ALTERED REGULATION OF PTEN BY MUTAGENESIS AND p85 BINDING

A Thesis Submitted to the College of

Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Biochemistry

University of Saskatchewan

Saskatoon

By

M. CHRIS PASTOR

Keywords: PTEN, p85, PI3K, signal transduction, cancer

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Dr. Deborah H. Anderson
Saskatchewan Cancer Agency
Cancer Research Unit
20 Campus Drive
University of Saskatchewan
Saskatoon, Saskatchewan
S7N 4H4

ABSTRACT

Growth and proliferation are normal functions of cells mediated in part *via* receptor tyrosine kinases such as the epidermal growth factor (EGF) receptor (EGFR). The EGFR binds the extracellular signaling ligand EGF and transduces the signal into the cell. Phosphatidylinositol 3'-kinase (PI3K) responds to EGFR activation and initiates downstream signaling cascades responsible for cell cycle entry, proliferation and inhibition of apoptosis. Cell cycle arrest is required to stop cell growth and proliferation as well as allow apoptosis, if required. The phosphatase and tensin homologue deleted on chromosome ten (PTEN) directly opposes PI3K signaling since its substrate is the PI3K product phosphatidylinositol 3,4,5-trisphosphate. PI3K is a heterodimer composed of a p85 regulatory subunit and a p110 catalytic subunit. PTEN is an essential tumor suppressor protein. Absence of PTEN has been associated with several types of cancer.

Our laboratory has characterized new specialized functions for the p85 protein. One function discovered was the ability of p85 to enhance PTEN lipid phosphatase activity. In this thesis PTEN activity is shown to be enhanced at least 3.5 fold *in vitro* by an equimolar amount of p85.

We performed an analysis of PTEN using seven PTEN mutants. Two types of mutants were created: i) regulatory or possible regulatory phosphorylation sites were substituted to mimic both phosphorylated and non-phosphorylated states and ii) alanine substitution of basic amino acid residues. The phosphorylation sites altered were the casein kinase 2 phosphorylation sites in the regulatory domain and tyrosine 336, a proposed regulatory phosphorylation site. Three mutants involving alanine substitution for basic amino acid residues included one mutant in the PASE domain and two more mutants in the C2 domain. It was observed that GFP-PTEN translocates to the plasma membrane upon EGF stimulation. The mimic of constitutive phosphorylation of

the Casein kinase 2 sites resulted in cytoplasmic localization whereas the non-phosphorylated mimic was plasma membrane localized regardless of EGFR activation status. Neutralization of positive charge in the PASE and C2 domains seriously impeded the ability of PTEN to bind to phosphorylated phosphatidylinositol lipids and abolished the ability of the protein to translocate to the plasma membrane in response to receptor activation. Located within a cluster of positively charged lysine residues in the C2 domain is a potential phosphorylation site at tyrosine 336. The phosphorylation mimic showed decreased binding to some membrane lipids compared to the non-phosphorylated mimic.

The results we generated are consistent with a current model for PTEN regulation that proposes PTEN is localized to the cytoplasm in quiescent cells and dephosphorylation of the regulatory domain occurs upon EGF stimulation allowing translocation to the plasma membrane. The model proposes that dephosphorylation of the casein kinase 2 sites unmasks regions of positive charge that interact with the anionic plasma membrane. Furthermore, the results suggested that at the plasma membrane p85 interacts with PTEN to increase lipid phosphatase activity and may be involved in targeting PTEN to the activated receptor where PI3,4,5P_3 lipids are being produced.

ACKNOWLEDGMENTS

I would like to acknowledge my friends and family for their support. I am sincerely grateful to my advisory committee for their guidance and advice. Many thanks to the graduate students, summer students and project students I had the opportunity to work with, especially Stacey Knafelc, a summer student whom I personally supervised, who contributed greatly by expressing and purifying PTEN mutant proteins. Expert technical support was provided by Andrea Hawrysh and Kristy James. Both were exceptional at maintaining a very organized laboratory and helping out when needed. I would like to acknowledge the Neuropsychiatry Research Unit, which graciously allowed free access to their confocal microscope. Finally, I would like to thank my supervisor Deborah H. Anderson for providing the opportunity to work in such a great environment. Her guidance, patience and foresight provided great ideas for experiments. This thesis would never have been realized without her insightful suggestions.

DEDICATION

This thesis is dedicated to family and friends.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AEBSF	4-(2-aminoethyl)-benzenesulfonylfluoride
AMP	adenosine mono-phosphate
ATP	adenosine tri-phosphate
BCR	breakpoint cluster region
αMEM	alpha modified eagle's medium
BH	BCR homology
CAMP	cyclic AMP
CBP	CREB-binding protein
CREB	CAMP response element binding protein
DTT	dithiothreitol
E. coli	*Escherichia coli*
EGF	epidermal growth factor
EGFR	EGF receptor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FKHR	Forkhead family of transcription factors
GAP	GTPase activating protein
GDP	guanosine diphosphate
GFP	green fluorescent protein
Grb2	growth-factor-receptor-bound protein 2
GS	glycogen synthase
GSK3	glycogen synthase kinase-3
GST	glutathione S-transferase
GTP	guanosine triphosphate
HB-EGF	heparin binding EGF
HEK293	human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IGFR	Insulin-like growth factor receptor
IPTG	isopropyl β-D-thiogalactopyranoside
LB	Luria-Bertani
LBA	LB ampicillin
LBK	LB kanamycin
LPA	lysophosphatidic acid
LPC	lysophosphocholine
MAPK	mitogen activated protein kinase
MAGI	membrane associated guanylate kinase inverted
Mdm2	mouse double minute 2
MEK	MAPK/ERK kinase
MMAC1	mutated in multiple advanced cancers 1
mTOR	mammalian target of rapamycin
NEDD4-1	neural precursor cell expressed, developmentally downregulated-4-1
(NF)-κβ	nuclear factor kappa beta

LIST OF ABBREVIATIONS (continued)

NLS	nuclear localization signal
NRG	neuregulin
PA	phosphatidic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCAF	p300/CBP-associated factor
PDK1	phosphoinositide-dependent kinase 1
PDZ	PSD-95/Dlg/ZO-1
PE	phosphatidylethanolamine
PH	plekstrin homology
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3 kinase
PI3P	PI 3 phosphate
PI4P	PI 4 phosphate
PI5P	PI 5 phosphate
$PI3,4P_2$	PI 3,4 bisphosphate
$PI3,5P_2$	PI 3,5 bisphosphate
$PI4,5P_2$	PI 4,5 bisphosphate
$PI3,4,5P_3$	PI 3,4,5 trisphosphate
PM	plasma membrane
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPS	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine
PS	phosphatidylserine
P/S	penicillin G/streptomycin
PTB	phosphotyrosine binding
PTEN	phosphatase and tensin homolog deleted on chromosome ten
RFP	red fluorescent protein
RTK	receptor tyrosine kinase
SDS	sodium dodecyl sulfate
SHP-1	SH2-containing protein tyrosine phosphatase-1
SH2	src homology 2
SH3	src homology 3
SOS	son of sevenless
S1P	sphingosine 1-phosphate
TBS	Tris-buffered saline
TBST	TBS and Tween-20
TCEP	tris(2-carbosyethyl)phosphine
TEP1	TGF-beta-regulated and epithelial cell-enriched phosphatase
TGFα	transforming growth factor-alpha
wt	wild type

Amino Acids

A	Alanine	M	Methionine
C	Cysteine	N	Asparagine
D	Aspartic Acid	P	Proline
E	Glutamic Acid	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histidine	T	Threonine
I	Isoleucine	V	Valine
K	Lysine	W	Tryptophan
L	Leucine	Y	Tyrosine

1.0 INTRODUCTION

1.1 Signal Transduction

In order for any multicellular organism to function there needs to be mechanisms to control the array of individual cells in relation to the organism as a whole. Specialized cell types have evolved with unique structural characteristics and functions. In order for these cells to effectively carry out the functions required of them, a communication network is required. Through this network the organism as a whole is able to mediate the function and behavior of the individual cells. The signals sent through the network are numerous and include messages to regulate differentiation, proliferation and cell death (apoptosis). Each cell is, by definition, a functional unit isolated by the presence of a semi-permeable lipid bilayer called the plasma membrane. Although some signaling molecules, such as small chemicals and drugs, are capable of traversing the plasma membrane, many cannot (Schuller, 1991). In some instances, signal factors activate lipid-gated transmembrane ion channels allowing entrance or exit of specific ions, which allows regulation of cellular function (Gready *et al.*, 1997). Some signaling molecules require a receptor located on the cell surface that spans the intermembrane space to convey information about the extracellular environment (Gururaj and Kumar, 2005; Schuller, 1991). The event of an extracellular signaling molecule binding a cell surface receptor results in a conformational change to the cytoplasmic portion of the receptor. Thus the signaling molecule

itself remains extracellular while the essence of the message is transferred, or transduced, inside the cell. The integration, execution and attenuation of the signal inside the cell occurs *via* a cascade of second messengers involving various molecular interactions (Kolch, 2000).

Important signaling biomolecules are produced by an assortment of sources and delivered by various processes. These biomolecules may originate from adjacent cells, distant organs or even the external environment. Signaling molecules that do not diffuse through the cell membrane include polypeptide growth factors, hormones, neuropeptides, neurotransmitters, the surfaces of neighboring cells, cytokines and the molecules of the extracellular matrix (Schuller, 1991). There are a plethora of signaling molecules involved in regulating the behavior and function of individual cells (Gururaj and Kumar, 2005). Correspondingly there is diverse set of receptors that bind these signaling molecules and transduce specific signals (Gururaj and Kumar, 2005). Specific cell types are in part defined by the types of receptors they express. How, or even if, a cell responds to a particular external stimulus is dictated in part by the particular receptors it expresses. Once a signal is received by a receptor, transduction occurs *via* a change in activation status and/or localization of a variety of cytoplasmic enzymes (Yu *et al.*, 1998). The signal is often amplified as one receptor may activate several enzymes and each enzyme acts on several substrate molecules that act as second messengers. The set of molecules involved in transduction and amplification of the signal are of varying size and composition and include small lipids, short peptides, proteins and large macromolecular multi-protein complexes. The signaling proteins found in cells are often conserved, but the specific responses and modulation of the signal may vary with cell type.

The interactions between molecules are often reversible and are tightly controlled. Regulation usually involves negative feedback loops that are initiated *via* the transduction of the original

signal. Multiple levels of regulation exist to ensure that the initial signal is not an exaggerated or insufficient response to the desired signal (Yu *et al.*, 2003).

Loss of function or total loss of proteins involved in signal transduction pathways are implicated in the pathophysiology of many diseases, including cancers (Yu *et al.*, 2003). Regulatory abnormalities are often caused by mutations or total deletion of genes that encode signal transduction proteins. Regulation of signal transduction molecules may involve a wide range of posttranslational modifications including the addition of functional groups such as lipids, methyls, ubiquitin, sugars, acetyls or phosphate groups. Hydrolytic cleavage at specific sites or total proteolytic degradation may also be incorporated in the regulation of signaling. With regard to signal transduction, the most utilized and best studied form of regulation is the addition and removal of phosphate groups to free hydroxyls by kinases and phosphatases, respectively.

1.2 Receptor Tyrosine Kinases

In order for a signal such as a hormone or growth factor to exert its effect on a cell, it must bind its specific receptor on the cell surface. One such family of cell receptors has intrinsic kinase activity and these receptors utilize ATP to transfer the γ phosphate group to the hydroxyl of a tyrosine amino acid residue (Adamson and Rees, 1981). This group of cell signaling receptors is collectively termed receptor tyrosine kinases (RTKs). According to current databases (IUPHAR) there are twenty classifications of RTKs that have been identified to date (Vanhoutte, 2006). Receptors are typically very large proteins possessing a transmembrane domain that allows the protein to span the plasma membrane and thus transduce the extracellular signal by causing changes inside the cell. The changes induced by RTK signaling are widespread and can include processes such as differentiation, altered metabolism, proliferation or cell

survival (Simon, 2000). Also, an RTK signal in one cell type may have an entirely different function in another cell type. This is especially true with regard to undifferentiated cells, such as fibroblasts, versus fully differentiated cells, such as epithelia (Halfon *et al.*, 2000).

Upon ligand binding, RTKs are activated by dimerization of subunits, which results in autophosphorylation of tyrosine residues within the cytoplasmic domains (Pandey *et al.*, 1994). Many RTK types include multiple isoforms, so dimerization may include homodimerization or heterodimerization (Pandey *et al.*, 1994). Phosphorylation of the cytoplasmic tyrosine residues results, in some cases, in the recruitment of signaling molecules to the receptor. Typically, translocation to the receptor positions RTK binding proteins in proximity to their substrate and may also increase their activity, which results in the initiation of various downstream cascades. The duration a ligand binds to its receptor is variable and some signals have quite a long duration whereas others, such as growth factor signaling, are very transient (Marshall, 1995). A common thread between all types of RTKs is that through transduction of the original signal, transcription factors are activated or inactivated, either directly or indirectly, to activate or repress transcription of specific genes (Simon, 2000). In normal cells, regardless of function, specific patterns of proliferation are adhered to (George, 2003). Strict control of these patterns is partly controlled by RTK signaling pathways that transduce extracellular information into intracellular signals (George, 2003). Normal cells rely on efficient and tightly controlled RTK signaling to carry out their normal physiological roles including metabolism, growth and proliferation (George, 2003). Mutations in RTKs, or components of the signaling pathways they regulate, can drive unregulated cell growth and may repress the ability undergo apoptosis (Nam and Parang, 2003). RTKs and downstream signaling proteins in those pathways are often deregulated in cancer cells due to their inherent ability to promote proliferative signaling.

The epidermal growth factor (EGF) receptor (EGFR) is a well studied subclass of RTK that has been associated with human cancers (Stoscheck and King, 1986). EGFRs consist of a cytoplasmic protein tyrosine kinase domain, a transmembrane domain and extracellular ligand binding domain containing several α-solenoid or β-helix folds (Jorissen *et al.*, 2003) (Figure 1.1). Humans have four subclasses of EGFR that have been classified types 1-4 (Navolanic *et al.*, 2003). Type 1 is the EGFR/Her1 receptor and types 2-4 are classified Her2, Her3 and Her4 (George, 2003). The four subclasses of EGFR are capable of homodimerization (Olayioye *et al.*, 2000). As well, types 1, 3 and 4 are capable of heterodimerization with type 2 (Figure 1.2 a) (Olayioye *et al.*, 2000). There are also two main classes of EGF ligand, EGF and neuregulin (NRG) (Navolanic *et al.*, 2003). The EGF ligand family includes: EGF, heparin binding EGF (HB-EGF), transforming growth factor α (TGFα), β cellulin, amphiregulin and epiregulin (Navolanic *et al.*, 2003). The NRG ligand family consists of NRG α, NRG β, NRG 2α, NRG 2β, NRG 3α and NRG 3β. Hetero- and homodimerization of the receptor classes combined with the two classes of EGF ligand provides a fairly high degree of specificity regarding signal type (Burgess *et al.*, 2003; Jorissen *et al.*, 2003; Navolanic *et al.*, 2003). Not every type of EGF ligand binds every receptor combination (Figure 1.2 b) (Olayioye *et al.*, 2000). The EGFR only binds EGF family ligands (Olayioye *et al.*, 2000). However, β cellulin, HB-EGF and epiregulin also bind Her4 (Olayioye *et al.*, 2000). The NRG ligand family does not bind the EGFR, but does bind the Her3 and Her4 receptors (Olayioye *et al.*, 2000).

Upon EGF binding, the EGFR homodimerizes and undergoes autophosphorylation of several tyrosines located within the cytosolic portion of the receptor (Earp *et al.*, 1995; Moriki *et al.*, 2001). Phosphorylation of these cytosolic tyrosine residues creates docking sites for proteins that contain the appropriate recognition domain (Pawson *et al.*, 2001). Several proteins are

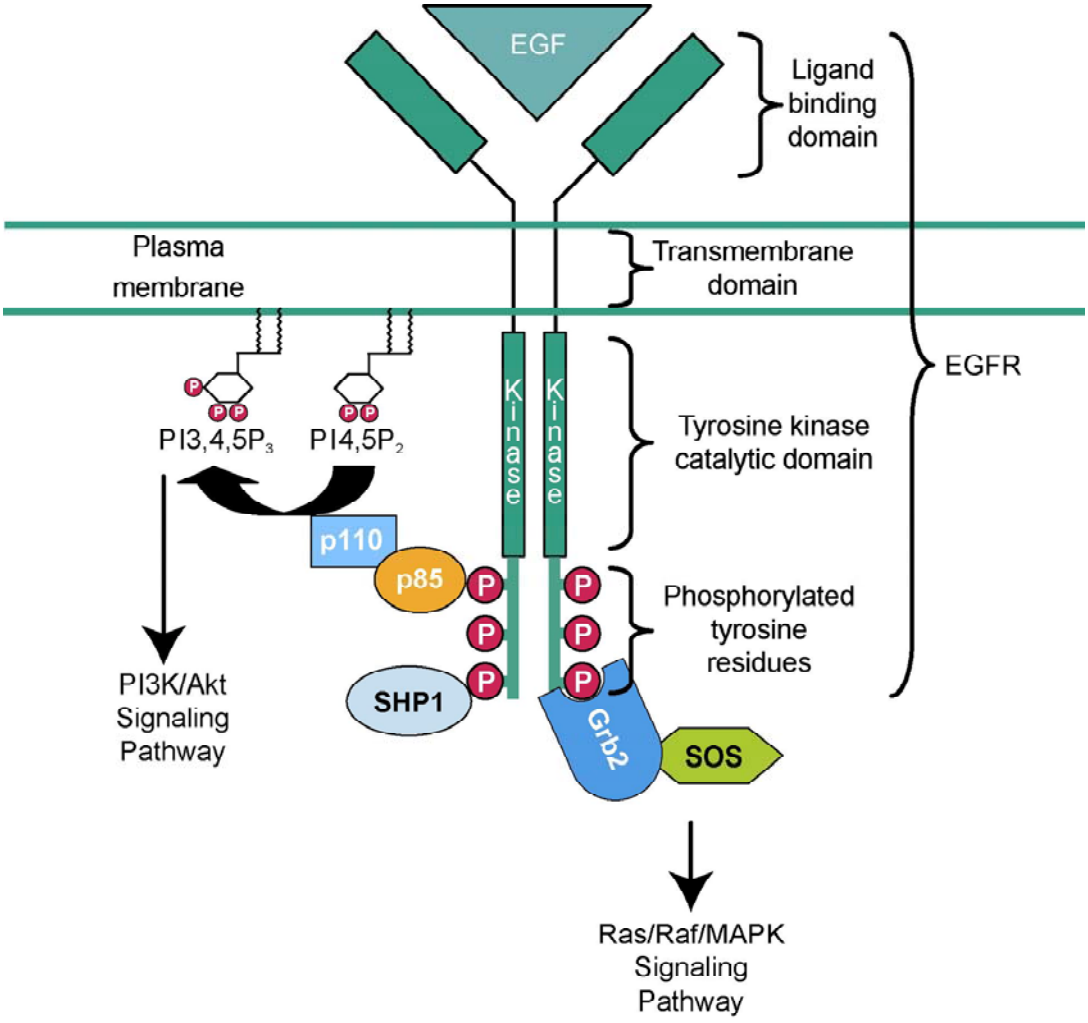
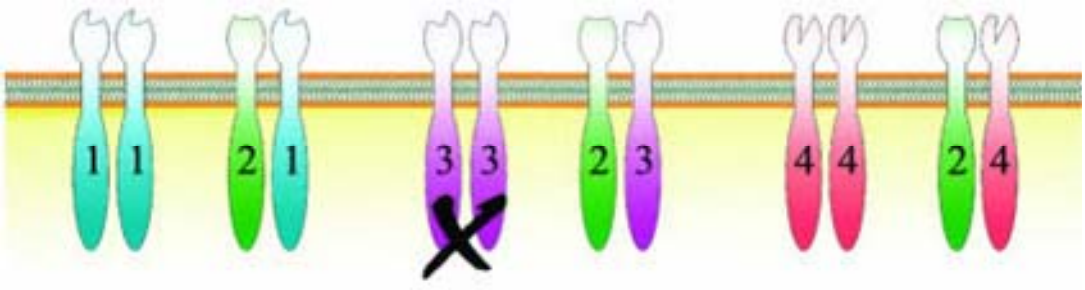


Figure 1.1: Domain structure of the EGFR and schematic including some EGFR binding proteins. The EGFR contains several cytosolic tyrosine residues, a protein tyrosine kinase domain, a transmembrane domain and an extracellular ligand binding domain. Proteins that contain SH2 domains such as Grb2, p85 and SHP1, bind to the phosphotyrosines of the EGFR in response to ligand binding.

(a)



(b)

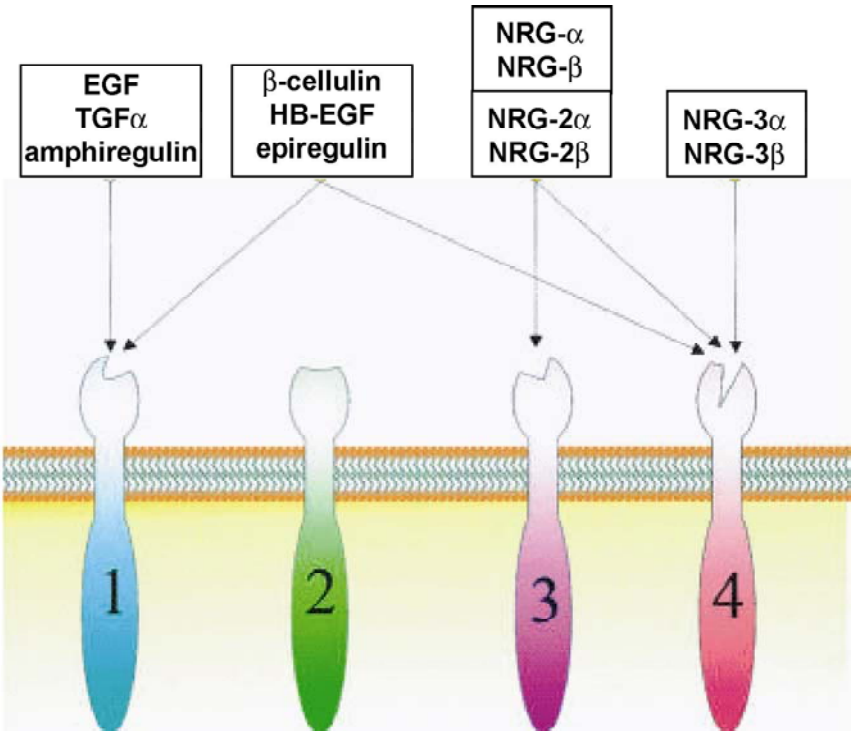


Figure 1.2: Heterodimerization of EGFR isoforms and binding preference of EGF and EGF-like ligands. (a) Her2 itself does not homodimerize but heterodimerizes with EGFR (1), Her3 (3) and Her4 (4). Her3 is capable of homodimerization, but has no associated signaling (x). Her4 is capable of homo- and heterodimerization. (b) schematic of EGF ligands and their receptors. EGFR (1) binds the EGF ligand family, EGF, TGFα, amphiregulin, β-cellulin, HB-EGF and epiregulin, but not the NRG ligand family. β-cellulin, HB-EGF and epiregulin all bind Her4 as well as EGFR. The NRG ligand family does not bind the EGFR. Modified from Olayioye (2000).

capable of interacting with these phosphotyrosines either *via* src homology 2 (SH2) domains or phosphotyrosine binding (PTB) domains (Jones and Dumont, 1999; Ricketts *et al.*, 1996). The difference in binding specificity between SH2 and PTB domains is that SH2 domains bind motifs that extend C terminal from a phosphotyrosine (e.g. pYXXM, where pY is a phosphotyrosine, X is any amino acid and M is methionine) whereas PTB domains bind motifs that extend amino terminal from a phosphotyrosine (NPXpY) (Navolanic *et al.*, 2003). An abundant amount of proteins containing SH2 and/or PTB domains are capable of binding phosphorylated EGFR of which only a selected few are shown (Figure 1.1).

Reversal or attenuation of RTK signaling is essential for effective transduction of the signal as hyperactivity of these cascades leads to uncontrolled cell growth. Activated RTKs lead to expression and activation of proteins that promote cell cycle progression and apoptotic inhibition (Navolanic *et al.*, 2003). Overexpression of EGFR results in dimerization and autophosphorylation in the absence of ligand, which also leads to cell transformation due to over-activity of pathways that promote cell survival and cell cycle progression (Chazin *et al.*, 1992; Di Fiore *et al.*, 1987). The simplest biochemical mechanism of attenuation is the removal of the phosphate group from the tyrosine by protein tyrosine phosphatases that contain SH2 domains, such as SHP-1 (Ostman and Bohmer, 2001). Another mechanism of opposing downstream signaling is through serine phosphorylation to disrupt the effect of the phosphotyrosines (Jorissen *et al.*, 2003). Additionally the entire receptor and ligand complex may be endocytosed and targeted for degradation (Sweeney and Carraway, 2004). Finally, fine tuning of the signal can be accomplished *via* protein interactions immediately downstream of the receptor.

Two important pathways that mediate parallel EGFR signaling cascades are the Ras/Raf/

mitogen activated protein kinase (MAPK) and phosphatidylinositol 3'-kinase (PI3K)/Akt pathways. The Ras/Raf/MAPK pathway is a proliferation, differentiation and pro-survival signaling cascade that is ubiquitously utilized in many cell types and is evolutionarily conserved (Figure 1.3) (Kolch, 2002; Yuryev *et al.*, 2000). Ras is a guanine nucleotide binding protein constitutively localized to the plasma membrane due to a lipid anchor added posttranslationally (Bollag and McCormick, 1991). Upon growth factor binding and receptor autophosphorylation, the PTB domain containing protein Shc is recruited to the receptor and phosphorylated on Tyr317 (van der Geer and Pawson, 1995). This creates a docking site for the SH2 domain-containing protein growth factor receptor-bound protein 2 (Grb2), which may alternatively bind directly to the activated receptor as shown in figure 1.1 (Heldin *et al.*, 1998). Grb2 functions as an adapter protein that localizes the guanine nucleotide exchange protein, son of sevenless (SOS), to the plasma membrane (Campbell *et al.*, 1998). At the plasma membrane, SOS mediates the exchange of Ras-bound GDP for GTP (Lodish, 1995). The conformational change induced by the change to Ras-GTP results in high-affinity binding to the Raf family of serine/threonine kinases. Raf proteins are cytosolic in quiescent cells, but are targeted to the plasma membrane by direct interaction with Ras-GTP (Avruch *et al.*, 1994). Raf becomes activated at the membrane and stimulates the dual specificity kinases MAPK/extracellular signal-regulated kinase (ERK) kinases (MEK) 1 and 2 by serine phosphorylation (Schubbert *et al.*, 2007). MEK phosphorylates MAPK on both a threonine and a tyrosine residue (Fukuhara *et al.*, 2000). Phosphorylated MAPK is activated, translocates to the nucleus and upregulates various transcription factors, involved in cell growth and proliferation through phosphorylation (Cyert, 2001).

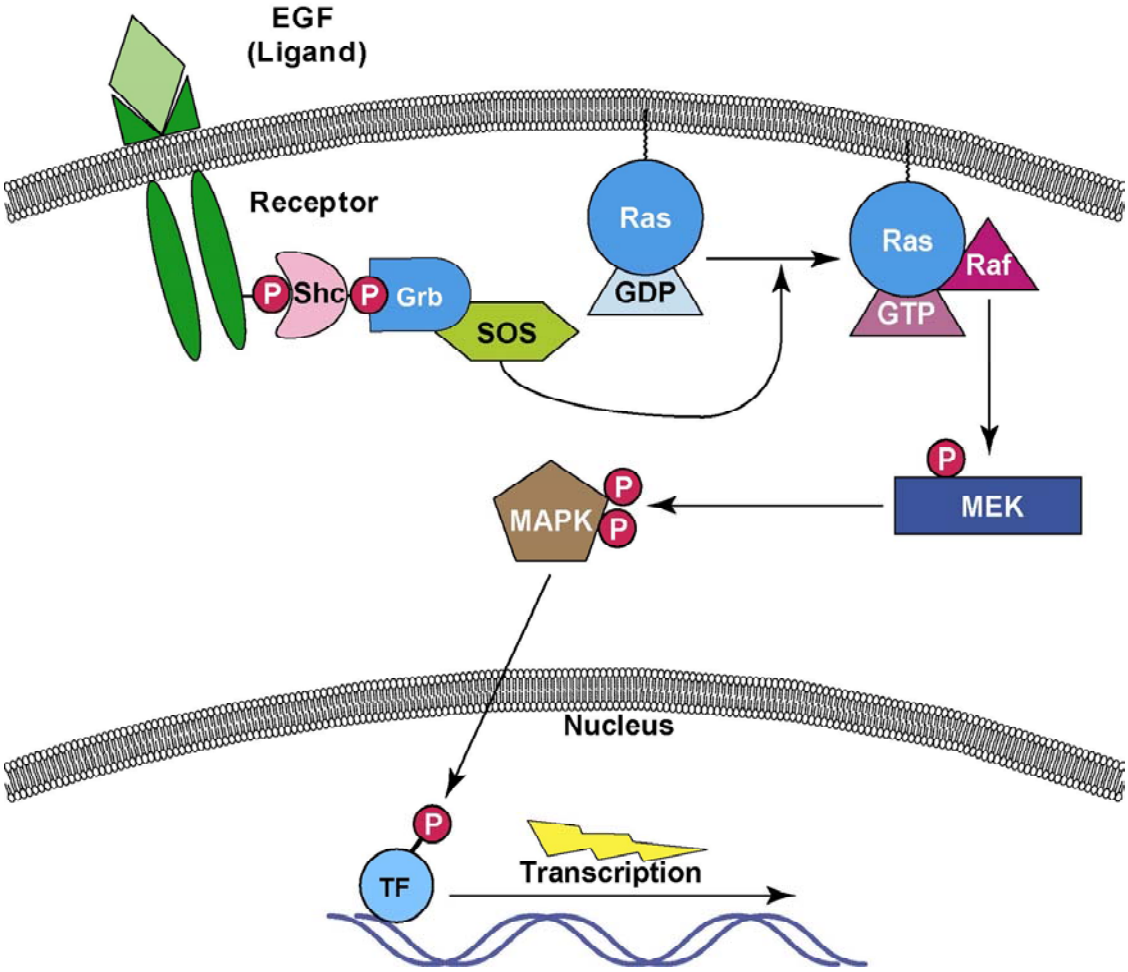


Figure 1.3: The Ras/Raf/MAPK signal transduction pathway. Binding of EGF to the EGFR results in dimerization and autophosphorylation of tyrosine residues creating docking sites for PTB and SH2 domains such as Shc. Phosphorylation of the adapter protein Shc recruits the Grb2/SOS complex. SOS stimulates the exchange of Ras-bound GDP for GTP, which permits binding of Raf. After binding to Ras, Raf phosphorylates MEK, which in turn phosphorylates and activates MAPK. MAPK translocates to the nucleus and activates transcription factors *via* phosphorylation. The transcription factors activated by MAPK are involved in cellular responses such as proliferation. Note that this is a simplified representation of the Ras/Raf/MAPK pathway. TF = transcription factor.

1.3 PI3K/Akt Signaling Pathway

The PI3K/Akt pathway is a well-studied signaling cascade parallel to the Ras/Raf/MAP pathway. PI3K/Akt signaling is activated when EGF binds to its receptor located on the cell surface. This event results in autophosphorylation of the receptor and recruitment of PI3K to the receptor (Figure 1.1) (Cantley, 2002; Lo and Hung, 2006). Although three classes of PI3Ks exist in higher eukaryotes, it is the class I enzymes that respond to growth factor stimulation and carry out phosphorylation of D-3 phosphoinositides (Cantley, 2002). Class I PI3K are heterodimers composed of an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110) (Cantley, 2002). Three genes encode for the p85 protein, *Pik3r1*, *Pik3r2* and *Pik3r3* (Fruman *et al.*, 1998). Three isoforms are encoded by the *Pik3r1* gene: p85α, p55α and p50α (Figure 1.4) (Fruman *et al.*, 1998). Isoforms p85β and p55γ are encoded by separate genes Pik3r2 and *Pik3r3*, respectively (Fruman *et al.*, 1998). The p85α isoform is the more abundant form of p85 and henceforth p85α will be referred to as p85. All five isoforms contain two SH2 domains, which form stable complexes with phosphotyrosine sites present on activated EGFRs (McGlade, 1992). SH2 domains bind the consensus sequence of pYXXM (Funaki *et al.*, 2000). All five isoforms also have an inter-SH2 region that spans 200 amino acid residues (Klippel *et al.*, 1993). The full-length p85 protein contains an N-terminal Src homology 3 (SH3) domain, which mediates binding to the proline-rich consensus sequence XPpXP (where P is always a proline and p is often a proline residue) (Klippel *et al.*, 1993). The break point cluster region (BCR) gene was originally identified as being involved in the translocation of chromosome 22 in chronic myelocytic leukemia patients (Heisterkamp *et al*., 1985). Later, the product of the BCR gene was determined to be a Rho GTPase activating protein (GAP) (Ridley *et al.*, 1993). A BCR homology (BH) domain was discovered in p85 (Funaki *et al.*, 2000) and it has been

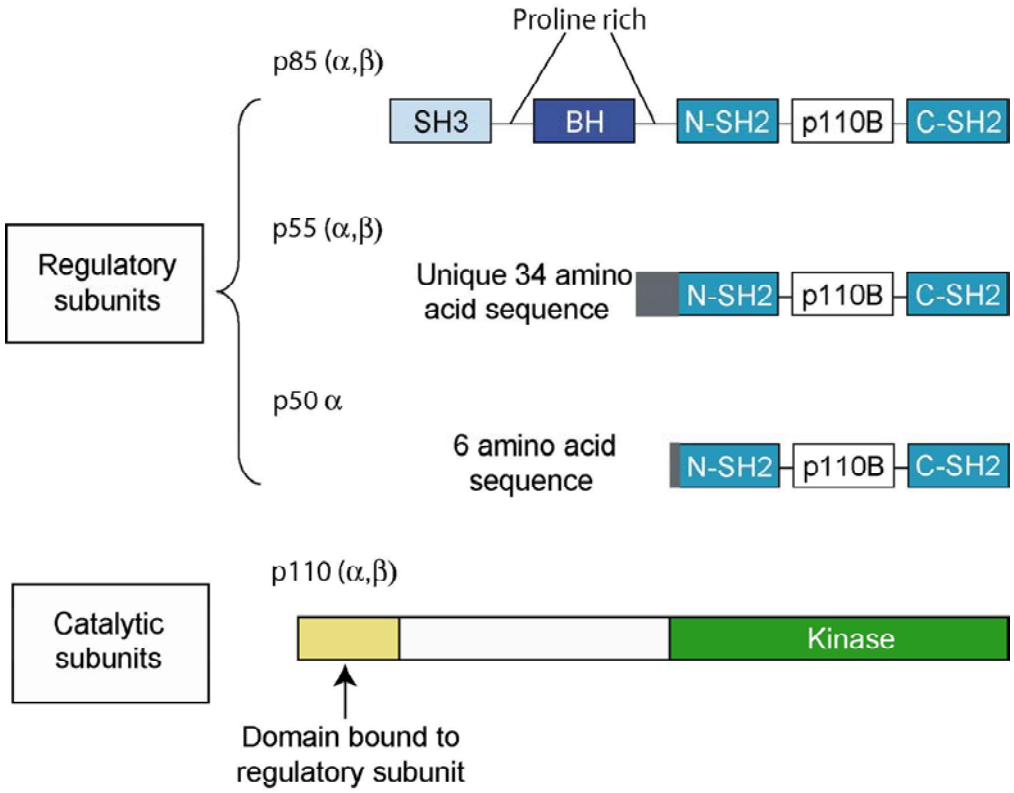


Figure 1.4: Domain structure of p85 and p110. The p85 protein has two full length isoforms, α and β, as well as three smaller isoforms p55 α and β as well as p50. Full length p85 has an SH3 domain, two proline-rich regions, BH domain, N-SH2 domain, p110 binding domain and C-SH2 domain. The smaller p85 isoforms are lacking the SH3 and BH domains. The p110 protein is the catalytic subunit of PI3K and has a p85 binding domain and a kinase domain. Adapted from Funaki *et al*., (2000).

demonstrated that p85 has GAP activity (Chamberlain *et al.*, 2004).

The catalytic activity of PI3K is carried out by the p110 subunit, which adds a phosphate group to the plasma membrane lipid component phosphatidylinositol 4,5 bisphosphate ($PI4,5P_2$) at the D3 position of the inositol ring creating the product phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$) (Sulis and Parsons, 2003). Four separate genes, *Pik3CA*, *Pik3CB*, *Pik3CD* and *Pik3CG* encode for four isoforms of p110: p110α, p110β, p110δ and p110γ (Funaki *et al.*, 2000). The delta (δ) isoform of p110 has not been shown to bind p85 (Foukas *et al.*, 2004). Since the p85:p110 interaction is quite strong, this suggests specialized functions for the delta isoform (Foukas et al., 2004). The gamma (γ) isoform is expressed predominantly in leukocytes, suggesting a unique role for p110 in this cell type (Foukas *et al.*, 2004; Funaki *et al.*, 2000). Henceforth p110α will be referred to as p110, since it is the isoform that pertains to this body of work. The p110 protein consists of an N-terminal p85 binding domain and a C-terminal lipid kinase domain (Figure 1.4) (Klippel *et al.*, 1994).

The p85:p110 interaction controls three key functions for the PI3K heterodimer. The p85 protein stabilizes p110, regulates enzymatic function and acts as an adapter protein to position p110 at the cell membrane in proximity to its lipid substrate (Cuevas *et al.*, 2001; Foukas *et al.*, 2004; Klippel *et al.*, 1994; Klippel *et al.*, 1996). The p110 protein is not stable at biological temperatures in the absence of p85. The p85:p110 interaction is very stable and no dissociation has been observed (Yu *et al.*, 1998). The enzymatic activity of p110 has been shown to be constitutively active (at lower temperatures than biologically relevant), but p85 inhibits this activity (Yu *et al.*, 1998). Finally, p110 does not contain any SH2 or PTB domains, so its localization to the plasma membrane where its substrate is located is a function of p85 (Klippel *et al.*, 1996). The p85 protein has two SH2 domains that have been shown to bind activated

RTKs, such as the EGFR. Upon the SH2 domains binding to the EGFR, the inhibition of p110/PI3K activity by p85 is relieved (Funaki *et al.*, 2000). Thus, in response to activation of the EGFR, the p85:p110 complex relocalizes to the plasma membrane and inhibition of PI3K activity is relieved.

Investigation of the enzymatic activity of p110 revealed that it is a dual specificity kinase possessing 3' phosphoinositide activity as well as protein serine kinase activity (Stephens *et al*., 1993). It is the conversion of $PI4,5P_2$ to $PI3,4,5P_3$ that is the most biologically relevant activity with regard to the PI3K/Akt pathway, but the protein serine kinase activity, which autophosphorylates p85 at Ser608 may also be important for regulation of the PI3K enzyme (Dhand *et al*., 1994; Stephens *et al*., 1993). Ser608 on p85 was identified as a regulatory phosphorylation site for PI3K (Foukas *et al*., 2004). Foukas and colleagues demonstrated that it is the p110 subunit that catalyzes this reaction in response to activation of other RTKs such as the platelet derived growth factor receptor as well as the insulin-like growth factor receptor (Foukas *et al*., 2004). The kinetics of Ser608 phosphorylation are slow in response to RTK activation, suggesting that inhibition of a phosphatase(s) occurs (Foukas et al., 2004). Furthermore, when cells are treated with the protein serine/threonine phosphatase inhibitor okadaic acid, the cellular levels of Ser608-phosphorylated p85 (pSer608-p85) increase (Foukas *et al*., 2004). Yu and colleagues clearly demonstrated that p85 binding to p110 was sufficient for inhibition of PI3K lipid phosphatase activity regardless of Ser608 phosphorylation status (Yu *et al*., 1998). Ser608 phosphorylation may be involved in a negative feedback mechanism to switch off PI3K activity. It has been demonstrated that when the p85 SH2 domains bind phosphotyrosines, the lipid kinase activity of PI3K is activated (Carpenter *et al*., 1993). Thus, in

the cytoplasm it is possible that a phosphatase removes the Ser608 phosphate. Upon receptor activation, the Ser608 phosphatase is either inhibited or simply remains cytoplasmic and no longer has access to its p85 substrate, which has translocated to the plasma membrane. At the plasma membrane, the SH2 domains of p85 bind the phosphorylated tyrosine residues and a conformational change activates p110 activity. The p110 protein phosphorylates its lipid substrate as well as the p85 protein on Ser608 to attenuate the signal (Figure 1.5 a).

Another important PI3K regulatory phosphorylation site is Tyr688 on p85. Tyr688 is phosphorylated by the Src-family kinases Lck and Abl in response to cytokine signaling (von Willebrand *et al.*, 1998). When p85 is phosphorylated at Tyr688 (pTyr688), the binding properties of the SH2 domains are altered (von Willebrand *et al.*, 1998). Cuevas *et al.* demonstrated that p85 phosphorylated on Tyr688 is associated with higher lipid kinase activity than the nonphosphorylated state (Cuevas *et al.*, 2001). Interestingly, the phosphatase identified for dephosphorylating p85 at Tyr688 was the SH2 containing tyrosine phosphatase 1 (SHP1) (Cuevas *et al.*, 1999). These authors proposed two models for PI3K regulation involving pTyr688 and activated RTKs (Cuevas *et al.*, 2001). Their first model was an intramolecular regulatory mechanism in which Tyr688 masks the SH2 domains and inhibits enzymatic activity and pTyr688 relieves inhibition. The second model described an intermolecular interaction. When a PI3K molecule binds the active RTK, pTyr688 serves as a docking site for the SH2 domains of a second PI3K molecule. The second PI3K also becomes phosphorylated at Tyr688 and serves as a docking site for a third PI3K molecule, etc., thus amplifying of the original signal being transduced (Cuevas *et al.*, 2001). Based on the other evidence in the literature that pTyr688 is not required for PI3K localization or activation (Carpenter *et al.*, 1993), neither of the

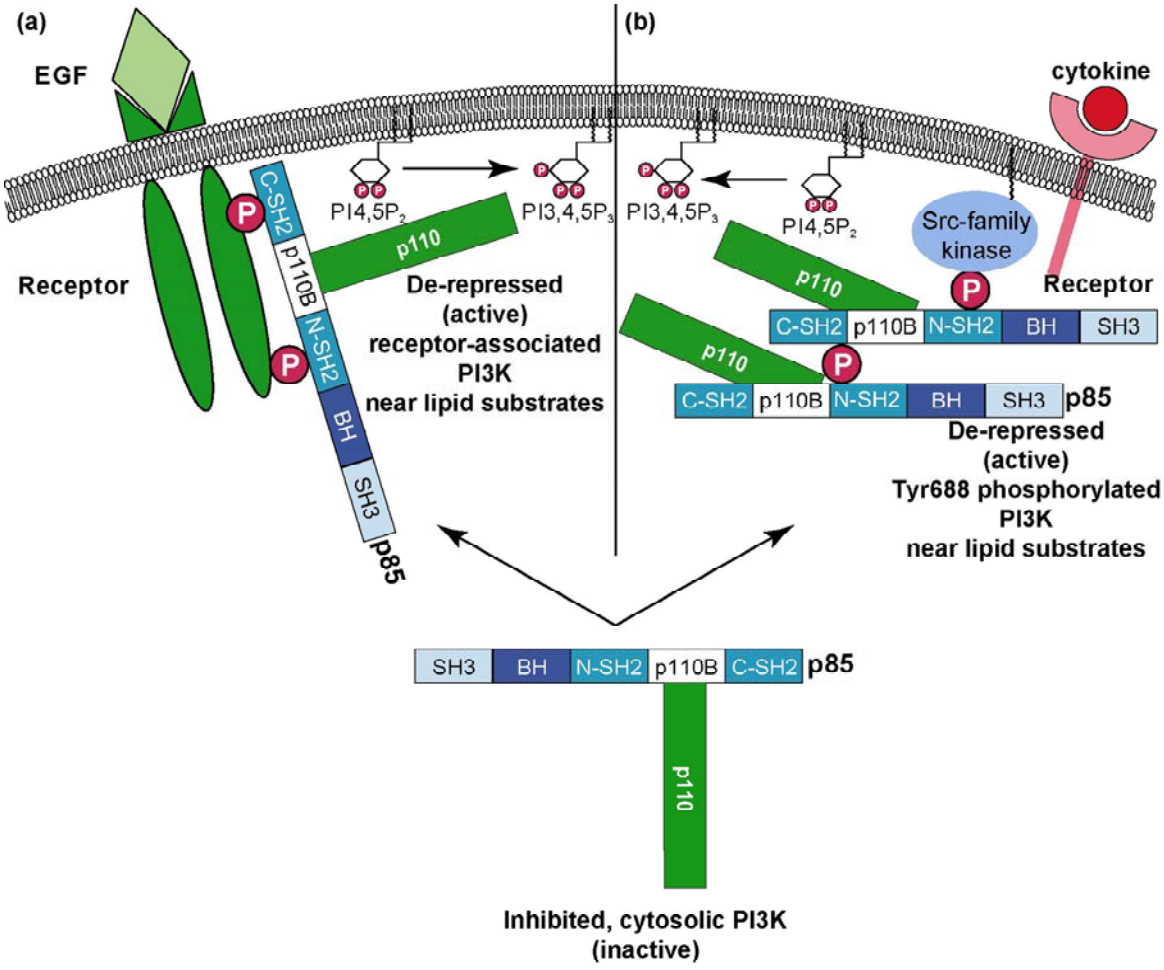
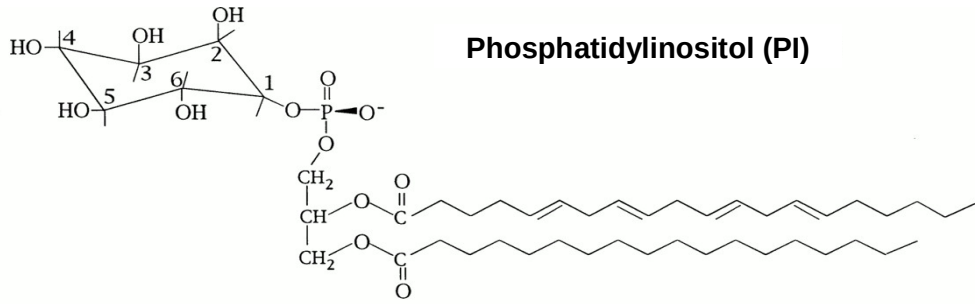


Figure 1.5: Models of PI3K signaling in response to RTK and cytokine receptor activation. (a) In the cytosol PI3K is catalytically inactive. Upon RTK activation PI3K translocates to the plasma membrane and is activated upon receptor binding. (b) Cytokine receptor activation results in tyrosine phosphorylation of Src-family kinases. PI3K is recruited to the Src-family kinase, which phosphorylates it at Tyr688 and activates enzymatic activity. Cuevas *et al.* proposed that Tyr688 phosphorylation also provides an SH2 docking site for a second PI3K molecule, which gets Tyr688 phosphorylated providing a docking site for a third PI3K etc. (Cuevas *et al.*, 2001).

models concerning RTK signaling proposed by Cuevas *et. al.* are valid. However, the Cuevas intermolecular model may have some validity with regard to cytokine signaling. I propose an adaptation of their model with regard to PI3K involvement in cytokine signaling (Figure 1.5 b). Cytokine receptor activation results in tyrosine phosphorylation of Src family kinases. PI3K is recruited to the membrane-localized Src family kinase and is phosphorylated at Tyr688. As proposed by Cuevas and colleagues (2001), the pTyr688 may be a docking site for another PI3K, which is then phosphorylated on Tyr688 creating a docking site for another PI3K molecule, etc.

Phosphatidylinositol (PI) constitutes only 10% of the total lipid content in eukaryotic cell membranes and thus has a minor role in the structure of the cell plasma membrane (Rameh and Cantley, 1999). The inositol ring of PI contains five free hydroxyls with the potential to become phosphorylated by lipid kinases such as PI3K (Figure 1.6) (Fruman *et al.*, 1998). PI is well recognized for its importance in cell signaling because it is the precursor to all phosphoinositide second messengers, such as PI3,4,5P_3 (Rameh and Cantley, 1999).

Upon phosphorylation of PI4,5P_2 by PI3K, the product PI3,4,5P_3 interacts with the PH domain containing-protein, phosphoinositide-dependent kinase-1 (PDK1) (Figure 1.7). PDK1 is a serine/threonine kinase critical for Akt activation (Cohen *et al.*, 1997). Akt also has a PH domain and translocates to the plasma membrane in response to an increase in PI3,4,5P_3 lipids (Kikani *et al.*, 2005). At the plasma membrane, Akt is phosphorylated by PDK1 at residue Thr308 in the activation loop (Anderson *et al.*, 1998) and possibly at Ser473 as well (Song *et al.*, 2005, Ueki *et al.*, 2002) (Figure 1.7). Phosphorylation of Thr308 is not a prerequisite for Ser473 phosphorylation or vice versa (Alessi *et al.*, 1996). However, phosphorylation of both residues



Monophosphoinositides	**PI3P, PI4,P, PI5P**
Bisphosphoinositides	**PI3,4P_2, PI3,5P_2, PI4,5P_2**
Trisphosphoinositides	**PI3,4,5P_3**

Figure 1.6: Chemical structure of phosphatidylinositol. Phosphatidylinositol (PI) is a plasma membrane lipid component. PI is also important as it is the precursor to important lipid second messengers. Note the free hydroxyls indicated at positions 2-6 of the inositol ring. The phosphoinositides that have been characterized in mammalian cells are PI3P, PI4P, PI5P, PI3,4P_2, PI3,5P_2, PI4,5P_2, and PI3,4,5P_3. PI4,5P_2 is a substrate for PI3K, which adds a phosphate to the 3' hydroxyl to create PI3,4,5P_3. PI3,4,5P_3 is a potent second messenger that recruits Akt to the plasma membrane initiating several proliferative and anti-apoptotic pathways. Phosphatase PTEN dephosphorylates PI3,4,5P_3 at the 3' position. Arrows represent upregulation whereas squares denote downregulation or inhibition. Adapted from Fruman *et al.* (1998).

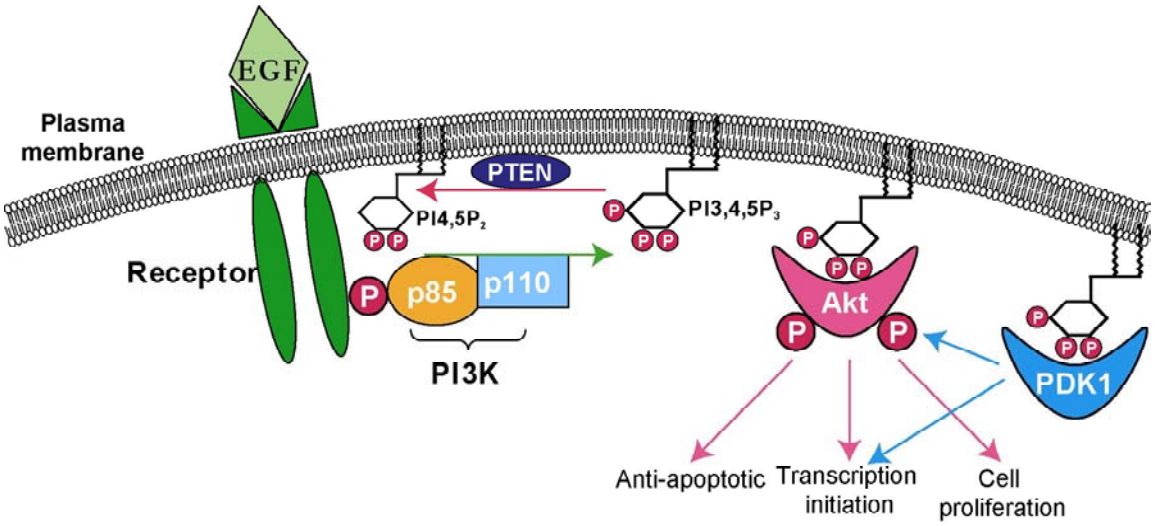


Figure 1.7: Schematic diagram of the PI3K/Akt Signal Transduction Pathway. Upon EGF binding to the EGFR, PI3K is recruited to the plasma membrane by the p85 subunit. PI4,5P2 is phosphorylated by p110 at the D-3 position on the inositol ring, creating the product PI3,4,5P3. Tumor suppressor PTEN phosphatase catalyzes the reverse reaction. PI3,4,5P3 recruits PH domain containing proteins Akt and PDK1. PDK1 phosphorylates Akt in the activation loop. Akt signaling inhibits apoptotic signals, initiates transcription and promotes cell proliferation. Note that this is a simplified schematic of the PI3K/Akt signal transduction pathway, with Akt targets described in more detail in a subsequent figure.

is required for high activation of Akt (Alessi *et al*., 1996). Currently, some ambiguity exists regarding the kinase responsible for Ser473 phosphorylation. Initially PDK1 was the kinase proposed to have this function, but other kinases may be involved, including: the integrin linked kinase (ILK), mTOR RICTOR, or even Akt itself (Hresko and Mueckler, 2005; Okamura *et al*., 2007; Song *et al*., 2005). Given that Akt is ubiquitously expressed, it stands to reason that it would have several activators that have tissue and/or signal specific activity. Active Akt results in signaling cascades that have endpoints such as initiation of transcription and promotion of cell proliferation (Song *et al*., 2005).

Although there are three isoforms of Akt, all consist of a conserved domain structure including an N-terminal PH domain, a C-terminal regulatory domain and a central serine/threonine kinase domain (Song *et al*., 2005). Current models of Akt signaling include activation of eight distinct pathways involved in cell survival and proliferation (Figure 1.8). Two pathways are involved in direct regulation of apoptosis, five more include transcriptional control of apoptosis, and one pathway involves metabolic regulation of apoptosis *via* glucose metabolism (Figure 1.8) (Song *et al*., 2005).

One Akt target is the protein BAD, which is a member of the Bcl-2 family of proteins. BAD and Bcl-2-associated_x_protein (Bax) alternatively bind to Bcl-2 or its homolog Bcl-xL. When BAD is bound to Bcl-2 or Bcl-xL, Bax localizes to the mitchondrial membrane resulting in the release of cytochrome c from the organelle (del Peso *et al*., 1997). The release of cytochrome c from the mitochondria is a trigger of the apoptosome. When BAD is phosphorylated by Akt, it loses the ability to bind Bcl-2 or Bcl-xL, which results in the sequestering of Bax by these proteins and inhibition of apoptosis (Datta *et al*., 1999; Datta *et al*., 1997).

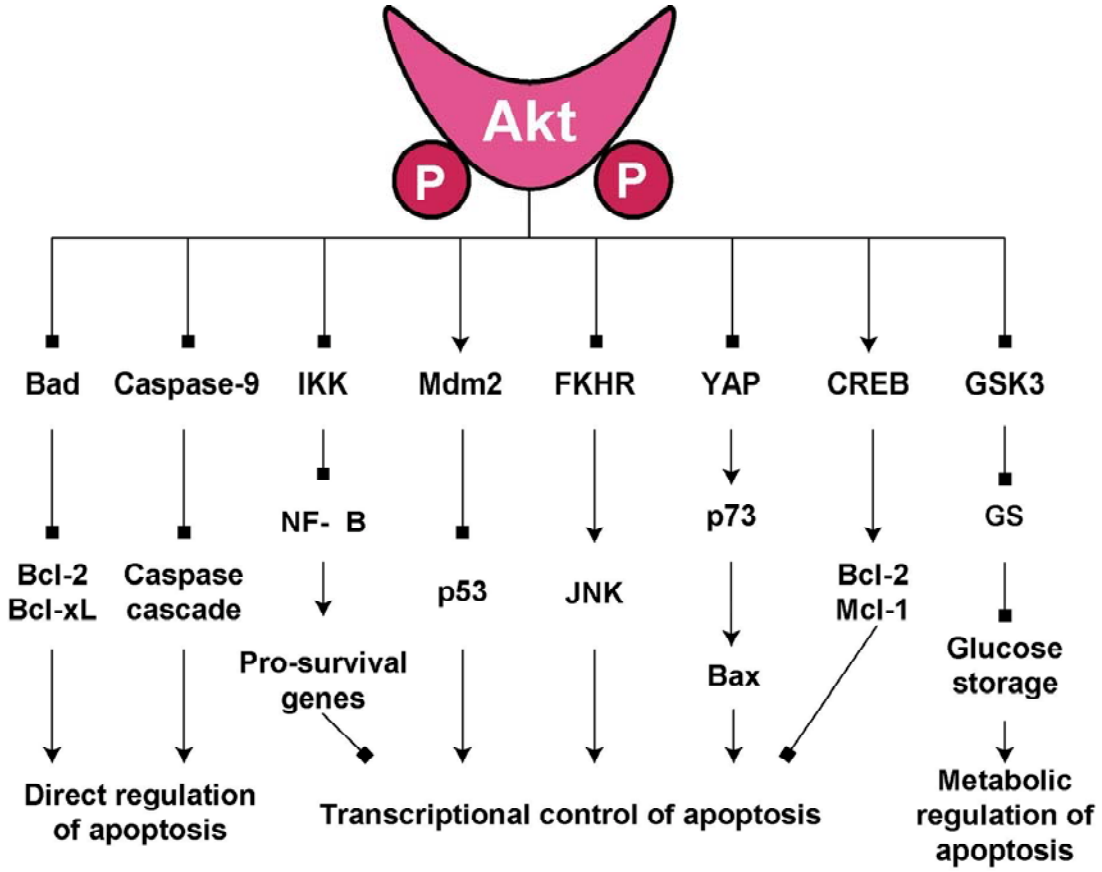


Figure 1.8: Multiple mechanisms of cell survival regulation by Akt. Upon receptor activation and autophosphorylation PI3K is recruited to the membrane where it adds a phosphate group to PI4,5P2 creating PI3,4,5P3. PH domain containing proteins such as Akt and PDK1, are recruited to the membrane by PI3,4,5P3. PDK1 phosphorylates Akt and activates it. Active Akt has eight distinct downstream signal pathways. Akt inhibits apoptosis by phosphorylating the proteins BAD and Caspase-9. Akt also regulates several transcription factors either directly or indirectly. Glucose storage is activated by Akt by phosphorylation and inactivation of GSK3. Active GSK3 phosphorylates and inhibits glycogen synthase (GS). Arrows denote upregulation wheareas squares represent down regulation or inhibition. Modified from Song *et al.* (2005).

Caspases are a family of proteases that are quickly activated by proteolytic cleavage (Cho and Choi, 2002). Caspase-9 is activated by cytochrome c release from the mitochondria (Donepudi and Grutter, 2002). Caspase-9 is a member of a large multi-protein complex termed the apoptosome (Yuan *et a*l., 1993). When human caspase-9 is phosphorylated by Akt, its conformation interferes with its ability to trigger apoptosis *via* this cascade because it can no longer associate with the other proteins in the apoptosome (Donepudi and Grutter, 2002).

Akt is capable of directly up-regulating or down-regulating several transcription factors involved in anti-apoptotic or pro-apoptotic gene expression, respectively. Akt directly phosphorylates the foxO forkhead family of transcription factors (FKHR), decreasing expression of their pro-apoptotic target genes (Song *et al*., 2005). Akt also phosphorylates cyclic AMP response element binding protein (CREB), which results in increased transcription of anti-apoptotic genes (Song *et al*., 2005). Akt indirectly regulates the transcription factors nuclear factor (NF)-κβ, p53 and p73 through phosphorylation of upstream proteins. NF-κβ is involved in transcription of pro-survival genes whereas p53 and p73 are both involved in activating genes involved in apoptosis (Song *et al*., 2005). Akt relieves inhibition of NF-κB and inhibits p53 and p73 (Song *et al*., 2005).

Insulin signaling is mediated *via* the insulin RTK. In the presence of insulin, Akt is involved in the metabolic regulation of cell survival through phosphorylation of glycogen synthase (GS) kinase 3 (GSK3) (Song *et al*., 2005). GS is the rate-limiting enzyme of glycogen synthesis and when phosphorylated it is inactive (Welsh et al., 1996). When GSK3 is phosphorylated by Akt, it is less active and increases glycogen synthesis by relieving inhibition of GS (Cross *et al*.,

1995). It was previously well established that inhibition of GSK3 is protective against apoptosis (Pap and Cooper, 1998), however emerging evidence is suggesting that GSK3 has anti-apoptotic functions (Meares and Jope, 2007). GSK3 has several targets, besides its major substrate GS, including other signaling enzymes and transcription factors. Recently, it was demonstrated that GSK3 contains a nuclear localization signal (NLS) and that phosphorylated GSK3 remains cytoplasmic in complexes with other proteins (Meares and Jope, 2007). It has been reported that nuclear GSK3 promotes transcriptional activity of NF-κB, a pro-survival transcription factor (Hoeflich *et al*., 2000; Steinbrecher *et al*., 2005). Thus, phosphorylated GSK3 is cytoplasmic, preventing apoptosis and dephosphorylated GSK3 is nuclear and promotes transcription of a pro-survival transcription factor (Meares and Jope, 2007).

1.4 PTEN Biological Function

Various enzymes downstream of Akt contribute to PI3K/Akt signaling. Phosphatase and tensin homolog deleted on chromosome ten (PTEN) inhibits all downstream PI3K pathway signaling at the source before amplification and thus it is the most important enzyme involved in regulating the PI3K/Akt signaling pathway (Sulis and Parsons, 2003). PTEN, previously referred to as MMAC1 or TEP1, is a dual specificity lipid and protein tyrosine phosphatase (Sulis and Parsons, 2003). The principle substrate of PTEN is $PI3,4,5P_3$, the lipid second messenger generated by PI3K (Maehama and Dixon, 1998; Myers *et al*., 1998; Stambolic *et al*., 1998). PTEN prevents activation of Akt in the absence of growth factor, suggesting that PTEN is either inactivated upon growth factor stimulation or is activated by growth factor depletion (Cantley and Neel, 1999). It is this lipid phosphatase activity that is the most biologically

relevant in qualifying PTEN as a tumor suppressor protein (Furnari *et al*., 1998). Mutants that retain protein tyrosine phosphatase activity, but are unable to dephosphorylate $PI3,4,5P_3$, have been found in tumors (Furnari *et al*., 1998; Lee *et al*., 1999; Myers *et al*., 1998). Mutations such as G129E (glycine 129 changed to glutamate) and H93X (histidine 93 changed to any amino acid) have been observed frequently in cancers (Lee *et al*., 1999).

PTEN is located on chromosome 10 at locus q23 and it is the deletion of this region in many cancers that results in loss of PTEN expression (Maehama and Dixon, 1999). Heterozygous loss of PTEN leaves cells prone to tumor development (Trotman *et al*., 2003). Single allele deletions are often observed in primary tumors, whereas the loss of the second PTEN allele is associated with advanced cancer and metastasis (Di Cristofano and Pandolfi, 2000; Trotman *et al*., 2003). Many human metastatic cancers, including glioblastoma, melanoma, prostate and endometrial carcinoma, exhibit loss of PTEN expression (Miller *et al*., 2002; Ouchi *et al*., 2000). As well, a lack of PTEN activity can also result in non-malignant lesions in many of these tissues (Suzuki *et al*., 1998).

PTEN gene deletion mice have been generated as a model for human cancers. Knockout of PTEN in mice [PTEN(-/-)] is embryonic lethal (Simpson and Parsons, 2001). Deletion of a single allele, or heterozygous PTEN [PTEN(+/-)] mice are viable but are prone to developing a variety of tumors, consistent with the established involvement of PTEN in tumor suppression (Di Cristofano *et al*., 1998; Podsypanina *et al*., 1999; Stambolic *et al*., 2000; Suzuki *et al*., 1998). The location of these tumors include tissues of the breast, prostate, lung, endometrium, brain, thyroid, liver, lymphoid cancers and melanoma (Cantley and Neel, 1999; Goberdhan and Wilson, 2003; Sansal and Sellers, 2004; Simpson and Parsons, 2001; Vivanco and Sawyers, 2002).

Heterozygous *Pik3r1* [+/-] (encodes the α isoforms of p85, p55 & p50) that also contain half the normal level of PTEN, i.e. PTEN(+/-), also display increased intestinal polyp incidence (~2-fold) and number (>3 per animal) compared to PTEN(+/-) mice (Luo *et al*., 2005). This observation suggests that p85 may have a role in modulating tumor suppression by PTEN (Luo *et al*., 2005).

1.5 The PTEN-p53 Tumor Suppression Connection

PTEN is well recognized as a regulator of the PI3K/Akt pathway signaling and was initially suspected to remain in the cytoplasm in quiescent cells (Baker, 2007). A growing body of evidence is implicating new PTEN functions in the nucleus (Baker, 2007). The proto-oncogene murine double minute 2 (mdm2) is a downstream target of Akt signaling (Figure 1.9) (Woods and Vousden, 2001). Mdm2 is a negative regulator of the well-studied tumor suppressor protein p53. In contrast, PTEN lipid phosphatase activity has been shown to be a positive regulator of p53 through its attenuation of Akt signaling (Woods and Vousden, 2001). However, PTEN also interacts with p53 in a phosphatase-independent manner and is involved in the acetylation of p53 (Woods and Vousden, 2001).

The p53 protein has been the most studied protein involved in apoptosis and is often deleted or non-functional in cancers. Activation of p53 occurs in response to cell stress and it functions as a transcription factor that up-regulates the transcription of genes whose products are necessary for cell cycle arrest and/or apoptosis (Mayo *et al*., 2002). Given the nature of p53's primary function to arrest the cell cycle and induce apoptosis, it has a short half-life and is not very abundant in normal cells (Bates and Vousden, 1999; Levine, 1997). The cellular stresses that

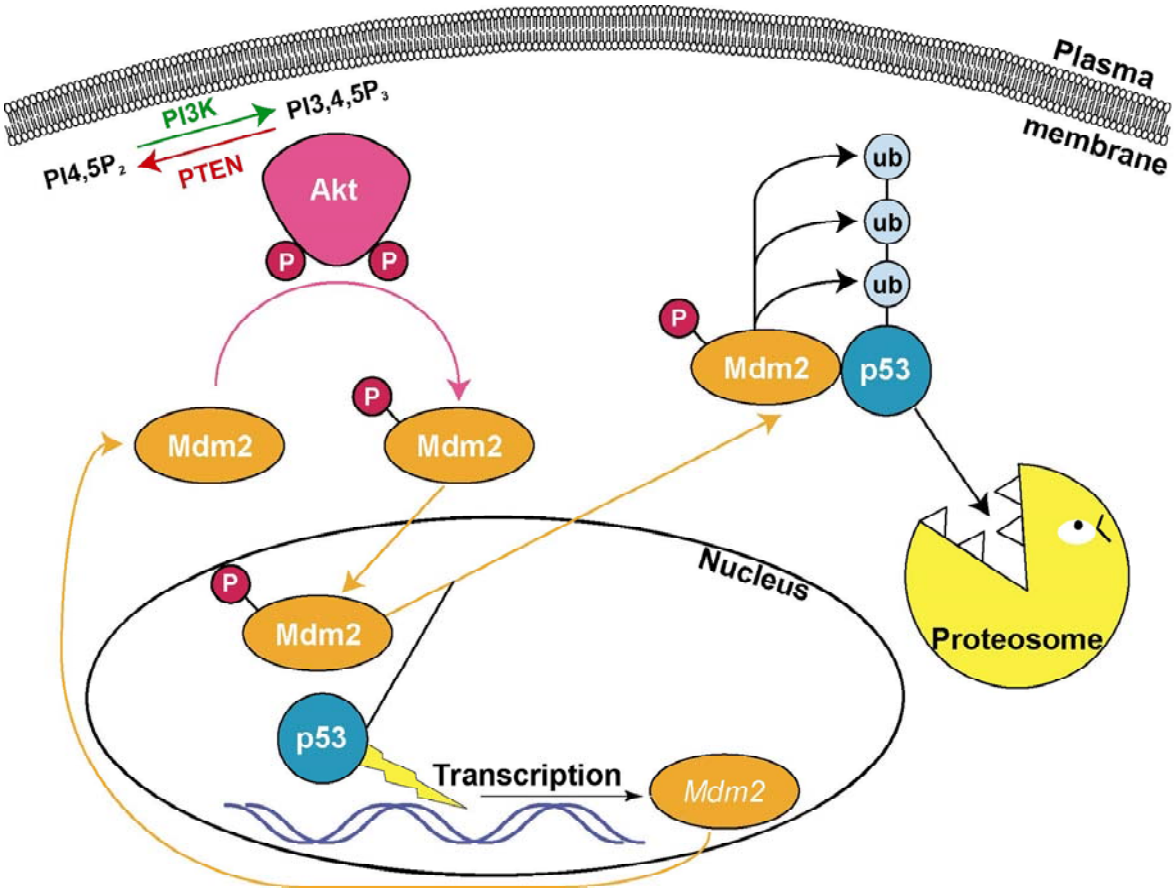


Figure 1.9: Regulation of p53 transcription and cellular concentration by mdm2. Cellular stresses result in activation of p53. p53 initiates transcription of apoptotic gene products. A negative feedback loop is initiated by p53 *via* transcriptional activation of *mdm2*. Activated Akt promotes translocation of mdm2 into the nucleus through phosphorylation of mdm2. mdm2 binds nuclear p53, shuttles it to the cytoplasm and targets it for ubiquitin-mediated degradation.

activate p53 can be transient in nature, thus activation of p53 need not necessarily lead to cell death. Both regulation of p53 transcriptional activity as well as cellular protein stability is accomplished by the proto-oncogene *mdm*2 (Freedman *et al*., 1999). The regulation of p53 relies on an autoregulatory feedback loop in which p53 positively regulates mdm2 and in turn, mdm2 negatively regulates p53 (Wu *et al*., 1993). Expression of p53 induces mdm2 expression, which binds the transcriptional activation domain of p53 and blocks the ability of p53 to recruit additional factors necessary for induction of gene expression (Chen *et al*., 1993; Momand *et al*., 1992; Oliner *et al*., 1993). Upon formation of the mdm2:p53 complex, p53 transcriptional activity is halted and the complex leaves the nucleus (Kubbutat *et al*., 1999; Roth *et al*., 1998; Tao and Levine, 1999). In the cytoplasm, p53 is targeted for ubiquitin-mediated degradation by mdm2, which is an E3 ubiquitin ligase (Honda *et al*., 1997; Kubbutat *et al*., 1997).

PI3K/Akt signaling promotes the translocation of mdm2 from the cytoplasm to the nucleus. The mdm2 protein is a substrate of the kinase Akt, which phosphorylates mdm2 at serine residues 166 and 186 (Mayo and Donner, 2001). These serine residues are in proximity to a nuclear localization sequence and phosphorylation unmasks this region, allowing localization to the nucleus (Mayo and Donner, 2001). PTEN is intrinsically linked to the regulation of p53 *via* negative regulation of PI3K/Akt signaling.

PTEN also regulates p53 by a phosphorylation-independent mechanism *via* direct binding to the p53 protein in the nucleus (Freeman *et al*., 2003). PTEN-knockout cells have dramatically reduced p53 levels; however, in the presence of the phosphatase inactive mutant PTEN-C124S, the level of p53 is increased (Freeman *et al*., 2003). Furthermore, in addition to stabilizing and

increasing cellular levels of p53, PTEN also increases p53 promoter activity (Freeman *et al*., 2003).

The p53 protein is acetylated at several lysine residues located in the C-terminal regulatory domain (Gu and Roeder, 1997). Acetylation is accomplished by the acetyltransferase p300/CBP in response to DNA damage and is implicated in p53 stability as well as the activation of p53 sequence-specific DNA binding (Gu and Roeder, 1997; Li *et al*., 2002; Liu *et al*., 1999; Lohrum *et a*l., 2001; Luo *et al*., 2004). Acetylation of p53 is reversible and de-acetylation activity is provided by the PID-MTA2-HDAC1 complex (Luo *et al*., 2000) and Sir2α (Luo *et al*., 2001; Vaziri *et al*., 2001).

PTEN stimulates p300-mediated acetylation through a phosphatase-independent mechanism by forming a PTEN-p300 complex in the nucleus (Li *et al*., 2006). Overexpression of either PTEN or p300 results in higher p53 transcriptional activity (Freeman *et al*., 2003; Gu and Roeder, 1997). However, overexpression of both PTEN and p300 synergistically increases p53 transcriptional activation because acetylation of p53 greatly enhances PTEN binding (Li *et al*., 2006).

1.6 PTEN Structure and Function

The physical properties of PTEN are a direct consequence of the domain structure of PTEN (Figure 1.10 a). PTEN has an approximate mass of 55 kDa and is composed of 403 amino acids. PTEN has been described as having a domain structure consisting of a phosphatase (PASE) domain, required for phosphatase activity, a C2 domain involved in non-specific plasma membrane targeting, a regulatory domain containing PEST sequences and phosphorylation sites

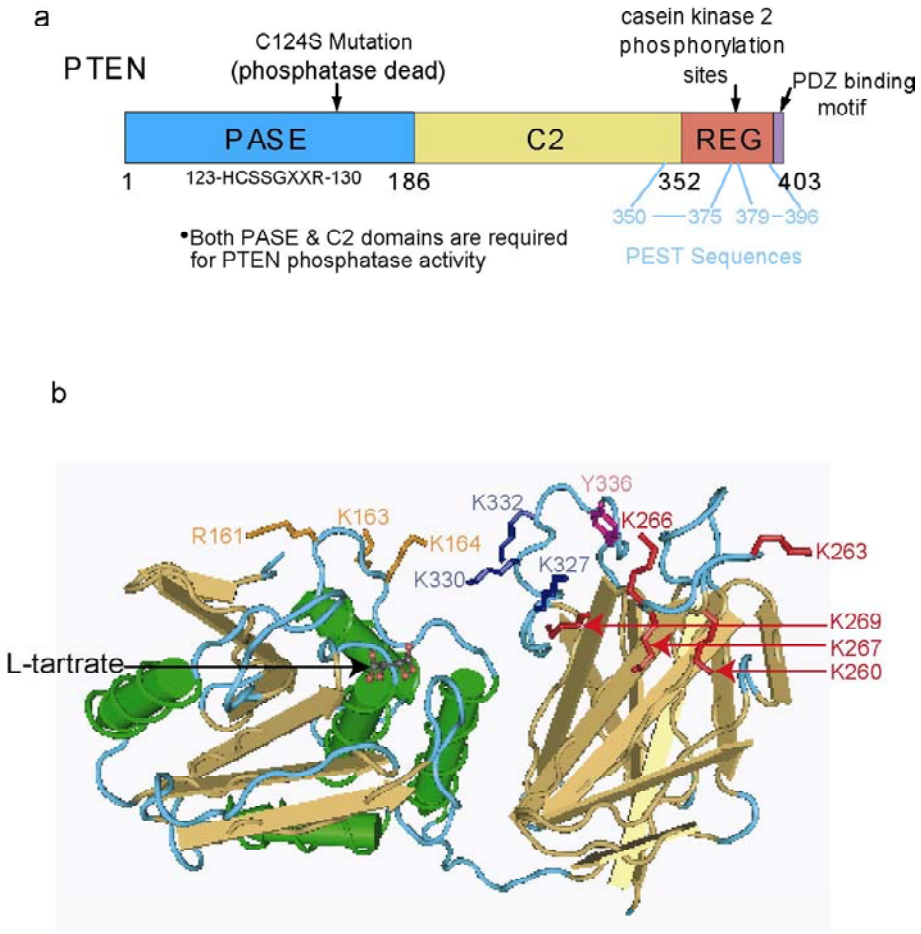


Figure 1.10: Domain structure of PTEN and partial crystal structure illustrating clusters of basic amino acids. (a) Domain structure of PTEN phosphatase. PASE = phosphatase domain, C2 = C2 lipid binding domain, REG = regulatory domain, PDZB = PDZ binding motif. **(b)** Crystal structure of phosphatase and C2 domain of PTEN. (L-tartrate is bound to the active site). Obtained from: Lee *et al.* (1999). Produced by the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov. Visualized, labeled and side chain color coding was performed using Cn3D v4.1.

and a PDZ binding domain (Figure 1.10 a). Although PTEN has traditionally been described in this "domain" fashion, an emerging body of evidence is revealing that the PASE domain is essential for plasma membrane binding and the C2 domain is essential for phosphatase activity (Das *et al*., 2003). Therefore, although PASE and C2 are referred to as domains, they should be considered as regions within a whole functional protein. In addition, the partial crystal structure of the PTEN PASE and C2 domains shows extensive contacts between these two domains, suggesting their functions may be interdependent (Figure 1.10 b) (Lee *et al*., 1999).

The PASE domain (aa 1-185) is in the N-terminal region and contains a phosphatase motif (HCXXGXXR; aa 123-130), which is required for catalytic activity (Simpson and Parsons, 2001). As with all phosphatases, changing the cysteine to a serine in the phosphatase motif results in an enzymatically dead mutant (C124S = Cys at position 124 mutated to Ser) (Barford, 1996). The full-length PTEN protein was tested for lipid binding affinity using a heterogeneous mixture of the anionic plasma membrane mimicking lipid vesicles 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) and compared to the PASE domain (aa 1-185) (Das et al., 2003). The affinity of the PASE domain (aa 1-185) for POPC/POPS vesicles was compared to full-length PTEN. Full-length PTEN had ~31-fold higher affinity for the vesicles than the PASE domain alone (Das et al., 2003). Two clusters of positive charge exist within the PASE domain consisting of R11/K13/R14/R15 and R161/K163/K164 (Figure 1.10 b) (Das *et al*., 2003; Lee *et al*., 1999). Both regions were mutated to alanine independently (PTEN-PASE-RKRR/AAAA and PTEN-PASE-RKK/AAA, respectively) and tested for binding to lipid vesicles (Das *et al*., 2003). Mutations of both regions resulted in greatly reduced lipid binding, *i.e.* 76- and 22-fold,

respectively, but only the PTEN-PASE-RKK/AAA retained phosphatase activity (Das *et al*., 2003). Previous experiments altering regions of positive charge in the PASE domain had been executed by Lee and colleagues (1999) to examine the effects on intrinsic lipid phosphatase activity. They created single mutations in the PASE domain at Thr167, Glu171, Lys128 and His93 (Lee *et al*., 1999). In all instances, decreases in PTEN lipid phosphatase activity were observed in the range of 60-75% less lipid phosphatase activity (Lee *et al*., 1999).

PTEN has a C2 domain that is located from residues 186 to 351. Typically, C2 domains are Ca^{2+}-dependent lipid binding domains found in proteins involved in cell signaling and membrane trafficking (Das *et al*., 2003). The PTEN C2 domain is similar to some phosphatidylinositol specific phospholipases' C2 domains (Georgescu *et al*., 1999). However, the C2 domain of PTEN lacks all but one Ca^{2+}-binding residues (Asp268) and has been implicated in Ca^{2+}-independent membrane localization as well as phospholipid binding (Georgescu *et al*., 1999; Lee *et al*., 1999; Simpson and Parsons, 2001). Similar to the PASE domain, the isolated C2 domain also had significantly lower binding affinity (~30-fold decrease) for lipid vesicles POPC/POPS compared to full-length PTEN (Das et al., 2003). Lee and colleagues also mutated two sequences in the C2 domain (263-K-M-L-K-K-D-K-269 to 263-A-A-G-A-A-D-A-269 and 327-K-A-N-K-D-K-A-N-A-335 to 327-A-A-G-A-D-A-A-N-A-335) and in both cases observed decreased affinity for phosphatidylcholine (PC) lipid vesicles (Lee *et al*., 1999). Cells expressing these mutants had a comparable phenotype to tumor-derived cells (Lee *et al*., 1999).

Located near the C-terminal end from residues 350-375 and residues 379-396 are PEST sequences, which are degradation motifs frequently found in proteins that are degraded *via* the ubiquitin-mediated proteosomal pathway (Baker, 2007). However, deletion of the PEST

sequences does not result in more PTEN protein, but, in fact, leads to decreased protein expression, suggesting that the PEST sequences must be required for proper protein folding (Simpson and Parsons, 2001; Vazquez *et al.*, 2000). Recently, the ubiquitin ligase Nedd4-1 has been implicated in PTEN ubiquitin-mediated degradation and may even be functioning as an oncogene in some cancers due to its ability to decrease cellular PTEN levels (Wang *et al.*, 2007).

The C-terminal region of PTEN has a regulatory domain that contains phosphorylation sites for casein kinase 2 that are phosphorylated in a hierarchical fashion with the primary sites being S370 and S385 followed by residues S380, T382, T383 and possibly T366 (Torres and Pulido, 2001). Phosphorylation of PTEN stabilizes it and localizes it to the cytoplasm (Das *et al.*, 2003; Torres and Pulido, 2001). It has been proposed that upon phosphorylation, the regulatory domain blocks the ability of PTEN to localize to the plasma membrane *via* an electrostatic interaction with the phosphatase domain (Torres and Pulido, 2001). Mutations resulting in constitutive dephosphorylation of S380, T382 and T383 or deletion of these residues results in a constitutively plasma membrane-localized PTEN mutant (Das *et al.*, 2003). The phosphorylation sites are important for the stability and activity of PTEN as mutations in the C-terminal region of PTEN reverse the tumor suppressing phenotype (Georgescu *et al.*, 1999). Phosphorylation of S370 and S385 by casein kinase 2 inhibits phosphatase activity (Miller *et al.*, 2002) and interestingly also inhibits cleavage of PTEN by the protease caspase-3 (Torres *et al.*, 2003). Therefore a phosphatase is required to dephosphorylate and activate PTEN so it can interact with the plasma membrane and then be quickly degraded. Currently no phosphatase that has PTEN as a substrate has been identified. Cells expressing a PTEN mutant lacking the C-

terminal regulatory domain have increased PTEN activity (Vazquez *et al*., 2001) and constitutive plasma membrane localization of the protein (Das *et al*., 2003).

PTEN has also been shown to translocate into the nucleus where it is acetylated (Okumura *et al*., 2006). Since PTEN interacts with p53 as well as p300 in the nucleus, there are several suggestions as to how the PTEN protein enters the nucleus including passive diffusion, a C-terminal nuclear import signal (Lian and Di Cristofano, 2005), an N-terminal nuclear import signal (Gil *et al*., 2006) or it may be carried in by another protein.

PCAF (p300/CBP-associated factor) interacts with PTEN at amino acids 186-202 (Okumura et al., 2006), a region previously characterized as the hinge region between the N-terminal phosphatase domain and the C-terminal C2 domain (Lee *et al*., 1999). Wild type (wt) PTEN and PCAF co-localize to the nucleus in untreated cells, but PTEN Δ186-202 is excluded from the nucleus, suggesting that PCAF chaperones PTEN into the nucleus (Okumura *et al*., 2006). A more recent study demonstrated that PTEN is mono-ubiquitinated by Nedd4-1 at lysine 289 and also translocates to the nucleus (Trotman *et al*., 2007). PTEN is acetylated by PCAF at lysine residues 125 and 128, which interferes with PTEN phosphatase signaling and diminishes the ability of PTEN to arrest cell cycle entry (Okumura *et al*., 2006).

A number of elegant experiments have been executed by the Cho laboratory at the University of Chicago involving PTEN mutants and truncations fused to green fluorescent protein (GFP) (Das *et al*., 2003). All of the PTEN variants that included the PASE domain also incorporated a C124A (cysteine to alanine) mutation in the phosphatase motif in order to abolish PTEN phosphatase activity because active PTEN was not visible for imaging (Das *et al*., 2003). Presumably, the inability to visualize enzymatically active PTEN was that some aspect of PTEN

signaling results in expedient degradation after translocation to the plasma membrane (Das *et al*., 2003). All of the images were generated in the context of normal unstimulated HEK293 cells, which had the growth medium replaced with 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4 (Das *et al*., 2003). GFP-PTEN-C124A was observed in the cytoplasm; however, removal of the regulatory domain (aa 353-403) resulted in constitutive plasma membrane localization of the protein. Interestingly, deletion of the regulatory domain together with mutation of a cluster of basic amino acids in the PASE domain (R161/K163/R164) abolished plasma membrane localization. Similar to deletion of the regulatory domain, substitution of amino acids S380, T382 and T383 to alanine (STT/AAA; can not be phosphorylated) also resulted in constitutive plasma membrane localization (Das *et al*., 2003). Mutation of the same amino acids to glutamate (STT/EEE; phosphorylation mimic) resulted in cytoplasmic localization (Das *et al*., 2003). The isolated C2 domain was also distributed throughout the cytoplasm.

In tissue extracts, PTEN exists within a large multi-protein complex termed the "PTEN associated complex" (PAC) (Wu *et al*., 2000a). The PAC contains the proteins MAGI-2, PTEN and p85 (Figure 1.11) (Wu *et al*., 2000a). PTEN has a PSD-95/Dlg/ZO-1 (PDZ) binding motif that facilitates binding to one of the PDZ domains of the scaffolding protein membrane associated guanylate kinase inverted-2 (MAGI-2) and MAGI-3, members of the MAGUK protein family (Wu *et al*., 2000a). MAGI-2 contains six PDZ domains, two WW domains (bind proline-rich sequences) and a guanylate kinase-like (GuK) domain that is catalytically inactive (Wu *et al*., 2000a). MAGI proteins are cytoplasmic in quiescent cells, but translocate to the plasma membrane upon receptor activation (Xu *et al*., 2001), where they bind to various

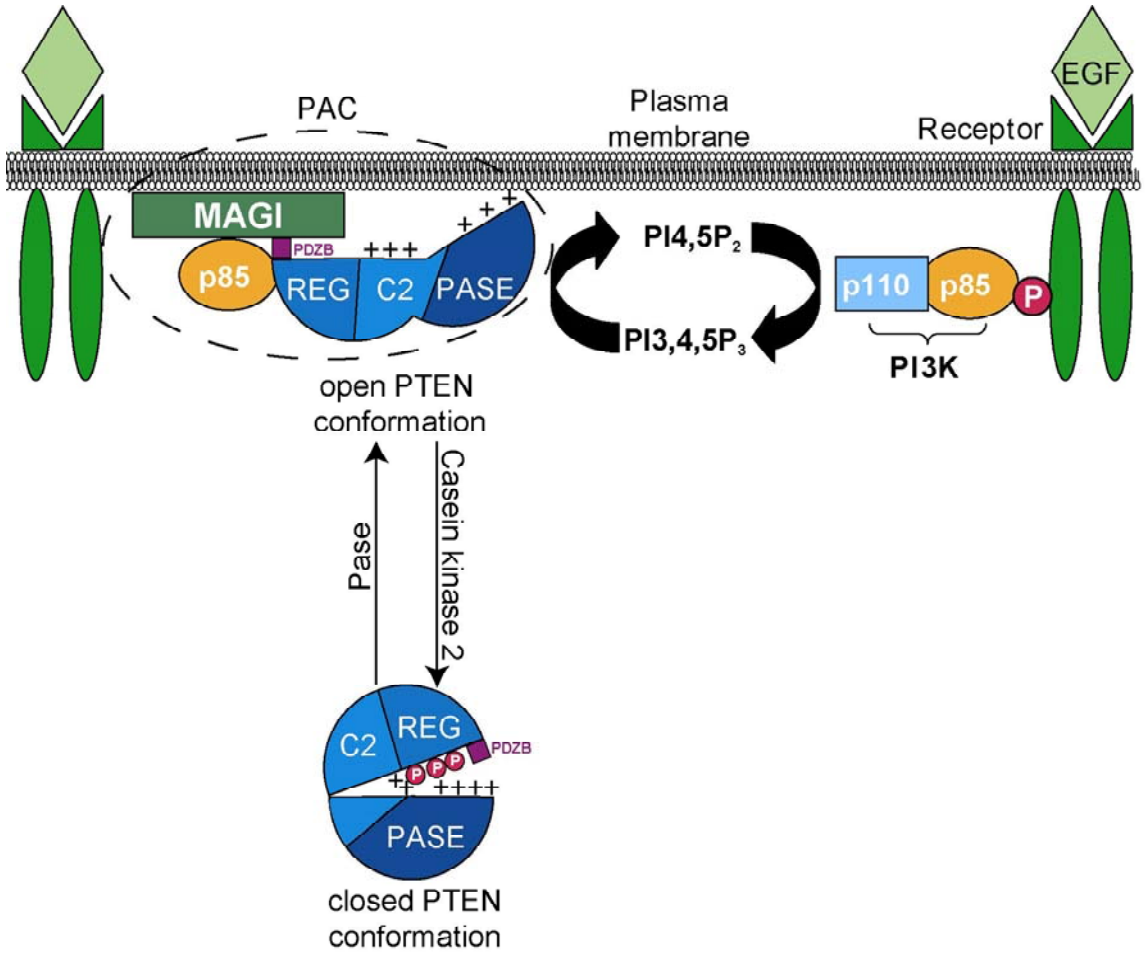


Figure 1.11: Model of the relationship between free p85, PI3K hetero dimer and PTEN in a cell. Casein kinase 2 phosphorylates the regulatory domain of PTEN, which results in the cytosolic closed conformation. The reverse reaction is catalyzed by an unidentified phosphatase, which causes PTEN to adopt an open conformation and non-specifically localize to the plasma membrane. At the plasma membrane, the scaffolding protein MAGI forms a complex with p85 and PTEN that may localize PTEN near activated receptors where it can dephosphorylate its substrate $PI3,4,5P_3$.

receptors, including G-protein coupled receptors, ion channel receptors and some RTKs *via* their PDZ domains (Kaech *et al*., 1998; Shiratsuchi *et al*., 1998; Wu *et al*., 2000b).

Recently, a graduate student in our laboratory, Ryaz Chagpar, has shown that p85 binds to PTEN and increases PTEN lipid phosphatase activity (Chagpar, 2004). Since the cellular concentration of p85 is higher than that of p110, with approximately 70% of the p85 in a cell associated with p110 (Ueki et al., 2002), we hypothesized that the free p85 may be involved in PTEN regulation. We have incorporated this information into a model for PTEN regulation (Figure 1.11). Casein kinase 2 phosphorylates the regulatory domain of PTEN resulting in the cytoplasmic closed conformation. The reverse reaction is catalyzed by an unidentified phosphatase. PTEN adopts an open conformation and non-specifically localizes to the plasma membrane through electrostatic interactions between the basic surfaces on the PASE and C2 domains and the anionic lipids of the plasma membrane. At the plasma membrane, the scaffolding protein, MAGI-2 forms a complex with p85 and PTEN that may allow p85 to positively regulate PTEN activity. While MAGI-2 proteins have not yet been shown to bind to RTKs in mammalian cells, we hypothesize that they do *via* one of their six PDZ domains, and that this would serve to localize PTEN at the receptor sites where PI3,4,5P_3 lipids have recently been generated. In order for RTK signaling to be efficient, highly regulated and expedient attenuation is required. Localization and activation of PTEN within the PAC would allow for only a transient activation of the PI3K/Akt pathway resulting in rapid attenuation of RTK signaling.

2.0 RATIONALE AND OBJECTIVES

One well-established role for the p85 regulatory subunit of PI3K is to stabilize, regulate and target the p110 PI3K catalytic subunit to the plasma membrane upon growth factor stimulation. The molar ratio for p85:p110 in a cell is not 1:1 as approximately only 70% of cellular p85 is associated with p110 (Ueki *et al*., 2002). Some of the "free" p85 may be involved in attenuating growth factor signaling by acting as a dominant negative because p85 that is not in a complex with p110 is still capable of binding the phosphotyrosines of an activated RTK. Thus, p85 can occupy a binding site that the catalytically active p85:p110 could otherwise have occupied. Our laboratory has shown that p85 has the ability to bind to and upregulate the lipid phosphatase activity of PTEN (Chagpar, 2004) and that this requires the N-terminal SH3 + BH domains of p85 (D. Anderson, unpublished observations).

Characterization of functional domains within PTEN demonstrates that the binding to membrane lipids and lipid substrate requires both the PASE and C2 domains (Das *et al*., 2003). The regulatory domain does not play an important role in the physical lipid binding properties of PTEN, but is responsible for transition between the proposed open and closed conformations of PTEN. Removal of the regulatory domain or even alanine substitution of the S380/T382/T383 phosphorylation sites results in a constitutively plasma membrane-localized protein (Das *et al*., 2003).

A cluster of positively charged residues in the PASE domain of PTEN have been identified to be important in non-specific membrane targeting. When this cluster of basic residues is mutated to alanine and the regulatory domain is removed, the ability to localize to the plasma membrane is abolished (Das *et al*., 2003). We examined the partial crystal structure of PTEN and observed that two more clusters of positively charged residues were on the same surface of the protein but were located in the C2 domain of PTEN (Lee *et al*., 1999). Further analysis of the PTEN crystal structure revealed Tyr336 located on the same basic surface of the C2 domain (Lee *et al*., 1999). This residue may have implications in regulation of disengagement of PTEN from the plasma membrane.

Hypothesis: The p85 protein increases PTEN lipid phosphatase activity. Regulation of PTEN is dependent on whether the protein is in an open or closed conformation. The casein kinase 2 phosphorylation sites, *i.e.* S380/T382/T383, regulate which conformation PTEN adopts (phosphorylation = closed; no phosphorylation = open). PTEN has positively charged surfaces important for plasma membrane targeting. In the closed conformation, the phosphate groups in the regulatory domain mask clusters of positive charge located in the PASE and C2 domains and PTEN remains cytoplasmic. Dephosphorylation of these regulatory phosphorylation sites allows PTEN to adopt an open conformation and localize non-specifically to the plasma membrane upon growth factor stimulation. At the plasma membrane PTEN is targeted to the active receptor where it is in proximity to its substrate and PTEN activity is enhanced by the p85 protein.

Objectives: The focus of this thesis was to characterize in more detail the activation of PTEN lipid phosphatase activity by p85 *in vitro* and to carry out a structure/function analysis of PTEN. The specific objectives are as follows:

1. Modify an existing PTEN enzymatic assay to measure activation by p85.

2. Structure/function analysis of PTEN – Site-directed mutagenesis was employed to create phosphorylated and unphosphorylated mimics of regulatory domain phosphorylation sites (S380/T382/T383) and a potential phosphorylation site (Y336) that may have a role in plasma membrane localization. The technique was also used to substitute clusters of positively charged residues, thought to be important for an electrostatic interaction with the anionic plasma membrane, with alanine residues.

2.1 Test lipid binding properties of wild type PTEN and the PTEN mutants.

2.2 Assess the subcellular localization of wild type PTEN and PTEN mutants under unstimulated and EGF stimulated conditions.

3.0 MATERIALS & METHODS

3.1 Materials

3.1.1 Mammalian cells

Human embryonic kidney 293 (HEK293) cells were obtained from American Type Cell Culture.

3.1.2 Bacterial strains & growth media

Plasmid DNA was amplified using *Escherichia coli* (*E. coli*) strain TOP10 [F^-, *mcr*A, D(*mrr-hsd*RMS-*mcr*BC), f80*lac*ZDM15 D*lac*X74, *deo*R, *rec*A1, *ara*D139 D(*ara-leu*)7697, *gal*K, *rps*L(StrR), *end*A1, *nup*G] (Invitrogen). Overexpression of recombinant GST-p85 proteins was performed using an *E. coli* strain deficient in protease activity, BL21 (DE3) [*E. coli B*, F^-, *dcm*, *ompT*, *hsdS*($r_B^- m_B^-$), *gal*] (Pharmacia). Overexpression of His_6-PTEN, His_6-PTEN-C124S and the various His_6-PTEN-C124S mutants was accomplished using M15[pREP4] derived from *E. coli* strain K12 Nal^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+ (Qiagen). All bacterial strains were grown in Millar's Luria-Bertani broth (LB, Sigma) containing 10.0 g tryptone, 5.0 g yeast extract and 10.0 g NaCl per litre. The media was supplemented with either 100 μg/mL ampicillin (LBA) or 50 μg/mL of kanamycin (LBK) depending on whether the

transforming plasmid contained ampicillin resistance (Amp^R) or kanamycin resistance (Kan^R), respectively. Bacterial cells were grown at 37 °C in a shaking incubator unless otherwise noted.

3.1.3 Immunoblot detection antibodies

For brevity and conciseness, all antibodies are listed in Table 3.1.

3.1.4 PTEN enzyme fluorescent substrate

The fluorescently-tagged $PI3,4,5P_3$ lipid substrate was NBD6-PtdIns(3,4,5)P3 and was obtained from Echelon Biosciences; catalogue number C-39N6.

3.1.5 Plasmids and vectors

The full-length PTEN cDNA encoding amino acids 1-403 was cloned by R. Chagpar (University of Saskatchewan) from HeLa cells using RT-PCR and subcloned into the pGEX-6P1 vector (Amersham Pharmacia). The phosphatase-inactivating mutation C124S was introduced using site-directed mutagenesis (Chagpar, 2004). The PTENwt aa 1-403 and PTEN-C124S aa 1-403 cDNA genes were subcloned from pGEX-6P1 to the pQE9 vector (Qiagen) by D. Anderson (Saskatchewan Cancer Agency). The pQE9-PTENwt and pQE9-PTEN-C124S were used to express His_6-PTEN fusion proteins in bacteria. The mutants PTEN-C124S-STT/AAA, PTEN-C124S-STT/EEE, PTEN-C124S-C2-K3A, PTEN-C124S-C2-K5A, PTEN-C124S-PASE-RKK/AAA, PTEN-C124S-Y336A and PTEN-C124S-Y336E were generated in the pEGFP (Invitrogen) vector and subcloned into to pQE9 by S. Knafelc (University of Saskatchewan).

Table 3.1: Primary and secondary antibodies used to probe protein and lipid blots

Antibody + Concentration	Company, Catalog #	Figures
PTEN A2B1 mouse monoclonal 1μg/mL	Sana Cruz Biotechnology, sc7974	4.3, 4.4
PTEN A2B1 mouse momoclonal horseradish peroxidase (HRP) 1μg/mL	Santa Cruz, Biotechnology, sc7974	4.1, 4.4
IRDye 680 conjugatede goat (polyclonal) anti-mouse IgG 1:2000	LI-COR Biosciences, 926-32220	4.3, 4.4
p85 NSH@ mouse monoclonal 1:500	Upstate Biotechnology 05-217	4.2
Goat anti-mouse IgG HRP 1:500	Santa Cruz Biotechnology, sc2055	4.2

The bovine p85α wild type protein aa 1-724 was obtained in the pGEX-2T vector from M. Waterfield (London, UK). The mammalian expression vector pEGFP was obtained from Invitrogen. Subcloning of the PTEN wt aa 1-403 and PTEN-C124S aa 1-403 cDNAs was performed by R. Chagpar.

3.1.6 Lipid Blots

Lipid blots (PIP strips) were obtained from Echelon Biosciences and are nitrocellulose membranes spotted with 100 pmol of lipid/spot (catalogue #P6001). The lipids spotted were: lysophosphatidic acid (LPA), lysophosphocholine (LPC), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI), PI 3-phosphate (PI3P), PI4P, PI5P, PI3,4P_2, PI3,5P_2, PI4,5P_2, PI3,4,5P_3, and sphingosine 1-phosphate (S1P).

3.1.7 Other reagents and supplies

Chemicals used were purchased from VWR or Sigma and were of analytical grade or higher quality unless otherwise noted.

3.2 Methods

3.2.1 DNA methods

3.2.1.1 Plasmid preparation

Generally, all plasmids with the p85α or PTEN cDNA inserts were transformed into competent E. coli, selected for on LB agar containing the appropriate antibiotic (ampicillin for pGEX and pQE9, kanamycin for pEGFP), grown overnight in LB containing the appropriate antibiotic and stored at -80 °C in 20 % glycerol (Elbing, 2002). Plasmid DNA of high yield and purity was obtained from overnight cultures using a QIAprep® Spin Miniprep Kit (catalogue #27104) or a HiSpeed® Plasmid Maxi Kit (catalogue #12662) obtained from Qiagen. The DNA purification kits were used in accordance with the manufacturer's instructions with the alteration that DNA was eluted with water instead of elution buffer. This change was made because dH2O did not contain salts that may have to be considered later when using the DNA for transfections. Mini-preps were eluted by applying 20 µl dH2O to the column, incubating at 55 °C for 5 minutes followed by centrifugation at 18,000 x g. Since only 20 µl of dH2O was used instead of the recommended 50 µl, the eluted DNA and water was reapplied to the column a second time, incubated at 55 °C for 5 minutes and centrifuged at 18,000 x g. Maxi-preps were eluted using 500 µL of dH20 instead of elution buffer. DNA quantification was determined by performing a 1:25 dilution in dH2O and reading the absorbance at a wavelength of 260 nm. Relative purity was performed by calculating the ratio of the absorbance at 260 nm divided by the absorbance at 280 nm using an Amersham Pharmacia Ultraspec 3100 spectrophotometer programmed for DNA quantification mode.

3.2.1.2 Site-directed mutagenesis

Seven PTEN C124S mutants were created using site-directed mutagenesis. The starting template for all of the mutants was pEGFP-PTEN-C124S aa 1-403 (Chagpar, 2004). All of the primers used were custom-ordered from Invitrogen Life Technologies in sense/anti-sense pairs. For brevity only the sense primer names and sequences will be noted as in all instances the reverse complement primer was identically named with the exception of the 3' designation preceding the name instead of 5' (Figure 3.1).

Mutagenesis reactions were performed in 50 µl of *Pfu* buffer –$MgSO_4$ (Fermentas) with 2.5 U of *Pfu* DNA polymerase (Fermentas), 1 ng template DNA, 2.5 ng each of sense and anti-sense primers and 50 µM dNTPs. The thermocycler program used was as follows: 95 °C for 1 minute followed by 14 cycles of 95 °C for 30 seconds, 54 °C for one minute and 68 °C for 17 minutes. A single final extension step of 72 °C for 7 minutes was added to the end of the program. Tubes were centrifuged 18,000 x g for 30 s and 10 U of *Dpn* I (Fermentas) was added and mixed by pipetting the mixture several times. The mixture was incubated at 37 °C for 1 h to allow the *Dpn* I to digest the methylated wild type template DNA. Forty µl was used to transform competent TOP10 bacteria (Elbing, 2002), which were plated onto warm LB agar plates containing 15 µg/ml kanamycin. The plates were incubated overnight at 37 °C and the smallest colonies were inoculated into 5 mL of LBK and grown overnight at 37 °C in a shaking incubator on a setting of 250-300 rpm. Plasmid DNA was prepared as stated previously. The plasmid DNA was submitted to the DNA Technologies Unit at the Plant Biotechnology Institute (Saskatchewan) for sequencing to verify that the desired mutation(s) were introduced. The primers included with

Mutant Name	Primer Name	Primer Sequence
		S/E^{380} T/E^{382}T/E^{383}
GFP-PTEN-C124S-STT/EEE	5' PTEN T382E	GA TAT TCT GAC GAA ACT GAC TCT G
	5' PTEN T382/T383E	GA TAT TCT GAC GAA GAA GAC TCT G
	5' PTEN S380/T382/T383E	CAT TAT AGA TAT GAA GAC GAA GAA GAC
		K/A^{327} K/A^{330} K/A^{332}
GFP-PTEN-C124S-C2K3A	5' PTEN K330A	C AAA GCA AAT GCA GAC AAA GCC
	5' PTEN K327/K330A	GAT CTT GAC GCA GCA AAT GCA G
	5' PTEN K327/K330/K332A	GCA AAT GCA GAC GCA GCC AAC CGA TAC
		K/A^{260} K/A^{263} K/A^{266}K/A^{267} K/A^{269}
GFP-PTEN-C124S-C2K5A	5' PTEN K266A	C AAG ATG CTA GCA AAG GAC AAA ATG
	5' PTEN K263/K266A	C AAA CAG AAC GCG ATG CTA GCA AAG
	5' PTEN K260/K263A	G TTC TTC CAC GCA CAG AAC GCG
	5' PTEN K266/K267A	G ATG CTA GCA GCG GAC AAA ATG TTT CAC
	5' PTEN K266/K267/K269A	CTA GCA GCG GAC GCA ATG TTT CAC
		R/A^{161} R/A^{163}R/A^{164}
GFP-PTEN-C124S-PASE-RKK/AAA	5' PTEN R161A	GAA GTA AGG ACC GCA GAC AAA AAG GG
	5' PTEN R161/K163A	GG ACC GCA GAC GCA AAG GGA GTA AC
	5' PTEN R161/K163/K164/A	GG ACC GCA GAC GCA GCG GGA GTA ACT ATT C

Figure 3.1: Four GFP-PTEN-C124S mutants generated in sequential mutagenesis reactions. One amino acid changer per mutagenesis reaction performed in the order the primers are shown. Underlining denotes what the nucleotides were changed to. The amino acids encoded are given above the primer sequences (wild type residue/mutant residue) Intermediate mutants were labeled according to the primer name shown. Each sequential step was verified by DNA sequencing and positive mutations became the template for the next round of mutagenesis. The anti-sense primers are the reverse compliment of the sense sequence shown.

the plasmid DNA for the sequencing reaction were 5' seqEFP and 5' PTENC124S.

The two mutants GFP-PTEN-C124S-Y336E and GFP-PTEN-C124S-Y336A were generated using a single set of degenerate primers that would alter the codon for tyrosine at position 336 from UAC to either GAA or GCA, resulting in an amino acid change to glutamic acid or alanine, respectively. The sense primer used for these mutants was 5'PTEN Y336E/A and the sequence was 5' GCC AAC CGA G(**A**/**C**)A TTT TCT CCA AAT.

The GFP-PTEN-C124S-STT/AAA mutant was generated using a single set of primers to change all three codons. The primer used was named 5'PTENCKII-U and its sequence was 5' CAT TAT AGA TAT **G**CT GAC **G**CC **G**CT GAC TCT GAT CC.

The remaining four mutants that had multiple amino acid changes were generated using a sequential approach using one set of primers per triplet codon change (Figure 3.1). These four mutants were GFP-PTEN-C124S-STT/EEE, GFP-PTEN-C124S-C2K3A, GFP-PTEN-C124S-C2K5A and GFP-PTEN-C124S-PASE-RKK/AAA. Upon successful mutagenesis, the new mutant was labeled according to the primer name for the mutation made e.g. GFP-PTEN-C124S-T382E. After verification by DNA sequencing GFP-PTEN-C124S-T382E became the template for the next round of mutagenesis. The primers were designed to overlap such that the set of primers to mutate the next amino acid incorporated the previous mutation and the resultant mutant was named GFP-PTEN-C124S-T382/T383E and so on for the remaining mutation as well as the three other GFP-PTEN-C124S mutants.

3.2.1.3 Subcloning

Subcloning the PTEN mutants from the EGFP vector to the pQE9 vector was accomplished by Stacey Knafelc (Summer student, 2006), by digesting 2 μg each of pEGFP-PTEN-mutant vector and pQE9 vector in One-Phor-All Buffer PLUS (GE Healthcare, NJ USA) for 1h at 37 °C using 50 U of *Bgl* II and 60 U *Sal* I purchased from New England Biolabs (MA, USA). Fragments were electrophoresed using a 1% low melt agarose gel (Sigma, catalogue # 9012-36-6). The appropriate pQE9 vector and PTEN insert DNA fragments were excised from the gel and purified using a Qiagen gel extraction kit in accordance with the manufacturer's instructions except that the final elution was performed with dH_2O. Ligation into the pQE9 vector was accomplished using Quick ligase in accordance with the manufacturer's instructions (New England Biolabs, MA, USA). The entire ligation mixture was used to transform competent TOP10 cells as previously stated (Elbing, 2002).

3.2.2 Protein analysis

3.2.2.1 Induction and affinity purification of His_6-fusion proteins

His_6-PTEN proteins were expressed and purified by Stacey Knafelc (Summer student 2006). The PTEN wt and mutants C124S, C124S-STT/AAA, C124S-STT/EEE, C124S-C2-K3A, C124S-C2-K5A, C124S-PASE-RKK/AAA, C124S-Y336A and C124S-Y336E were expressed individually using the pQE9 vector transformed into M15pREP4 cells. pQE9-PTEN-C124S was previously generated by D Anderson (Saskatchewan Cancer Agency, Canada). The M15pREP4 cells containing the respective plasmids were each inoculated into 100 mL of LB growth medium

containing ampicillin and grown overnight at 37 °C in a shaking incubator. Sixteen – twenty four hours after inoculation, the culture was added to 1L of similar media and grown to an optical density of 0.6 at A_{600}. The culture was cooled to room temperature and protein production was induced using isopropyl β-D-thiogalactopyranoside (IPTG; Sigma catalogue # I-5502) at a final concentration of 0.3 mM. Induction was carried out overnight at room temperature. The bacterial cultures were subjected to centrifugation at 5,000 x g for 10 minutes. Lysis of the pellets was performed in 8 ml of lysis buffer per 1.4 g of cells. The lysis buffer was 50 mM NaH_2P0_4 pH 8.0 containing 300 mM NaCl, 10 mM imidazole, 1 mg/ml lysozyme, 1 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) and 5 mM β-mercaptoethanol. The suspensions were vortexed, incubated for 1 h at 4 °C, and 25 ml aliquots were sonicated 3 times for 10 seconds at 4 °C at a setting of 2.5 with a Branson Sonifier 450 fitted with a micro tip. Centrifugation at 24,000 x g removed cell debris and the supernatant was subjected to affinity chromatography. It was discovered that using the Qiagen Ni-NTA agarose purification kit, but substituting Talon metal affinity resin (Clontech) for the Ni-NTA resin, resulted in the highest yield of relatively pure protein. Thus the Qiagen protocol was employed using the Clontech resin. The lysate and resin were divided into two 50 mL tubes (Falcon) and nutated at 4 °C for 1 h. The tubes were centrifuged at 4 °C at 834 x g for 5 minutes in a Sorvall RT6000D benchtop centrifuge equipped with a H1000B rotor. The supernatant was removed and the resin was washed with Qiagen wash buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris Cl, pH 6.3, 1 mM tris(2-carbosyethyl)phosphine (TCEP) added just before use), centrifuged at 834 x g and the supernatant removed. A Bradford protein determination (BioRad) was performed on the wash

buffer supernatant and multiple washes were performed until no protein was detected in the wash buffer (Qiagen) supernatant. The resin was transferred to a TALON 2 mL disposable column (Clontech) and 10 mL of Qiagen elution buffer (8 M urea,100 mM NaH_2PO_4, 10 mM Tris Cl, 10 mM imidazole, pH 6.3, 1 mM TCEP added just before use) was applied to the column. One mL aliquots were collected from the column and the protein concentration of each aliquot was verified using a Bradford assay. The content and purity of each aliquot was assessed by using SDS-PAGE (10%) followed by Coomassie Blue stain (Sasse, 2003). Typically, elution aliquots 2-8 were pooled and applied to an Amicon Ultra Concentrator dual membrane with a 10,000 Da molecular weight cut off (Millipore, MA, USA). The concentrator was used in accordance with the manufacturer's instructions to buffer-exchange the His_6-PTEN proteins into PTEN storage buffer (50 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl, 2 mM DTT added fresh).

Protein concentration was determined using the Bradford assay. Analysis of the purity of the recombinant protein was performed using SDS-PAGE followed by staining with Coomassie Blue stain (Elbing, 2002). The proteins were also resolved by SDS-PAGE (10%) and transferred to nitrocellulose using an Owl semi-dry transfer apparatus. The nitrocellulose membrane was soaked in dH_2O for 15 minutes prior to transferring. The resolved polyacrylamide gel and eight pieces of 3MM paper (Whatman) cut to the same size as the gel were incubated in transfer buffer (50 mM Tris, 37 mM glycine, 1.3 mM SDS, pH 9.2) for 15 minutes. The transfer apparatus required dH_2O on the graphite electrode and was assembled according to the manufacturer's instructions. The transfer was performed at constant milliamperage of 400 for 15 minutes. The

nitrocellulose membrane was blocked in TBST containing milk (5% w/v) for 1 h. The blot was probed overnight with a horseradish peroxidase-conjugated PTEN monoclonal antibody at a concentration of 1 μg/mL in TBST containing milk (5% w/v) and visualized with chemiluminescence according to the manufacturer's instructions (Perkin Elmer). Film was obtained from Kodak (X-OMAT AR, 8 x 10 inches, catalogue # 165 1454).

3.2.2.2 Induction and affinity purification of GST-fusion proteins

The pGEX-2T-p85 plasmid was transferred into BL21 (DE3) cells that were treated in the same fashion as the pQE9-PTEN plasmids with the alteration that the induction was performed with 0.1 mM IPTG at room temperature for 5 h. The cells were collected and lysed in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3) containing AEBSF (1 mM), aprotinin (10 μg/ml) and leupeptin (10 μg/ml) as previously stated with the alteration that the affinity chromatography was performed using glutathione-Sepharose resin, and the p85 protein was cleaved from the GST portion and beads with Thrombin (Sigma) according to the method previously published (Current Protocols in Molecular Biology, Section 16.4.8). The immobilized GST-p85 on beads was cleaved with Thrombin (5 U/mg protein) in PBS overnight at room temperature. Protein concentrating, quantification and verification of purity were performed using the same method as used for the His_6-PTEN proteins. For concentrating, a concentrator with a 30,000 Da molecular weight cut-off was used. The Bradford assay was used to determine protein concentration and purity was assessed using SDS-PAGE and Coomassie Blue staining.

3.2.2.3 Fluorescent PTEN enzyme activity assay

The fluorescent PTEN enzyme activity assay was adapted from Taylor and Dixon (2001). The reaction was performed in a total volume of 20 µl of 50 mM Tris HCl pH 8.0, 1 mM EDTA, 150 mM NaCl with 2 mM DTT (added fresh) with 1.5 µg fluorescent lipid substrate [NBD6-Ptdns(3,4,5)P_3] and different concentrations of proteins (His_6PTEN +/- p85). The reactions were quenched with 100 µl of acetone and dried in a "speed vac" with warming. Dried lipids were resuspended in 9 µl of 5:5:2 methanol:2-propanol:glacial acetic acid and spotted onto a 20 x 20 cm LK6 silica gel 60 Å thin layer chromatography (TLC) plate (catalog #4865-820, Whatman Inc.). The TLC plate was developed for 20 minutes in a sealed chromatography chamber equilibrated with the following developing solvent: 70:50:20:20:20 chloroform:methanol:acetone:glacial acetic acid:dH_2O. The TLC plate was visualized using a BioRad Gel Doc UV transilluminator and fluorescent spot quantification was performed using BioRad Quantity One software. Local background subtraction was used. For the PTEN titration, the average across three independent replicates was plotted using Graphpad Prism software v4.0. It was determined that 0.56 µM His_6-PTEN would convert approximately 40% of 1.5 µg of fluorescent PI3,4,5P_3 substrate, NBD6-Ptdns(3,4,5)P_3, to PI4,5P_2 product in a reaction time of 20 minutes at 37 °C. To measure the effect of p85 on PTEN activity, p85 was added at concentrations of 0.14, 0.28, 0.56 and 1.12 µM, keeping a constant concentration of His_6-PTEN protein (0.56 µM). Duplicate p85 addition experiments were plotted separately using GraphPad Prism 4.0.

3.2.2.4 Lipid blot binding

The nitrocellulose membranes spotted with various lipids (see section 3.1.6) obtained from Echelon Biosciences were blocked for 1 h at room temperature in blocking buffer (fatty acid-free BSA 3% w/v [Sigma; catalogue #A0281] in 10 mM Tris/HCl pH 8.0, 150 mM NaCl and 0.1% v/v Tween-20). The blots were incubated overnight at 4 °C with 2 µg His6-PTEN in 2 ml of blocking buffer. The membranes were washed six times with blocking buffer for 5 minutes per wash on a Rocking Platform (VWR). The blots were probed with 1 µg/ml PTEN A2B1 antibody in blocking buffer for 1 h at room temperature. The blots were washed again as before and probed with a 1:2000 dilution of IRDye 680 goat anti-mouse antibody in blocking buffer for 1 h at room temperature. The blots were washed and scanned using the Odyssey imaging system (LI-COR Biosciences; Nebraska, USA) at 700 nm. The brightness and contrast were set to level 50 and a sensitivity setting of 5 in the linear mode. Quantification was performed using the Odyssey v2.1 software. The blots were normalized to $PI4,5P_2$ levels in the PTEN-C124S samples across the replicates because it was the spot with the least variation. For each PTEN protein, at least three independent experiments were carried out and a new lipid blot nitrocellulose membrane was used. The lipid blot data was analyzed using the Odyssey 2.1 software for quantification with local background subtraction. Statistical differences were calculated using a matched pairs t-test with a confidence interval of at least 95%. The values calculated are listed in Appendix 1.

3.2.3 Microscopy

3.2.3.1 Cell culture technique

HEK293 cells were maintained in Alpha Modified Eagle's Medium (αMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, VWR), 100 U/ml penicillin G and 100 μg/ml streptomycin (1% P/S). The cells were grown at a temperature of 37 °C with the level of CO_2 enriched to 5%.

3.2.3.2 Transfection of HEK293 cells with pEGFP-PTEN-C124S plasmids

HEK293 cells, at a concentration of 200,000 cells/mL, were seeded onto #1.5 coverslips (VWR) in a tissue culture plate, 6 well (Falcon), in culture media and the cells were grown overnight. Briefly, 1 μg of DNA was transfected into cells using Lipofectamine Plus (Invitrogen) in accordance with the manufacturer's instructions optimized for 6 well tissue culture plates. The DNA, 100 μL of Opti-MEM (Invitrogen; catalogue # 11058), and 6 μl of PLUS reagent (Invitrogen) were incubated at room temperature for 15 minutes. Four μl of Lipofectamine was added to 100 μl of Opti-MEM and this was added to the DNA mixture. After incubating for 15 minutes at room temperature, 800 μl of warm serum-free αMEM was added. The cells were washed with 500 μL of warm serum free αMEM and the transfection media was added. After a 5 h incubation at 37 °C and 5% CO_2, 1 mL of αMEM containing 20% FBS was added to the 1 mL of serum-free transfection media in the well resulting in a final concentration of 10% FBS. Twenty four h after adding the transfection media, 1 mL of the media was removed, and epidermal growth factor (Sigma; catalogue # E-9644) was added to a concentration of 80 nM. The EGF containing media was used to stimulate the cells for 5 minutes. Following

stimulation, cells were immediately washed once with 0.5 mL of ice-cold PBS and fixed with 4% paraformaldehyde (Electron Microscopy Sciences, PA, USA) for 30 minutes at room temperature. Coverslips were mounted directly on slides using Prolong Gold mounting media (Invitrogen).

3.2.3.3 Confocal image acquisition

Imaging of the cells was performed by using an Olympus FluoView 300 point-scanning, point-detection, confocal laser-scanning microscope (Neuropsychiatry Research Unit, University of Saskatchewan). The 10x objective lens was used in fluorescence mode to locate cells expressing the GFP-PTEN proteins. The objective lens was changed to 40x and the confocal mode was activated as well as 4x digital zoom was employed. A 688 nm argon positive ion laser was used to excite the GFP. A dichroic mirror that split the wavelength at 570 nm was used to send light <570 nm to the channel 1 detector (>570 to the channel 2 detector). A barrier filter was used to allow only wavelengths of light in the range of 505 nm to 525 nm through to the detector. GFP has an excitation wavelength of 488 nm and an emission wavelength of 509 nm. The focal plane was adjusted to the middle of the cell by performing quick low resolution scans at 1 micron intervals starting at the bottom of the cell and continuing through the top. Middle layers of the cell were imaged using the Kalman scanning mode to take 5 consecutive scans of the same area and overlay them to reduce background and generate a clearer image. All final images were the result of a 5 line-scan average combined using the FluoView software. Adobe Photoshop v2.0 was used to adjust the levels of fluorescence intensity.

4.0 RESULTS

Three aspects of PTEN were characterized. First, PTEN activity was assayed *in vitro* and the ability of p85 to enhance PTEN lipid phosphatase activity was assessed. Second, PTEN lipid binding specificity was examined utilizing lipid blots, which consisted of nitrocellulose membranes with purified lipids spotted onto them. Finally, PTEN subcellular localization was studied in HEK293 cells using GFP-PTEN-C124S fusion proteins and visualized using confocal microscopy.

4.1 PTEN phosphatase activity

4.1.1 Characterization of PTEN phosphatase activity and the effect p85 had on that activity *in vitro*

In order to investigate the effect of p85 on PTEN activity, a PTEN activity assay was first established. A study of the enzymatic activity of PTEN required high quality and relatively pure protein. The most efficient way to obtain bacterially-expressed protein of high purity was by using affinity chromatography. PTEN was expressed and purified using a histidine tag consisting of six histidine residues fused to the N-terminus of the protein. The histidine residues were captured using their affinity for nickel beads. The His_6-PTEN was eluted from the beads using imidazole. A phosphatase-dead PTEN mutant (Cys 124 changed to Ser) was purified

similarly (His_6-PTEN-C124S). The purity of the His_6PTEN preparations were verified after resolution by SDS-PAGE and Coomassie blue staining (Figure 4.1 a). The immunoreactivity of each protein was confirmed by immunoblot analysis with anti-PTEN antibodies (Figure 4.1 b).

To characterize the enzymatic activity of His_6-PTEN *in vitro*, we used a fluorescently-labeled PTEN substrate, phosphatidylinositol 3,4,5-trisphosphate ($PI3,4,5P_3$) (Taylor and Dixon, 2001). Briefly, 3 μg of His_6-PTEN (final concentration of 3.18 μM) was incubated with 1.5 μg of fluorescently-tagged $PI3,4,5P_3$ for 20 minutes to allow complete dephosphorylation of the 3' phosphate group. Thin layer chromatography (TLC) was used to resolve the substrate, $PI3,4,5P_3$, and product, phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$), which were visualized using an ultraviolet transilluminator.

Further experiments to assess both positive and negative effects of p85 on PTEN activity required the ability to detect increased or decreased PTEN activity. Therefore, enzymatic activity was assayed at different concentrations of PTEN (Figure 4.1 c). This titration of the PTEN enzyme allowed the selection of a lower concentration of PTEN (0.56 μM) to be used in subsequent assays with p85 to assess its regulatory effects. The fluorescent $PI3,4,5P_3$ substrate was not titrated because for quantification purposes, it was better to have the same amount of substrate in each lane on the TLC. As a control, a known phosphatase-dead PTEN (PTEN-C124S) was used to demonstrate lack of lipid phosphatase activity (Figure 4.1 c).

Once titration of the concentration of PTEN was completed, the next step was to examine the effect of p85 on PTEN activity. A His_6-PTEN concentration of 0.56 μM was chosen since this converted approximately 40% of the $PI3,4,5P_3$ substrate to $PI4,5P_2$ product using the standard

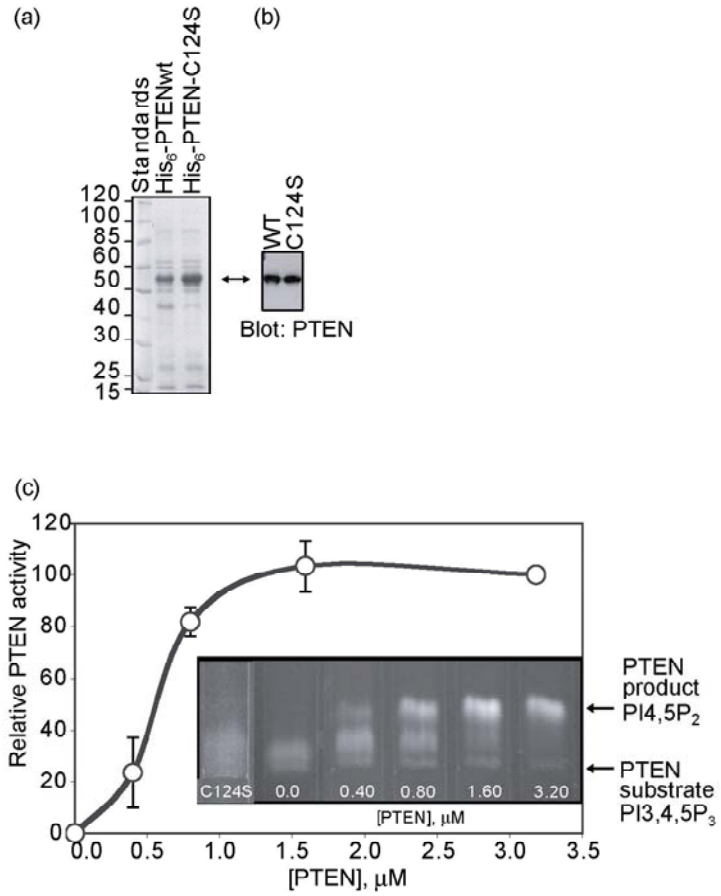


Figure 4.1: Characterization of PTEN lipid phosphatase activity toward the substrate PI3,4,5P$_3$ *in vitro*. (a-b) His$_6$ tagged PTEN wild type (wt) and phosphatase dead (C124S) proteins were bacterially expressed and purified using affinity chromatography. The proteins were resolved by SDS-PAGE stained with Coomassie blue (a) as well as transferred to nitrocellulose and probed with mouse anti-PTEN antibody conjugated to horseradish peroxidase. Visualization was accomplished using chemiluminesence (b). (c) The indicated concentrations of PTEN were assayed using a fluorescent PI3,4,5P$_3$ lipid substrate. The resulting product, PI4,5P$_2$, was resolved from the PI3,4,5P$_3$ substrate by TLC and then visualized under UV light (inset). Biorad Quantity One software was used to quantify the product using local background subtraction and normalization to the 0 PTEN lane (the mean of three independent experiments is plotted and the bars represent the standard error of the mean).

assay conditions. At this concentration of His_6-PTEN, it would be apparent whether addition of p85 enhanced, inhibited or had no effect on His_6-PTEN activity. The p85 protein was expressed as a GST fusion protein, captured on glutathione Sepharose beads and cleaved from the GST using Thrombin protease (Figure 4.2 a). The band that resolved at approximately 85 kDa was verified to be p85 by immunoblot analysis (Figure 4.2 b)

To examine the quantitative effect that p85 had on His_6-PTEN enzymatic activity *in vitro*, p85 was added to the fluorescent PTEN enzyme assay at increasing concentrations (Figure 4.2 c). PTEN assay controls showed that the p85 preparation (1.12 μM) did not exhibit PTEN lipid phosphatase activity. Similar results were observed in a replicate experiment. As the p85 concentration increased, so did His_6-PTEN activity to a maximum activation at a 1:1 molar ratio (0.56 μM for both p85 and PTEN). Furthermore, the PTEN activation by p85 addition was approximately 3.5-fold when compared to the PTEN activity in the absence of p85. These results clearly show that p85 can stimulate PTEN activity at least 3.5-fold *in vitro*, suggesting that p85 may be a positive regulator of PTEN.

4.2 PTEN plasma membrane lipid binding

4.2.1 PTEN proteins bound preferentially to phosphorylated phosphatidylinositol lipids

PTEN has been proposed to remain in the cytoplasm and move to the membrane in response to some aspect of activation of PI3K signaling, such as growth factor stimulation (Cantley and Neel, 1999). The ability of PTEN to bind to different lipids has not previously been examined, especially phosphatidylinositol phosphates. In order to investigate PTEN binding specificity to a

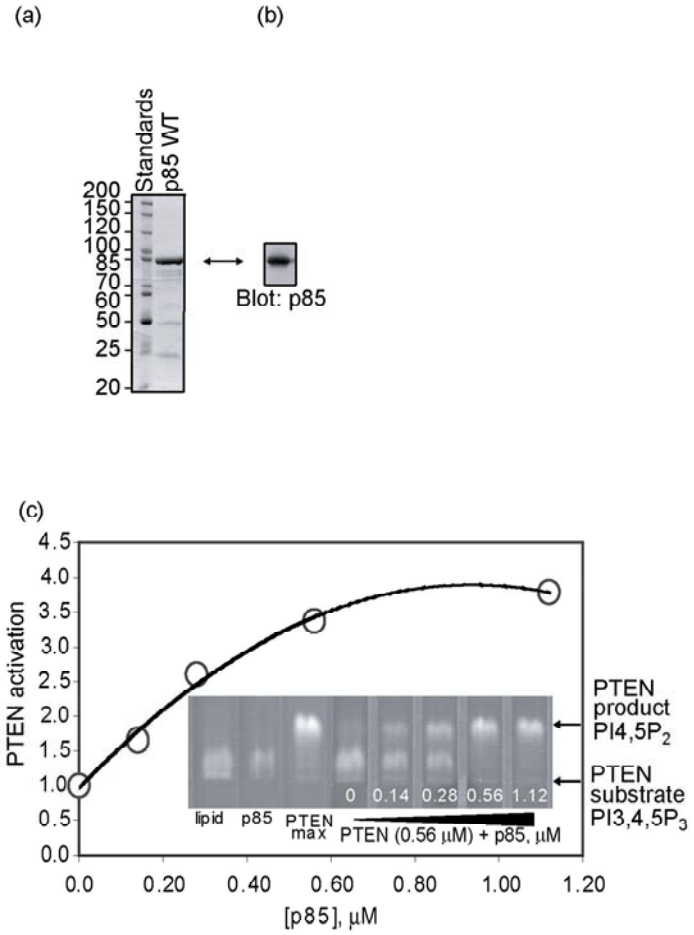


Figure 4.2: PTEN dephosphorylation of PI3,4,5P$_3$ is enhanced by the presence of p85 protein. His$_6$-tagged PTEN fusion proteins were purified as stated previously. The p85 protein was bacterially-expressed and purified as GST-p85. Affinity chromatography was used to capture the GST tag. The GST was cleaved from p85 using Thrombin protease. Proteins were resolved by SDS-PAGE stained with Coomassie blue (a) or transferred to nitrocellulose and probed with mouse anti p85-NSH2 antibody and visualized using chemiluminesence (b). (c) PTEN activity was assayed in the presence of increasing p85 concentrations (inset). As controls, lipid, p85 (1.12 µM) and a high concentration of PTEN (3.18 µM; PTEN max) were also assayed individually and confirmed that the p85 preparation did not possess PTEN activity in the absence of added PTEN (inset). Similar results were observed in a replicate experiment.

variety of plasma membrane lipids, we used purified His_6-PTEN and His_6-PTEN-C124S protein (Figure 4.1 a). We hypothesized that the phosphatase-dead His_6-PTEN-C124S mutant protein would be a substrate-trapping mutant and give a stronger, yet similar, binding profile to the wild type protein. Also, in order to perform future experiments imaging PTEN localization in living cells, the C124S mutation would be required since enzymatically-active PTEN is rapidly degraded following translocation to the plasma membrane (Das *et al.*, 2003).

The His_6-PTEN and His_6-PTEN-C124S proteins were used to probe lipid blots in a fashion similar to immunoblotting (Figure 4.3 a). After scanning using the Odyssey system, the supplied software was used to quantify the intensity of each spot. The mean and standard error of the mean across three independent replicates was used to generate a bar graph to compare the binding profile of His_6-PTEN to His_6-PTEN-C124S (Figure 4.3 b). A matched pairs t-test was used to determine the significance of difference. The results indicated that the PTEN-C124S mutation did bind better to PI3P; PI4P; PI5P; PI3,4P_2; PI3,5P_2; PI4,5P_2; PI3,4,5P_3 and PA. Both His_6-PTEN and His_6-PTEN-C124S proteins bound to phosphorylated PI lipids and PA but not to LPA, LPC, PE, PC, S1P and PS. We wanted to determine if PTEN-C124S was in fact a substrate-trapping mutant, expected to bind better than, but to the same lipid targets as, the wild type PTEN. To rule out that the C124S mutation resulted in a completely altered binding profile, the spot intensities in each set of replicates for each different protein were normalized to the PI4,5P_2 spot and plotted on a bar graph (Figure 4.3 c). Using a matched pairs t-test, it was determined that the PTEN-C124S binding profile was not significantly different than that of the wild type active PTEN ($p < 0.01$). The normalized data was used for the t-tests with the

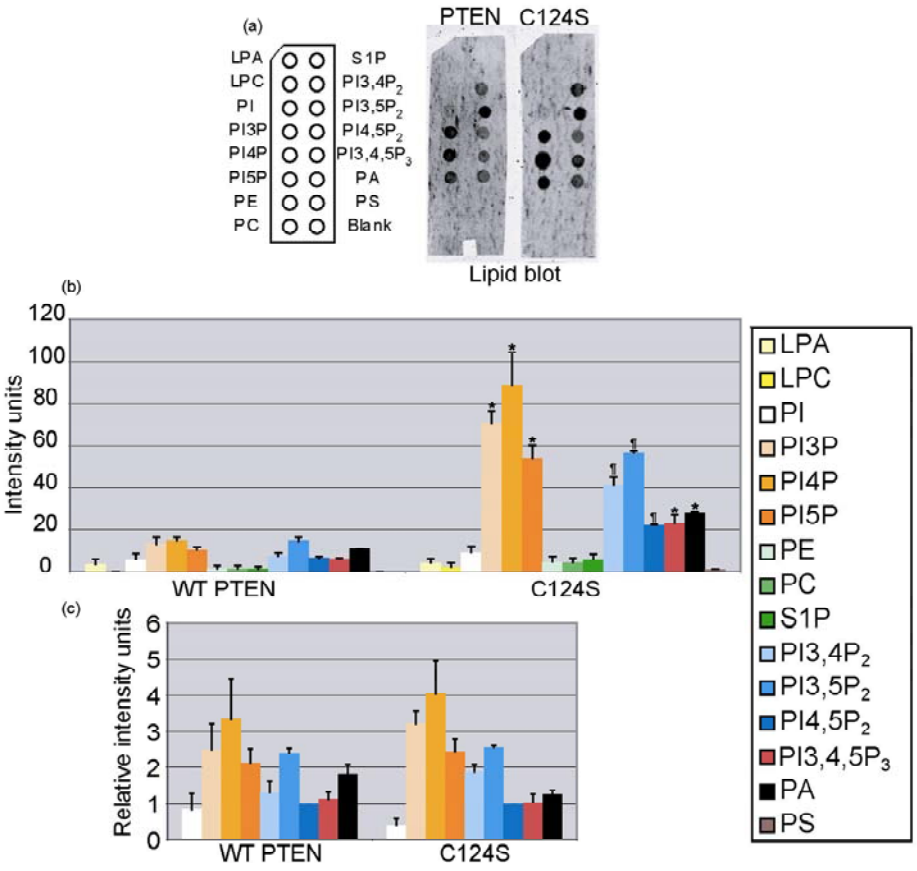


Figure 4.3: Wild type His_6-PTEN and the phosphatase dead His_6-PTEN-C124S both bind preferentially to phosphorylated PI lipids. His_6-PTEN and the enzymatic dead His_6-PTEN-C124S were bacterially expressed and purified as stated previously. (a) Nitrocellulose membranes spotted with the indicated lipids (100 pmol/spot) were blocked for 1 h using 3% fatty acid free BSA in TBST. The lipid blots were incubated with the indicated mutant PTEN proteins, washed and probed with mouse anti-PTEN(A2B1) primary antibody followed by goat anti-mouse secondary antibody conjugated to an infrared dye. The blots were visualized using the Odyssey imaging system and the intensity of the individual spots was quantified using the Odyssey software v2.1. (b) Bars represent the mean intensity of three independent replicates and error bars are the standard error of the mean. A matched pairs t-test was used to calculate significance of difference (* $p<0.05$; ¶ $p<0.01$). (c) Data was normalized to $PI4,5P_2$ separately for each blot and analyzed as in panel b. No significant differences in the lipid binding profile for wtPTEN and the C124S mutant were found ($p<0.01$). LPA = lysophosphatidic acid; LPC = lysophosphocholine; PI = phosphatidylinositol; PI3P = phosphatidylinositol 3-phosphate; PI4P = phosphatidylinostiol 4-phosphate; PI5P = phosphatidylinositol 5-phosphate; PE= phosphatidylethanolamine; PC = phosphatidylcholine; S1P = sphingosine 1-phosphate; $PI3,4P_2$ = phosphatidylinositol 3,4-bisphosphate; $PI3,5P_2$ = phosphatidylinositol 3,5-bisphosphate; $PI4,5P_2$ = phosphatidylinositol 4,5-bisphosphate; $PI3,4,5P_3$ = phosphatidylinositol 3,4,5-trisphosphate; PA = phosphatidic acid; PS = phosphatidylserine.

exception of the PI,45P_2 spot intensities. In this instance the raw data had to be used since it is not possible to compare two data sets with no variation. Subsequent experiments generating additional mutations in PTEN also contained the C124S mutation since this modification was required in subsequent experiments for stable PTEN expression in mammalian cells. These results confirm that the lipid binding specificities are not altered by the C124S mutation.

4.2.2 Bacterially-expressed His_6-PTEN-C124S mutants have altered binding to some membrane phosphatidylinositol phosphate lipids.

Given the current state of knowledge about PTEN phosphorylation by casein kinase-2 (Tolkacheva *et al*., 2001; Torres and Pulido, 2001; Vazquez *et al*., 2001; Vazquez *et al*., 2000) and the importance of the PASE and C2 domains in plasma membrane binding (Das et al., 2003) we strategically selected amino acids to be mutated to test the function of the proposed PTEN regulatory regions based on a published partial crystal structure of PTEN (Lee *et al*., 1999). Two types of mutations were made involving either phosphorylation sites or clusters of positively-charged basic amino acids. Phosphorylation sites were altered to mimic the phosphorylated state and dephosphorylated state by substitution of the regulatory phosphorylation sites with glutamate or alanine, respectively. Clusters of positive charge were removed by substituting basic amino acid residues in these clusters with alanine. Seven distinct His_6-PTEN-C124S mutants were generated (Table 4.1). Two regulatory casein kinase 2 phosphorylation site mutations were created to mimic the phosphorylated and dephosphorylated states. These distinct mutants involved substitution of basic residues in the C2 and PASE domains to disrupt positively charged surfaces. Finally, tyrosine 336 was altered to mimic both phosphorylated and non-phosphorylated states.

Table 4.1 Histidine tagged PTEN-C124S mutants

Mutant Name	Amino Acids Mutated
His_6-PTEN-C124S-STT/AAA	S380A/T382A/T383A
His_6-PTEN-C124S-STT/EEE	S380E/T382E/T383E
His_6-PTEN-C124S-PASE-RKK/AAA	R161A/K163A/K164A
His_6-PTEN-C124S-C2-K5/A	K260A/K263A/K266A/K267A/K269A
His_6-PTEN-C124S-C2-K3/A	K327A/K330A/K332A
His_6-PTEN-C124S-Y336A	Y336A
His_6-PTEN-C124S-Y336E	Y336E

The serine and threonine residues within the regulatory domain are S380, T382 and T383 and are phosphorylated by casein kinase 2. Site-directed mutagenesis was used to alter these phosphorylation sites to mimic either a constitutive dephosphorylated state, STT/AAA, or constitutive phosphorylated state, STT/EEE. The STT/AAA mutant has been shown to constitutively localize to the plasma membrane independent of growth factor treatment (Das *et al.*, 2003).

Clusters of basic amino acid residues in the PASE and C2 domains impart regions of positive charge, which are important for plasma membrane localization. The mutations made in the PASE and C2 domains were selected to neutralize these regions of positive charge to test their proposed role in binding to the plasma membrane *via* non-specific electrostatic interactions. One group of positive amino acid residues was located in the PASE domain. The C2 domain contains an N-terminal cluster of five basic amino acids and a C-terminal cluster of three basic amino acids. The PASE group of amino acid residues included R161, K163 and K164. The N-terminal cluster of positive amino acids mutated in the C2 domain were five lysines located at residues 260, 263, 266, 267 and 269 and the C-terminal lysines substituted by alanine were located at residues 327, 330 and 332. Furthermore, the partial crystal structure PTEN revealed that all eleven of these residues are located on the same surface, consistent with their suggested role in plasma membrane binding (Lee *et al.*, 1999). PTEN has 23 tyrosine residues located within consensus sequences for phosphorylation, leading to speculation that tyrosine phosphorylation may be an important modifier of PTEN behavior (Lu *et al.*, 2003). Difficulty arises when trying to study phosphorylation of a phosphatase such as PTEN because it is also a protein tyrosine phosphatase. Thus it may be capable of autodephosphorylation in the absence

of other proteins. Some studies have mutated PTEN tyrosine residues to characterize what, if any, regulatory effects a particular tyrosine may have in cells (Miller *et al.*, 2002). Of particular interest was the tyrosine at position 336, which is located within the clusters of basic residues in the C2 domain. Phosphorylation of this residue could have an important role in regulating PTEN plasma membrane localization. Mutations of the tyrosine at position 336 had been previously generated to change the residue to an alanine or a glutamate to mimic unphosphorylated or phosphorylated states, respectively.

The PTEN-C124S mutant proteins were expressed and purified under my supervision by S. Knafelc (Summer Student, 2006) (Figure 4.4 a-b). The His_6-PTEN C124S mutants were used to probe lipid blots as stated previously. The blots were scanned using the Odyssey imaging system (figure 4.4 c). The results indicated that the His_6-PTEN-C124S mutant proteins as well as the control His_6-PTEN-C124S protein do not bind to non-phosphatidylinositol phospholipids with significant intensity. This is same result obtained previously for the His_6-PTEN wt and His_6-PTEN-C124S proteins (Figure 4.2). Therefore, in the subsequent data analysis these values were not included. Phosphatidylinositol was included because it is the precursor molecule to all the phosphorylated PI molecules and phosphatidic acid was also included because some significant differences were observed (Figure 4.5).

The His_6-PTEN-C124S-STT/AAA protein had no significant differences in lipid binding preference when compared to the bacterially-expressed control His_6-PTEN-C124S. The His_6-PTEN-C124S-STT/EEE mutant demonstrated some slightly impaired binding to the PI, PI4P, PI3,4P_2, and PA lipids. His_6-PTEN-C124S-PASE-RKK/AAA had significantly impaired

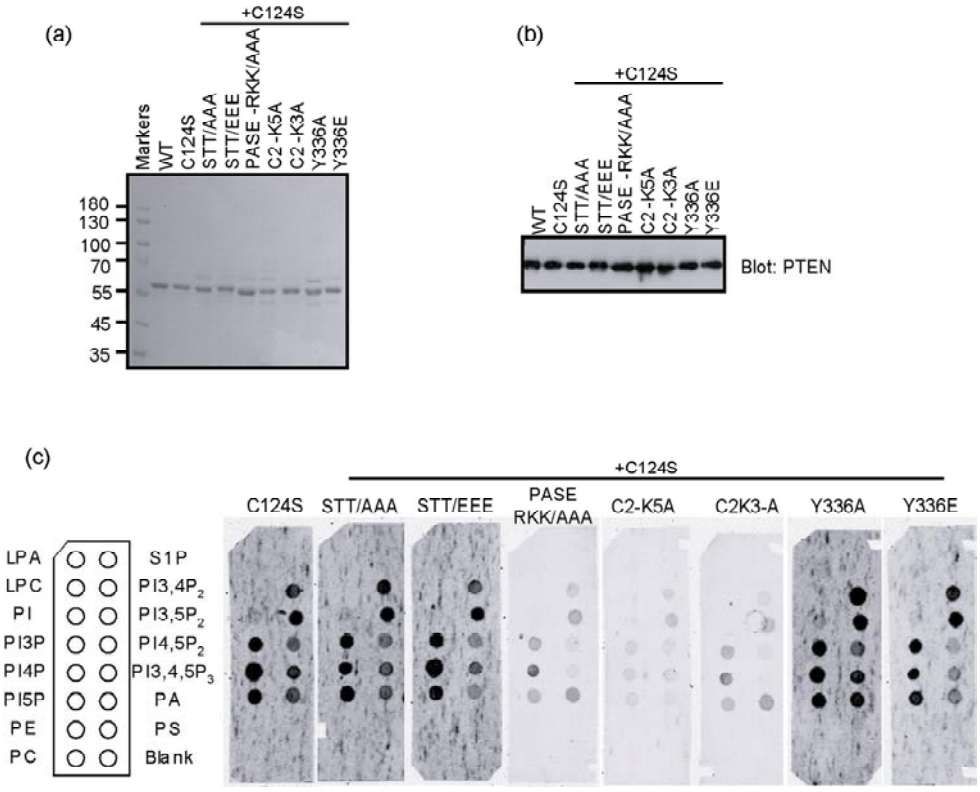


Figure 4.4: His_6-PTEN-C124S mutants have altered binding to specific membrane lipids. The data shown in panels a and b was generated by S. Knafelc under my supervision. (a) His_6-PTEN-C124S mutant proteins were bacterially-expressed and purified using Talon beads. The His_6-PTEN-C124S mutant proteins were resolved by SDS-PAGE (10%) and stained with Coomassie blue. (b) Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose blots were probed with a mouse anti-PTEN horseradish peroxidase-conjugated antibody and visualized by chemiluminescence. (c) Nitrocellulose membranes containing the indicated lipids (100 pmol/spot) were blocked and probed with the indicated His_6-PTEN-C124S mutants . Bound proteins were detected using mouse anti-PTEN antibodies, followed by rabbit anti-mouse antibodies conjugated with a infrared tag and were visualized simultaneously with the Odyssey imaging system (LI-COR IRDYE-680, n=4). LPA = lysophosphatidic acid; LPC = lysophosphocholine; PI = phosphatidylinositol; PI3P = phosphatidylinositol 3-phosphate; PI4P = phosphatidylinostiol 4-phosphate; PI5P = phosphatidylinositol 5-phosphate; PE= phosphatidylethanolamine; PC = phosphatidylcholine; S1P = sphingosine 1-phosphate; $PI3,4P_2$ = phosphatidylinositol 3,4-bisphosphate; $PI3,5P_2$ = phosphatidylinositol 3,5-bisphosphate; $PI4,5P_2$ = phosphatidylinositol 4,5-bisphosphate; $PI3,4,5P_3$ = phosphatidylinositol 3,4,5-trisphosphate; PA = phosphatidic acid; PS = phosphatidylserine.

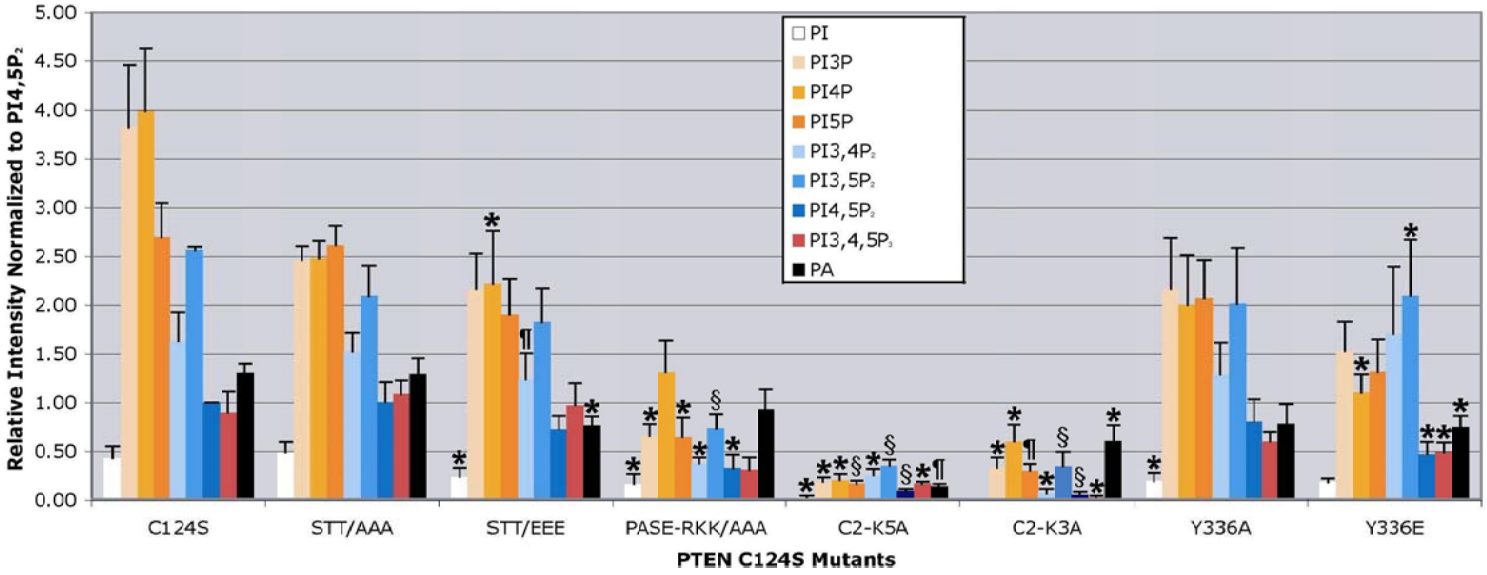


Figure 4.5: Quantification of lipid binding preferences of mutant His_6-PTEN-C124S proteins. Utilizing the Odyssey software v2.1, the intensity of the lipid membrane spots were quantified and normalized to the $PI4,5P_2$ spot of the His_6-PTEN-C124S blot. The mean of 4 independent replicates +/- the standard error of the mean was plotted on a bar graph. Matched pairs t-tests were performed to calculate the significance of binding difference between the His_6-PTEN-C124S-mutant proteins and the control His_6-PTEN-C124S protein (* $p<0.05$; ¶ $p<0.01$; § $p<0.001$).

binding to all lipids with the exception of PI4P and PA. Both C2 domain mutants, His_6-PTEN-C124S-C2-K5A and His_6-PTEN-C124S-C2-K3A, had significantly impaired binding to all lipids. His_6-PTEN-C124S-Y336A had little difference in binding except for slightly less binding to PI and PA. The His_6-PTEN-C124S-Y336E displayed significantly reduced binding to PI4P, $PI3,5P_2$, $PI4,5P_2$, $PI3,4,5P_3$ and PA. This suggests that a negative charge at this residue within the positively charged C2 domain surface interferes somewhat with PTEN binding to specific lipids.

4.3 PTEN cellular localization

4.3.1 PTEN cellular localization in response to epidermal growth factor stimulation

In order to assess PTEN subcellular localization, each PTEN mutant was expressed as an enhanced green fluorescent protein (eGFP) fusion protein. Since active PTEN is quickly degraded after translocation to the plasma membrane (Das *et al.*, 2003), it was necessary to use the phosphatase-dead C124S versions of these mutants in order to capture images using a scanning laser confocal microscope.

Although GFP-PTEN-C124S fusion proteins have been expressed and imaged in cells previously, no one had examined the effect of growth factor stimulation on PTEN localization. One previous report clearly demonstrated that removal of the casein kinase 2 phosphorylation sites in the regulatory domain (S380, T382 and T383) by mutation to alanine or deletion resulted in a GFP-PTEN-C124S mutant that was constitutively localized to the plasma membrane (Das *et al.*, 2003). Since we hypothesized that PTEN moves to the plasma membrane upon growth factor stimulation, the PTEN-C124S-STT/AAA mutant was an ideal control for a plasma

membrane localized protein. GFP-PTEN-C124S or GFP-PTEN-C124S-STT/AAA were visualized in the context of regular versus EGF stimulated conditions (Figure 4.6 a) The images generated confirmed that GFP-PTEN-C124S does appear to become less cytoplasmic and more concentrated at the plasma membrane upon growth factor stimulation (Figure 4.6 b).

4.3.2 Importance of Positive Charge for PTEN Subcellular Localization

Mutants had already been generated and bacterially-expressed, previously in the laboratory, as recombinant proteins for use in lipid binding assays. Some of the same mutants were expressed in a mammalian system using the pEGFP mammalian expression vector. Of particular interest were the PTEN-C124S mutants that had seriously altered binding to lipids as observed in the lipid binding experiments; these were: PTEN-C124S-PASE-RKK/AAA, PTEN-C124S-C2-K5A and PTEN-C124S-C2-K3A. PTEN-C124S-STT/EEE was also tested. Unfortunately, the GFP-PTEN-C124S-C2-K5A plasmid did not express well and no images were acquired. Similar experiments to the GFP-PTEN-C124S subcellular localization experiment in Figure 4.6 were performed. This set of experiments gave us an opportunity to test the mutants in the context of a mammalian system capable of performing posttranslational modifications and regulation, including phosphorylation events.

The mutants examined, C2-K3A, PASE RKK/AAA and STT/EEE, did not show definitive membrane localization upon growth factor stimulation (Figure 4.6 c-e). In all three instances, the experimental cell (growth factor treated) appeared similar in size, shape and GFP dispersion compared to the control cell (no growth factor). In no instance did the growth factor treated cells resemble the plasma membrane localization control GFP-PTEN-C124S-STT/AAA. These results

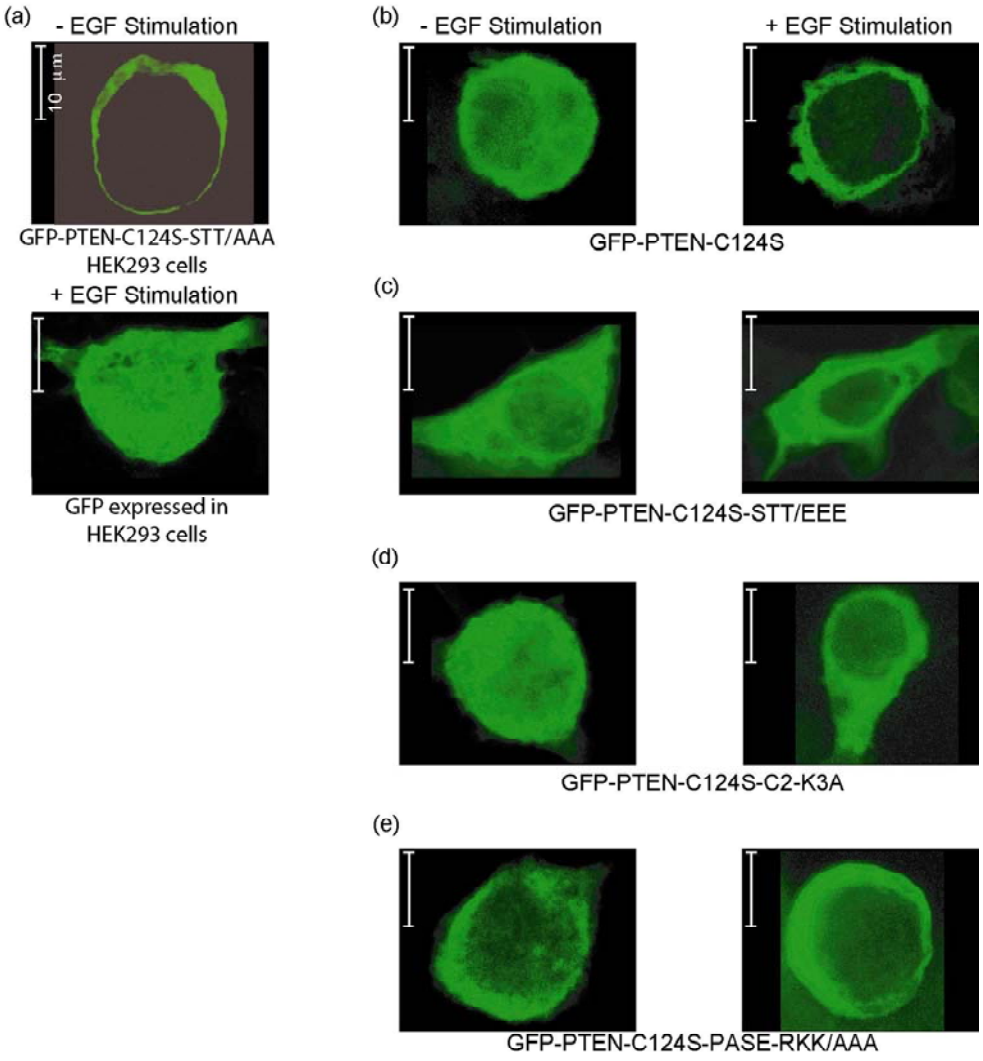


Figure 4.6: Confocal micrographs of GFP-PTEN-C124S mutant proteins. Plasmids encoding GFP-PTEN-C124S fusion proteins were transiently transfected into HEK293 cells seeded onto coverslips and allowed to grow for 24 h. Treatment with EGF (5 min) was performed as indicated and the cells were fixed and mounted to slides. Visualization was performed using confocal microscopy and sizing bars represent 10 microns. (a) GFP-PTEN-C124S-STT/AAA constitutively localizes to the plasma membrane and thus was a positive control for plasma membrane localization. (b-e) Plasmids encoding the indicated GFP-PTEN-C124S and GFP-PTEN-C124S mutants were treated as above and stimulated with growth factor as indicated. Scale bar = 10 µm.

suggested that the positive charge imparted by clusters of basic amino acids in the PASE and C2 domains were required for the plasma membrane localization of PTEN in response to EGF stimulation.

5.0 DISCUSSION

5.1 PTEN structure/function studies

5.1.1 Phosphorylation of PTEN

A complete understanding of PTEN regulation is still under investigation. Regulation of PTEN levels have been linked to ubiquitination by the ubiquitin ligase neural precursor cell expressed, developmentally downregulated-4-1 (NEDD4-1) (Wang *et al.*, 2007). NEDD4-1 polyubiquitinates PTEN, resulting in ubiquitin-mediated proteasomal degradation (Wang *et al.*, 2007). Acetylation of PTEN by the histone acetyltransferase p300/CBP-associated factor (PCAF), decreased PTEN lipid phosphatase activity (Okumura *et al.*, 2006). Acetylation and ubiquitination have not been implicated in the open/closed conformation of PTEN. Regulation *via* phosphorylation is the most relevant with regard to our previous studies of EGFR signaling, open/closed conformation model, and relevance to the pathogenesis of cancer (Das *et al.*, 2003; Torres and Pulido, 2001). Several phosphorylation sites have been identified within PTEN that contain serines, threonines or tyrosines (Miller *et al.*, 2002). Casein kinase 2 has been shown to phosphorylate PTEN at five specific sites in the regulatory domain in a hierarchical fashion, with the primary sites being Ser370 and Ser385 followed by Ser380, Thr382 and Thr383 (Torres and Pulido, 2001). It has been demonstrated that phosphorylation of Ser370 and Ser385 inhibits both the lipid phosphatase activity and caspase-dependent proteosomal degradation of PTEN (Miller

et al., 2002; Torres and Pulido, 2001). This finding suggests that PTEN is stabilized when it is inactivated since only active PTEN can be degraded. Investigation of the last three casein kinase 2 phosphorylation sites led to the discovery that these sites are important for the regulation of the plasma membrane localization of PTEN. Removal of the regulatory domain (aa 353-403) or even mutation of residues 380, 382 and 383 to alanine (PTEN-C124S-STT/AAA) resulted in constitutive plasma membrane localization (Das *et al.*, 2003). The same effect could not be observed with active PTEN because after translocation to the plasma membrane PTENwt is rapidly degraded (Das *et al.*, 2003). This data, along with the crystal structure of PTEN, helped develop the open/closed conformation model of PTEN regulation (Das *et al.*, 2003; Lee *et al.*, 1999).

It has been proposed that when phosphorylated in the regulatory domain, PTEN adopts a closed conformation. The regulatory domain is folded over and interacts with clusters of positive charges in the PASE and C2 domains. The closed conformation of PTEN is localized to the cytosol (Figure 5.1). PTEN adopts the open conformation in response to dephosphorylation of the casein kinase 2 sites in the regulatory domain. The PASE and C2 regions are unmasked as the interaction with the regulatory domain is disrupted.

We substituted the regulatory domain phosphorylation sites with alanine (PTEN-C124S-STT/AAA) or glutamic acid (PTEN-C124S-STT/EEE). We wanted to test if these mutations altered the preference for selected plasma membrane lipids. The plasma membrane lipids that were chosen included a variety of phosphorylated phosphatidylinositol lipids. Since the His_6-PTEN-C124S protein was bacterially-expressed and purified, it contained none of the post-translational modifications, such as phosphorylation, found in cells and had indistinguishable

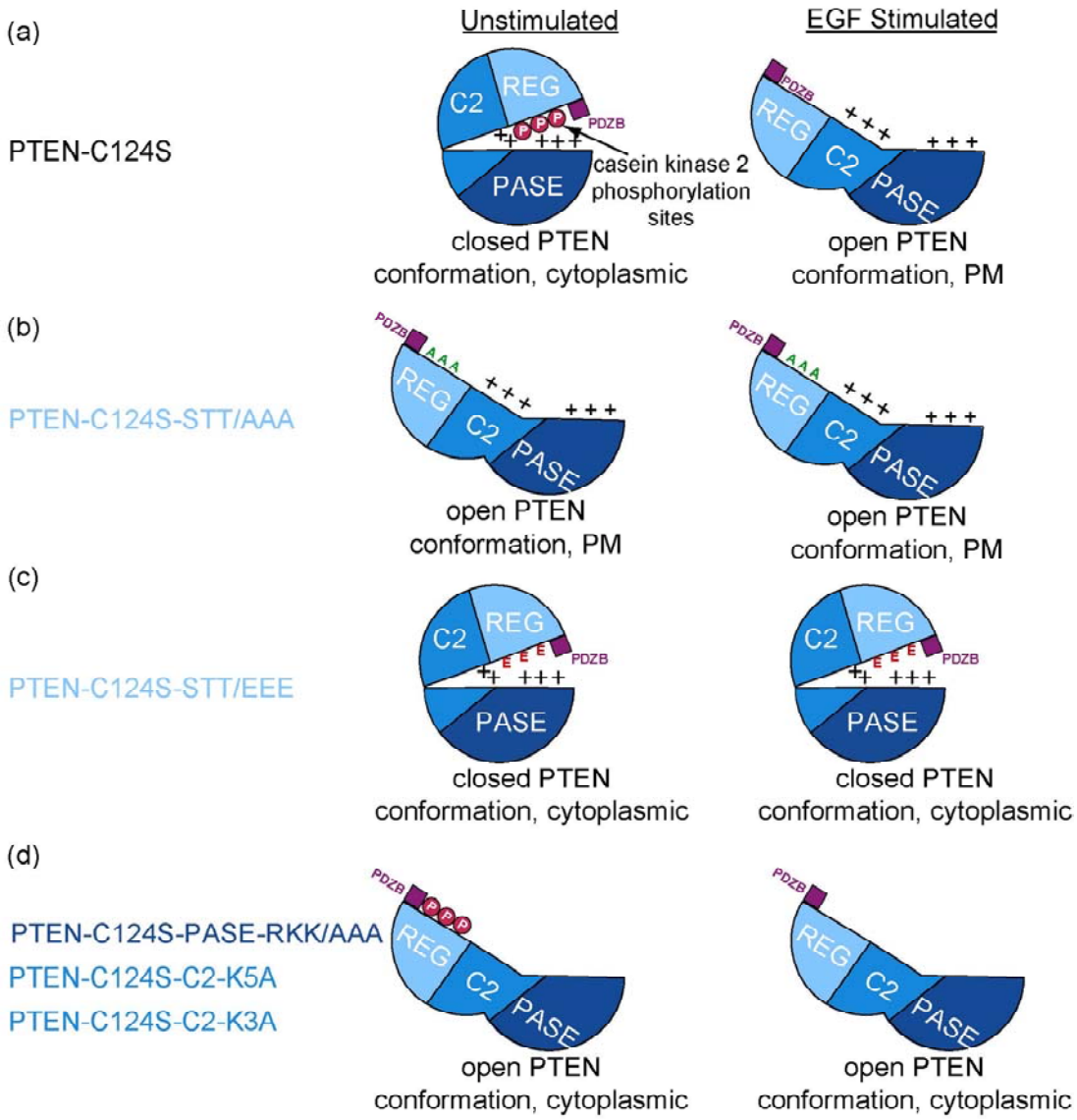


Figure 5.1: Depictions of the PTEN-C124S mutants in their respective open/closed conformation in unstimulated and EGF-stimulated conditions. (a) PTEN-C124S is closed and cytoplasmic in unstimulated cells. Upon EGFR activation PTEN adopts the open conformation. (b) PTEN-C124S-STT/AAA has the casein kinase 2 phosphorylation sites changed to alanine and is constitutively open and plasma membrane (PM) localized regardless of EGFR status. (c) PTEN-C124S-STT/EEE is a mimic of constitutive casein kinase 2 phosphorylation and always in the closed conformation and restricted to the cytoplasm. (d) Mutation of the clusters of positive charge in the PTEN-C124S-PASE-RKK/AAA, PTEN-C124S-C2-K5A, and PTEN-C124S-C2-K3A results in open conformation PTEN that does not localize to the plasma membrane, but is still phosphorylated by casein kinase 2.

lipid binding preference compared to the His_6-PTEN-C124S-STT/AAA mutant protein. The His_6-PTEN-C124S-STT/EEE mutant had a similar lipid binding profile to the His_6-PTEN-C124S protein. There were statistically significant decreases in lipid binding preference towards the PI, PI4P, and $PI3,4P_2$ lipids. Although there was a statistical difference between the His_6-PTEN-C124S-STT/EEE and His_6-PTEN-C124S proteins, the general characteristics of the data show a very similar profile. This suggests that in the context of bacterially-expressed recombinant proteins the casein kinase 2 phosphorylation sites do not greatly alter binding preference to the lipids tested.

We expressed the same mutants in HEK293 cells as GFP fusion proteins to assess the effects of the mutations in the context of growth factor stimulation. The GFP-PTEN-C124S-STT/AAA mutant had been previously established to constitutively localize to the plasma membrane and was used as a positive control for plasma membrane localization (Das *et al*., 2003). In quiescent cells, the GFP-PTEN-C124S protein was cytoplasmic and upon stimulation with EGF, it translocated to the plasma membrane. According to our hypothesis, the GFP-PTEN-C124S-STT/EEE mutant would remain in the cytoplasm in a closed conformation regardless of growth factor stimulation. The results observed were consistent with our hypothesis. The GFP-PTEN-C124S-STT/EEE mutant was cytoplasmic in both the unstimulated and EGF-stimulated cells. Previous evidence demonstrated that casein kinase 2 phosphorylation of PTEN serves to inhibit lipid phosphatase activity and stabilize the protein (Torres and Pulido, 2001). Our data suggests a second function for casein kinase 2 phosphorylation, that being to restrict PTEN to the cytoplasm. Preliminary phosphatase activity experiments performed *in vitro* with purified

protein generated data that has shown PTEN-STT/EEE retains phosphatase activity, suggesting that phosphorylation at these sites regulates PTEN plasma membrane localization *via* open/closed conformation and not intrinsic PTEN lipid phosphatase enzymatic activity (S. Knafelc, unpublished observations).

Upon examination of the partial crystal structure of PTEN, we also identified a tyrosine residue within the C2 domain. The tyrosine is located at position 336 and its orientation in the three dimensional structure placed it on the same surface of the protein as several clusters of positively charged amino acids. Using site-directed mutagenesis, we generated a dephosphorylation and phosphorylation mimic by mutating Tyr336 to Ala and Glu, respectively. The two mutants generated, His_6-PTEN-C124S-Y336A and His_6-PTEN-C124S-Y336E, were tested for lipid binding preferences and compared to the His_6-PTEN-C124S control protein as described earlier. The Y336A mutant displayed some slightly decreased preference for binding to the PI lipid, but otherwise exhibited no significant difference from the control. The His_6-PTEN-C124S-Y336E mutant had statistically significant decreased binding to the PI3P, PI4P, PI4,5P_2, PI3,4,5P_3 and PA lipids, suggesting that the extra negative charge imparted by the glutamate did interfere with lipid binding. Note that the Y336E mutation decreased binding to both the substrate (PI3,4,5P_3) and product (PI4,5P_2) of PTEN, suggesting that if this site were phosphorylated it could attenuate PTEN function. These data suggest that Y336 has the potential to be a regulatory site for PTEN since if it was phosphorylated, PTEN lipid binding would be impaired. Since an actual phosphate group at this position imparts a greater negative charge than a glutamate, the decreased binding may be even more marked. It is tempting to speculate that this is the mechanism by which PTEN is disengaged from the plasma membrane (Figure 5.2).

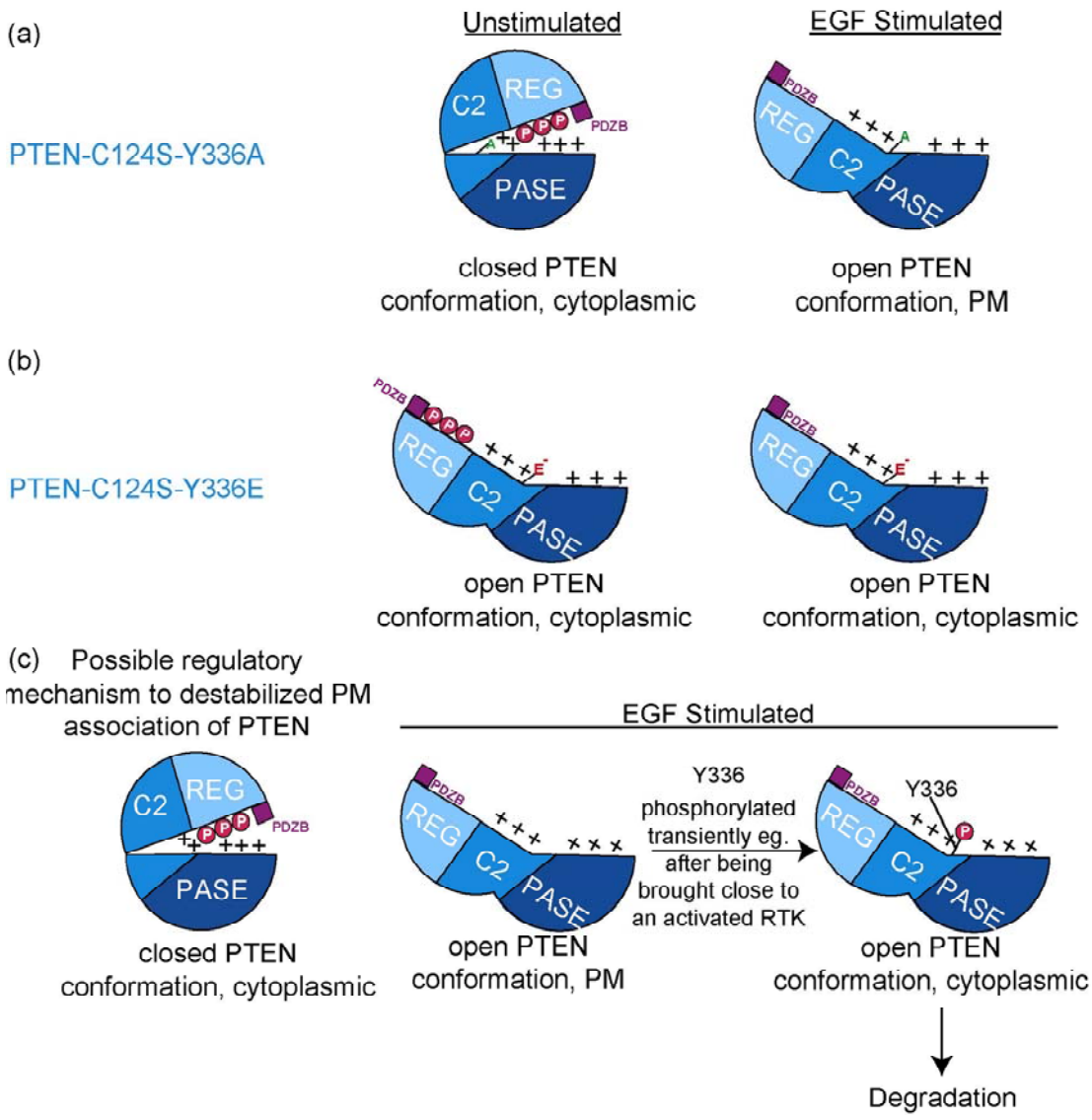


Figure 5.2: Proposed models of PTEN-C124S-Y336 mutants' open/closed conformation in unstimulated and EGF stimulated conditions. (a) PTEN-C124S-Y336A is closed and cytoplasmic in unstimulated cells. Upon EGFR activation PTEN adopts the open conformation and can translocate to the plasma membrane (PM). (b) PTEN-C124S-Y336E is open, but cytoplasmic in both unstimulated and stimulated cells. (c) Our proposed regulatory feature for Y336 is that it may be phosphorylated by an RTK, thus disengaging PTEN from the PM.

However, whether Y336 is phosphorylated in cells has yet to be determined.

5.1.2 The role of basic amino acid residues in plasma membrane targeting

The model of PTEN open and closed conformations proposes that the regulatory domain, when phosphorylated, is involved in masking positively charged surfaces in the PASE and C2 domains. Upon dephosphorylation, PTEN adopts the open conformation and is capable of an electrostatic interaction with the plasma membrane. C2 domain-containing proteins are typically involved in membrane binding, however the C2 domain of PTEN has been shown to be insufficient to result in plasma membrane localization (Das *et al*., 2003). A cluster of basic amino acids in the PASE domain (R161/K163/R164) were previously tested for involvement in plasma membrane targeting (Das *et al*., 2003). It was observed that when the positive charge of this cluster was neutralized (PASE-RKK/AAA), the affinity for anionic lipid vesicles was greatly reduced. In addition, a PTEN-PASE-RKK/AAA mutant that also lacked the regulatory domain was localized to the cytoplasm of HEK293 cells, consistent with the proposed role of the basic residues in plasma membrane association (Das *et al*., 2003). From the partial crystal structure of PTEN, there are two other clusters of positive charge located in the C2 domain on the same surface as the PASE domain cluster (Lee *et al*., 1999). Lee and colleagues (1999) mutated a series of residues in the C2 domain. The residues mutated were 263-K-M-L-K-K-D-K-269 to 263-A-A-G-A-A-D-A-269 and 327-K-A-N-K-D-K-A-N-335 to 327-A-A-G-A-D-A-A-N-A-335. They observed decreased affinity for PC lipid vesicles with these two mutants. When expressed in cells, these same mutants also displayed a phenotype similar to cells derived from PTEN mutant L345G tumors (Lee *et al*., 1999). The tumor phenotype was characterized by increased

proliferation and decreased contact inhibition compared to wild type PTEN-expressing cells (Lee *et al.*, 1999). Since PTEN had been previously tested for binding to plasma membrane mimicking anionic lipid vesicles POPC and POPS, we wanted to test the PTEN-C124S-PASE-RKK/AAA mutant as well as the PTEN-C124S-C2-K5A and PTEN-C124S-C2-K3A mutants for their ability to bind a broader range of membrane lipids including phosphatidylinositol lipids. The His_6-PTEN-C124S-PASE-RKK/AAA, His_6-PTEN-C124S-C2-K5A and His_6-PTEN-C124S-C2-K3A mutants were compared to the His_6-PTEN-C124S control protein using the lipid blots. All three mutants showed severely impaired lipid binding ability compared to His_6-PTEN-C124S. The PTEN-PASE-RKK mutant had previously been tested for lipid phosphatase activity and was found to retain activity (Das *et al.*, 2003). Preliminary data have demonstrated that the His_6-PTEN-C2-K5A and His_6-PTEN-C2-K3A mutants do not have lipid phosphatase activity (S. Knafelc, unpublished observations). This further illustrated that the PASE and C2 domains are both important for functional lipid phosphatase activity and lipid binding. Although the mutants did not display lipid phosphatase activity, it is important to note that they were expressed at similar levels to the other PTEN proteins in bacteria and were soluble. This suggests that the decreased lipid binding were not a result of poorly folded protein. Further investigation of the mutant proteins could be obtained by using circular dichroism measurements and/or limited trypsin digestion to verify they are properly folded proteins.

The three mutants were also expressed as GFP-fusion proteins in HEK293 cells and their localization within the cell was visualized before and after treatment with EGF using confocal microscopy. The ability of the GFP-PTEN-C124S-PASE-RKK/AAA and the GFP-PTEN-C124S-C2-K3A mutants to localize to the plasma membrane in response to EGF stimulation was

impaired. This result is consistent with the model that the clusters of positive charge in these domains are required for lipid binding and targeting to the plasma membrane.

The GFP-PTEN-C124S-C2-K5A mutant was poorly expressed in the HEK293 cells. This mutant may be quickly degraded in cells. The reason for this could be that the regulatory casein kinase 2 sites are still present even though a significant amount of positively charged residues have been substituted for alanine. When the casein kinase 2 sites are phosphorylated, these three phosphate groups likely interact with the positive charge cluster in the C2 domain. Since the PTEN-C124S-C2-K5A mutant lacks this positive charge cluster, there may be insufficient electrostatic forces to facilitate an intramolecular interaction between the C2 domain and the regulatory domain. Thus, the protein may not adopt the closed conformation. PTEN that is phosphorylated at the casein kinase 2 sites and in an open conformation in the cytoplasm, may be unstable. Another possibility for the poor expression of GFP-PTEN-C124S-C2-K5A in cells, is that there are simply too many mutations to express a properly folded protein. Although the PTEN-C124S-C2-K5A was expressed and purified as a soluble recombinant protein in bacteria, posttranslational modifications that occur in a mammalian cell system may affect protein stability.

The open/closed conformation model for PTEN proposes that PTEN in the open conformation localizes to the plasma membrane. The PASE domain mutations as well as the C2-K3A mutations resulted in mutant proteins that failed to localize to the plasma membrane upon growth factor stimulation. Thus the data generated are consistent with the current model.

5.2 PTEN and p85

PTEN was originally cloned in 1997 and over the past decade a large body of evidence has been compiled linking the PTEN phosphatase with tumor suppression. Although PTEN is a dual specificity phosphatase, possessing both protein phosphatase and 3' phosphoinositide phosphatase activity, it is only the latter function that has been linked to tumor suppression (Wu *et al.*, 2003). PTEN attenuates the PI3K/Akt signaling pathway, which is activated in response to growth factor activation. When a growth factor, such as EGF, binds to its receptor, autophosphorylation of tyrosine residues creates docking sites for SH2 domain-containing proteins. The regulatory subunit of PI3K, p85, contains two SH2 domains that allow it to recruit the catalytic subunit, p110, to the plasma membrane in response to EGFR activation. PI3K catalyzes the phosphorylation of $PI4,5P_2$ to $PI3,4,5P_3$. The lipid second messenger $PI3,4,5P_3$ interacts with the PH domains of Akt and PDK1, promoting the activation of Akt by PDK1 *via* phosphorylation. PTEN antagonizes PI3K/Akt signaling by removal of the 3' phosphate from the second messenger $PI3,4,5P_3$. A decrease in the cellular concentration of $PI3,4,5P_3$ results in less Akt and PDK1 at the plasma membrane. This allows cells to undergo apoptosis or exit the cell cycle.

PTEN and p85:p110 are known to carry out opposing functions in RTK signaling. The p85 protein is involved in the activation of the PI3K/Akt pathway whereas the PTEN protein deactivates this pathway. Experimental evidence now suggests a more integrated regulation of p110-PI3K and PTEN. The two enzymes and their opposing forces on opposite sides of a

molecular switch are connected by the p85 protein. Our laboratory previously identified that p85 and PTEN interact and that p85 enhances PTEN enzymatic activity *in vitro* (Chagpar, 2004). Another laboratory observed that PTEN and p85 can be isolated from tissues with the scaffolding protein MAGI-2 in a multi-protein complex termed the PTEN-associated complex (PAC) (Wu *et al.*, 2000a). This suggested that p85 and PTEN may have an interconnected function in cells. Increased epithelial transformation has been frequently observed in PTEN (+/-) mice (Di Cristofano *et al.*, 1998; Podsypanina *et al.*, 1999; Suzuki *et al.*, 1998). PTEN (+/-) mice that also have half the normal expression of *Pik3r1* (p85α, p55α, p50α) displayed increased intestinal polyp incidence (~2-fold) and number (>3-fold) compared with PTEN (+/-) mice. This suggests that p85 has a role in tumor suppression, at least under conditions of reduced PTEN expression (Luo *et al.*, 2005).

Based on the evidence generated by Ryaz Chagpar in our laboratory, combined with the collective knowledge of the literature, we decided to directly investigate the nature of the PTEN and p85 interaction. Others in the laboratory performed GST pulldown experiments and demonstrated that GST-PTEN-C124S binds to p85. Using lysates containing espressed p85 domains, D. Anderson observed that a region within the BH domain of p85 facilitates binding to PTEN. As described in section 4.1, a PTEN lipid phosphatase assay was modified to assess the activation of PTEN by p85. The p85 protein was added in increasing concentration and the maximum activation observed was at a 1:1 molar ratio of p85:PTEN. A 3.5-fold activation was observed at this molar ratio. The p85 protein was also assayed independently of PTEN as a control to demonstrate that p85 alone does not alter the lipid substrate.

PTEN binds the MAGI-2 protein at the PDZ-2 domain (Tolkacheva *et al.*, 2001). This interaction has been demonstrated to be biologically relevant as expression of MAGI-2 enhances PTEN signaling, but has no effect in the presence of a PTEN mutant unable to bind MAGI-2 (Wu *et al.*, 2000a). The nature of p85 involvement with MAGI-2 is not yet characterized. However, MAGI-2 contains two WW domains, which bind proline-rich regions (Wu *et al.*, 2000b). The p85 protein has two regions on either side of the BH domain that are rich in proline residues. We hypothesize that p85 interacts with the two WW domains of MAGI-2 *via* these proline sequences (Figure 5.3). MAGI-2 and p85 are both cytoplasmic proteins that translocate to the plasma membrane in response to activated RTKs, such as the EGFR (Klippel *et al.*, 1993; Xu *et al.*, 2001). The PTEN-associated complex (PAC) has been isolated at the plasma membrane (Vazquez *et al.*, 2001). The current model of p85/PTEN interaction in cells is that MAGI-2, p85 and PTEN are recruited to the plasma membrane independently of each other upon RTK activation. Once at the plasma membrane, PTEN, p85 and possibly other proteins, such as RTKs, bind to the scaffolding protein MAGI-2. We hypothesize that MAGI-2 binding serves two key functions. First, it facilitates enhancement of PTEN activity by p85. Second, it targets PTEN to the activated EGFR where its substrate is being produced by PI3K. We hypothesize that an interaction occurs between a PDZ binding domain within MAGI-2 (a component of the PAC) and a PDZ binding motif at the C-terminus of the EGFR. This would localize PTEN to the site where PI3K is actively generating PI3,4,5P_3 second messengers. The data in this thesis using the Y336E mutant of PTEN further suggests a possible mechanism by which PTEN is disengaged from the plasma membrane. Phosphorylation of PTEN Y336, by an RTK, may

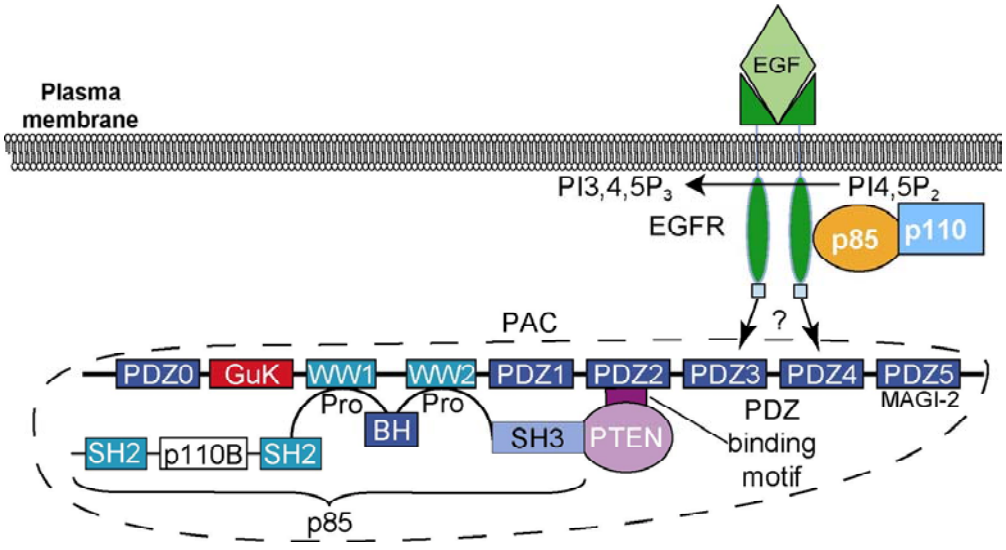


Figure 5.3: Proposed model for the interaction between the PTEN, p85 and MAGI-2 proteins in the PTEN-associated complex. A PTEN-associated complex (PAC) has been isolated from tissue extracts containing PTEN, p85 and MAGI-2. MAGI-2 is a scaffolding protein that is cytosolic in quiescent cells, but translocates to the plasma membrane upon growth factor stimulation. MAGI-2 has 6 PDZ domains. PTEN is known to bind to the PDZ2 domain of MAGI-2. It is possible that p85 binds to one or both WW domains *via* proline-rich regions. MAGI-2 may interact with a PDZ binding motif of the activated receptor. MAGI-2 also has a guanylate kinase-like (Guk) domain that is catalytically inactive.

release PTEN from the plasma membrane once PI3K signaling has been terminated.

5.3 Future directions

The next step in the analysis of PTEN is to examine if phosphorylation at position Y336 is biologically relevant. Tandem mass spectrometry could identify if Y336 is a phosphorylation site on PTEN and if it is phosphorylated in the presence or absence of EGF. Antibodies specific for PTEN phosphorylated on Y336 could be used to probe whole cell lysates generated at various time points during EGF stimulation. This would determine if the cellular concentration of PTEN-Y336-phosphorylated changes over the course of EGF stimulation. Confocal microscopy experiments using the PTEN mutants GFP-PTEN-C124S-Y336A and GFP-PTEN-C124S-Y336E in cells in the context of growth factor stimulation would be performed to examine if there is altered plasma membrane localization upon growth factor stimulation. These experiments may provide some insight into Y336 as a possible regulatory site for plasma membrane delocalization if the Y336E mutation impairs membrane localization upon growth factor stimulation. Since confocal microscopy generates qualitative data, some quantitative results to corroborate the images are required. The PTEN-C124S-Y336A and PTEN-C124S-Y336E mutants can be bacterially-expressed as recombinant proteins and be tested for their affinity to select membrane phosphatidylinositol phosphate lipids using a surface plasmon resonance (Biacore) system. These experiments will provide direct measurements of the affinity of these mutants for selected membrane lipids. Further testing of the Y336 mutants will involve phosphatase active versions (PTEN-Y336A and PTEN-Y336E) of the mutants that can be expressed in bacteria, purified and assayed for lipid phosphatase activity. This experiment compares the activity of the unphosphorylated and phosphorylated mimics to examine if the

Y336 residue has a role in the regulation of intrinsic lipid phosphatase activity. In order to examine the effects of the Y336 mutants that mimic constitutive unphosphorylated and phosphorylated states in cells, these PTEN mutants will be overexpressed, whole cell lysates generated, and immunoblot analysis implemented to compare phosphorylated Akt levels. The PTEN-Y336A mutant may result in lower phosphorylated Akt levels due to increased PTEN signaling relative to the PTEN-Y336E mutant.

It has been established that PTEN binds to p85 as well as MAGI-2. To map the region of PTEN that binds to p85, regions of PTEN expressed as GST fusion proteins can be used in GST pulldown experiments with whole cell lysates as a source of p85 protein. This information can be used to generate a PTEN mutant incapable of binding to p85 (PTENΔp85). PTEN interacts with the PDZ2 domain of MAGI-2 *via* a PDZ binding motif. Two additional PTEN mutants can also be generated that are incapable of binding MAGI-2 (i.e. PTEN without its PDZ binding motif - PTENΔMAGI-2) and a double mutant that does not interact with p85 and MAGI-2 (PTENΔp85-MAGI-2). These three PTEN mutants can be assayed *in vitro* for lipid phosphatase activity to confirm that they are properly folded, functional proteins. Each can also be expressed as GFP fusion proteins and their ability to translocate to the plasma membrane upon growth factor stimulation can be assessed in HEK293 cells. This experiment will test the current model that PTEN non-specifically localizes to the plasma membrane upon receptor activation independently of MAGI-2 and/or p85. Each mutant may also be expressed individually and phosphorylated Akt levels can be compared quite accurately using the Odyssey imaging system for quantitative western blot analyses. If our model is correct, then the levels of phosphorylated Akt will be lower for PTENΔp85, PTENΔMAGI-2, and PTENΔp85-MAGI-2 versus PTENwt.

Some comparisons may also be drawn between phosphorylated Akt levels among the three mutants as well.

PI3K is recruited to activated receptors upon growth factor stimulation by the SH2 domains of p85, where the p110 subunit generates the $PI3,4,5P_3$ product. Our model proposes that MAGI-2 targets PTEN to this $PI3,4,5P_3$ substrate by simultaneously interacting with PDZ binding motifs on PTEN (known) as well as the receptor. In order to test this model in cells, the MAGI-2 protein can be expressed as a red fluorescent protein (RFP) fusion protein, and using fluorescent dye conjugated anti-EGFR antibodies, we can assess whether MAGI-2 and EGFR are co-localized in EGF-stimulated cells. Furthermore, we can express both RFP-MAGI-2 and GFP-PTEN-C124S to examine if the two proteins are co-localized in quiescent cells as well as in response to growth factor stimulation. Since fluorescence microscopy is not sufficient to make convincing arguments about the interaction of PTEN and MAGI-2 in cells, co-immunoprecipitations can be performed with the wild type proteins as well as with non-binding mutants to complement the microscopy data.

5.4 Conclusion

Mapping of homozygous deletions on human chromosome 10q23 led to the isolation of the PTEN gene in 1997 as a candidate tumor suppressor (Steck *et al.*, 1997). Since its discovery, it has been identified as an important tumor suppressor and its characteristics and biological function have been well-studied. The lipid phosphatase activity of PTEN has been established as the key attenuator of the PI3K/Akt pathway.

Although the function of PTEN has been known for some time now, insight into regulation of this important protein is only now emerging. Our laboratory, through the study of the Ras/Raf/MAPK pathway and the role of p85, discovered that PTEN enzymatic activity is enhanced by p85. Although the work in this thesis is not comprehensive, the characterization of PTEN activity in the presence of p85 has given further insight into p85 being the central regulator of the PI3K/Akt pathway through its dual regulation of both the p110 lipid kinase and the corresponding PTEN lipid phosphatase.

Previous confocal microscopy work involving GFP-PTEN-C124S fusion proteins inspired us to assess PTEN subcellular localization in the context of EGF stimulation as well as perform some structure/function analyses of PTEN. The previous work combined with our data is helping to build a strong case for the model of PTEN involving the casein kinase 2 phosphorylation sites regulating open and closed conformations of PTEN. The data we generated suggest that clusters of positive charge in the PASE and C2 domains are important for lipid binding, especially to phosphorylated phosphatidylinositol lipids, as well as for plasma membrane targeting in response to growth factor stimulation.

Although the work demonstrated in this thesis does not comprehensively elucidate the regulation of PTEN or fully explain the structural nature of the protein, the data presented do provide some insight into these two aspects of PTEN. Greater understanding of all aspects of PTEN is critical for the development of therapeutic interventions that will benefit patients with cancer.

6.0 REFERENCES

Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. Embo J *15*, 6541-6551.

Anderson, K. E., Coadwell, J., Stephens, L. R., and Hawkins, P. T. (1998). Translocation of PDK-1 to the plasma membrane is important in allowing PDK-1 to activate protein kinase B. Curr Biol *8*, 684-691.

Baker, S. J. (2007). PTEN enters the nuclear age. Cell *128*, 25-28.

Barford, D. (1996). Molecular mechanisms of the protein serine/threonine phosphatases. Trends Biochem Sci *21*, 407-412.

Bates, S., and Vousden, K. H. (1999). Mechanisms of p53-mediated apoptosis. Cell Mol Life Sci *55*, 28-37.

Cantley, L. C., and Neel, B. G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci U S A *96*, 4240-4245.

Carpenter, C. L., Auger, K. R., Chanudhuri, M., Yoakim, M., Schaffhausen, B., Shoelson, S., and Cantley, L. C. (1993). Phosphoinositide 3-kinase is activated by phosphopeptides that bind to the SH2 domains of the 85-kDa subunit. J Biol Chem *268*, 9478-9483.

Chagpar, R. B. (2004) Role of A-Raf and p85 in regulation of the PTEN/PI3K Signaling Axis, M. Sc Thesis, University of Saskatchewan, Saskatoon.

Chen, J., Marechal, V., and Levine, A. J. (1993). Mapping of the p53 and mdm-2 interaction domains. Mol Cell Biol *13*, 4107-4114.

Cho, S. G., and Choi, E. J. (2002). Apoptotic signaling pathways: caspases and stress-activated protein kinases. J Biochem Mol Biol *35*, 24-27.

Cohen, P., Alessi, D. R., and Cross, D. A. (1997). PDK1, one of the missing links in insulin signal transduction? FEBS Lett *410*, 3-10.

Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785-789.

Cuevas, B., Lu, Y., Watt, S., Kumar, R., Zhang, J., Siminovitch, K. A., and Mills, G. B. (1999). SHP-1 regulates Lck-induced phosphatidylinositol 3-kinase phosphorylation and activity. J Biol Chem *274*, 27583-27589.

Cuevas, B. D., Lu, Y., Mao, M., Zhang, J., LaPushin, R., Siminovitch, K., and Mills, G. B. (2001). Tyrosine phosphorylation of p85 relieves its inhibitory activity on phosphatidylinositol 3-kinase. J Biol Chem *276*, 27455-27461.

Das, S., Dixon, J. E., and Cho, W. (2003). Membrane-binding and activation mechanism of PTEN. Proc Natl Acad Sci U S A *100*, 7491-7496.

Datta, S. R., Brunet, A., and Greenberg, M. E. (1999). Cellular survival: a play in three Akts. Genes Dev *13*, 2905-2927.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.

del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science *278*, 687-689.

Dhand, R., Hiles, I., Panayotou, G., Roche, S., Fry, M. J., Gout, I., Totty, N. F., Truong, O., Vicendo, P., Yonezawa, K., and et al. (1994). PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. Embo J *13*, 522-533.

Di Cristofano, A., and Pandolfi, P. P. (2000). The multiple roles of PTEN in tumor suppression. Cell *100*, 387-390.

Di Cristofano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P. P. (1998). Pten is essential for embryonic development and tumour suppression. Nat Genet *19*, 348-355.

Donepudi, M., and Grutter, M. G. (2002). Structure and zymogen activation of caspases. Biophys Chem *101-102*, 145-153.

Elbing, K. a. B., Roger (2002). E. coli, Plasmids, and Bacteriophages. In Current Protocols in Molecular Biology (John Wiley & Sons Inc.), pp. 1-1.3.6.

Foukas, L. C., Beeton, C. A., Jensen, J., Phillips, W. A., and Shepherd, P. R. (2004). Regulation of phosphoinositide 3-kinase by its intrinsic serine kinase activity in vivo. Mol Cell Biol *24*, 966-975.

Freedman, D. A., Wu, L., and Levine, A. J. (1999). Functions of the MDM2 oncoprotein. Cell Mol Life Sci *55*, 96-107.

Freeman, D. J., Li, A. G., Wei, G., Li, H. H., Kertesz, N., Lesche, R., Whale, A. D., Martinez-Diaz, H., Rozengurt, N., Cardiff, R. D., *et al.* (2003). PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. Cancer Cell *3*, 117-130.

Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998). Phosphoinositide kinases. Annu Rev Biochem *67*, 481-507.

Funaki, M., Katagiri, H., Inukai, K., Kikuchi, M., and Asano, T. (2000). Structure and function of phosphatidylinositol-3,4 kinase. Cell Signal *12*, 135-142.

Furnari, F. B., Huang, H. J., and Cavenee, W. K. (1998). The phosphoinositol phosphatase activity of PTEN mediates a serum-sensitive G1 growth arrest in glioma cells. Cancer Res *58*, 5002-5008.

Georgescu, M. M., Kirsch, K. H., Akagi, T., Shishido, T., and Hanafusa, H. (1999). The tumor-suppressor activity of PTEN is regulated by its carboxyl-terminal region. Proc Natl Acad Sci U S A *96*, 10182-10187.

Gil, A., Andres-Pons, A., Fernandez, E., Valiente, M., Torres, J., Cervera, J., and Pulido, R. (2006). Nuclear localization of PTEN by a Ran-dependent mechanism enhances apoptosis: Involvement of an N-terminal nuclear localization domain and multiple nuclear exclusion motifs. Mol Biol Cell *17*, 4002-4013.

Goberdhan, D. C., and Wilson, C. (2003). PTEN: tumour suppressor, multifunctional growth regulator and more. Hum Mol Genet *12 Spec No 2*, R239-248.

Gu, W., and Roeder, R. G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell *90*, 595-606.

Hoeflich, K. P., Luo, J., Rubie, E. A., Tsao, M. S., Jin, O., and Woodgett, J. R. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature *406*, 86-90.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett *420*, 25-27.

Hresko, R. C., and Mueckler, M. (2005). mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. J Biol Chem *280*, 40406-40416.

Kaech, S. M., Whitfield, C. W., and Kim, S. K. (1998). The LIN-2/LIN-7/LIN-10 complex mediates basolateral membrane localization of the C. elegans EGF receptor LET-23 in vulval epithelial cells. Cell *94*, 761-771.

Kikani, C. K., Dong, L. Q., and Liu, F. (2005). "New"-clear functions of PDK1: beyond a master kinase in the cytosol? J Cell Biochem *96*, 1157-1162.

Klippel, A., Escobedo, J. A., Hirano, M., and Williams, L. T. (1994). The interaction of small domains between the subunits of phosphatidylinositol 3-kinase determines enzyme activity. Mol Cell Biol *14*, 2675-2685.

Klippel, A., Escobedo, J. A., Hu, Q., and Williams, L. T. (1993). A region of the 85-kilodalton (kDa) subunit of phosphatidylinositol 3-kinase binds the 110-kDa catalytic subunit in vivo. Mol Cell Biol *13*, 5560-5566.

Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Kubbutat, M. H., Ludwig, R. L., Levine, A. J., and Vousden, K. H. (1999). Analysis of the degradation function of Mdm2. Cell Growth Differ *10*, 87-92.

Lee, J. O., Yang, H., Georgescu, M. M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J. E., Pandolfi, P., and Pavletich, N. P. (1999). Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. Cell *99*, 323-334.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-331.

Li, A. G., Piluso, L. G., Cai, X., Wei, G., Sellers, W. R., and Liu, X. (2006). Mechanistic insights into maintenance of high p53 acetylation by PTEN. Mol Cell *23*, 575-587.

Li, M., Luo, J., Brooks, C. L., and Gu, W. (2002). Acetylation of p53 inhibits its ubiquitination by Mdm2. J Biol Chem *277*, 50607-50611.

Lian, Z., and Di Cristofano, A. (2005). Class reunion: PTEN joins the nuclear crew. Oncogene *24*, 7394-7400.

Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D., and Berger, S. L. (1999). p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. Mol Cell Biol *19*, 1202-1209.

Lohrum, M. A., Woods, D. B., Ludwig, R. L., Balint, E., and Vousden, K. H. (2001). C-terminal ubiquitination of p53 contributes to nuclear export. Mol Cell Biol *21*, 8521-8532.

Lu, Y., Yu, Q., Liu, J. H., Zhang, J., Wang, H., Koul, D., McMurray, J. S., Fang, X., Yung, W. K., Siminovitch, K. A., and Mills, G. B. (2003). Src family protein-tyrosine kinases alter the function of PTEN to regulate phosphatidylinositol 3-kinase/AKT cascades. J Biol Chem *278*, 40057-40066.

Luo, J., Li, M., Tang, Y., Laszkowska, M., Roeder, R. G., and Gu, W. (2004). Acetylation of p53 augments its site-specific DNA binding both in vitro and in vivo. Proc Natl Acad Sci U S A *101*, 2259-2264.

Luo, J., Nikolaev, A. Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. Cell *107*, 137-148.

Luo, J., Sobkiw, C. L., Logsdon, N. M., Watt, J. M., Signoretti, S., O'Connell, F., Shin, E., Shim, Y., Pao, L., Neel, B. G., *et al.* (2005). Modulation of epithelial neoplasia and lymphoid

hyperplasia in PTEN+/- mice by the p85 regulatory subunits of phosphoinositide 3-kinase. Proc Natl Acad Sci U S A *102*, 10238-10243.

Luo, J., Su, F., Chen, D., Shiloh, A., and Gu, W. (2000). Deacetylation of p53 modulates its effect on cell growth and apoptosis. Nature *408*, 377-381.

Maehama, T., and Dixon, J. E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem *273*, 13375-13378.

Maehama, T., and Dixon, J. E. (1999). PTEN: a tumour suppressor that functions as a phospholipid phosphatase. Trends Cell Biol *9*, 125-128.

Mayo, L. D., Dixon, J. E., Durden, D. L., Tonks, N. K., and Donner, D. B. (2002). PTEN protects p53 from Mdm2 and sensitizes cancer cells to chemotherapy. J Biol Chem *277*, 5484-5489.

Mayo, L. D., and Donner, D. B. (2001). A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc Natl Acad Sci U S A *98*, 11598-11603.

Meares, G. P., and Jope, R. R. (2007). Resolution of the nuclear localization mechanism of glycogen synthase kinase-3: Functional effects in apoptosis. J Biol Chem.

Miller, S. J., Lou, D. Y., Seldin, D. C., Lane, W. S., and Neel, B. G. (2002). Direct identification of PTEN phosphorylation sites. FEBS Lett *528*, 145-153.

Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell *69*, 1237-1245.

Myers, M. P., Pass, I., Batty, I. H., Van der Kaay, J., Stolarov, J. P., Hemmings, B. A., Wigler, M. H., Downes, C. P., and Tonks, N. K. (1998). The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc Natl Acad Sci U S A *95*, 13513-13518.

Okamura, M., Yamaji, S., Nagashima, Y., Nishikawa, M., Yoshimoto, N., Kido, Y., Iemoto, Y., Aoki, I., and Ishigatsubo, Y. (2007). Prognostic value of integrin beta1-ILK-pAkt signaling pathway in non-small cell lung cancer. Hum Pathol.

Okumura, K., Mendoza, M., Bachoo, R. M., DePinho, R. A., Cavenee, W. K., and Furnari, F. B. (2006). PCAF modulates PTEN activity. J Biol Chem *281*, 26562-26568.

Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature *362*, 857-860.

Ouchi, T., Lee, S. W., Ouchi, M., Aaronson, S. A., and Horvath, C. M. (2000). Collaboration of signal transducer and activator of transcription 1 (STAT1) and BRCA1 in differential regulation of IFN-gamma target genes. Proc Natl Acad Sci U S A *97*, 5208-5213.

Pap, M., and Cooper, G. M. (1998). Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. J Biol Chem *273*, 19929-19932.

Podsypanina, K., Ellenson, L. H., Nemes, A., Gu, J., Tamura, M., Yamada, K. M., Cordon-Cardo, C., Catoretti, G., Fisher, P. E., and Parsons, R. (1999). Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proc Natl Acad Sci U S A *96*, 1563-1568.

Rameh, L. E., and Cantley, L. C. (1999). The role of phosphoinositide 3-kinase lipid products in cell function. J Biol Chem *274*, 8347-8350.

Roth, J., Dobbelstein, M., Freedman, D. A., Shenk, T., and Levine, A. J. (1998). Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. Embo J *17*, 554-564.

Sansal, I., and Sellers, W. R. (2004). The biology and clinical relevance of the PTEN tumor suppressor pathway. J Clin Oncol *22*, 2954-2963.

Sasse, J. a. G. (2003). Detection of Proteins. In Current Protocols in Molecular Biology (John Wiley &Sons Inc.), pp. 10.16.11-10.16.12.

Shiratsuchi, T., Oda, K., Nishimori, H., Suzuki, M., Takahashi, E., Tokino, T., and Nakamura, Y. (1998). Cloning and characterization of BAP3 (BAI-associated protein 3), a C2 domain-containing protein that interacts with BAI1. Biochem Biophys Res Commun *251*, 158-165.

Simpson, L., and Parsons, R. (2001). PTEN: life as a tumor suppressor. Exp Cell Res *264*, 29-41.

Song, G., Ouyang, G., and Bao, S. (2005). The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med *9*, 59-71.

Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell *95*, 29-39.

Stambolic, V., Tsao, M. S., Macpherson, D., Suzuki, A., Chapman, W. B., and Mak, T. W. (2000). High incidence of breast and endometrial neoplasia resembling human Cowden syndrome in pten+/- mice. Cancer Res *60*, 3605-3611.

Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., *et al.* (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat Genet *15*, 356-362.

Steinbrecher, K. A., Wilson, W., 3rd, Cogswell, P. C., and Baldwin, A. S. (2005). Glycogen synthase kinase 3beta functions to specify gene-specific, NF-kappaB-dependent transcription. Mol Cell Biol *25*, 8444-8455.

Stephens, L., Eguinoa, A., Corey, S., Jackson, T., and Hawkins, P. T. (1993). Receptor stimulated accumulation of phosphatidylinositol (3,4,5)-trisphosphate by G-protein mediated pathways in human myeloid derived cells. Embo J *12*, 2265-2273.

Sulis, M. L., and Parsons, R. (2003). PTEN: from pathology to biology. Trends Cell Biol *13*, 478-483.

Suzuki, A., de la Pompa, J. L., Stambolic, V., Elia, A. J., Sasaki, T., del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., *et al.* (1998). High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Curr Biol *8*, 1169-1178.

Tao, W., and Levine, A. J. (1999). P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. Proc Natl Acad Sci U S A *96*, 6937-6941.

Taylor, G. S., and Dixon, J. E. (2001). An assay for phosphoinositide phosphatases utilizing fluorescent substrates. Anal Biochem *295*, 122-126.

Tolkacheva, T., Boddapati, M., Sanfiz, A., Tsuchida, K., Kimmelman, A. C., and Chan, A. M. (2001). Regulation of PTEN binding to MAGI-2 by two putative phosphorylation sites at threonine 382 and 383. Cancer Res *61*, 4985-4989.

Torres, J., and Pulido, R. (2001). The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. J Biol Chem *276*, 993-998.

Torres, J., Rodriguez, J., Myers, M. P., Valiente, M., Graves, J. D., Tonks, N. K., and Pulido, R. (2003). Phosphorylation-regulated cleavage of the tumor suppressor PTEN by caspase-3: implications for the control of protein stability and PTEN-protein interactions. J Biol Chem *278*, 30652-30660.

Trotman, L. C., Niki, M., Dotan, Z. A., Koutcher, J. A., Di Cristofano, A., Xiao, A., Khoo, A. S., Roy-Burman, P., Greenberg, N. M., Van Dyke, T., *et al.* (2003). Pten dose dictates cancer progression in the prostate. PLoS Biol *1*, E59.

Trotman, L. C., Wang, X., Alimonti, A., Chen, Z., Teruya-Feldstein, J., Yang, H., Pavletich, N. P., Carver, B. S., Cordon-Cardo, C., Erdjument-Bromage, H., *et al.* (2007). Ubiquitination regulates PTEN nuclear import and tumor suppression. Cell *128*, 141-156.

Ueki, K., Fruman, D. A., Brachmann, S. M., Tseng, Y. H., Cantley, L. C., and Kahn, C. R. (2002). Molecular balance between the regulatory and catalytic subunits of phosphoinositide 3-kinase regulates cell signaling and survival. Mol Cell Biol *22*, 965-977.

Vaziri, H., Dessain, S. K., Ng Eaton, E., Imai, S. I., Frye, R. A., Pandita, T. K., Guarente, L., and Weinberg, R. A. (2001). hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. Cell *107*, 149-159.

Vazquez, F., Grossman, S. R., Takahashi, Y., Rokas, M. V., Nakamura, N., and Sellers, W. R. (2001). Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex. J Biol Chem *276*, 48627-48630.

Vazquez, F., Ramaswamy, S., Nakamura, N., and Sellers, W. R. (2000). Phosphorylation of the PTEN tail regulates protein stability and function. Mol Cell Biol *20*, 5010-5018.

Vivanco, I., and Sawyers, C. L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer *2*, 489-501.

von Willebrand, M., Williams, S., Saxena, M., Gilman, J., Tailor, P., Jascur, T., Amarante-Mendes, G. P., Green, D. R., and Mustelin, T. (1998). Modification of phosphatidylinositol 3-kinase SH2 domain binding properties by Abl- or Lck-mediated tyrosine phosphorylation at Tyr-688. J Biol Chem *273*, 3994-4000.

Wang, X., Trotman, L. C., Koppie, T., Alimonti, A., Chen, Z., Gao, Z., Wang, J., Erdjument-Bromage, H., Tempst, P., Cordon-Cardo, C., *et al.* (2007). NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. Cell *128*, 129-139.

Welsh, G. I., Wilson, C., and Proud, C. G. (1996). GSK3: a SHAGGY frog story. Trends Cell Biol *6*, 274-279.

Woods, D. B., and Vousden, K. H. (2001). Regulation of p53 function. Exp Cell Res *264*, 56-66.

Wu, H., Goel, V., and Haluska, F. G. (2003). PTEN signaling pathways in melanoma. Oncogene *22*, 3113-3122.

Wu, X., Bayle, J. H., Olson, D., and Levine, A. J. (1993). The p53-mdm-2 autoregulatory feedback loop. Genes Dev *7*, 1126-1132.

Wu, X., Hepner, K., Castelino-Prabhu, S., Do, D., Kaye, M. B., Yuan, X. J., Wood, J., Ross, C., Sawyers, C. L., and Whang, Y. E. (2000a). Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain containing scaffold protein MAGI-2. Proc Natl Acad Sci U S A *97*, 4233-4238.

Wu, Y., Dowbenko, D., Spencer, S., Laura, R., Lee, J., Gu, Q., and Lasky, L. A. (2000b). Interaction of the tumor suppressor PTEN/MMAC with a PDZ domain of MAGI3, a novel membrane-associated guanylate kinase. J Biol Chem *275*, 21477-21485.

Xu, J., Paquet, M., Lau, A. G., Wood, J. D., Ross, C. A., and Hall, R. A. (2001). beta 1-adrenergic receptor association with the synaptic scaffolding protein membrane-associated guanylate kinase inverted-2 (MAGI-2). Differential regulation of receptor internalization by MAGI-2 and PSD-95. J Biol Chem *276*, 41310-41317.

Yu, J., Zhang, Y., McIlroy, J., Rordorf-Nikolic, T., Orr, G. A., and Backer, J. M. (1998). Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. Mol Cell Biol *18*, 1379-1387.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H. M., and Horvitz, H. R. (1993). The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell *75*, 641-652.

APPENDIX I

Table A1: Normalized values of intensity of lipid spots. Data was collected using the Odyssey imaging system and supplied software. Values in each replicate were normalized to the C124S PI4,5P_2 lipid spot values.

C124S	Replicate 1	Replicate 2	Replicate 3	Replicate 4		
PI	0.28	0.21	0.75	0.48		
PI3P	3.76	2.59	3.33	5.60		
PI4P	5.60	4.08	2.46	3.83		
PI5P	2.90	1.74	2.71	3.43		
PI3,4P2	1.84	2.26	1.57	0.86		
PI3,5P2	2.63	2.46	2.59	2.58		
PI4,5P2	1.00	1.00	1.00	1.00		
PI3,4,5P3	1.50	0.80	0.83	0.46		
PA	1.40	1.04	1.36	1.43		
STT/AAA	Replicate 1	Replicate 2	Replicate 3	Replicate 4	test statistic	statistically significant if test statistic is <0.05
PI	0.17	0.55	0.57	0.67	0.670	> 0.05
PI3P	2.80	2.31	2.58	2.15	0.152	> 0.05
PI4P	2.04	2.85	2.68	2.34	0.148	> 0.05
PI5P	2.90	2.27	3.01	2.27	0.842	> 0.05
PI3,4P2	1.92	1.24	1.78	1.15	0.752	> 0.05
PI3,5P2	2.41	1.36	2.76	1.85	0.195	> 0.05
PI4,5P2	0.71	1.15	1.52	0.64	0.982	> 0.05
PI3,4,5P3	1.03	1.20	1.39	0.74	0.467	> 0.05
PA	0.93	1.26	1.72	1.26	0.945	> 0.05
STT/EEE						
PI	0.03	0.17	0.47	0.29	0.034	< 0.05
PI3P	2.65	2.79	2.03	1.20	0.189	> 0.05
PI4P	3.72	2.26	1.67	1.23	0.018	< 0.05
PI5P	2.05	2.81	1.70	1.05	0.345	> 0.05
PI3,4P2	1.54	1.75	1.12	0.54	0.004	< 0.05
PI3,5P2	2.50	2.19	1.71	0.90	0.128	> 0.05
PI4,5P2	0.90	0.94	0.73	0.35	0.138	> 0.05

STT/EEE (continued)	Replicate 1	Replicate 2	Replicate 3	Replicate 4	test statistic	statistically significant if test statistic is <0.05
PI3,4,5P3	1.31	1.35	0.86	0.38	0.682	> 0.05
PA	0.87	0.90	0.81	0.49	0.047	< 0.05
PASE RKK/AAA						
PI	0.02	0.03	0.46	0.14	0.003	< 0.05
PI3P	0.49	0.63	1.00	0.53	0.020	< 0.05
PI4P	1.05	1.07	2.27	0.86	0.060	> 0.05
PI5P	0.47	0.49	1.24	0.40	0.016	< 0.05
PI3,4P2	0.26	0.39	0.55	0.30	0.023	< 0.05
PI3,5P2	0.64	0.76	1.12	0.43	0.001	< 0.05
PI4,5P2	0.16	0.28	0.74	0.12	0.018	< 0.05
PI3,4,5P3	0.11	0.32	0.67	0.13	0.121	> 0.05
PA	0.77	0.83	1.53	0.60	0.192	> 0.05
C2-K5A						
PI	0.06	0.00	0.04	0.03	0.042	< 0.05
PI3P	0.19	0.13	0.32	0.09	0.012	< 0.05
PI4P	0.15	0.18	0.39	0.09	0.012	< 0.05
PI5P	0.17	0.16	0.25	0.09	0.006	< 0.05
PI3,4P2	0.16	0.26	0.44	0.16	0.018	< 0.05
PI3,5P2	0.36	0.27	0.53	0.25	0.000	< 0.05
PI4,5P2	0.15	0.07	0.11	0.06	0.000	< 0.05
PI3,4,5P3	0.18	0.11	0.19	0.20	0.045	< 0.05
PA	0.11	0.12	0.21	0.11	0.001	< 0.05
C2-K3A						
PI	0.00	0.00	0.00	0.00	0.037	< 0.05
PI3P	0.45	0.23	0.56	0.09	0.016	< 0.05
PI4P	0.72	0.37	1.04	0.27	0.018	< 0.05
PI5P	0.36	0.19	0.48	0.17	0.007	< 0.05
PI3,4P2	0.04	0.04	0.20	0.00	0.013	< 0.05
PI3,5P2	0.51	0.14	0.69	0.05	0.000	< 0.05
PI4,5P2	0.09	0.00	0.13	0.00	0.000	< 0.05
PI3,4,5P3	0.03	0.00	0.08	0.00	0.027	< 0.05
PA	0.77	0.28	0.98	0.39	0.015	< 0.05
Y336A						
PI	0.05	0.03	0.34	0.32	0.024	< 0.05
PI3P	1.21	3.53	1.48	2.38	0.165	> 0.05

Y336A (continued)	Replicate 1	Replicate 2	Replicate 3	Replicate 4	test statistic	statistically significant if test statistic is <0.05
PI4P	1.04	2.63	1.20	3.08	0.103	> 0.05
PI5P	1.42	3.03	1.42	2.34	0.396	> 0.05
PI3,4P2	0.86	2.11	0.67	1.46	0.408	> 0.05
PI3,5P2	1.11	3.20	0.94	2.74	0.413	> 0.05
PI4,5P2	0.32	1.19	0.50	1.17	0.429	> 0.05
PI3,4,5P3	0.38	0.81	0.47	0.71	0.377	> 0.05
PA	0.47	0.54	0.73	1.35	0.054	> 0.05
Y336E						
PI	0.18	0.19	0.09	0.28	0.180	> 0.05
PI3P	2.35	1.02	1.59	1.16	0.050	> 0.05
PI4P	1.49	0.86	1.35	0.72	0.020	< 0.05
PI5P	2.26	1.27	0.76	0.98	0.066	> 0.05
PI3,4P2	2.01	0.49	3.51	0.81	0.925	> 0.05
PI3,5P2	3.10	1.07	3.09	1.14	0.460	< 0.05
PI4,5P2	0.37	0.36	0.85	0.31	0.026	< 0.05
PI3,4,5P3	0.78	0.41	0.48	0.30	0.041	< 0.05
PA	1.06	0.81	0.58	0.56	0.040	< 0.05