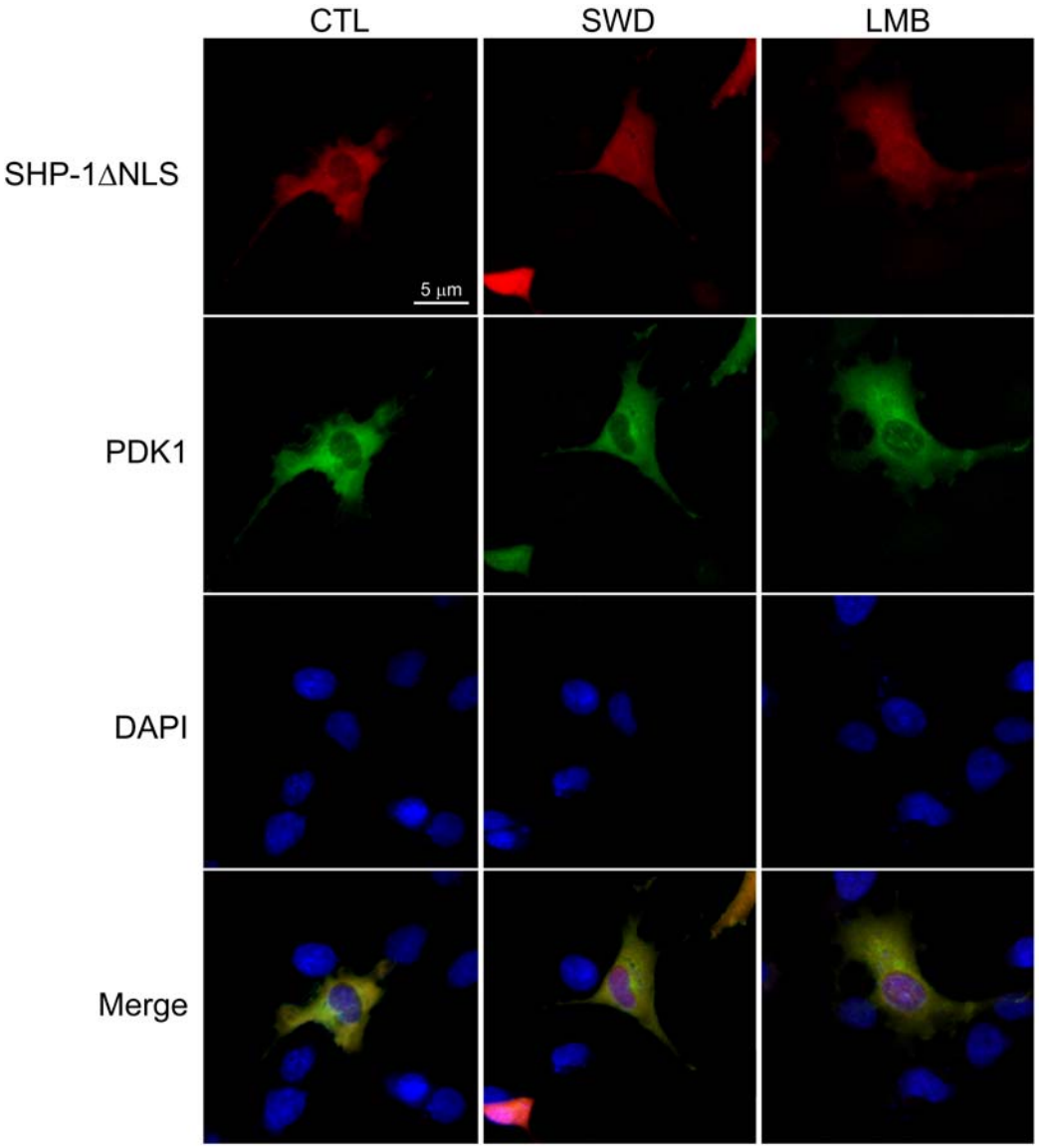


Figure 3.64: Deletion of the SHP-1 NLS reduced the nuclear localization of RFP-SHP-1ΔNLS and EGFP-PDK1 in response to SWD. C6 cells were cotransfected with pEGPF-PDK1 (PDK1) and pRFP-SHP-1ΔNLS (SHP-1ΔNLS) and grown in serum-free medium (SWD) for 30 min or treated with leptomycin-B (LMB; 50 nM, 3 h). Cells were prepared and analyzed as described in Figure 3.61. Excitation/emission wavelengths (nm) are RFP (red): 557/585, EGFP (green): 488/507 and DAPI (blue): 358/461. Data are representative of two independent experiments.

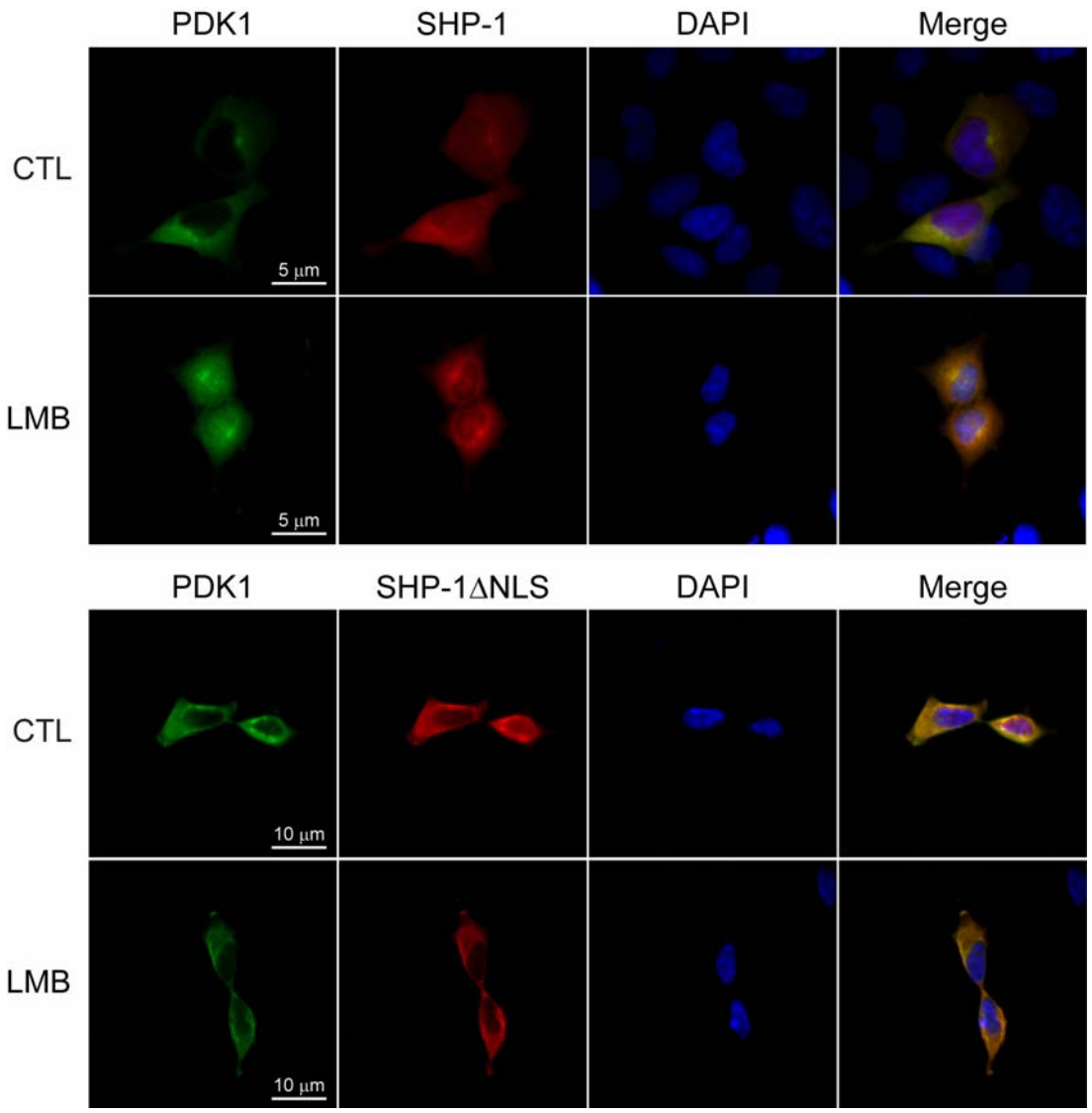


Figure 3.65: Deletion of the SHP-1 NLS inhibits the nuclear accumulation of RFP-SHP-1ΔNLS and EGFP-PDK1 in HEK293A cells. Cells were cotransfected with pEGFP-PDK1 (PDK1) and pRFP-SHP-1 (SHP-1) or pRFP-SHP-1ΔNLS (SHP-1ΔNLS). 24 h post-transfection cells were treated with leptomycin-B (LMB; 50 nM, 4 h). Cells were prepared and analyzed as described in Figure 3.61. Excitation/emission wavelengths (nm) are RFP (red): 557/585, EGFP (green): 488/507 and DAPI (blue): 358/461. Data are representative of three independent experiments.

3.9 Usage of PC12 cells to determine the function of the SHP-1:PDK1 complex.

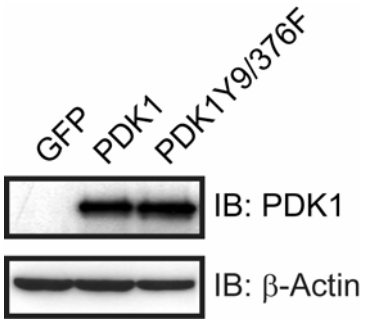
The removal of serum from C6 cells is likely to affect concurrently many signalling pathways and cellular processes, *i.e.* metabolism, which would make any observation difficult to interpret. PDK1 and SHP-1 have been implicated independently in a variety of cell functions, including differentiation. PDK1 mediates differentiation of adipocytes (Yin et al., 2006). In addition, SHP-1 has been implicated in the differentiation of astrocytes (Massa et al., 2004; Wishcamper et al., 2001) and PC12 cells (Marsh et al., 2003; Vambutas et al., 1995). Furthermore, nuclear PDK1 has been implicated in the effects of NGF in PC12 cells (Martelli et al., 2003) and in COS-7 cells overexpressing the NGF receptor, TrkA (Salinas et al., 2000).

3.9.1 The SHP-1:PDK1 complex shuttles to the nucleus during differentiation of PC12 cells.

RFP-SHP-1 and RFP-SHP-1ΔNLS, EGFP-PDK1 and EGFP-Y9/376F were overexpressed in PC12 cells (Figure 3.66). Stimulation of PC12 cells with NGF for 30 minutes did not affect the phenotype of vector-transfected cell cultures. The co-expression of SHP-1 and PDK1 was sufficient to induce modest neurite extension in vehicle-treated cultures (CTL) and more so in NGF-treated cultures (Figure 3.67). Overexpression of the SHP-1ΔNLS protein blocked the effect of NGF (Figure 3.67). Longer treatment of PC12 cells with NGF (*e.g.* two days) began to induce neurite extension in vector-transfected cultures; however, coexpression of SHP-1 and PDK1 significantly enhanced the effect of NGF (Figure 3.68). The SHP-1ΔNLS protein blocked the effects of NGF, as did the overexpression of the PDK1Y9/376F mutant (Figure 3.68).

This series of experiments was repeated and included LMB treatment to test whether SHP-1 and PDK1 enter the nucleus during stimulation of cells with NGF. Nuclear accumulation of SHP-1 and PDK1 proteins was observed in cultures overexpressing the wildtype SHP-1 and PDK1 proteins as well as in cultures overexpressing the wildtype SHP-1 and PDK1Y9/376F proteins (Figure 3.69). In contrast, there was significantly less nuclear accumulation of SHP-1 and PDK1 signals in cultures overexpressing the SHP-1ΔNLS protein (Figure 3.69). Stimulation with NGF

A.



B.

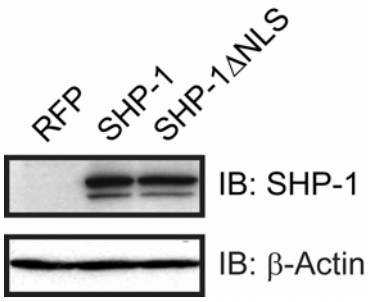


Figure 3.66: Expression of EGFP1-PDK1 and RFP-SHP-1 proteins in PC12 cells. Cells were transfected with (**A**) pEGFP (GFP) or pEGFP-PDK1 (PDK1), or pEGFP-Y9/376F (Y9/376F) and (**B**) pRFP (RFP) or pRFP-SHP-1 (SHP-1), or pRFP-SHP-1ΔNLS (SHP-1ΔNLS). 24 h post-transfection proteins (10 μg of protein per lane) were resolved by SDS-PAGE and membranes were probed using antibodies for PDK1, SHP-1 and equal loading was monitored using β-actin.

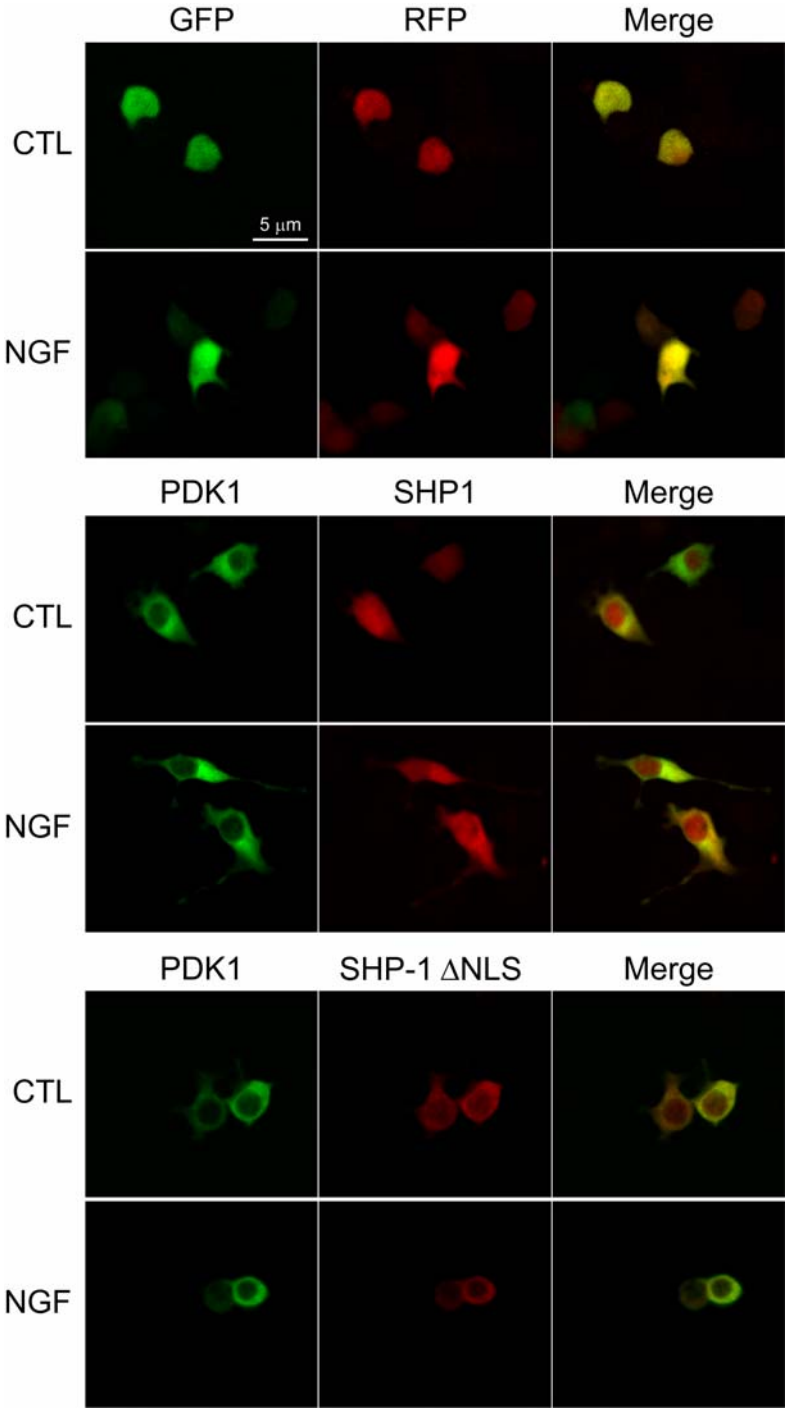


Figure 3.67: Effect of NGF stimulation on SHP-1:PDK1 nuclear localization in PC12 cells. Cells were cotransfected with pEGFP (GFP) and pRFP (RFP) or pEGFP-PDK1 (PDK1) and pRFP-SHP-1 (SHP-1) or pEGFP-PDK1 (PDK1) and pRFP-SHP-1ΔNLS. 24 h post-transfection cells were treated with NGF (50 nM, 30 min). Cells were fixed and analyzed using an Olympus FV300 confocal microscope. Excitiation/emission wavelengths (nm) are RFP (red): 557/585 and EGFP (green): 488/507. Data are representative of two independent experiments.

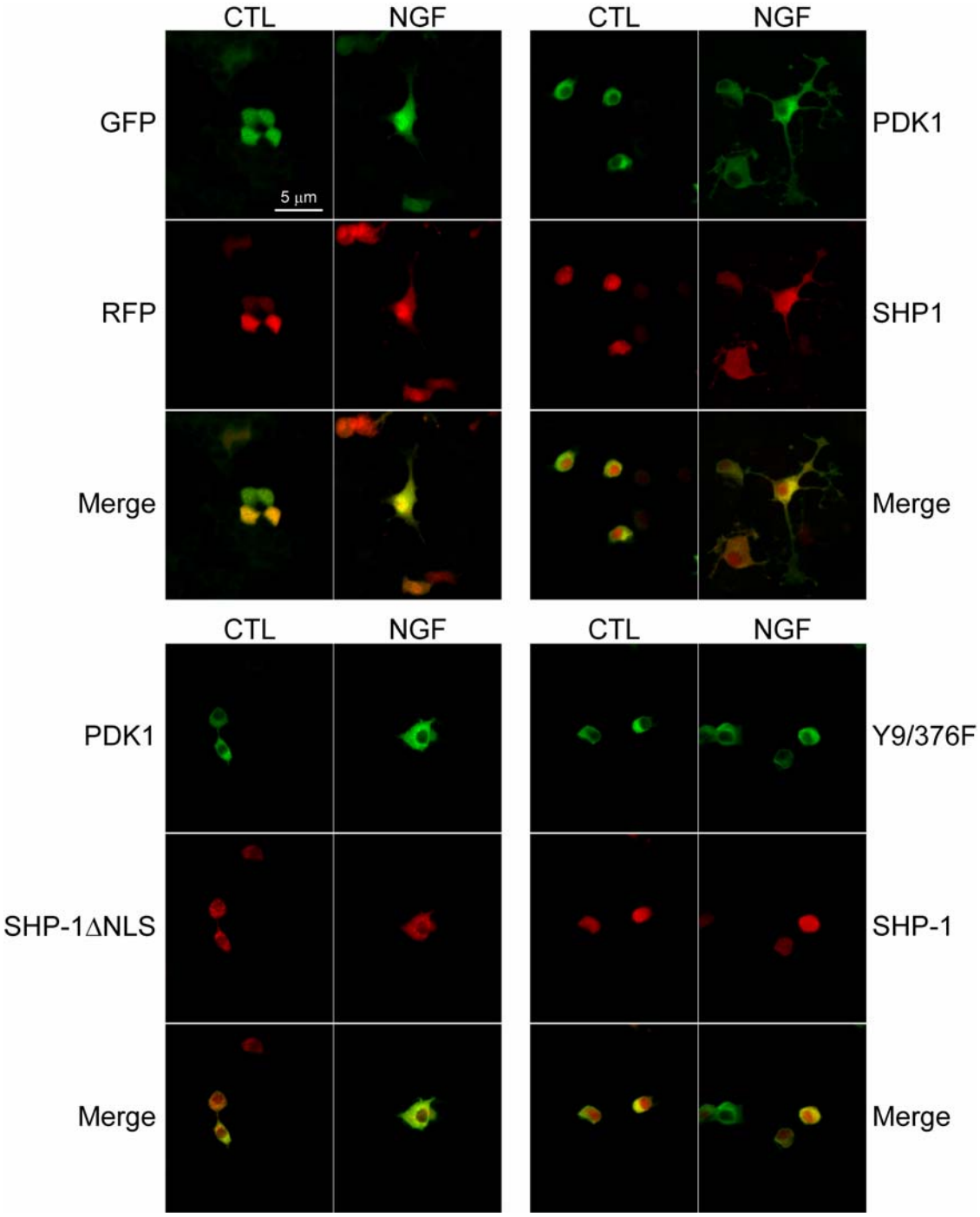


Figure 3.68: Effect of NGF on SHP-1:PDK1 nuclear localization in PC12 cells. Cells were cotransfected with pEGFP (GFP) and pRFP (RFP) or pEGFP-PDK1 (PDK1) and pRFP-SHP-1 (SHP-1) or pRFP-SHP-1ΔNLS (SHP-1ΔNLS) or pEGFP-PDK1Y9/376F and SHP-1. 24 h post-transfection cell were treated with NGF (50 nM, 2 days). Cells were prepared and analyzed as described in Figure 3.67. Excitation/emission wavelengths (nm) are RFP (red): 557/585 and EGFP (green): 488/507. Data are representative of two independent experiments.

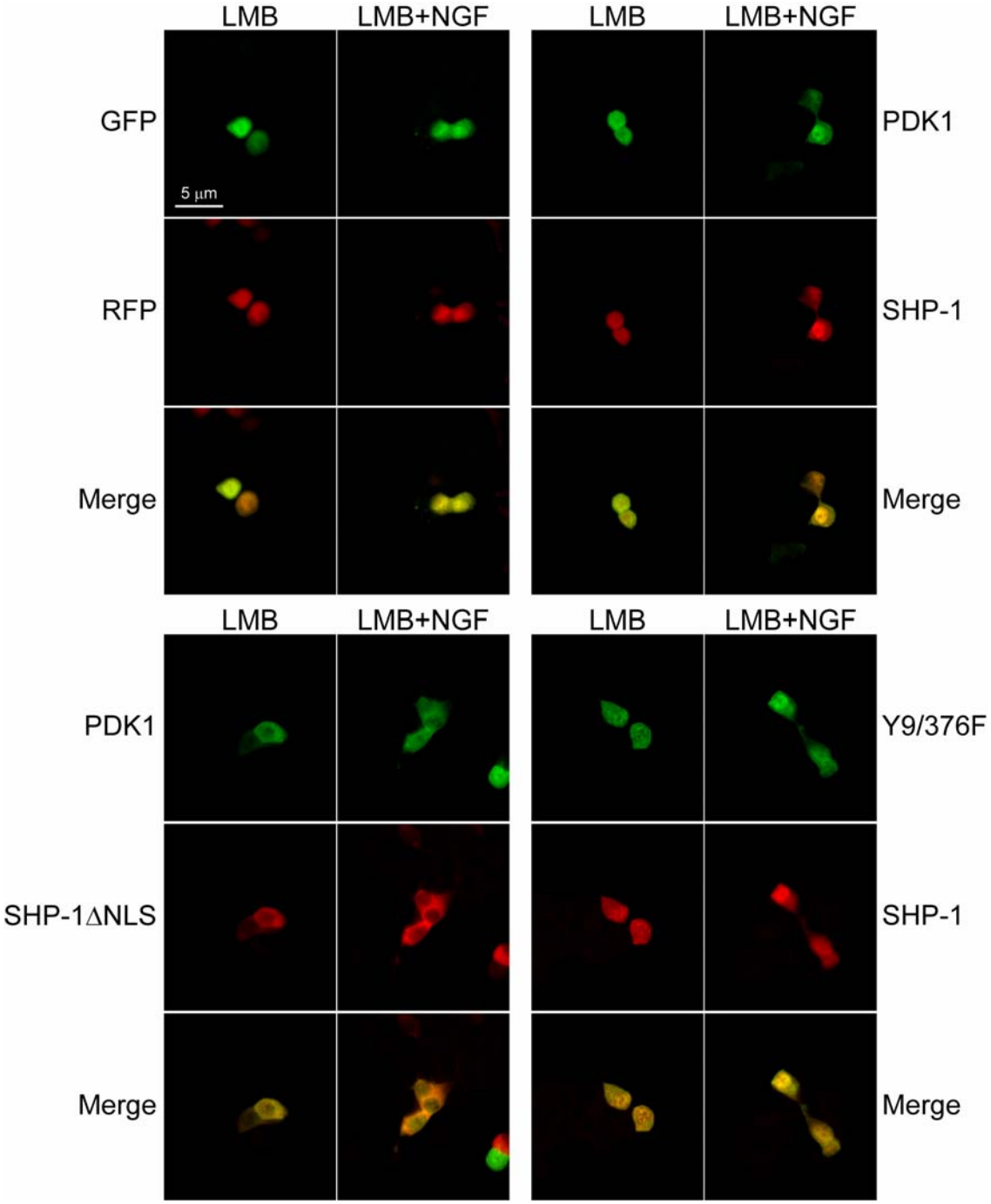


Figure 3.69: SHP-1:PDK1 accumulate in the nucleus of PC12 cells when treated with LMB. Cells were cotransfected with pEGFP (GFP) and pRFP (RFP) or pEGFP-PDK1 (PDK1) and pRFP-SHP-1 (SHP-1) or pRFP-SHP-1ΔNLS (SHP-1ΔNLS) or pEGFP-PDK1Y9/376F (PDK1Y9/376F) and pRFP-SHP-1 (SHP-1). 24 h post-transfection cells were treated with leptomycin-B (LMB; 20 nM, 3 h) and NGF (50 nM, 30 min) Cells were prepared and analyzed as described in Figure 3.67. Excitation/emission wavelengths (nm) are RFP (red): 557/585 and EGFP (green): 488/507. Data are representative of two independent experiments.

(30 min) resulted in additional accumulation of SHP-1 and PDK1 in cells overexpressing the wildtype SHP-1 and PDK1 (or PDK1Y9/376F) proteins (Figure 3.69). The reduction of nuclear localization of proteins in cells overexpressing SHP-1ΔNLS was not affected by NGF treatment (Figure 3.69).

3.9.2 The association between SHP-1 and PDK1 is maintained during NGF treatment of PC12 cells.

NGF has been shown to increase the tyrosine phosphorylation status of PDK1. PC12 cells were treated with NGF and proteins were examined for phosphorylation changes in PDK1 and whether the interaction with SHP-1 was affected as a result of treatments. Treatment of PC12 cells with NGF for either 30 minutes or two days did not affect the association between SHP-1 and PDK1 (Figure 3.70). NGF treatment did, however, cause a decrease in PDK1 mobility and a corresponding increase in Akt phosphorylation (Figure 3.70).

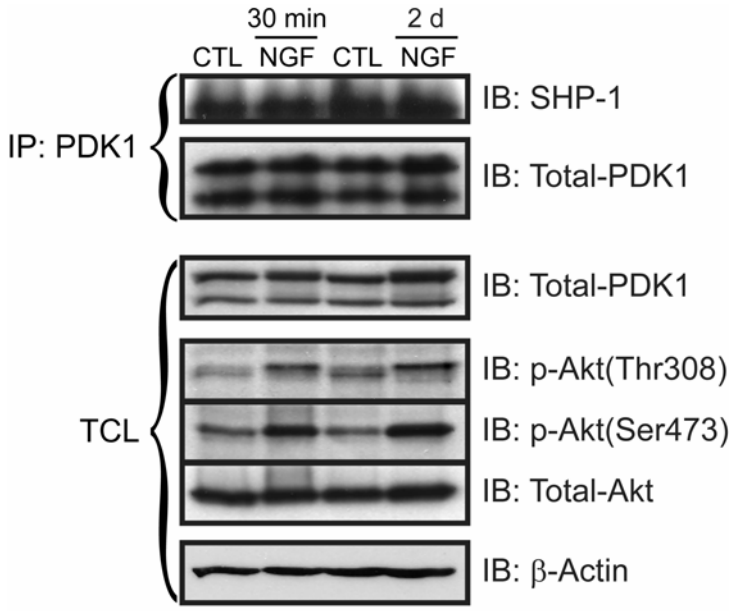


Figure 3.70: The association between SHP-1:PDK1 is maintained with NGF treatment of PC12 cells. Cells were treated with NGF (50 nM, 30 min or 2 d). Proteins (300 μg) were immunoprecipitated (IP) with anti-PDK1 antibody and the immunoprecipitates were resolved by SDS-PAGE and probed for SHP-1 and for total PDK1. Corresponding protein lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with anti-total PDK1, anti-Akt(Thr308), anti-Akt(Ser473), anti-total Akt and anti-β-actin. Data are representative of one experiment.

4. DISCUSSION

SHP-1 has been implicated as a negative regulator of cellular events, most often in cancer and immune cells. The PI3K/PDK1/Akt signalling pathway is credited with regulating growth, proliferation and differentiation in cells in the periphery as well as in the central nervous system (CNS); it is this signalling pathway that is most often targeted by SHP-1, yet there is a paucity of data regarding the role of SHP-1 in regulating the PI3K/PDK1/Akt pathway in the CNS.

This thesis provides evidence for an association between SHP-1 and PDK1. This association is dependent on the tyrosine phosphorylation of PDK1 and plays a critical role in regulating PDK1 function and localization. Most importantly, this thesis provides clear evidence for a SHP-1:PDK1 nucleo-cytoplasmic shuttling complex that relies on the SHP-1-NLS and the PDK1-NES. This complex is constitutively present in several cell lines and plays a critical role during cellular stress, *i.e.* SWD or NGF-mediated differentiation.

4.1 Response of the PI3K pathway in C6 cells grown in serum-free medium.

Akt phosphorylation need not always directly reflect PI3K function. Our laboratory has recently shown that activation of the sigma2 receptor system by the antipsychotic drug haloperidol induces PI3K activity and a concurrent loss of Akt phosphorylation (Dai *et al.*, 2007, in press). Furthermore, differentiation of C6 glioma cell cultures using dibutyryl-cAMP (in combination with serum withdrawal) can induce PI3K activity (Roymans et al., 2001), while inhibiting the phosphorylation of Akt (Van Kolen and Slegers, 2004). A similar uncoupling is observed in differentiated 3T3-L1 adipocytes chronically treated with GH (Takano et al., 2001). It is the contention of this laboratory that this “uncoupling” of PI3K activity and Akt phosphorylation has been observed before, but is not published. I chose to investigate the contribution of SHP-1 to this phenomenon.

Removal of serum from the culture medium results in the expected change in morphology (Baranska et al., 2004), *e.g.* from a fibroblast-like flat shape to a rounded astrocyte-like phenotype. Furthermore, this corresponds with cell cycle arrest and a reduction in proliferation, thereby confirming previous reports (Chou and Langan, 2003; Fan, 1983; Heldin et al., 1980). Both cell cycle progression and differentiation are known to involve PI3K activation of effector molecules such as Akt and Erk1/2 (Datta et al., 1999; Zhang and Liu, 2002). Initial characterization of the PI3K signalling pathway revealed an increase in p85/PI3K-associated lipid kinase activity and the concurrent loss of Akt phosphorylation, reflecting similarly reported paradoxical observations (Roymans et al., 2001; Takano et al., 2001; Van Kolen and Slegers, 2004) however, the concurrent loss of p85 phosphorylation is unexpected as tyrosine phosphorylation of p85 is thought to be an obligate condition for PI3K activation or for PI3K recruitment to RTKs (Cuevas et al., 2001; Yu et al., 1998c). Overexpression of the p85 protein, p85Y688D, which mimics tyrosine phosphorylation and is known to increase Akt phosphorylation presumably by affecting PI3K activity (Cuevas et al., 2001), increases Akt phosphorylation, but does not reverse the effect of serum withdrawal (SWD) on C6 cell proliferation. Furthermore, the maintained association of endogenous p85 with Gab1, a scaffolding protein thought to recruit p85 to RTKs (Ingham et al., 2001), suggests that the events and machinery necessary for PI3K activation are intact. Confocal microscopy confirmed the increased production of $PI3,4,5P_3$ in this model; however, these $PI3,4,5P_3$ are not localized at the plasma membrane as would be expected of a cell line with constitutively active PI3K (Maher et al., 2001; Roymans et al., 2001), but rather are concentrated in the perinuclear region. In keeping with this, p85, whose distribution under control conditions is diffuse and throughout the cell, including in the nucleus, is also concentrated in the perinuclear region during SWD.

The redistribution of p85(PI3K) and $PI3,4,5P_3$ to the perinuclear region of C6 cells grown in serum-free medium could explain why overexpression of the catalytic dead PTENC124S mutant (which is incapable of dephosphorylating $PI3,4,5P_3$) does not protect against the loss of Akt phosphorylation that occurs in response to SWD. Indeed, PTEN preferentially targets $PI3,4,5P_3$ at the plasma membrane, but not the nuclear pool (Lindsay et al., 2006). Similarly, the inability of the plasma membrane-directed

myristoylated PDK1 (myr-PDK1) to protect against the loss of Akt phosphorylation in reponse to SWD may be due to the redistribution of $PI3,4,5P_3$ synthesis, and corresponding redistribution of Akt, to the nucleus. Obviously, a plasma-membrane bound PDK1 could not activate a nuclear pool of Akt. Although not examined in this thesis, it is possible that myr-PDK1 can be expressed in other cellular compartments besides the plasma membrane (Scheid et al., 2002). Despite all the required machinery present in the nuclear region, Akt is not phosphorylated. This may not be that surprising and part of the story may be suggested by the weak distribution of FOXO3a to the nucleus of cells in response to SWD. Indeed, nuclear FOXO31 is associated with apoptosis (Brunet et al., 1999), yet removal of serum is more often associated with differentiation, and not apoptosis (Chou and Langan, 2003; Fan, 1983). This could therefore be indicating events required to set up the nucleus for differentiation. However, the data in this thesis could also be indicating that additional events may be occurring within the nucleus to disrupt proper Akt phosphorylation. Possible explanations could be that (i) PDK1 is not active in the nucleus of these cells; (ii) SHP-1 sterically hinders PDK1; (iii) myr-PDK1 may not be anchored on the same aspect of the nuclear membrane as $PI3,4,5P_3$ synthesis; (iv) or it could be something much less obvious such as a difference in pH, which is definately heterogeneous across subcellular compartments, particularly the nucleus and cytoplasm (Masuda et al., 1998), and which could affect SHP-1 conformation and activity (Thangaraju et al., 1999). Obviously, further studies are warranted.

PDGF stimulation of C6 cells grown in serum-free medium recruits p85 to the PDGF receptor (PDGFR), as expected (Yu et al., 1998c), and protects against the loss of Akt phosphorylation. PDGF supplemented in serum-free medium also promotes cell cycle progression compared to serum-free medium alone, confirming previous observations with this growth factor (Heldin et al., 1980) and it (but not EGF) does so by inducing a re-distribution of $PI3,4,5P_3$ to the plasma membrane. This suggests that p85, and presumably the PI3K complex, is not being recruited to this RTK during SWD. The lack of effect of the overexpressed p85Y688D mutant, which has been associated with both PI3K activation and recruitment to tyrosine phosphorylated proteins, suggests that the observed loss of p85 tyrosine phosphorylation during SWD may extend to other

tyrosine residues that are equally important for proper p85/PI3K function and localization. This is supported indirectly by the intact association between p85 and the docking protein Gab1 (presumably still associated with the PI3K complex) as well as by the ability of PDGF stimulation to maintain the localization of a pool of p85 to the plasma membrane during SWD. Obviously combinations of various phosphorylated and dephosphorylated tyrosine residues on p85 could dictate its function in a context-dependent manner. This intriging notion also warrants investigation.

4.2 Tyrosine-phosphorylated PDK1 interacts with SHP-1.

The decrease in PDK1 mobility during SWD indicates a post-translational modification. The phosphorylation of the PDK1(Ser241) residue commonly associated with PDK1 activation (Casamayor et al., 1999) is not a contributing factor. This is clearly supported by the inability of overexpressed PDK1Ser241Glu phosphorylation mimic to protect against the loss of Akt phosphorylation. This is not that surprising given reports of a similar disconnect between PDK1(Ser241) and activation of Akt (Chen et al., 2001; Riojas et al., 2006).

A review of the literature revealed that tyrosine phosphorylation of PDK1 also contributes to the regulation of its activity and ultimately that of its downstream effectors (Fiory et al., 2005; Grillo et al., 2000; Park et al., 2001). There is evidence that H_2O_2, pervanadate and c-Abl all increase PDK1 tyrosine phosphorylation and its activity towards its substrates SGK and Akt (Grillo et al., 2000; Park et al., 2001). The present series of experiments reveals that SWD, pervanadate and Src kinase increase PDK1 tyrosine phosphorylation. While Src kinase and pervanadate are both known to induce tyrosine phosphorylation of PDK1, they exert their effects *via* PI3K-independent and -dependent manners, respectively (Grillo et al., 2000; Prasad et al., 2000). The mobility shift in PDK1 induced by SWD is also PI3K-independent (*i.e.* LY294002-insensitive). Regardless of the approach used, they all increase the association between SHP-1 and a higher molecular weight PDK1 species.

While the effect of pervanadate is the strongest, the effects of pervanadate and SWD are additive suggesting that these approaches target different residues on PDK1. Furthermore, treatment with pervanadate increases Akt phosphorylation and this effect

dominates during SWD. These effects may be reflecting the properties of pervanadate as a general tyrosine phosphatase inhibitor, *i.e.* it can also target other phosphatases such as SHP-2 (Xu et al., 2002) and SHIP (Phee et al., 2000), thus, making it difficult to attribute specific effects to endogenous SHP-1.

The association between SHP-1 and PDK1 is not unexpected given that PDK1 is a Src substrate (Park et al., 2001) and that SHP-1 is a general phosphatase of Src substrates (Frank et al., 2004). There is at least one other known common substrate of Src and SHP-1, $p120^{ctn}$, which is recognized as a prominent Src substrate (Mariner et al., 2001). The Src phosphorylation sites on $p120^{ctn}$ have been mapped and SHP-1 can bind to, and dephosphorylate, $p120^{ctn}$ (Mariner et al., 2001). In this thesis, a role for SHP-1 in regulating PDK1 phosphorylation is demonstrated by the decrease of endogenous PDK1 tyrosine phosphorylation in C6 cell cultures co-expressing activated Src and wildtype SHP-1, but not in cultures co-expressing the catalytically inactive mutant SHP-1C455S. Examination of the PDK1 protein sequence reveals that Y9, Y373 and Y376, known targets of Src kinase (Park et al., 2001), are contained within putative ITIMs [pYXX(V/I/L)] and, thus, are potential targets of SHP-1 (Yang et al., 2000; Yang et al., 1998). The *PhosphoMotif Finder* on the Human Protein Reference Database (http://www.hprd.org/) identifies several residues, including the three mentioned above, as being potential targets for Src kinase, but also, that Y373 and Y376 (and, in addition Y333), are also potential targets for SHP-1 (Appendix I). Overexpression of the corresponding tyrosine to phenylalanine substitution mutants of PDK1 (*i.e* Y9F, Y333F, Y373F, Y376F) do not exert any consistent effects on their own on either C6 cell proliferation or cell cycle, supporting the hypothesis that a combination of tyrosines regulates PDK1 function (Park et al., 2001). However, overexpression of these same mutants in cells treated with either pervanadate or SWD reveals that Y9 and Y376 exert similar effects on Akt phosphorylation, and that these effects contrast those of Y333 and Y373. The importance of the Y9 and Y376 residues is underscored by the reduced association between the Y9/376F double mutant and SHP-1 in reponse to SWD or in the presence of activated Src. The loss of association between these two proteins is not complete, indicating that other tyrosines (possibly Tyr273, which resides within an IXYXXV motif) contribute to the interaction. However, the importance of the Y9 and

Y376 residues is further demonstrated by the dominant-negative inhibitory effect of the double PDK1Y9/376F mutant on Akt phosphorylation. These obervations are seemingly counter-intuitive, especially if viewed from within the paradigm that the predominant role of SHP-1 in the cell is that of a negative regulator of PI3K/PDK1/Akt function. However, there is evidence that SHP-1 functions beyond simply being a phosphatase. Indeed, SHP-1 can bind to phopholipids, which may confer structural changes that facilitate substrate access to the active site (Frank et al., 1999; Zhao et al., 1993). In addition, the presence of a nuclear localization signal (NLS) enables it to translocate to the nucleus (Craggs and Kellie, 2001) and presumably target a distinct pool of substrates/functions.

It is already known that tyrosine phosphorylation of PDK1 is not only important for its activation, but also for membrane localization (Park et al., 2001). The current data suggest that SHP-1 contributes to this event.

5.3 Cellular localization of SHP-1 and PDK1

The mechanism that underlies the nuclear localization of PDK1 is not known, but what is known is that it can relocalize to this subcellular compartment upon stimulation with growth factors (*i.e.* insulin, NGF and IGF-1), all of which increase PDK1 tyrosine phosphorylation (Lim et al., 2003; Salinas et al., 2000; Scheid et al., 2005). While IGF-1 can induce the nuclear localization of endogenous PDK1, it is perplexing that there is not a proportional increase in nuclear accumulation of overexpressed PDK1 with IGF-1 stimulation (Scheid et al., 2005). As stated above, the mechanism by which PDK1 is imported into the nucleus is not known; yet, if the association between SHP-1 and PDK1 goes beyond a simple regulation of cytoplasmic signalling cascades, then it is not unreasonable to accept that the import of PDK1 to the nucleus could occur *via* a "piggy-back" mechanism, based on SHP-1 (by virtue of its NLS) (Craggs and Kellie, 2001) as an import vehicle and that the nuclear localization of overexpressed PDK1 is limited if the endogenous pool of SHP-1 is saturated.

In fact, SHP-1 and PDK1 do colocalize to the nucleus with SWD, a condition that modesly increases the tyrosine phosphorylation of PDK1 and its interaction with SHP-1 (Figure 4.1). Interestingly, PDK1 localization to the plasma membrane can be

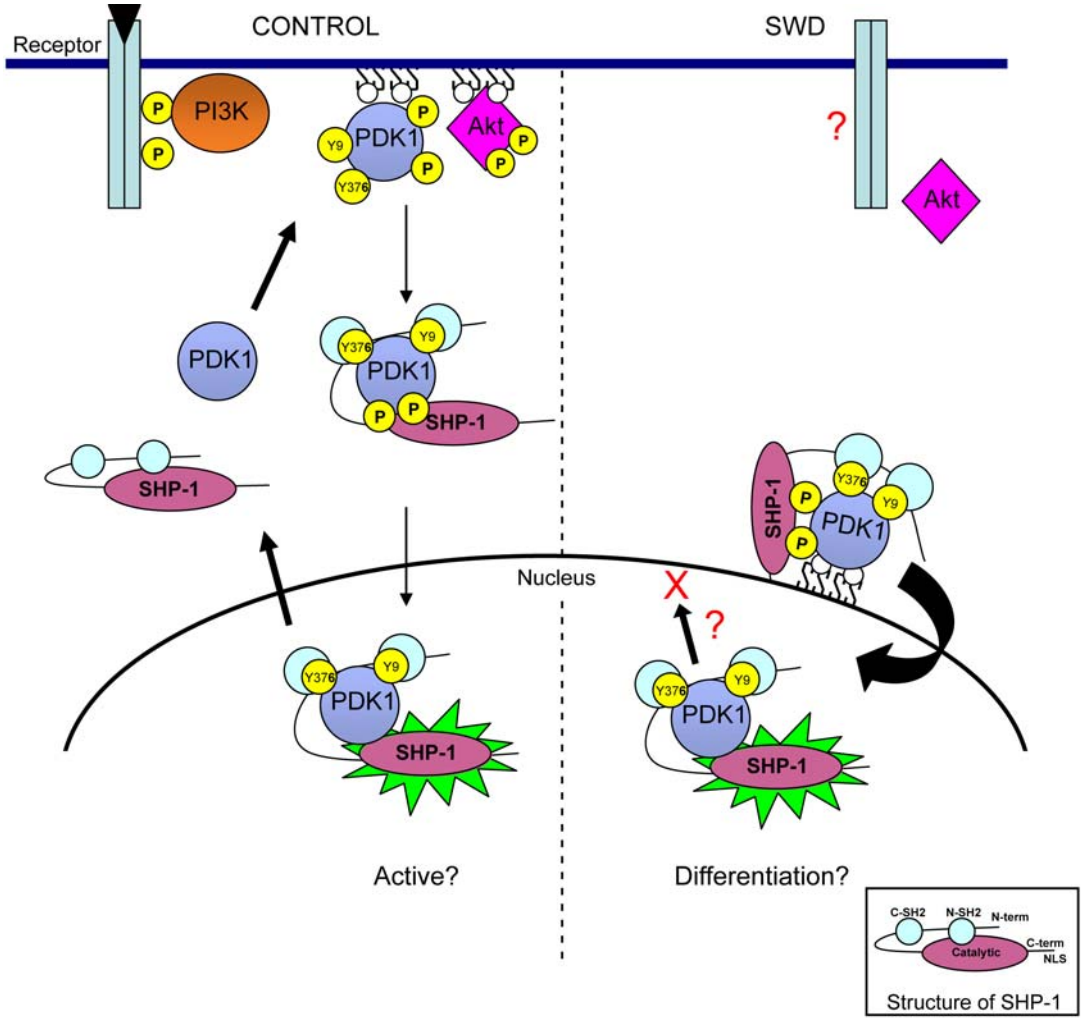


Figure 4.1: Model for SHP-1:PDK1 nuclear shuttling in response to SWD in C6 cells. (Left) Activated PI3K generates PI3,4P_2 and PI3,3,5P_3 which localize PDK1 to the plasma membrane. PDK1 is phosphorylated on putative ITIMs, including Y9 and Y376, which leads to an interaction with SHP-1. PDK1 is then able to "piggy-back" to the nucleus, an event mediated by the nuclear localization signal (NLS) of SHP-1. In the nucleus PDK1 is dephosphorylated (on Y9 and/or Y376?). SHP-1:PDK1 then shuttles to the cytosol, an event mediated by the nuclear export signal of PDK1. (Right) In response to serum withdrawal (SWD) PI3K-associated lipid production occurs near the nucleus which localizes PDK1 to this lipid pool. PDK1 is phosphorylated, particularly on Y9 and Y376 which leads to an interaction with SHP-1. The SHP-1PDK1 complex is not exported (mechanism unknown) from the nucleus leading the complex accumulation (fast rate of protein shuttling: thick arrows; slow rate of protein shuttling: thin arrows).

disrupted in the presence of increased levels of intracellular cAMP (Kim et al., 2001). The mechanism by which cAMP impairs PDK1 localization to the plasma membrane has not been described, however, it is possible a similar mechanism is occurring in reponse to SWD which increases the levels of cellular cAMP as discussed previously (Baranska et al., 2004).

The nuclear accumulation of both SHP-1 and PDK1 in cells treated with A23187 (induces Ca^{2+}-calmodulin-dependent activation of the NPC; Sweitzer and Hanover, 1996) or with leptomycin-B (LMB: inhibits the nuclear pore complex (NPC)-export machinery; Kutay and Guttinger, 2005) implicates the NPC in their transport across the nuclear membrane. This is corroborated by the exclusion of both molecules from the nucleus of cell treated with BAPTA-AM (inhibits the assembly of the NPC; (Macaulay and Forbes, 1996). The combined molecular weight of an SHP-1:PDK1 complex is well above the 25-40 kDa threshold for passive diffusion and, therefore, undoubtedly requires energy-dependent active transport across the NPC (*i.e.* RanGTP-dependent) (Fried and Kutay, 2003). A pivotal role for SHP-1 in this transport is demonstrated by the reduction in nuclear accumulation of PDK1 in cells overexpressing the SHP-1 protein truncated at the C-terminal NLS (SHP-1ΔNLS). However, the effect of the SHP-1ΔNLS in C6 cells is not absolute, whereas it is in HEK293 cells. This suggests that the nuclear import of SHP-1 in HEK293 cells depends exclusively on the C-terminal NLS, whereas in C6 cells the N-terminal NLS (Yang et al., 2002b) may also contribute.

Other proteins able to contribute to PDK1 nuclear import can not be discounted. There are several other proteins which contain a NLS that could act in a similar way as SHP-1. For example, the *PhosphoMotif Finder* on the Human Protein Reference Database identified PDK1 as having phosphorylation motifs for the NLS-containing tyrosine phosphatase, TCPTP (Lorenzen et al., 1995; Tillmann et al., 1994) and the NLS-containing serine/threonine kinase MAPKAPK-2 (Stokoe et al., 1993). Although these proteins do not contain SH2 domains, they could potentially target PDK1 to the nucleus as part of a larger, multimeric protein complex.

The fact that SHP-1 and PDK1 accumulate in the nucleus of LMB-treated cells suggests that the SHP-1:PDK1 complex shuttles between the cytoplasm and the nucleus under resting conditions, with the speed, or rate, of export being significantly greater

than the rate of import. What controls the rate of nuclear shuttling of the SHP-1:PDK1 complex? If the NLS of SHP-1 controls the nuclear import of the SHP-1:PDK1 complex and the tyrosine phosphorylation state of PDK1 drives that association with SHP-1, then it is not unreasonable to consider that the tyrosine phosphorylation state of PDK1 is also contributing to nuclear shuttling of this complex. In the nuclear extracts of both resting cells and in cells grown in serum-free medium, SHP-1 associates with a lower molecular weight form of PDK1, suggesting SHP-1-associated PDK1 is in a dephosphorylated state in the nucleus (Figure 4.1). Overexpressing the substitution mutants (Y9F, Y376F, or Y9/376F) in cells grown in serum-free medium does not result in the expected nuclear accumulation of PDK1; however, endogenous SHP-1 expression is more diffuse throughout these cells and can be found in the nucleus as well. A similar effect is observed in cells coexpressing the Y9/376F mutant with SHP-1 and activated Src. This initially suggested that the interaction between SHP-1 and PDK1 is disrupted due to the mutations on Y9/376; this is supported by the subsequent observation of a nuclear accumulation of both SHP-1 and PDK1Y9/376F in cells treated with LMB. By extension, this may also be indicating that the Y9/376 residues on PDK1 are influential in the rate of export of PDK1 from the nucleus.

The dephosphorylation of Y9 and Y376 on PDK1 also affected SHP-1 export (recall: SHP-1 remains in the nucleus, while PDK1 Y9F, Y376F and Y9/376F mutants are cytoplasmic). Given that SHP-1 interacts less with PDK1 dephosphorylated on Y9/376, and SHP-1 is "left-behind" in the nucleus of cells overexpressing this mutant, argues that these sites are important for the association of SHP-1 with PDK1 and not only for regulating its own rate of export from the nucleus, but also regulating the rate of export of SHP-1. Does SHP-1 "piggy-back" out of the nucleus or is it left behind (Figure 4.1)? The retention of SHP-1 in the nuclear matrix when co-expressed with the PDK1Y9/376F protein is also revealing. Indeed, Lyn, a Src kinase family member, is associated with the nuclear matrix, and not in the chromatin fraction of the nucleus, and is maximally activated at the G1/S transition phase (Radha et al., 1996). If any conclusions can be drawn from the fact that SHP-1 can associate with, and inactivate, such Src kinases such as Lck (Chiang and Sefton, 2001; Frank et al., 2004) and Lyn (Daigle et al., 2002), and that PDK1 can be targeted by Src kinases (Grillo et al., 2000;

Park et al., 2001), then it is possible that Lyn is activated during serum withdrawal (and the ensuing G1 arrest), which would promote the phosphorylation of PDK1 and its retention within the nucleus.

Both SHP-1 and PDK1 have been implicated in differentiation, which invariably involves a nuclear event (Marsh et al., 2003; Martelli et al., 2003; Vambutas et al., 1995). The function of nuclear shuttling of SHP-1 and PDK1 was considered using NGF-induced differentiation of PC12 cells. This model was used instead of SWD in C6 cells (also shown to induce differentiation), because NGF targets the TrkA-receptor specifically, and presumably is more selective as to which signalling pathways are affected, unlike SWD in C6 cells which could be affecting a number of receptors and many signalling pathways.

PC12 cells coexpressing SHP-1 and PDK1 are more sensitive to acute and subchronic NGF treatment as observed by the presence of vast neuritic branching, which usually is only observed after a longer period of treatment (5-14 days). The sensitivity of these cells to NGF is blocked by the overexpressed SHP-1ΔNLS protein or the overexpressed PDK1 Y9/376F mutant, implicating important roles for both SHP-1 and PDK1 in NGF-induced differentiation of PC12 cells (Figure 4.2). However, their roles may not be strictly limited to signalling. For example, the role of SHP-1 may be that of a carrier (if any conclusion can be drawn from the fact that SHP-1 is nuclear when co-expressed with mutated PDK1, yet NGF does not stimulate differentiation in these cells). The actual differentiation process may rely on PDK1 or it could rely on both SHP-1 and PDK1 needing to reside in the nucleus for a critical interval and the rapid export of the PDK1Y9/376F mutant mitigates its effect on the machinery involved in differentiation. Whatever the case, it is obvious that if PDK1 (or a SHP-1:PDK1 complex) does not reside within the nucleus long enough, then differentiation does not occur.

NGF is reported to increase the tyrosine phosphorylation of PDK1 in HEK293 cells stably expressing the TrkA receptor (Prasad et al., 2000). There is an observable decrease in mobility on PDK1 in PC12 cells treated with NGF, however this does not reflect any change in tyrosine phosphorylation on PDK1 in these cells (which constitutively express the TrkA receptor; (Kaplan et al., 1991). While it is difficult to

reconcile these contrasting observations, it may simply be that tyrosine phosphorylation of PDK1 is minimal, yet sufficient, to allow for constitutive association with SHP-1 and for nucleo-cytoplasmic shuttling, an event that may exist simply as a means of priming the nuclear machinery or that may serve to maintain normal PC12 growth and function.

Nuclear shuttling of the SHP-1:PDK1 complex is a conserved event, occurring in glial, neuronal and peripheral cell lines. What might be attracting these proteins to the nuclear membrane in the first place? There are two possible models: first, the tyrosine phosphorylation of PDK1 induced by growth factor stimulation or conditions of stress results in the recruitment and binding of SHP-1, and together they are directed to the NPC by importins. In this model, SHP-1 is simply functioning to bring PDK1 to the nucleus where it can be dephosphorylated and recycled to the cytosol for modulation of its targets. A second possibility is that SHP-1:PDK1 shuttling may occur through direction of nuclear PI3K production of 3'-phospholipids, which is observed in NGF-induced differentiation of PC12 cells (Martelli et al., 2003) and is observed in response to SWD in C6 cells (current data). Both SHP-1 and PDK1 have binding affinities for $PI3,4,5P_3$ (Frank et al., 1999; Vanhaesebroeck and Alessi, 2000). They may be recruited to the nuclear envelope where they are able to interact and, due to proximity, are recognized by the NPC and rapidly imported in the nucleus (Figure 4.2). It is possible that both models are correct, but simply context-dependent. Whatever the case, it is clear that SHP-1 and PDK1 contribute to differentiation in these cells and it is therefore highly possible that these two proteins existing in a complex can contribute to differentiation in many other cell types (glial, neuronal and non-neuronal).

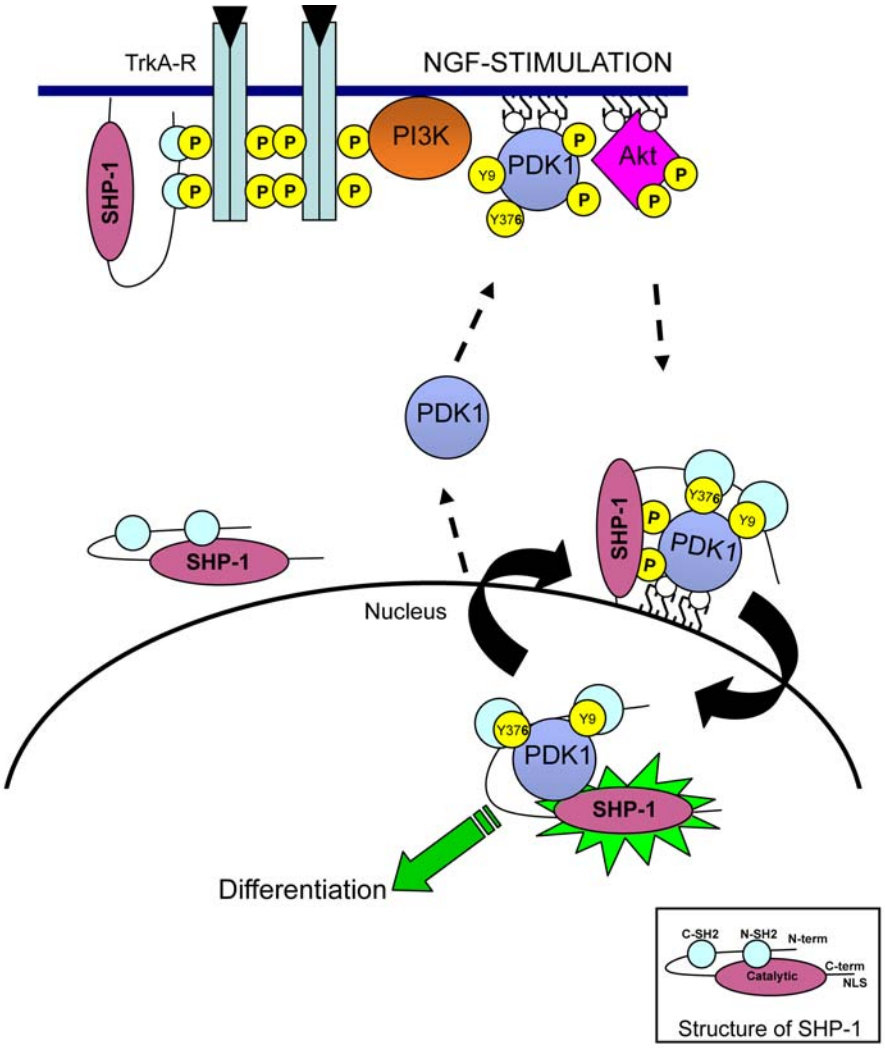


Figure 4.2: Model for SHP-1:PDK1 nuclear shuttling in response to NGF stimulation of PC12 cells. In response to NGF stimulation, PI3K is active at the plasma membrane, but more so at the nuclear membrane. PDK1 is associated predominantly with the nuclear membrane where it is phosphorylated, particularly on Y9 and Y376 (two putative ITIMs) which leads to an interaction with SHP-1. PDK1 is then able to "piggy-back" to the nucleus, an event mediated by the nuclear localization signal (NLS) of SHP-1. In the nucleus SHP-1:PDK1 complex mediates NGF-induced PC12 differentiation. PDK1 is then dephosphorylated possibly by SHP-1 or another phosphatase then shuttles to the cytosol, an event mediated by the nuclear export signal of PDK1. Shuttling from the outer nuclear membrane into the nucleus occurs at a fast rate (indicated by the thick arrows) in comparison to shuttling from the plasma membrane which possibly occurs to a lesser extent in response to NGF treatment (dashed arrows).

6. FUTURE DIRECTIONS

This work demonstrates that SHP-1 can regulate the tyrosine phosphorylation and subcellular distribution of PDK1. Moreover, the rate of SHP-1:PDK1 nucleo-cytoplasmic shuttling can be determined by the type of stimulation, *i.e.* SWD causes a nuclear accumulation of the SHP-1:PDK1 complex and NGF stimulation does not induce a noticeable accumulation of either protein, but does require a SHP-1:PDK1 nuclear event. These findings suggest that distinct events regulate the rate of SHP-1:PDK1 nuclear shuttling and that tyrosine phosphorylation is an important contributing factor. How the tyrosine phosphorylation of PDK1 affects SHP-1:PDK1 nuclear shuttling needs to be investigated further. The interaction between SHP-1 and PDK1 is demonstrated in this work to be phosphotyrosine-dependent. Introduction of point mutations (R to A) into the SH2 domains of SHP-1 would determine their roles and/or which SH2 domain(s) mediates the interaction with PDK1.

The data presented in this thesis do not identify any nuclear targets of the SHP-1:PDK1 complex, however, such targets must exist. The PI3K/Ak pathway is active and produces $PI3,4,5P_3$ in the nucleus of NGF-differentiated PC12 cells and several downstream targets of PDK1 and SHP-1 are also present in the nucleus (Neri et al., 2002; Ram and Waxman, 1997). The SHP-1:PDK1 complex promotes differentiation and may be important for cell cycle arrest. Identifying the nuclear function of PDK1 and SHP-1 as well as their nuclear targets would surely further our understanding of regulation of nuclear events.

Some of the data, although not characterized as extensively, indicate that PDK1 may regulate the nuclear export of SHP-1. It is interesting that there are distinct differences with the subcellular distribution of SHP-1 between hematopoietic and non-hematopoietic cell lines (Brumell et al., 1997; Ganesan et al., 2003; He et al., 2005). The expression, or rate, of nuclear PDK1 shuttling may provide some insight to differences in SHP-1 distribution. Understanding how nucleo-cytoplasmic shuttling of SHP-1 is

regulated may present better models to understanding differences in the regulation of these cells. There is still more to learn about nuclear SHP-1, such as its function, the mechanism involved in nuclear targeting and import, and even how it may be exported from the nucleus.

Neurotrophins such as NGF or brain-derived neurotrophic factor (BDNF) are involved in a variety of neuromodulatory processes in the brain, including neuronal survival, neurite outgrowth and synapse formation (Altar and DiStefano, 1998). Growth factors such as NGF and BDNF are abnormally regulated in models of psychiatric diseases such as schizophrenia and depression (Angelucci et al., 2004). Recently, certain second generation antipsychotic (Angelucci et al., 2005) and antidepressant (Mallei et al., 2002; Nibuya et al., 1995) drugs have been shown to alter the brain levels of neurotrophins and promote differentiation of neurons (Lu and Dwyer, 2005; Malberg and Blendy, 2005). Interestingly, most antidepressant drugs increase intracellular levels of cAMP through activation of adrenoceptors or serotonin receptors, but it is important to note that not all subtypes of these receptors are coupled to the adenylyl cyclase-cAMP-PKA pathway (Malberg and Blendy, 2005). Thus, the proposed mechanism involving SHP-1 and PDK1 in mediating differentiation may also provide more insight into how some of these psychiatric drugs are mediating differentiation and, more importantly, neurogenesis. Furthermore, a better understanding of the mechanisms underlying the effects of the differentiation/neurogenesis induced by some psychiatric drugs might lead to new therapeutics.

6. APPENDIX I

Human Protein Reference Database

You are at: HPRD

Query
Browse
Blast
FAQs
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Human Proteinpedia
Pathways
PhosphoMotif Finder
Become a "Molecule Authority"

PhosphoMotif Finder

Your query protein contains 34 Tyrosine kinase / phosphatase motifs described in the literature and are underlined below.

MARTTSQL**YDAV**PIQSSVVLCSCPSPSMVRTQTESSTPPGIPGGSRQGPAMDGTAAEPRPGAGSLQHAQPPPQPRKKRPEDFKFGKILGEGSFSTVVLARELATS**REYA**I
KILEKRHIIKENKVPYVTRERDVMSRLDHPFFVKLYFTFQDD**EKLYFGLSYA**KNGELLKYIRKIGSFDETCTRF**YT**AEIVSA**LEYL**HGKGIIHRDLKPENILLNEDMHIQ
ITDFGTAKVLSPESKQARANSFVGTAQYVSPELLTEKSACKSSDLWALGCII**YQLV**AGLPPFRAG**NEYL**IFQKIIK**LEYD**FPEKFFPKARDLVEKLLVLDATKRLGCEEM
EGYGPLKAHPFFESVTWENLHQQTPPKLTAYLPAMSEDD**EDCYGNYDNL**LSQFGCMQVSSSSSSHSLSASDTGLPQRSGSNI**EQY**IHDLDSNSFELDLQFSEDEKRLLLE
KQAGGNPWHQFVENNLILKMGPVDKRKGLFARRRQLLLTEGPHL**YY**VDPVNKVLKGEIPWSQELRPEAKNFKTFFVHTP**NRTYY**LMDPSGNAHKWCRKIQEVWRQRYQSH
PDAAVQ

Sort by Position in query protein

PhosphoMotif

	Position in query protein	Sequence in query protein	Corresponding motif described in the literature (phosphorylated residues in red)	Features of motif described in the literature	Link to original article describing the motif
1	9 - 10	YD	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
2	9 - 12	YDAV	pYXX[L/I/V]	JAK2 kinase substrate motif	PubMed
3	9 - 12	YDAV	pY[E/D]X[I/L/V/M]	ALK kinase substrate motif	PubMed
4	106 - 109	REYA	X[E/D]pYX	EGFR kinase substrate motif	PubMed
5	107 - 108	EY	[E/D/Y]pY	TC-PTP phosphatase substrate motif	PubMed
6	108 - 109	YA	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
7	153 - 156	EKLY	[E/D]XXpY	ALK kinase substrate motif	PubMed
8	156 - 159	YFGL	pYXX[L/I/V]	JAK2 kinase substrate motif	PubMed
9	156 - 161	YFGLSY	pYXXXX[F/Y]	ALK kinase substrate motif	PubMed
10	161 - 162	YA	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
11	185 - 186	YT	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
12	193 - 196	LEYL	X[E/D]pYX	EGFR kinase substrate motif	PubMed
13	193 - 196	LEYL	X[E/D]pY[I/L/V]	EGFR kinase substrate motif	PubMed
14	194 - 195	EY	[E/D/Y]pY	TC-PTP phosphatase substrate motif	PubMed
15	273 - 276	YQLV	pYXX[L/I/V]	JAK2 kinase substrate motif	PubMed
16	286 - 289	NEYL	X[E/D]pYX	EGFR kinase substrate motif	PubMed
17	286 - 289	NEYL	X[E/D]pY[I/L/V]	EGFR kinase substrate motif	PubMed
18	287 - 288	EY	[E/D/Y]pY	TC-PTP phosphatase substrate motif	PubMed
19	297 - 300	LEYD	X[E/D]pYX	EGFR kinase substrate motif	PubMed
20	298 - 299	EY	[E/D/Y]pY	TC-PTP phosphatase substrate motif	PubMed
21	299 - 300	YD	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
22	331 - 333	EGY	[E/D]XpY	SHP1 phosphatase substrate motif	PubMed
23	333 - 334	YG	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
24	333 - 336	YGPL	pYXX[L/I/V]	JAK2 kinase substrate motif	PubMed
25	370 - 373	EDCY	[E/D]XXpY	ALK kinase substrate motif	PubMed
26	371 - 373	DCY	[E/D]XpY	SHP1 phosphatase substrate motif	PubMed
27	373 - 374	YG	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
28	376 - 377	YD	pY[A/G/S/T/E/D]	Src kinase substrate motif	PubMed
29	376 - 379	YDNL	pYXX[L/I/V]	JAK2 kinase substrate motif	PubMed
30	376 - 379	YDNL	pY[E/D]X[I/L/V/M]	ALK kinase substrate motif	PubMed
31	413 - 415	EQY	[E/D]XpY	SHP1 phosphatase substrate motif	PubMed
32	485 - 486	YY	[E/D/Y]pY	TC-PTP phosphatase substrate motif	PubMed
33	520 - 523	NRTY	[L/N][R/I]TpY	PDGFR kinase substrate motif	PubMed
34	523 - 524	YY	[E/D/Y]pY	TC-PTP phosphatase substrate motif	PubMed

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MARTTSQLYDAVPIQSSVVLCSCPSPSMVRTQTESSTPPGIPGGSRQGPAMDGTAAEPRPGAGSLQHAQPPPQPRKKRPEDFKFGKILGEGSFSTVVLARELATSREYAI
KILEKRHIIKENKVPYVTRERDVMSRLDHPFFVKL**YFTF**QDDEKL**YFGL**SYAKNGELLKYIRKIGSFDETCTRFYTAEIVSALEYLHGKGIIHRDLKPENILLNEDMHIQ
ITDFGTAKVLSPESKQARANSFVGTAQ**YVSP**ELLTEKSACKSSDLWALGC**IIYQLV**AGLPPFRAGNEYLIFQKIIKLE**YDFP**EKFFPKARDLVEKLLVLDATKRLGCEEM
EGYGPLKAHPFFESVTWENLHQQTPPKLTAYLPAMSEDDEDC**YGNYDN**LLSQFGCMQVSSSSSSHSLSASDTGLPQRSGSNIEQYIHDLDSNSFELDLQFSEDEKRLLLE
KQAGGNPWHQFVENNLILKMGPVDKRKGLFARRRQLLLTEGPHLY**YVDP**VNKVLKGEIPWSQELRPEAKNFKTFFVHTP**NRTYYLM**DPSGNAHKWCRKIQEVWRQRYQSH
PDAAVQ

Sort by Position in query protein

PhosphoMotif

	Position in query protein	Sequence in query protein	Corresponding motif described in the literature (phosphorylated residues in red)	Features of motif described in the literature	Link to original article describing the motif
1	146 - 149	YFTF	pYFX[F/P/L/Y]	HCP SH2 domain binding motif	PubMed
2	156 - 159	YFGL	pYFX[F/P/L/Y]	HCP SH2 domain binding motif	PubMed
3	248 - 251	YVSP	pY[A/E/V][Y/F/E/S/N/V][P/F/I/H]	Itk SH2 domain binding motif	PubMed
4	248 - 251	YVSP	pYXXP	Crk SH2 domain binding motif	PubMed
5	248 - 251	YVSP	pYXXP	RasGAP C-terminal SH2 domain binding motif	PubMed
6	271 - 276	IIYQLV	[I/V]XpYXX[L/V]	SHP1 SH2 domain binding motif	PubMed
7	299 - 302	YDFP	pYXXP	Crk SH2 domain binding motif	PubMed
8	299 - 302	YDFP	pYXXP	RasGAP C-terminal SH2 domain binding motif	PubMed
9	373 - 375	YGN	pYXN	Grb2 SH2 domain binding motif	PubMed
10	376 - 378	YDN	pYXN	Grb2 SH2 domain binding motif	PubMed
11	486 - 489	YVDP	pYXXP	Crk SH2 domain binding motif	PubMed
12	486 - 489	YVDP	pYXXP	RasGAP C-terminal SH2 domain binding motif	PubMed
13	520 - 523	NRTY	NXXpY	FRIP PTB domain binding motif	PubMed
14	523 - 526	YYLM	pYXXM	PI3 Kinase p85 SH2 domain binding motif	PubMed
15	523 - 526	YYLM	pY[I/E/Y/L]X[I/L/M]	SHC SH2 domain binding motif	PubMed

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