DIFFERENTIAL REGULATION OF PHOSPHATIDYLINOSITOL PHOSPHATE 5 KINASE BETA DURING HYPERTONIC AND OXIDATIVE STRESS

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DEDICATION

I would like to thank my family, C.T., Nancy, Amy and Baby.

I would like to also thank the members of my Graduate Committee and my friends who have made graduate school and living in Texas an unforgettable and wonderful experience.

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by

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DIFFERENTIAL REGULATION OF PHOSPHATIDYL INOSITOL PHOSPHATE 5 KINASE BETA DURING HYPERTONIC AND OXIDATIVE STRESS

Mark Chen, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, Graduation Year

Helen L. Yin, M.D., Ph.D.

Of the total amount of phospholipids in the cell, phosphoinositides account for only a small fraction, yet they play an indispensable role in regulating cellular homeostasis. The phosphatidylinositol derivative, PI(4,5)P_2 (PIP_2)is an essential mediator of cellular processes such as signal transduction, membrane traffic, actin cytoskeleton, ion homeostasis, growth and apoptosis. Despite the importance of this signaling molecule in controlling a diverse array of functions, little is known about the regulation of the kinases that produce PIP_2 as well as its precursor, phosphatidylinositol 4 phosphate (PI4P).

Our laboratory found that hypertonic and oxidative stress increases and decreases PIP_2 levels, respectively. In this thesis, I sought to understand how these two different types of stresses alter PIP_2 by examining the effects on the phosphatidylinositol 5-phosphate 4 kinases that generate PIP_2. Using RNAi and in vitro kinase activity assays, I found that PIP5Kβ is regulated by both stimuli in opposite directions and is responsible for the net change in PIP_2 levels. Hypertonic stress activates PIP5Kβ through PP1 phosphatase dependent ser/thr dephosphorylation which leads to increased kinase activity and plasma membrane localization. The PIP_2 produced by PIP5Kβ during this event leads to an increase in actin polymerization and stress fiber formation. Conversely, oxidative stress leads to a decrease in PIP5Kβ activity, membrane detachment and stress fiber dissolution. Oxidative stress induces simultaneous tyrosine phosphorylation and ser/thr dephosphorylation, establishing that tyrosine phosphorylation decreases PIP5Kβ activity and is the dominant modification of PIP5Kβ that overrides the activating effects of ser/thr dephosphorylation. I further identified the tyrosine kinase Syk as the kinase responsible for PIP5Kβ phosphorylation during oxidative stress. The phosphorylation site was mapped to position Y105 which was shown to control both activity and localization of PIP5Kβ.

Oxidative stress also increased PI4P which is likely to be at least partly due to activation of a PI4K. Using RNAi and wortmannin to selectively inhibit the activity of different PI4K isoforms, I sought to identify the PI4K isoform that is responsible for this increase.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Chen MZ, Zhu X, Chen L, Wei YJ, Yin HL. Oxidative stress deactivates PIP5Kβ through Syk-dependent tyrosine phosphorylation. *(ms. submitted)*

Yamamoto M*, **Chen MZ***, Wang YJ, Sun HQ, Wei Y, Martinez M, Yin HL. Hypertonic stress increases phosphatidylinositol 4,5-bisphosphate levels by activating PIP5KIb. J Biol Chem. 2006 Oct 27;281(43):32630-8. *These authors contributed equally to this work.

Moffat BA, **Chen M**, Kariaapper MS, Hamstra DA, Hall DE, Stojanovska J, Johnson TD, Blaivas M, Kumar M, Chenevert TL, Rehemtulla A, Ross BD. Inhibition of vascular endothelial growth factor (VEGF)-A causes a paradoxical increase in tumor blood flow and up-regulation of VEGF-D. Clin Cancer Res. 2006 Mar 1;12(5):1525-32.

Hamstra DA, Lee KC, Tychewicz JM, Schepkin VD, Moffat BA, **Chen M**, Dornfeld KJ, Lawrence TS, Chenevert TL, Ross BD, Gelovani JT, Rehemtulla A. The use of 19F spectroscopy and diffusion-weighted MRI to evaluate differences in gene-dependent enzyme prodrug therapies. Mol Ther. 2004 Nov;10(5):916-28.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AP2	Adaptor Protein 2
ARF	ADP-Ribosylation Factor
BMMC	Bone Marrow Mast Cell
Ca^{2+}	Calcium
CalyA	Calyculin A
CBP	Cysteine Based Protease
Cyt	Cytosol
DAG	Diacylglycerol
DTT	Dithiothreitol
ER	Endoplasmic Reticulum
FA	Focal Adhesion
FAK	Focal Adhesion Kinase
GPCR	G Protein Coupled Receptor
GFP	Green Fluorescence Protein
GSH	Glutatione
GSSG	Oxidized Glutathione
HSP	High Speed Pellet
IP	Immunoprecipitation
IP_3	Inositol (3,4,5) Trisphosphate
KD	Knockdown

LSP	Low Speed Pellet
MEM	Membrane
PAO	Phenylarsine oxide
PDGF	Platlet Derived Growth Factor
PH	Pleckstrin Homology
PIP_2	Phosphatidylinositol (4,5) bisphosphate
PIP5K	Phosphatidylinositol 4-Phosphate 5 Kinase
PI3K	Phosphatidylinositol 3-Phosphate Kinase
PI4K	Phosphatidylinositol 4-Kinase
PI4P	Phosphatidylinositol 4 Phosphate
PKA	Protein Kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PM	Plasma Membrane
PPI	Phosphoinositides
RNAi	RNA interference
ROS	Reactive Oxidative Species
RTK	Receptor Tyrosine Kinase
RVI	Regulatory Volume Increase
SH-2	Src Homology 2
TLC	Thin Layer Chromatography
VEGF	Vascular Endothelial Growth Factor

VEGFR	VEGF Receptor
WASP	Wiskott-Aldrich Syndrome Protein

CHAPTER ONE
Introduction

1.1 CELLULAR STRESS

An important development in the evolution of life was the ability of the organism to cope with environmental stresses. Cells are constantly exposed to fluctuations in temperature, pH, nutrient availability, oxygen, osmotic pressure and reactive oxidative species. In this thesis, I have focused on the effects of hypertonic and oxidative stresses on the cell's phosphoinositide homeostasis and the actin cytoskeleton. I found that these stresses alter the homeostasis of phosphoinositides, which are important signaling molecules and regulators of the actin cytoskeleton in eukaryotes. In this introduction, I will first describe briefly these stresses, and the regulation of phosphoinositide homeostasis.

1.2 HYPERTONIC STRESS

Cells exposed to a hyperosmotic environment lose cellular water by passive diffusion (Burg et al. 2007). In mammals, cells in the renal inner medulla are exposed to the most extreme changes in osmolarity (Marsh and Azen 1975) . These cells have developed powerful mechanisms to counteract osmotic changes. However, these stress response factors are not restricted to kidney cells, because almost all cells are susceptible to these changes. There is increasing evidence that the mechanisms of coping with osmotic pressure are ubiquitous among all organisms and cell types. In the acute response

to hypertonic stress, cells undergo a process called regulatory volume increase (RVI). RVI consists of the rapid uptake of inorganic ions which causes an osmotic influx of water to balance the intracellular environment and restore cell volume (Wehner et al. 2003). Over a longer time period, cells adapt to their environment by undergoing transcriptional changes that allow accumulation of organic osmolytes to reduce the ionic strength and restore the normotonic state.

Some cellular processes are particularly sensitive to acute hypertonicity. Cell cycle arrest occurs regardless of the phase the cells are in (Michea et al. 2000). Cell death occurs when hypertonicity exceeds a certain cell-specific setpoint (Cidlowski et al. 1996; Dmitrieva and Burg 2007). Cells undergo the apoptotic program due to either mitochondrial depolarization or triggering of the extrinsic death receptor pathway (Jin and El-Deiry 2005). Hypertonic stress also induces DNA damage through DNA strand breakage (Dmitrieva et al. 2003). Transcription and translation are also affected by osmotic stress (Burg et al. 2007).

The immediate consequences of loss of cell water are decreased cellular volume, increased ionic strength and macromolecular crowding. Increased ionic strength affects biochemical processes. Macromolecular crowding generally leads to an elevation in the activity of enzymes and macromolecules (Garner and Burg 1994). The decrease in volume is typically accompanied by cytoskeletal rearrangement (Di Ciano et al. 2002a; Bustamante et al. 2003). The exact role of cytoskeletal rearrangement in osmoregulation remains unclear (Eggermont 2003b). It may function as a cell volume sensor or help combat the deleterious effects associated with cell shrinkage. Also the cytoskeleton may play a role in signal transduction from osmosensors to osmoregulators. The mechanism

underlying hypertonicity induced changes in the actin cytoskeleton is unknown. This will be the focus of the Chapter Two in this thesis.

1.3 OXIDATIVE STRESS

Eukaryotic cells are constantly exposed to reactive oxidative species (ROS) (Genestra 2007) that are produced as byproducts of cellular metabolic reactions. ROS are highly reactive free-radical and peroxides generated by the one electron reduction of molecular oxygen to form superoxide, (O_2^-). Further reduction of O_2^- forms hydrogen peroxide (H_2O_2).

Under normal conditions, ROS are generated locally and can act as *bona fide* signaling molecules (Genestra 2007). Signaling pathways that respond to changes in redox state include the NF-κB, MAPK, JNK, and Src (Genestra 2007). Many proteins have been implicated as targets of ROS; these include protein kinase C (PKC), guanylyl cyclase, phospholipase D (PLD) and ion channels. Vascular smooth muscle cell migration and remodeling are controlled by ROS signaling (Lee and Griendling 2008). ROS can also act as a protective mechanism against invading pathogens (Gaut et al. 2001). In these situations, ROS are generated as a respiratory burst by neutrophils and macrophages upon engagement with the microbial organism during phagocytosis. NADPH oxidase, which converts O_2 to O_2^- and other ROS, is activated locally in the phagosomes to kill the engulfed pathogens (Nauseef 2007).

ROS are small molecules that are rapidly diffusible. Excessive ROS can inappropriately injure cells (Sahnoun et al. 1997) (Cao et al. 1997). This is usually prevented by neutralizing ROS with oxidant scavengers, which accept the reactive

electrons, or by enzymes which degrade ROS into non-toxic byproducts (Faraci and Didion 2004). The proteins, superoxide dismutase (SOD), glutathione peroxidase, catalase, and thioredoxin act as anti-oxidants that prevent oxidation of the intracellular environment. The balance between ROS and anti-oxidant production is therefore critical for maintaining normal signaling without incurring cell injury.

Dysregulation of the balance between ROS and antioxidants can lead to DNA, protein and lipid oxidation which alters the functions of these molecules. Excessive ROS leads to apoptosis of the damaged cells. Chronic oxidative damage is implicated in the etiology of several disease pathologies including cancer, neurodegenerative diseases, hypertension and diabetes (Smith et al. 2007; Esper et al. 2008; Gupte and Wolin 2008; Kuro-o 2008). ROS are also believed to play a pivotal role in the process of aging in all organisms (Wolff and Dillin 2006). Acute oxidative stress is associated with inflammation, trauma and immunological cell responses. For example, massive dermal burn induced trauma, which is usually followed by a second inflammatory insult, increases ROS production and decreases antioxidant levels (Wright et al. 2000; Turnage et al. 2002). It has been suggested that the increase in ROS initially may be associated with a decrease in ATP and the consequent increase in AMP (Horton 2003). This is because AMP is catabolized to form hypoxanthine which eventually leads to the generation of H_2O_2 and other ROS. In addition, the subsequent inflammatory insult activates inflammatory cells to generate ROS that diffuse away from burn wound site to damage other organs at a distance. The lung microvasculature is particularly susceptible to ROS induced damage. Burn injury patients typically develop generalized lung edema due to the break down of the endothelial barrier which normally controls fluid movement

(Weis 2008). Studies find the endothelial actin cytoskeleton and the integrity of the adherens and tight junctions are compromised by exposure to ROS (Turnage et al. 2002).

An integral mechanism of protein regulation is through protein phosphorylation and dephosphorylation by protein kinases (PK) and phosphatases (PP). ROS directly regulate the function of many of these enzymes, particularly protein tyrosine kinases and phosphatases (PTK, PTP) (Denu and Tanner 1998; Aslan and Ozben 2003). All PTPs share a common cysteine residue in their catalytic site that participates in their enzymatic function (Meng et al. 2002). This residue is redox sensitive and its reduction by ROS inhibits the PTP activity.

The integrity of the actin cytoskeleton is affected by exposure to ROS (Dalle-Donne et al. 2001). The response is complicated and specific to the type of cell tested. The mechanism of regulation is unknown, although the phosphoinositide, phosphatidylinositol 4,5 bisphosphate [$PI(4,5)P_2$ or PIP_2] has been implicated. Oxidative stress affects PIP_2 turnover and homeostasis (Mesaeli et al. 2000). Reports from other groups as well as our own find that H_2O_2 leads to a decrease in cellular PIP_2 levels (Reimann et al. 1990; Mesaeli et al. 2000; Halstead et al. 2006). I investigated the underlying mechanism of the observation that H_2O_2 treatment of several different cell types caused a prominent change in PIP_2 turnover. This is described in Chapter 3.

1.4 PHOSPHOINOSTIDE LIPIDS AS SIGNALING MOLECULES

Phosphoinositides (PPIs) are phospholipids characterized by a fatty acid chain tail linked to an inositol head group by a glycerol linkage (Fig 1.1A). Their role as signaling molecules first gained attention in 1953 when Mabel and Lowell Hokin reported that stimulation of pancreatic tissue with carbachol was accompanied by enhanced turnover of a minor lipid product, phosphatidylinositol (Hokin and Hokin 1953; Irvine 2003). Subsequently, data from other labs expanded this observation by finding that second messengers were derived from hydrolysis of PIP_2 by phospholipase C to generate inositol 1,4,5-tris-phosphate (IP_3) and diacyglycerol (DAG) (Berridge 1993). It was further discovered that activation of G-protein coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) accelerated the turnover of these phospholipids. This led to an increased interest in PPIs as an integral step in signal transduction. The importance of PPIs became even more evident when it was realized that the inositol head group can be phosphorylated at three positions in multiple combinations (Michell 2008), and can have different functions including activation of signaling molecules and as docking sites for the recruitment of proteins to membranes

Since the discovery of PPIs, researchers have begun to realize the importance of these molecules as they relate not only to cellular physiology but also as they contribute to disease progression. PPI dysregulation has been implicated in a wide variety of diseases such as cancer, metabolic syndrome, hypertension and Alzheimers (Wymann and Schneiter 2008). Yet while the appreciation of these multi-faceted signaling molecules has only recently been recognized, large gaps remain in our understanding of how PPIs are synthesized, regulated and control cellular events.

1.5 PHOSPHOINOSITIDE BIOLOGY

The inositol headgroup, which most often occurs as *myo*-inositol, *cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol, can be phosphorylated on 3 known positions. The 3, 4, and 5 positions are phosphorylated either singly or combinatorially by dedicated lipid kinases. Furthermore, each position can be selectively dephosphorylated by lipid phosphatases (Cockroft 2000). PPIs are typically located on the cytoplasmic face of organelle membranes. All eukaryotes from yeast to humans possess PPIs while bacteria do not. Thus the composition of the plasma membrane (PM) is one of the delineating characteristics between eukaryotes and prokaryotes. While PPIs only consist of approximately 10% of total phospholipids in the mammalian cell, they play an indispensable function in the activity of the eukaryotic cell (Cockroft 2000).

PIP_2, which accounts for 5% of total PPIs, is a very versatile signaling molecule (Figure 1.1B). As mentioned earlier, PIP_2 can be cleaved by PLC upon agonist stimulation to form second messengers that participate in calcium signaling and PKC activation. PIP_2 also serves as a focal point for signaling complexes by acting as a scaffolding molecule to recruit proteins to the PM (Lemmon 2008). Ion channels have been identified as being regulated by PIP_2 as well (Li et al. 2005). Moreover, PIP_2 is an important mediator of membrane trafficking including endocytosis and exocytosis, and is also an integral regulator of the actin cytoskelelton (Yin and Janmey 2003). PIP_2 is also a substrate of the phosphatidylinositol 3 kinases (PI3K), which add a phosphate group to the D3 position to generate PI $(3,4,5)P_3$ (PIP_3). PIP_3 is a major mediator of cell growth

and survival (Engelman et al. 2006). Extracellular growth signals such as insulin activate PI3K. Upon PIP_3 formation, the cytosolic protein Akt is recruited to the plasma membrane where it continues signaling to the downstream ser/thr kinase, Tor. Dysregulation of PIP_3 homeostasis has been implicated in several cancers and growth related defects.

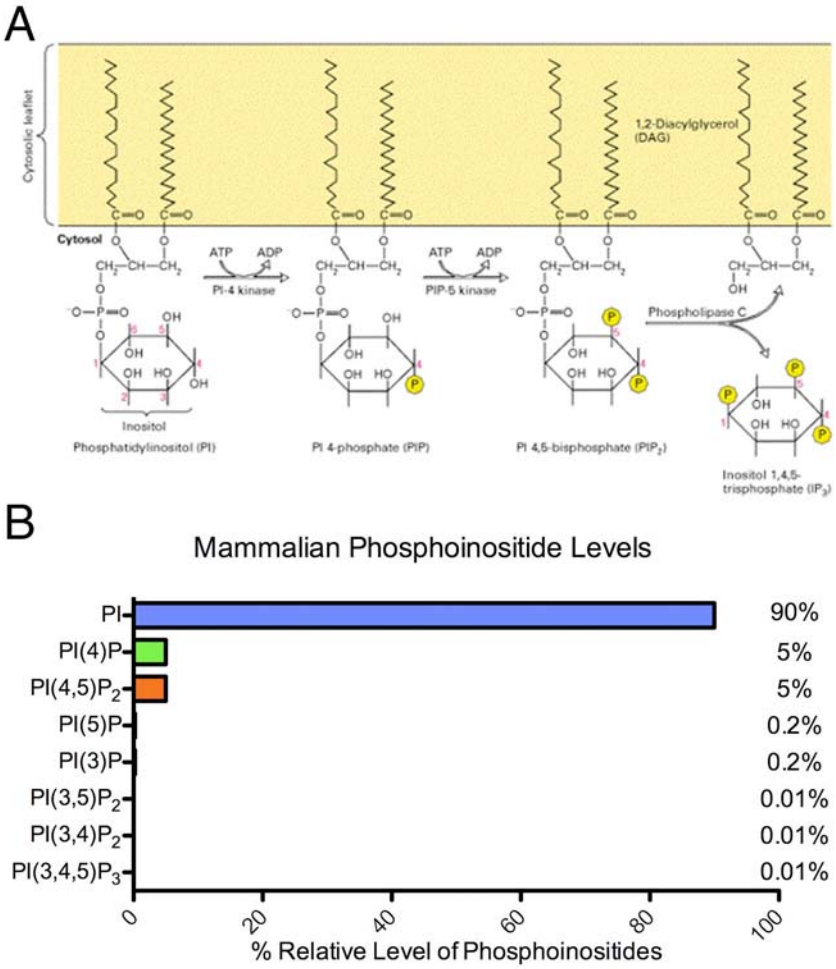


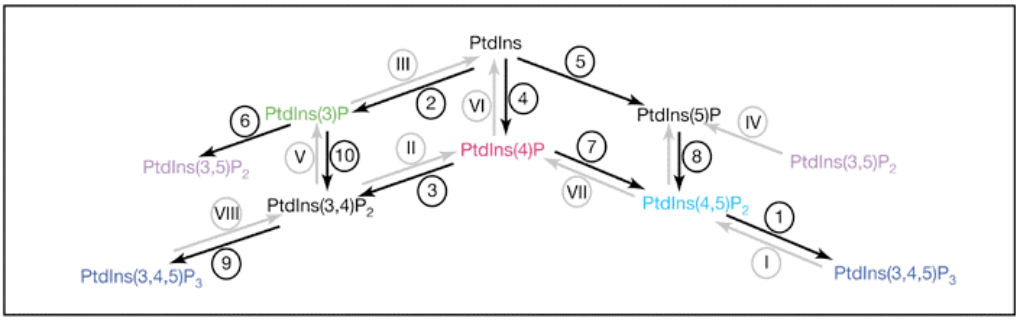
Figure 1.1- A Chemical structure of PPIs and its derivatives. B. Relative level of PPIs in mammalian cells vs. total level of PPIs (Cockroft 2000).

1.6 LIPID SIGNALING PARADIGM SHIFTS

The previously held notion was that PPIs were simply intermediates for the secondary messenger IP_3, DAG and PIP_3. The evolution in thinking of how PPIs played into the hierarchy of signaling occurred with the discovery that PPIs were not merely substrates for PLC by also regulators of membrane traffic and the cytoskeleton (Ling et al. 2006; Mao and Yin 2007). PPIs are physically and functionally segregated into pools that presumably have specific functions. Furthermore, there is an emerging realization that different types of PPI define each membrane organelle through recruitment and regulation of proteins associated with their respective organelles (De Matteis and Godi 2004**).** These pools are maintained by a balance of phosphorylation, dephosphorylation and lipid transfer (Figure 1.2). PIP_2 is thought to define the PM while PI4P has been implicated as a Golgi marker (Wang et al. 2003). PI3P is implicated as an early endosome marker, while $PI(3,5)P_2$ has been shown to be localized to the multi-vesicular body and late endosome.

Proteins bind to PPIs through their lipid binding motifs. Because PPIs possess acidic headgroups, the lipid binding domains on their binding proteins contain basic clusters of residues. These have a stereospecific conformation that dictates the specificity of PPI binding despite only having subtle differences in their phosphorylation positions. To date, ten lipid binding domain motifs have been identified (Lemmon 2008). Among these, the pleckstrin homology (PH) domain which has been identified in over 100

proteins to date, has been studied extensively. The use of fusing green fluorescence protein (GFP) to these domains has proved to be an invaluable tool for studying the spatial and temporal behavior of PPIs (Halet 2005). For instance, GFP-tagged PH domain of PLCδ (which binds PIP_2) has allowed researchers to study the dynamics of PIP_2 at the plasma membrane.



Phosphoinositide kinases

		yeast homologues	localization
PI(3)K			
1	P110α–δ		PM, N
2 3	PI(3)K-C2α		GC, E
2 3	PI(3)K-C2β		PM
2 3	PI(3)K-C2γ		GC
2	Vps34	Vps34	GC, E
PI(4)K			
4	PI(4)KIIα	Lsb6p[a]	GC, E
4	PI(4)KIIβ		PM, GC
4	PI(4)KIIIα	Stt4p	GC, ER
4	PI(4)KIIIβ	Pik1p	GC, N
PI(5)K			
5? 6	PIK-FYVE	Fab1p	LE
PIP(5)K			
7 9	PIP(5)Kα, β, γ	Mss4p	PM
PIP(4)K			
10 8	PIP(4)Kα, β		PM
10 8	PIP(4)Kγ		ER

Phosphoinositide phosphatates

		yeast homologues	localization
3-phosphatates			
I II	PTEN1, 2	Tep1p	PM, GC, N
I	TPIPα, β, γ		ER, GC
III	MTM1	Yjr110w	PM
IV III	MTMR1-8		PM, ND
4-phosphatates			
V	Type I, II		ND
VI	hSac1, 2, 3	Sac1p	GC, ER
VI	Synaptojanin 1, 2	Inp52p, Inp53p	CCV, Mi
5-phosphatates			
VII	5-phosphatase II		ND
VII	Ocrl		GC, E, Ly
VII	Synaptojanin	Inp52p, Inp53p	SV, Mi
VII	PIPP		PM
VIII	72-kDa-5-phosphatase		GC
VII	SKIP	Inp54p, Inp51p	ER, PM
VIII	SHIP 1, 2		PM, N
VIII	5-phosphatase IV		ND

Figure 1.2- Cycles of PPI phosphorylation and dephosphorylation by phosphoinositide kinases and phosphatases, respectively. Abbreviations: GC, Golgi complex; PM, plasma membrane; ER, endoplasmic

reticulum; N, nucleus; E, endosomes; LE, late endosoomes; Ly, lysosomes; SV, synaptic vesicles; CCV, clathrin-coated vesicles; Mi, mitochondria; ND, not determined. Taken from (De Matteis and Godi 2004).

1.7 GENERATION OF PIP_2

PIP_2 can be synthesized by several different means (Figure 1.2). The main mode of synthesis is through the phosphorylation of the D5 position of the inositol ring of PI4P by the type I phosphatidylinositol 4-phosphate 5-kinases (heretofore referred to as PIP5K). An alternate route is through the phosphorylation of the D4 position of PI5P by the type II PIP5K. The relative contribution of the type II kinase to the cellular PIP_2 is much less because there is much less PI5P than PI4P in cells (0.2% vs. 5% of total PPIs, respectively (Cockroft 2000). Also, studies in cells find the phosphate at the D5 position has a higher specific activity than at the D4 position, indicating that it is the last phosphate added to PIP_2 (King et al. 1989). Finally, PIP_2 can be synthesized by the dephosphorylation of the 3 position of $PI(3,4,5)P_3$ (PIP_3) by phosphatases such as MTM1 and PTEN (Wishart and Dixon 2002). However, the contribution of this reaction is very low since PIP_3 consists of only 0.01% of total PPIs.

The PIP5K gene is found in all eukaryotes. S. Cerevisia has a single PIP5K gene , while the NCBI EST database reports that *C. elegans*, *drosophila* both have three putative PIP5K genes. Mammals have 3 established PIP5K genes. These genes are named PIP5Kα, PIP5Kβ, and PIP5Kγ (Figure 1.3, Table 1) (Oude Weernink et al. 2004). Additionally, mammalian PIP5Kγ has 3 splice variants, PIP5Kγ87, PIP5Kγ90, and PIP5Kγ93. The human and mouse PIP5K genes are highly conserved and were cloned simultaneously (Mao and Yin 2007). Unfortunately, the human alpha isoform was

designated beta in the mouse nomenclature while the human beta was named mouse alpha. The gamma isoform was conserved. Genebank recently unified the nomenclature, using the human designation. Therefore, I will be using the human designation for my work as well.

The mammalian PIP5Ks share a highly conserved kinase domain that has about an 80% sequence identity. Their N- and C-terminal extensions are nonhomologous and may specify isoform specific functions (Figure 1.3). All PIP5Ks can be activated by phosphatidic acid (PA) (Skippen et al. 2002) and ser/thr dephosphorylation (Mao and Yin 2007). In addition, the small GTPase Rho, Rac, and Arf have been implicated as regulators of the PIP5Ks (Santarius et al. 2006). Although PIP5Ks's substrate (PI4P) and product (PIP_2) are lipids that by definition should reside in membranes, PIP5Ks are cytosolic proteins that are only partially membrane associated. Structural studies find that the type II PIP5Ks have a large flat positively charged patch which is postulated to mediate membrane association (Rao et al. 1998). It is believed that type I PIP5Ks also use a similar flat surface for docking on membranes. Type II PIP5Ks form homo dimmers which leads to its activation. Unpublished data from our lab indicates Type I PIP5Ks also form dimmers as well. The pathways and factors that regulate recruitment of PIP5K to the PM and exposure of this surface for docking are relatively unknown**.** Oxidative stress has been found to decrease PM localization of PIP5Kβ. but the mechanism of this regulation is unclear (Halstead et al. 2006). My work has identified tyrosine phosphorylation as the post-translational modification that regulates PIP5Kβ membrane association. This will be described in Chapter 3.

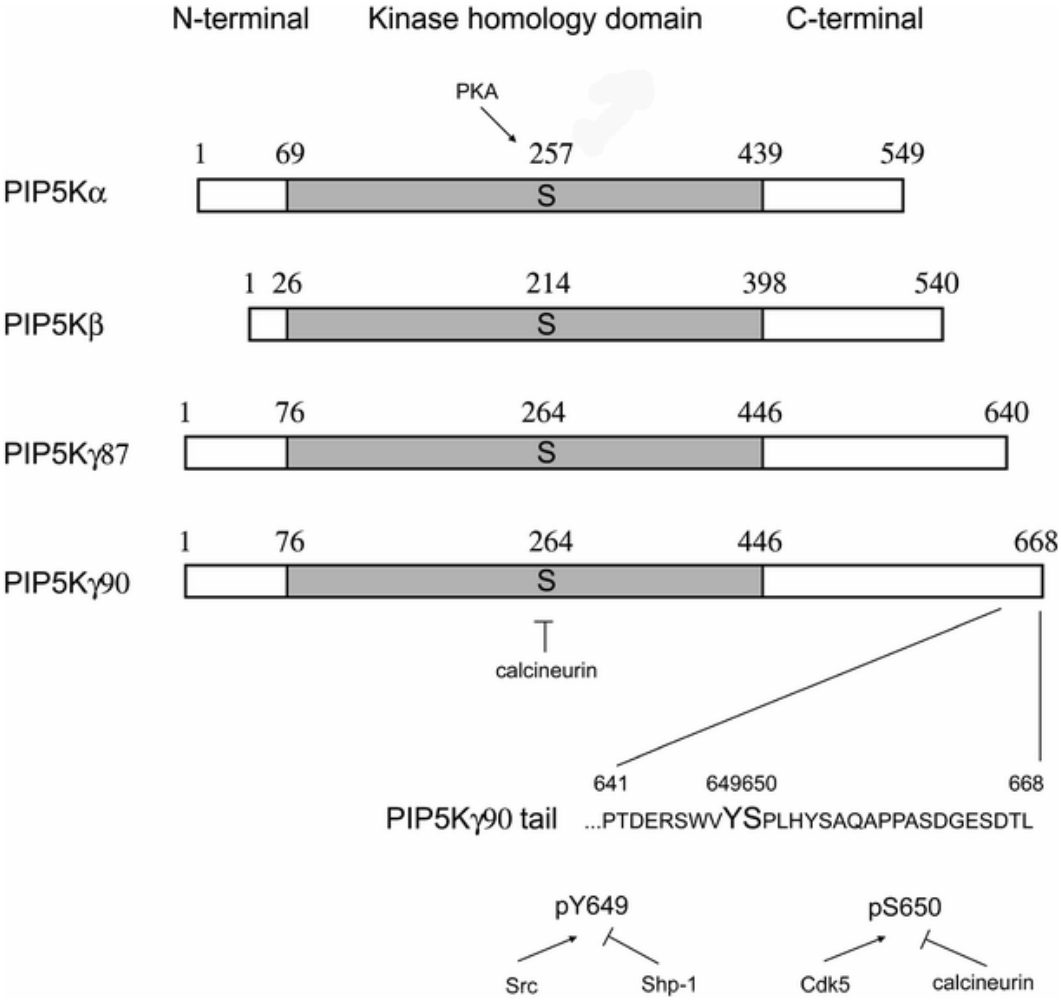


Figure 1.3-Structure of human Type I PIP5Ks. PIP5Ks share a highly conserved kinase core while their N and C-terminal tails are highly variable (Mao and Yin 2007).

Table 1.1-Summary of human and mouse PIP5Ks. α, β and γ are different genes. PIP5Kγ contains 3 splice variants.

Human isoforms and splice variants	Mouse counterparts	Molecular weight (kDa)	Number of residues in human (mouse)
hPIP5Kα	mPIP5Kβ	68	549 (546)
hPIP5Kβ	mPIP5Kα	68	540 (539)
hPIP5Kγ87	mPIP5Kγ635	87	640 (635)
hPIP5Kγ90	mPIP5Kγ661	90	668 (661)
hPIP5Kγ93	mPIP5Kγ688	93	NA (688)

1.8 REGULATION OF PIP5Ks BY SMALL GTPases

In vitro studies suggest that PIP5Ks are regulated in many ways. Some of the mechanisms of activation are not well understood and the physiological relevance is not

established. These include regulation with Rho family small GTPases or the second messenger, phosphatidic acid (PA), as well as by phosphorylation/dephosphorylation. PA is synthesized by two different pathways, either phospholipase D (PLD), or diacylglycerol kinase (DGK) (Jenkins and Frohman 2005) and has been found to stimulate Type I PIP5K but not Type II PIP5K activity (Jenkins et al. 1994). Both mammalian isoforms of PLD interact with PIP5Kβ and recruit it to vesicular compartments (Divecha et al. 2000). PLD1 is directly regulated by the small GTPases Arf and Rho. PLD derived PA is essential for maintenance of the Golgi and endo- and exo-cytic machinery (Aikawa and Martin 2003; Krauss et al. 2003; Donaldson and Honda 2005).

The Arf family has six different members (Munro 2005) and most Arfs have been found to regulate PIP5K activity (Santarius et al. 2006). Arf1 and Arf6 in particular have been shown to regulate membrane traffic and actin dynamics. While PIP_2 is thought of as a PM marker, PIP_2 may also be important for trafficking from the Golgi, since Arf1 recruits PIP5K, as well as PI4KIIIβ, a kinase that synthesizes PI4P (a PIP5K substrate) to the Golgi (Godi et al. 1999; Jones et al. 2000). Interestingly, the mechanism of Arf GEF, Arno's activation of Arf is dependent on recruitment to this GEF to membranes by PIP_2 binding (Paris et al. 1997). Because Arf proteins are capable of activating both PIP5K and PLD, while Arf activators are activated by PIP_2, this suggest a form of positive feedback regulation of Arf .

The first clues that there is a relation between Rho and PIP5K followed the discovery that Rho regulates FA assembly and actin stress fiber formation in response to PDGF stimulation (Ridley and Hall 1992). Since PDGF treatment also stimulates PIP_2

synthesis, it was concluded that Rho and PIP5K are in the same signaling pathway (Chong et al. 1994b). Treatment of cells with C3 exozyme, a Rho inhibitor lead to a decrease in PIP5K activity and subsequent decrease in IP_3 production and calcium mobilization. Furthermore, addition of activated RhoA to cell extracts stimulated PIP5K activity. These results established Rho as a *bona fide* PIP5K regulator.

Rac has also been implicated as a regulator of PIP5K. Expression of a constitutively active Rac mutant induces PIP_2-mediated actin polymerization (Hartwig et al. 1995) Furthermore, expression of PIP5Kβ induced the same actin polymerization phenotype as overexpressing Rac (Tolias et al. 2000). Rac directly binds and activates all PIP5K isoform in human embryonic kidney epithelial (HEK) cells (Weernink et al. 2004a). In addition, expression of a constitutively active Rac leads to both PIP5K membrane localization as well as an increase in PIP5K activity (Chatah and Abrams 2001).

Cdc42 is believed to regulate localized actin polymerization at filopodia (Kozma et al. 1995). This is presumably through the indirect activation of PIP5K activity (Weernink et al. 2004a). Cdc42 stimulates localized PIP_2 production that in turn recruits N-WASP and subsequent Arp2/3 activation. In addition, recent evidence suggest that PIP_2 recruits Cdc42 to the apical membrane where it regulates polarized epithelial development (Martin-Belmonte et al. 2007; Martin-Belmonte and Mostov 2007).

1.9 REGULATION OF PIP5Ks BY POST-TRANSLATIONAL MODIFICATIONS

PIP5Ks can also be regulated by phosphorylation. All PIP5K isoforms are ser/thr phosphorylated through autophosphorylation as well as by other conventional protein kinases. One study reports that ser/thr phosphorylation inhibits the lipid kinase activity of PIP5Ks while dephosphorylation by PP1 phosphatase *in vitro* increases activity (Itoh et al. 2000). Phosphorylation was performed *in vitro* and the physiological relevance is unknown. The same group found that the cAMP dependent protein kinase A (PKA) phosphorylates PIP5Kβ on ser 214 which is located within the kinase core (Park et al. 2001b). I confirmed that PIP5Ks are activated by ser/thr dephosphorylation and found that hypertonic stress increases cellular PIP_2 by activating PIP5Kβ through ser/thr dephosphorylation. However, I was not able to confirm that ser 214 was the site of phosphorylation. I have dedicated portions of my work towards understanding how phosphorylation regulates PIP5K activity and this is described in Chapters 2 and 3.

PIP5Ks are also regulated by tyrosine phosphorylation. Ling and colleagues found that the tyrosine kinase, Src phosphorylates PIP5Kγ90 on its C-terminal tail (Ling et al. 2003). This phosphorylation increases PIP5Kγ90's activity and promotes recruitment of talin into focal adhesions (FA). Another study finds that H_2O_2, which decreases PIP_2 and induces apoptosis, increases PIP5Kβ tyrosine phosphorylation and detachment from the PM (Halstead et al. 2006). The drop in PIP_2 is believed to induce apoptosis since overexpression of PIP5Kβ decreases the number of apoptotic cells. This study also found that H_2O_2 treatment decreases overall PIP5K activity, but did not

determine if all PIP5K isoforms are similarly affected (Rumenapp et al. 1998; Mesaeli et al. 2000). My work will seek to expand on these observations by identifying the PIP5K isoform that is deactivated during oxidative stress as well as the signaling cascade that regulates PIP5K activity.

1.10-1.12 PHYSIOLOGICAL ROLES OF PIP_2

1.10 ROLE OF PIP_2 IN IP_3 SIGNALING

The earliest role discovered for PIP_2 was as a target of PLC. PIP_2 cleavage by PLC produced two secondary signaling molecues, IP_3 and DAG, which were shown to activate calcium release from intracellular storage sites and activate protein kinase C (PKC), respectively (Michell 2008).

The PLC family is divided up into four subfamilies, named β, γ, δ and ε. PLC binds to PIP_2 through its PH domain and is activated by GPCRs or tyrosine kinases. Each PLC isoform is activated by a specific stimulus (Cockroft 2000). Just as specific PLC isoforms perform specialized functions, it is no surprise then that specific PIP5Ks produce distinct pools of PIP_2 that are substrates for PLC cleavage. Our laboratory found evidence of this by using RNAi to knockdown specific isoforms of PIP5Ksβ (Wang et al. 2004). Hela cells were stimulated with histamine, which binds a GPCR, to activate PLCβ and generate IP_3 production. The IP_3 response was blocked when PIP5Kγ. but not the other isoforms, was knocked down. Paradoxically, PIP5Kγ RNAi did not decrease total PIP_2. These results raise the possibility that PIP5Kγ generates a specialized pool of

PIP_2 that is used by PLCβ. Recently, PLC signaling from within the nucleus has been described in the literature (Bunce et al. 2006). Because PIP5Kα is the only known PIP5K localized to the nucleus, it is conceivable that this kinase generates a specific pool of PIP_2 for nuclear IP_3 signaling.

1.11 PIP_2 REGULATES MEMBRANE TRAFFIC

PIP_2 is particularly abundant at the PM and is therefore proposed to be a PM organelle marker. Studies using GFP-PLCδ PH (which binds PIP_2) have found that PIP_2 is concentrated at sites of endocytosis, exocytosis and phagocytosis. PIP_2 plays an integral role in mediating membrane trafficking (Martin 2001; Scott et al. 2005). It recruits many endocytic proteins to the PM through their PIP_2 binding PH motifs (Lemmon 2008). These include adaptor protein 2 (AP2), AP-180, epsin, and dynamin. Functional studies confirm that the formation of clathrin coated vesicles is a PIP_2 dependent process. Depletion of PIP_2 specifically at the PM by an inducible PM localized inositol 5-phosphatase blocks endocytosis through the inability of dynamin to be recruited to the sites of endocytosis (Zoncu et al. 2007). There is evidence in HeLa cells that PIP5Kβ is required for the receptor-mediated endocytosis of transferrin . Overexpression of PIP5Kβ increases transferrin uptake and the number of clathrin coated pits at the PM (Padron et al. 2003b). RNAi of PIP5Kβ blocked transferrin uptake. On the other hand, another isoform, PIP5Kγ, has been implicated in neurotransmitter trafficking in synapses (Nakano-Kobayashi et al. 2007). *In vitro* studies using brain

membrane extract and brain cytosol to form clathrin-coated pits found PIP5Kγ in the proximity of these structures (Wenk et al. 2001).

Arf stimulated PIP_2 production is implicated as a regulator of membrane traffic as well. ARF6 regulates both PLD and PIP5Kβ at the PM leading to clathrin-mediated endocytosis (Honda et al. 1999). Arf6 and PIP_2 act synergistically to form clathrin coated vesicles in synaptic preparations and recruit AP2 onto liposomes *in vitro*, directly implicating PIP_2 as a mediator of endocytosis. Expression of an Arf GEF, EFA6, induces the formation of PIP_2-positive membrane protrusions and endosomal structures. Similarly, expression of a constitutively active Arf6 mutant causes PIP_2 rich vesicles to accumulate in the recycling endosome compartment (Brown et al. 2001).

The balance of PIP_2 levels is tightly regulated by synthesis and degradation/hydrolysis. Like PIP_2 generation, PIP_2 hydrolysis is equally complex and many phosphatases have been implicated. Synaptojanin and OCRL are the two well characterized 5' phosphatases that dephosphorylates PIP_2 to generate PI4P. Their mutation has been linked to several diseases (Voronov et al. 2008). Synptojanin is primarily PM localzed and its mutation has been linked to bipolar disorder in humans (Halstead et al. 2005). Knockdown of synaptojanin, a 5' phosphatase, leads to neurological defects most likely due to changes in vesicle recycling. Synaptojanin$^{-/-}$ neurons exhibit a delay in vesicle recycling as well as impaired clathrin shedding from budding vesicles (McPherson et al. 1996). Recently, synaptojanin has been implicated as a factor contributing to Down's Syndrome. Increasing its expression through gene duplication leads to decreased PIP_2 and Down Syndrome like symptoms in mice (Voronov et al. 2008) OCRL is implicated as a regulator of membrane traffic through its

localization on the Golgi, endosomes and clathrin-positive intracellular structures (Dressman et al. 2000; Choudhury et al. 2005; Hyvola et al. 2006). OCRL is reported to be localized at early endosomes and clathrin-coated endocytic pits as well (Erdmann et al. 2007). OCRL is believed to interact with components that regulate signaling and sorting of cell surface receptors. Mutations in the OCRL gene leads to mental retardation and renal Fanconi syndrome presumably by altering membrane trafficking (Lowe 2005).

1.12 PIP_2 REGULATION OF ACTIN

The first observations connecting PIP_2 to actin regulation was the finding that PIP_2 induces the dissociation of profilin from actin to increase actin polymerization (Lassing and Lindberg 1985). Much work of the last two decades has focused on *in vitro* assays showing PPI binding to actin regulating proteins. Recently, a wealth of information linking PIP_2 to actin in the cellular context has established PIP_2 as a focal point of cytoskeletal organization.

PIP_2 directly regulates three types of actin-binding proteins that affect filament length: actin sequestering, filament severing, and capping proteins (Cockroft 2000). These proteins bind to PIP_2 through positively charged residues that often do not have a recognizable PH domain with a high degree of specificity. Profilin, an actin sequestering protein, is dissociated from actin by PIP_2, allowing actin to polymerize at the barbed end of the filament. Gelsolin, an actin severing protein that is attached to the "barbed" end of the severed filament, is dissociated from filament end by PIP_2 (Lin et al. 1997; McGough et al. 2003). CapZ and CapG, actin barbed end capping proteins, are dissociated from the actin by PIP_2. Cofilin, a weak actin severing protein and an actin monomer binding

protein, is also inhibited by PIP_2. Cofilin disassembles F-actin from the rear of the actin network which allows the actin monomers to recycle to the leading edge of the site of actin polymerization (Huang et al. 2006). (DesMarais et al. 2005).

PIP_2 also regulates membrane adhesion complexes which link the actin cytoskeleton to the extracellular matrix (Sheetz et al. 2006). Many proteins that are found in these complexes directly bind PIP_2. Vinclulin, a FA protein, is activated by PIP_2 which dissociates the intramolecular interactions between its head and tail to expose the ligand binding region (Gilmore and Burridge 1996). The ERM (ezrin, moesin, and radixin) family of membrane adhesion proteins maintain cell shape, adhesion and migration through either indirectly scaffolding proteins attached to transmembrane proteins or directly binding cytoplasmic tails to actin (Fievet et al. 2007). PIP_2 recruits the ERM proteins to the membrane and allows their subsequent association with other proteins and actin binding.

Studies manipulating levels of PIP_2 in the cell illustrate a cause and effect relationship between PPIs and the actin cytoskeleton. Overexpression of PIP5Kβ induces actin stress-fiber formation in several different cell types (Yamamoto et al. 2001; Auvinen et al. 2007). Stress-fiber formation is dependent on the activity of RhoA and Rac1, which regulate the activity of PIP_2 interacting proteins (Oude Weernink et al. 2004) and PIP5Ks. Forced overexpression of PIP5Kβ has also been linked to the formation of actin comets (Rozelle et al. 2000), which propel vesicles during trafficking events. These comet structures are formed by actin nucleation in the WASP:Arp2/3 pathway. PIP_2 is an essential regulator of WASP (Rohatgi et al. 2000) and WASP contains a non-canonical PIP_2 binding domain. The exact PIP5K that regulates WASP

activity is unclear but two PIP5Ks have been implicated. PIP5Kβ overexpression leads to actin comet formation in a WASP-dependent manner (Rozelle et al. 2000). Pessin and colleagues found that WASP regulates the trafficking of GLUT4 containing vesicles to the PM of adipocyes (Kanzaki et al. 2004).

PIP5Kα also seems to play other roles in actin polymerization and vesicle recycling. Overexpression of PIP5Kα or a constitutively active Arf6 mutant (which activates PIP5Ks) increases PIP_2 and actin on vacuolar structures that are unable to recycle back to the PM (Brown et al. 2001). This is presumably due to the actin being unable to depolymerize, thus immobilizing the vesicles in the intracellular compartment. The role of PIP5Kβ in actin polymerization seems to be cell specific. Expression in neuronal cells leads to neurite retraction through the loss of vinculin (van Horck et al. 2002). The expression of wt PIP5Kβ led to the loss of vinculin and neurite retraction while expression of a dominant negative PIP5Kβ induced neurite elongation. Overexpression in Cos-7 cells leads to formation of pine needle-like actin structures while expression in CV1 cells produces robust stress fiber formation (Shibasaki et al. 1997; Yamamoto et al. 2001).

Deletion studies from yeast and *drosophila* further support PIP5K's role in regulating actin. Deletion of the only known PIP5K gene in *S. cerevisiae*, MSS4, leads to abnormal polarized actin distribution (Desrivieres et al. 1998). In addition, unlike wt yeast cells, which are able to reform normal actin morphology after heat shock, the mutant yeast cannot reorganize its actin cytoskeleton after heat shock.

1.13 KNOCKOUT STUDIES DESCRIBE ISOFORM SPECIFIC ROLES FOR PIP5Ks

The existence of multiple PIP5K genes suggests a specialized role for each kinase. This is supported by the presence of unique sequences outside of their kinase core domain and distinct localization in cells. The possibility of isoform specific functions is confirmed by RNAi and, more recently by gene knockout.

The PIP5Kβ knockout mouse (human terminology, mouse PIP5Kα) develops normally and shows no reproductive abnormalities (Sasaki et al. 2005). The only known phenotype is defects in mast cell degranulation resulting in increased systemic anaphylactic response. PIP5Kβ$^{-/-}$ bone marrow mast cells (BMMC) degranulate excessively in response to IgE stimulation and this is due to a decrease in the cortical actin barrier that normally negatively regulates exocytsosis.

The PIP5Kγ$^{-/-}$ mouse (w/o all PIP5K splice variants) dies shortly after birth due to a suckling defect indicating a neurological abnormality (Di Paolo et al. 2004). The cultured neurons from these mice exhibit defects in synaptic transmission and vesicle trafficking. Because PIP5kγ90 is highly expressed in the neuronal synapse, this is not completely unexpected. The PIP5Kγ$^{-/-}$ cells also show a smaller pool of recycling vesicles as well as slower rates of endocytosis implicating the PIP_2 generated by this kinase in these events. Another study showed that PIP5Kγ$^{-/-}$ megakaryocytes have a defect in the attachment of the actin cytoskeleton to the PM (Wang et al. 2008)

The PIP5Kα$^{-/-}$ (human designation, mouse PIP5Kβ) has been generated by C. Abrams (personal communication) the manuscript is not yet published.

1.14 PIP_2-CONTAINING LIPID RAFTS

It is believed that in addition to even distribution of PPIs in membranes, there are also specialized PM PIP_2 pools that are believed to be functionally defined for specific purposes. Some of these specialized pools may be located in "lipid rafts" that are enriched in cholesterol and basic proteins such as GAP43, MARCKS, and CAP23 (Laux et al. 2000; Golub and Caroni 2005; McLaughlin and Murray 2005). These proteins contain lipid binding domains and are believed to sequester PIP_2 in a specialized region.

Bruton tyrosine kinase (Btk) has been implicated in regulating PIP_2 generation in lipid rafts. Btk associates with all isoforms of PIP5K through its PH domain (Carpenter 2004) and it shuttles PIP5Kβ to the PM in response to B cell receptor activation. The PIP_2 in these lipid rafts are essential for PLCγ mediated hydrolysis as well as for PIP_3 synthesis. PIP_2 containing lipid rafts are also implicated in microtubule-based cell motility and alpha-adrenergic receptor signaling (Golub and Caroni 2005; Morris et al. 2006).

1.15 PHOSPHATIDYLINOSITOL 4 KINASES

Phosphatidylinositol 4 phosphate (PI4P) is the obligatory substrate for type I PIP5Ks and is therefore a key player in PPI homeostasis. PI4P is synthesized by phosphatidylinositol 4-kinases (PI4K), which add a phosphate to the D4 position of phosphatidylinositol. Four different PI4Ks have been identified in mammals and three

have been discovered in yeast (Balla and Balla 2006). The PI4Ks are divided into two families, named type II and III based on their sequence homology and differential sensitivity to inhibitors (Table 1.2) Wortmannin, a fungal metabolite that inhibits PI3K at low doses, inhibits type III PI4Ks at higher doses but not type II PI4Ks (Heilmeyer et al. 2003). Type II PI4Ks is inhibited by adenosine in vitro.

Table 1.2-Summary of PI4Ks and inhibitor sensitivity.

	MW (kDa)	Wortmannin	(Ki) Adenosine	Yeast homolog
PI4KIIα	56	Insensitive	10-70 μM	Lsb6p
PI4KIIβ	56	Insensitive	10-70 μM	Lsb6p
PI4KIIIα	230	IC_{50} 50-300 nm	Millimolar	Stt4p
PI4KIIIβ	110	IC_{50} 50-300 nm	Millimolar	Pik1p

Modified from Balla and Balla, 2006.

Deletion studies from yeast find that their type III PI4Ks have non-redundant roles and distinct membrane localization. These results suggest that PI4P, like PIP_2, is likely to be partitioned into specialized pools. In addition, proteins with PI4P-selective lipid binding domains have been identified, suggesting that PI4P may have important roles, in addition to simply being a substrate for PIP5Ks (Carlton and Cullen 2005).

1.16 TYPE III PI4Ks

The type III PI4Ks have two isoforms. In mammals, they are called PI4KIIIα (230 kDa) and PI4KIIIβ (110 kDa). Genetic studies with yeast have highlighted their

isoform specific functions. Stt4p, the yeast PI4KIIIα, is localized at the PM where it supports PIP_2 production (Audhya and Emr 2002a). PIP_2 in yeast is necessary for signaling, endocytosis and the recruitment of Spo14p, the yeast PLD enzyme to the PM (Routt et al. 2005).

However, unlike in mammals, PIP_2 is not necessary for calcium signaling due to the lack of IP_3 receptors in this organism (Perera et al. 2004). Interestingly, while Stt4p is localized in the PM in yeast, PI4KIIIα appears to have a different localization in mammals. Immunofluorescence studies show that most of the PI4KIIIα is localized in the ER, pericentriolar area over the Golgi and the nucleolus (Nakagawa et al. 1996; Wong et al. 1997; Heilmeyer et al. 2003) although biochemical partitioning shows an enrichment at the plasma membrane (Ekblad and Jergil 2001). Regardless, there is evidence to suggest that PI4KIIIα functions at the PM (Balla et al. 2008). This is because wortmannin inhibits GPCR-mediated generation of IP_3 (Nakanishi et al. 1995) , and another inhibitor, phenylarsine oxide (PAO) preferentially inhibits PI4KIIIα but not the other PI4Ks also blocks signal generation (Balla et al. 2008).

Pik1p, the yeast PI4KIIIβ supplies 50% of total PI4P in yeast (Audhya et al. 2000). Arf1 was shown to mediate the recruitment of Pik1p to the Golgi (Walch-Solimena and Novick 1999). In the same study expression of a temperature sensitive allele of Pik1p lead to defects in the actin cytoskeleton and multinucleation suggesting a role in cytokinesis. The Rab-GTPase Ypt1p (Rab11 homolog) was identified in a screen to interact with Pik1p. It is believed that Pik1p is upstream of these GTPases and regulates their activity (Sciorra et al. 2005). As in yeast, the mammalian PI4KIIIβ has been shown to regulate Golgi to PM trafficking. It is recruited to the Golgi by Arf1-GTP

and is subject to multiple regulations. These include interaction with Frq1p and post-translational modification by the Golgi-associated PKDs.

PI4KIIIβ binds calcium binding protein, NCS-1 (Rajebhosale et al. 2003). This association enhances PI4KIIIβ activity, which in turn upregulates exocytosis of secreted proteins including insulin (Gromada et al. 2005). Studies in Madin-Darby canine kidney (MDCK) cells implicate PI4KIIIβ as key player in polarized vesicle trafficking (Bruns et al. 2002) because overexpression of a kinase dead PI4KIIIβ inhibits delivery of VSV-G to the basolateral membrane. PI4KIIIβ is phosphorylated on several sites including ser268 which is a target of PKD (Hausser et al. 2005). This phosphorylation is believed to occur at the Golgi. Phosphorylation at this site positively regulates its lipid kinase activity as well as transport of VSV-G, further lending credence to the notion PI4KIIIβ regulates secretion.

1.17 TYPE II PI4Ks

The Type II PI4K family is characterized by their sensitivity to adenosine in vitro (Balla and Balla 2006). The relative contribution of the type II PI4Ks differs between mammals and yeast. Lsb6p, the yeast equivalent of the type II PI4Ks contributes very little to the overall levels of PI4P (Shelton et al. 2003; Wang et al. 2003) however, its activity is important for regulating endosome motility (Chang et al. 2005). Mammalian PI4KIIα and β share a high degree of homology with each having a molecular weight of about 55kDa. Both type II PI4Ks are palmitoylated causing them to have a high degree of membrane association (Barylko et al. 2001; Jung et al. 2008). PI4KIIβ is localized to

endosomes and has a higher degree of cytosolic staining suggesting a difference in steady-state palmitoylation compared to PI4KIIα (Balla et al. 2002; Wei et al. 2002). PI4KIIα shows a strong Trans-Golgi Network (TGN) localization by immunoflouresence and evidence of ER localization by biochemical partitioning.(Wang et al. 2003; Waugh et al. 2003). Although neither kinases are found at high level at the PM, there is biochemical evidence suggesting that they contribute to the PIP_2 pool at the PM (Pike 1992). Additionally, PDGF stimulation leads to Rac mediated PI4KIIβ PM recruitment (Wei et al. 2002). Recent evidence finds membrane bound PI4KIIβ is phosphorylated at a ser residue but this does not apparently affect its localization or activity (Jung et al. 2008).

Emerging evidence suggests that PI4P acts as an organelle marker for the Golgi. A seminal finding by our lab showed that PI4KIIα supplies the PI4P necessary for AP-1 recruitment to the Golgi (Wang et al. 2003). This establishes PI4KIIα as an essential mediator of Golgi to PM trafficking. Recently, it was also discovered that PI4KIIα is necessary for the recruitment of AP-3 to late endosomes as well (Salazar et al. 2005).

PI4P is emerging as an integral signaling molecule in its own right and much data elucidating its role in cellular physiology remains to be obtained. For example, much less is known about the regulation of type II PI4Ks than type III PI4Ks. I found that oxidative stress increases PI4P not simply because of decrease in PIP_2 synthesis. These experiments will be described in chapter 3 and 4.

1.18 STATEMENT OF PURPOSE

The evidence linking disruption of PPI homeostasis to oxidative and hypertonic stress is compelling. Despite previous observations made by several other laboratories, the exact mechanism underlying these changes remains unknown. The goal of my work has been to elucidate the regulation of PIP5Ks during hypertonic and oxidative stress.

I started with the observation from our laboratory that hypertonicity and oxidative stress have opposing effects on PIP_2 levels in cells. I set out to examine how hypertonicity increases PIP_2 synthesis. Is this a PIP5K dependent process? If so, how is PIP5K regulated by this stimulus. The second part sought to understand the basis for the oxidative stress induced drop in PIP_2. I wanted to understand why PIP_2 was decreased by this stress. Is PIP5K inhibited during this event? If PIP5K is indeed affected, what were the signaling factors responsible for regulating PIP5K. In addition, I wanted to gain a deeper understanding of PIP5K post-translational regulation as well as isoform specific roles of PIP5Ks. In the process of answering these questions, I discovered novel forms of regulation of the PIP5Kβ isoform. Using cell biology, genetic, biochemical and pharmacological techniques, I identified the kinases and phosphatases that regulate these processes. The discoveries discussed in this body of work open up a new avenue in the understanding of PIP5K biology and may have therapeutic relevance.

CHAPTER TWO

HYPERTONIC STRESS INCREASES PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE LEVELS BY ACTIVATING PIP5Kβ

2.1 INTRODUCTION

The first clues hinting at a link between osmotic stress and regulation of PIP5K was found when it was discovered that Na/H and Na/Ca exchangers, ion channels that are sensitive to volume changes, were regulated by PIP_2 (Gillis et al. 2001). It was then found that osmotic stress led to increased levels of PIP_2 in isolated cardiac myocytes (Nasuhoglu et al. 2002b). Similar results were obtained with several other types of cells (Pedersen et al. 2001). This led me to examine the underlying mechanism of action leading to an increase in PIP_2 levels. Was this due to an increase in PIP5K activity? If so, was there a preferential activation of a specific PIP5K isoform.

In this chapter I show that PIP_2 levels increased rapidly in HeLa cells during hypertonic treatment. Depletion of the PIP5Kβ by RNA interference (RNAi) impaired both the PIP_2 and actin cytoskeletal responses. PIP5Kβ was recruited to membranes and was activated by hypertonic stress through ser/thr dephosphorylation. Calyculin A (caly A), a protein phosphatase 1 inhibitor, blocked the hypertonicity-induced PIP5Kβ dephosphorylation/activation as well as PIP_2 increase in cells. Urea, which raises osmolarity without inducing cell shrinkage, did not promote dephosphorylation nor increase PIP_2 levels. Disruption or stabilization of the actin cytoskeleton, or inhibition of the Rho kinase, neither blocked the PIP_2 increase nor PIP5Kβ dephosphorylation.

Therefore, PIP5KIβ is dephosphorylated in a volume-dependent manner by a caly A-sensitive protein phosphatase, which is activated upstream of actin remodeling and independently of Rho kinase activation. These results establish a cause-and-effect relation between PIP5KIβ dephosphorylation, lipid kinase activation and PIP_2 increase in cells. This PIP_2 increase can orchestrate multiple downstream responses, including the reorganization of the actin cytoskeleton.

The experiments in this section were performed by myself, two former postdoctoral fellows, Masasya Yamamoto, Yongjie Wei, and a research assistant, Manuel Martinez.

2.2 HYPERTONIC STRESS INCREASES PIP_2 LEVELS IN HELA CELLS

When HeLa cells were exposed to 250 mM sucrose for 10 min., ^{32}P-PIP_2 level increased to a greater extent than ^{32}P-PI(4)P (Fig. 2.1A and Table 2.1). Likewise, addition of 100 mM NaCl to normal culture medium also increased PIP_2 levels to a much higher extent than PI4P (Table 2.1). Therefore, hypertonic stress induced by these two stimuli preferentially increases PIP_2 level.

The increase in phosphoinositide levels was evident within 2 min. of sucrose stimulation, and reached a maximum by 10 min (Fig. 2.1A). While there was a subsequent slow decline, PIP_2 was still above prestimulation levels at 20 min. The rapid increase in ^{32}P-PIP_2 and ^{32}P-PI4P suggests that the change in phosphoinositide turnover/biosynthesis is an early response to hypertonic stimulation.

HPLC, which measures the amount of each lipid and can discern between the different types of phosphoinositides more clearly than the TLC method using standards

mixed in the reaction (Nasuhoglu et al. 2002a), confirmed that there was a 2-fold increase in PIP_2 (Fig. 2.1B and Table 1). Unexpectedly, there was almost no change in PI4P mass, in spite of a modest increase in its labeling by ^{32}P (Fig. 2.1A). No new peak corresponding to either PI3P, $PI(3,4)P_2$ or $PI(3,4,5)P_3$ was detected (Fig. 2.1B). The HeLa cell response profile establishes that PIP_2 is increased selectively. This increase is most likely to be due to the direct activation of PIP5KIs and/or inactivation of PIP_2 phosphatsases, and is unlikely to be due to increased availability of the PI4P substrate.

The sites of PIP_2 increase were identified by immunofluorescence microscopy. As shown previously (Wang et al. 2004), the PIP_2 antibody stained small punctae that lined the PM (PM pool) and a perinuclear region (internal organelle pool) (Fig. 2.1C). Hypertonic treatment increased anti-PIP_2 staining intensity, especially in large punctae that line the plasma membrane. Some of these punctae were at the tips of retraction fibers that were formed when the cell shrunk (see Fig. 2.2B). There is much controversy as to the location of PIP_2 within the cell. Indirect labeling experiments using PLCγ-PH domain indicates PIP_2 is exclusively localized at the plasma membrane while other studies have found a non-redundant role for the PIP_2 phosphatase, OCRL which is localized at the Golgi. Furthermore, PIP5Kα is reported to be located within the nucleus. This argues for the existence of PIP_2 at internal membranes. The PIP_2 antibody labeling suggests PIP_2 is indeed localized at other compartments besides the plasma membrane.

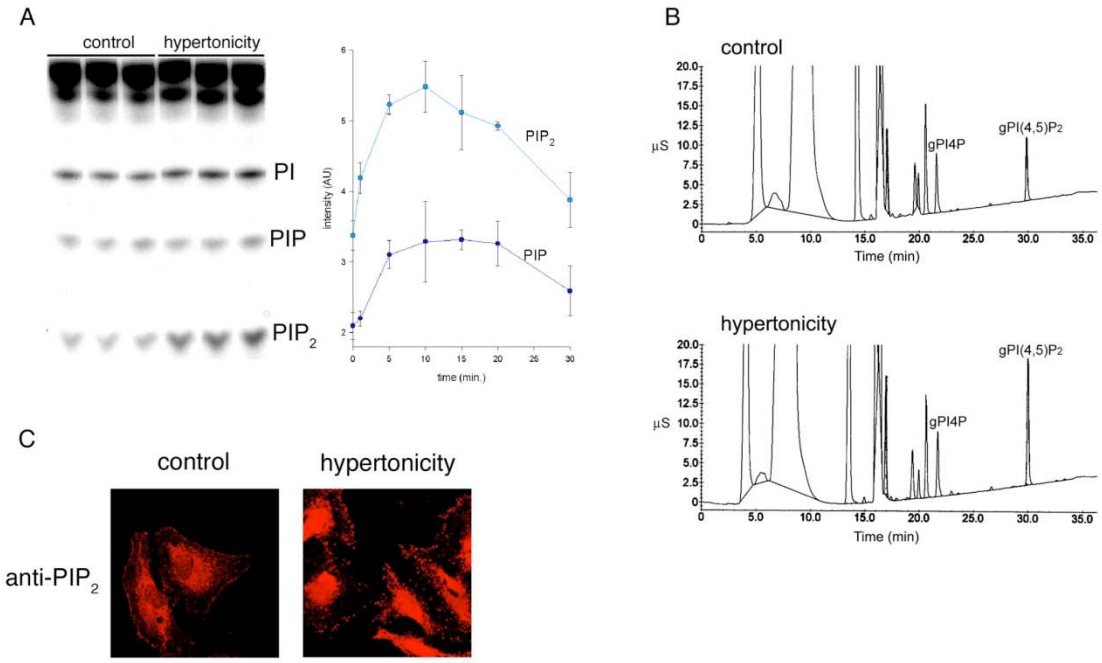


Figure 2.1.

Effects of hypertonic stress on PIP_2- HeLa cells were exposed to 250 mM sucrose for 10 min. and their phosphoinositides were analyzed. (A). TLC. HeLa cells were labeled with ^{32}P for 4 h and exposed to sucrose. Left panel, triplicate samples from a single experiment are shown. Data are representative of four independent experiments. ^{32}P-PIP_2 and ^{32}P-PI4P levels increased to 180±10% (n=8) and 121±10% (n=8) of control level, respectively. Right panel, time course of ^{32}P incorporation into PIP_2 and PI4P. The amount of ^{32}P, expressed in arbitrary intensity units, is shown as mean±SEM from three independent experiments, each done in triplicate. (B) HPLC quantitation. Phospholipids were deacylated and negatively charged glycerol head groups were eluted and detected online by suppressed conductivity (µS, microsiemen units). Glycerol-PIP_2 levels increased to 219±33% of control value (n=4). Results are from a single experiment, and are representative of three independent experiments. (C) PIP_2 distribution as detected by anti-PIP_2, with and without a 10 min. sucrose treatment.

Table 2.1. Effects of Osmotic Stress on Phosphatidylinositol Lipids

	Lipid Mass* (percent of isotonic control)			^{32}P Lipids** (percent of isotonic control)		
Treatment	PI	PI4P	PIP_2	PI	PI4P	PIP_2
250 mM sucrose	105±2 (n=4)	93±17 (n=4)	219±33 (n=4)	113±4 (n=3)	121±10(n=8)	180±10 (n=8)
100 mM NaCl	ND	ND	ND	128±7 (n=3)	151±18 (n=3)	253±48 (n=3)

* from HPLC analyses. ** from phosphorimager analyses of TLC data. Data are mean ± SEM, and expressed as percent of isotonic control. ND, not determined.

2.3 PIP5Kβ DEPLETION ATTENUATES THE HYPERTONICITY INDUCED PIP_2 AND ACTIN RESPONSES

Small interfering RNA oligos (siRNA) were used to identify if and which PIP5K isoform is primarily responsible for the hypertonic stress induced PIP_2 increase. As reported previously, PIP5Kβ depletion by RNAi (Padron et al. 2003a; Wang et al. 2004) decreased the basal ^{32}P-PIP_2 level to the greatest extent compared with depletion of the other PIP5K isoforms (Fig. 2.2A). PIP5Kβ completely blocked the hypertonicity-induced PIP_2 increase, while depletion of the other PIP5KIs had much less effect. These results showed that PIP5Kβ accounts for most of the hypertonicity induced PIP_2 increase. Therefore I focused on the behavior of PIP5Kβ the remainder of this section.

HeLa cells normally have long actin stress fibers and cortical actin filaments. After hypertonic stimulation, the stress fibers became thicker and retraction fibers were formed at the cell periphery as the cell shrunk (Fig. 2.2B). An increase in stress fibers and polymerized actin were confirmed biochemically by isolating the Triton-X100 insoluble cytoskeleton (Fig. 2.2C). The amount of actin in the Triton-insoluble low speed pellet

(LSP)(Yamamoto et al. 2001), which contains crosslinked actin filaments such as stress fibers, and the Triton-insoluble high speed pellet (HSP), which contains long actin filaments that are not crosslinked sufficiently to be sedimented by centrifugation were increased, while actin in the high speed supernatant (representing actin monomers and small oligomers) decreased. Therefore, hypertonicity promotes actin polymerization and crosslinking into stress fibers and/or networks.

PIP5Kβ depletion dramatically changed the cell shape and decreased the amount of stress fibers (Fig. 2.2B). Although hypertonic stress shrunk the PIP5Kβ RNAi-treated cells, it did not promote stress fiber formation. Since PIP5Kβ depletion blocked the actin polymerization/reorganization response, PIP5Kβ plays a major role in orchestrating the hypertonic stress fiber response.

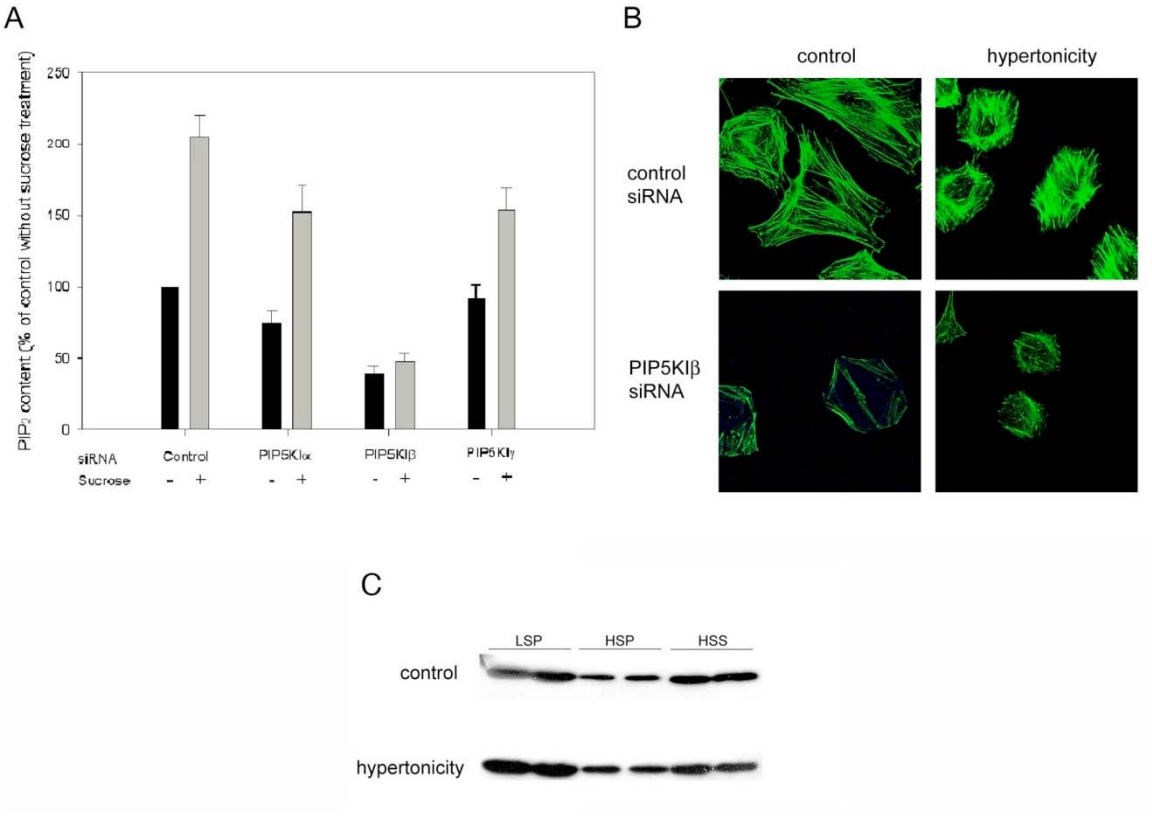


Figure 2.2

PIP5K RNAi inhibits the hypertonic PIP_2 and actin responses. (A) HeLa cells were transfected with siRNA oligos targeting the PIP5KIα, β and γ isoforms or an irrelevant sequence (control). Cells were stimulated with sucrose, and ^{32}P incorporation into PIP_2, was quantitated after TLC. Radioactivity of siRNA treated samples were expressed as a percentage of that from cells transfected with the control siRNA sample. Data is mean±SEM of five independent experiments. (B) Phalloidin actin staining. Control and PIP5Kβ RNAi cells with and without sucrose treatment were fixed, permeabilized and stained with FITC-phalloidin. (C). Partitioning of actin in Triton-soluble and -insoluble fractions. Two samples from each condition are shown, and the mean percentages of actin in each were calculated. The low speed pellets of control and sucrose-treated samples contained 23% and 33% of the total actin, respectively. The high-speed pellets of control and sucrose-treated samples were 12% and 19% of total, respectively.

2.4 PIP5Kβ IS RECRUITED TO MEMBRANES BY HYPERTONIC STRESS

Confocal immunofluorescence was next used to examine the effect of hypertonic stress on the subcellular distribution of PIP5Kβ. PIP5Kβ was both cytosolic and associated with plasma membrane and endomembranes under control conditions (Fig. 2D). Hypertonic stress increased the amount of PIP5Kβ at the cell periphery. Retraction fibers also contained PIP5Kβ. Previous studies using inducible PIP5Kβ shows a prominent plasma membrane localization under basal conditions. While some PIP5Kβ can be seen on the plasma membrane during our over expression studies, we do not see the same extent of association as these studies.

The increase in PIP5Kβ membrane association was confirmed by subcellular fractionation using two different methods. Centrifugation at 100,000xg showed that under isotonic conditions, approximately 45% of the kinase was recovered in the pellet (membranes) (Fig. 3A). HA-PIP5Kβ migrated as a doublet in both the supernatant (cytosol) and pellet, which, as will be shown later, was due to a difference in the extent of phosphorylation. Hypertonic stimulation collapsed the doublet into a single band, and increased the recovery of PIP5Kβ (70% of total) in the pellet fraction.

Multistep fractionation (Wei et al. 2002) provided additional information about the partitioning of PIP5Kβ among different organelle fractions (Fig. 3B). Under isotonic conditions, slightly less than half of the total PIP5Kβ was membrane associated, and of this, half was recovered in the plasma membrane (PM) enriched fraction (Fig. 3B). Sucrose stimulation decreased the percentage of PIP5Kβ in the cytosolic fraction by 60%, and almost doubled the percentage recovered in the PM and HSP fraction.

Therefore, the immunofluorescence and biochemical data both show that there is an increase PIP5Kβ membrane association.

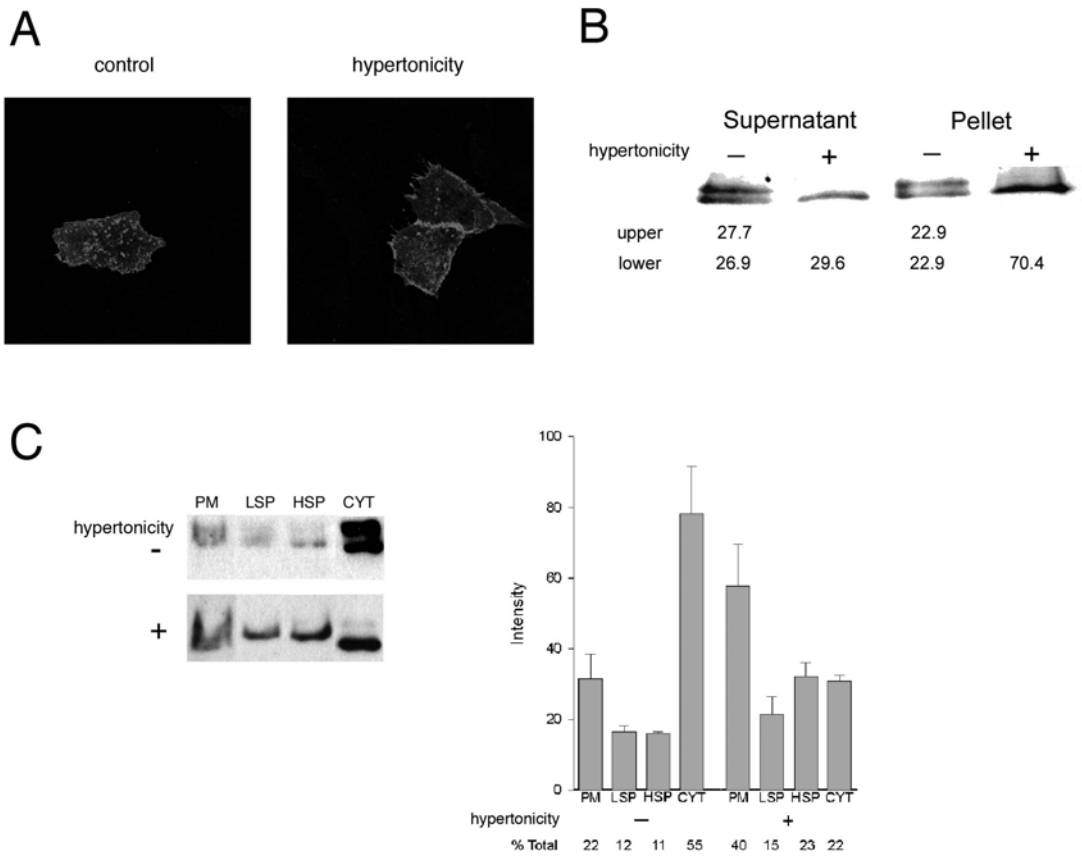


Figure 2.3

PIP5Kβ distribution in subcellular fractions. (A) HeLa cells overexpressing HA-PIP5Kβ were incubated with NaCl, and stained with anti-HA. (B) Cells overexpressing HA-PIP5Kβ at low levels were treated with sucrose and homogenized in the absence of detergents. Samples were electrophoresed in SDS-polyacrylamide gels and HA-PIP5Kβ was detected by Western blotting. The intensities of the bands were determined by quantitative densitometry. Partitioning between 100,000xg pellets and superanatants. The percentage of total HA-PIP5Kβ in each band of the HA-PIP5Kβ doublet is shown below the Western blot. (C). Multistep fractionation. A representative Western blot containing two samples for each condition is shown. The intensities of the bands (in arbitrary units) are plotted as mean±SEM of three different experiments. The percentage of total HA-PIP5Kβ in each fraction is indicated at the bottom.

2.5 HYPERTONIC STRESS DEPHOSPHORYLATES PIP5Kβ BUT NOT THE OTHER PIP5Ks

Park et al. (Park et al. 2001a) show that PIP5K isoforms are constitutively phosphorylated and that they can be activated by ser/thr dephosphorylation. Hypertonic stress collapsed the HA-PIP5Kβ doublet into a single band on SDS-polyacrylamide gels, which is consistent with dephosphorylation (Fig. 2.3B

). Dephosphorylation was confirmed by a decrease in ^{32}P labeling of the upper band in the doublet. Under isotonic conditions, both HA-PIP5Kβ bands were ^{32}P labeled, with the upper band being more phosphorylated versus the lower band (Fig. 2.4A). The ^{32}P intensity of the upper band decreased dramatically within 3 min. of sucrose treatment, while that of the lower band was not decreased to a similar extent. The results suggest that PIP5Kβ is constitutively phosphorylated at multiple sites, and that a subset of these sites is preferentially dephosphorylated as an early response to hypertonic stress. The time course of dephosphorylation of the upper band paralleled the rise in PIP_2 level (Fig. 2.1A), lending further support to the possibility that PIP5Kβ dephosphorylation increases PIP_2 levels in cells.

Unlike PIP5KIβ, neither PIP5Kα nor PIP5KIγ (both L and S variants) were dephosphorylated by hypertonic stimulation (Fig. 2.4B). PIP5Kβ is phosphorylated by Protein Kinase A on its S241 residue (Park et al. 2001a). However, HA-PIP5KβS241A mutant was dephosphorylated normally during hypertonic stimulation (data not shown). Therefore, S241 is not a major site of hypertonicity-induced dephosphorylation.

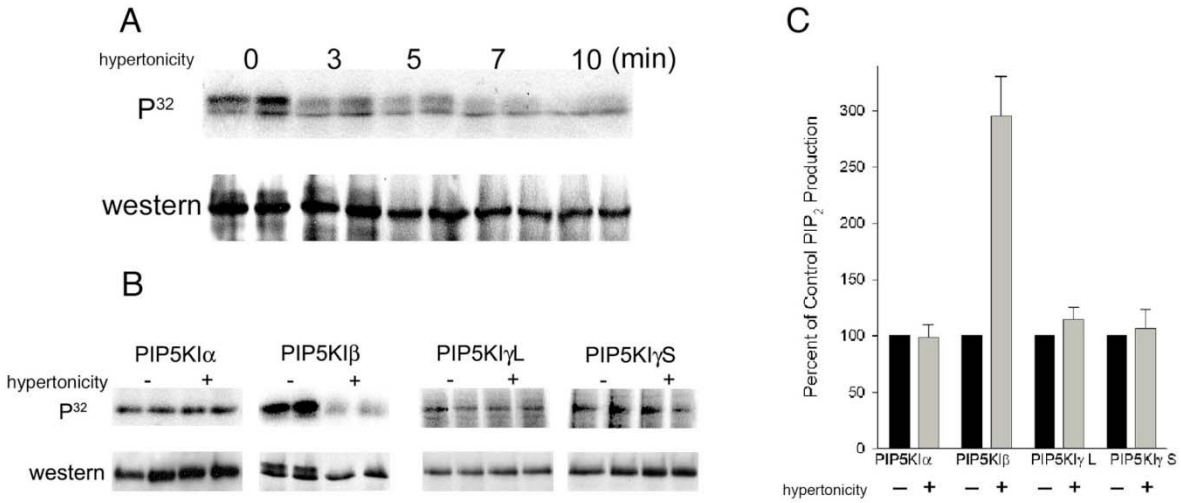


Figure 2.4

Hypertonic stress promotes the dephosphorylation of PIP5Kβ, but not the other PIP5Ks. (A) Time course of PIP5Kβ dephosphorylation. HeLa cells overexpressing HA-PIP5Kβ were labeled with ^{32}P and incubated with isotonic or hypertonic (250 mM sucrose) medium for the periods indicated. HA-PIP5Kβ was immunoprecipitated, and analyzed by Phosphorimager analysis and Western blotting. Duplicate samples are shown. (B). Phosphorylation of PIP5K isoforms. Experimental conditions were similar to that described above. (C). PIP5K activity. PIP5Ks were immunoprecipitated with anti-epitope antibodies, and used for *in vitro* lipid kinase assays and for western blotting. The amount of ^{32}P incorporated into PIP_2 was determined by Phosphorimager analysis after TLC. Kinase activity was normalized against the amount of immunoprecipitated PIP5Ks (based on Western blots, not shown) and the specific activity (^{32}P/protein) of sucrose-treated samples was expressed as percent of an equivalent sample from cells not exposed to hypertonic stress. Data were mean ± SEM of three different experiments, each done in duplicate.

2.6 EFFECTS OF PIP5Kβ DEPHOSPHORYLATION

The effect of hypertonic stress on PIP5Kβ's lipid kinase activity was examined using an *in vitro* lipid kinase assay. HA-PIP5Kβ immunoprecipitated from hypertonically stressed cells had three times higher lipid kinase activity than those from the unstimulated control, while the activities of PIP5Kα, γL and γS were not significantly altered (Fig. 2.4C). Furthermore, the relative activity of α and γ were much lower than that of PIP5Kβ. This establishes unequivocally PIP5Kβ contributes the major pool of PIP_2 and is selectively activated during hypertonic stress.

The next step was to examine the effect of inhibiting ser/thr protein phosphatases on the hypertonic response. The following ser/thr phosphatase inhibitors were tested: caly A inhibits PP1 and PP2A; Okadaic acid inhibits PP2A at 1-10 nm concentrations (IC_{50} 0.51 nM) and PP1 at higher concentrations (IC_{50} 42 nM).

Caly A increased the intensity of ^{32}P label in the upper band of the PIP5Kβ doublet under isotonic conditions and blocked the sucrose-induced dephosphorylation (Fig. 2.5A). Okadaic acid had no effect at 10 nM (data not shown), but did increase HA-PIP5Kβ basal phosphorylation and blocked dephosphorylation at 100 nM. The differential effects of caly A and okadaic acid on PIP5Kβ dephosphorylation suggest that the PP1 phosphatases promote PIP5Kβ dephosphorylation during hypertonic stress.

Caly A was used to evaluate the relationship between the hyperonicity induced PIP5Kβ dephosphorylation and lipid kinase activation. Caly A decreased basal PIP5Kβ activity by 63%, and blocked PIP5Kβ activation by sucrose (Fig. 2.5B). Caly A was also used to determine if PIP5Kβ dephosphorylation is a primary trigger for the hypertonic

PIP_2 response. Caly A dampened the PIP_2 response (Fig. 2.5C), and this effect was specific for PIP_2, because PI4P increased as previously observed. It is curious though that caly A had minimal effect on the cell's PIP_2 level under isotonic condition (Fig. 2.5C), even though it inhibited PIP5Kβ *in vitro* (Fig. 2.5B). It is possible that PIP_2 did not decrease in the calyculin A treated cells because of compensatory changes that restore the ambient isotonic PIP_2 level. However, these compensations are not able to raise PIP_2 to a sufficiently high level to compensate for the lack of PIP5Kβ activation during hypertonic stress. Taken together, the series of experiments establish that there is a cause and effect relationship between hypertonicity induced PIP5Kβ dephosphorylation, lipid kinase activation and PIP_2 increase in cells.

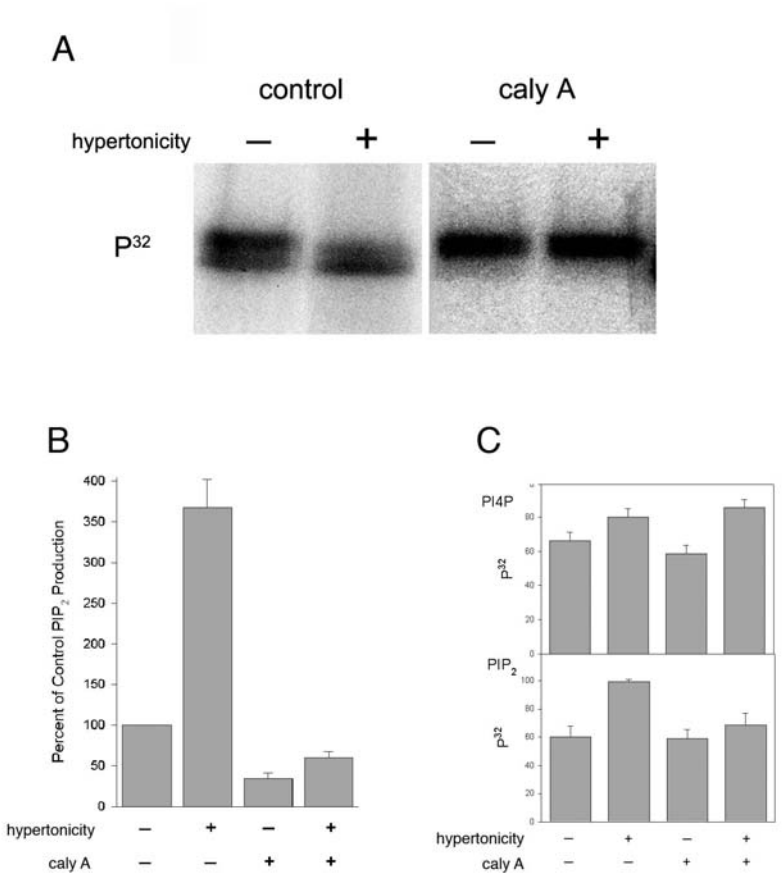


Figure 2.5

Effects of caly A. HeLa cells overexpressing HA-PIP5Kβ were exposed to 5 nM caly A for 15 min. prior to sucrose stimulation. (A). Phosphorylation. Cells were ^{32}P labeled, and HA-PIP5Kβ was immunoprecipitated with anti-HA antibody. (B). *In vitro* lipid kinase assay using immunoprecipitated HA-PIP5Kβ. ^{32}P incorporation into PI4P was normalized against the amount of immunoprecipitated kinase (determined by parallel western blots, not shown), and expressed as a percentage of control. Values are mean ± SEM of three independent experiments, each done in duplicate. (C). The phosphoinositide response in cells. The amounts of ^{32}P incorporated into the bands comigrating with the standard PI4P and PIP_2 on TLC were plotted in arbitrary units (mean ± SEM of three independent experiments).

2.7 EFFECTS OF PIP5Kβ DEPHOSPHORYLATION ON STEADY STATE MEMBRANE ASSOCIATION

Since hypertonicity induces PIP5KIβ dephosphorylation and also promotes its recruitment to membranes (Figs. 2.2-2.4), we examined the possibility that the less phosphorylated PIP5Kβ is preferentially membrane associated. However, the ratio of the two bands in the PIP5Kβ doublet in the 100,000xg supernatant and pellet fractions (Fig. 2.3A) were similar. Therefore, the more phosphorylated and less phosphorylated PIP5Kβ associate with membranes to a similar extent under the steady state isotonic conditions used here. In conclusion, the increase in membrane association during hypertonic stress cannot be simply attributed to dephosphorylation of ser/thr residues.

2.8 RELATIONSHIP BETWEEN THE HYPERTONICITY INDUCED PIP_2 RESPONSE, VOLUME CHANGE AND ACTIN REMODELING

Hypertonic NaCl or sucrose induces cell shrinkage and reorganization of the actin cytoskeleton. In contrast, 200 mM urea, which is cell permeant and therefore increases osmolarity without inducing cell shrinkage, neither increased PIP_2 nor PIP5Kβ dephosphorylation (Fig. 2.6A). Therefore, the PIP_2 and dephosphorylation responses are both dependent on volume changes.

Cell shrinkage deforms the actin cytoskeleton and imposes mechanical tension on the integrins (Kashani et al. 2001). Since the actin cytoskeleton can potentially act as a volume sensor and PIP5Ks are activated by integrin signaling (Chong et al. 1994a; Di Ciano et al. 2002b; Lewis et al. 2002; Dahl et al. 2003), we investigated the possibility

that PIP5Kβ dephosphorylation depends on signals transmitted by the actin cytoskeleton. Latrunculin A, which depolymerizes actin filaments, and jasplakinolide, which stabilizes actin filaments, were used to interfere with the cytoskeletal response. Neither blocked the PIP_2 increase nor PIP5Kβ dephosphorylation (Fig. 2.6B). Therefore, PIP5Kβ dephosphorylation and PIP_2 increase do not directly depend on cytoskeletal remodeling.

Under isotonic conditions, the small GTPase Rho and its downstream effector Rho kinase stimulate stress fiber formation, promote PIP5KI targeting and activation (Chong et al. 1994a; Chatah and Abrams 2001; Weernink et al. 2004b). Since Rho is activated by hypertonic stress (Ciano-Oliveira et al. 2003; Eggermont 2003a), we examined the possibility that Rho kinase activation promotes PIP5Kβ dephosphorylation. This was not the case because the Rho kinase inhibitor, Y27843, did not block PIP5Kβ dephosphorylation (Fig. 2.6C) even though actin stress fiber formation was inhibited (data not shown).

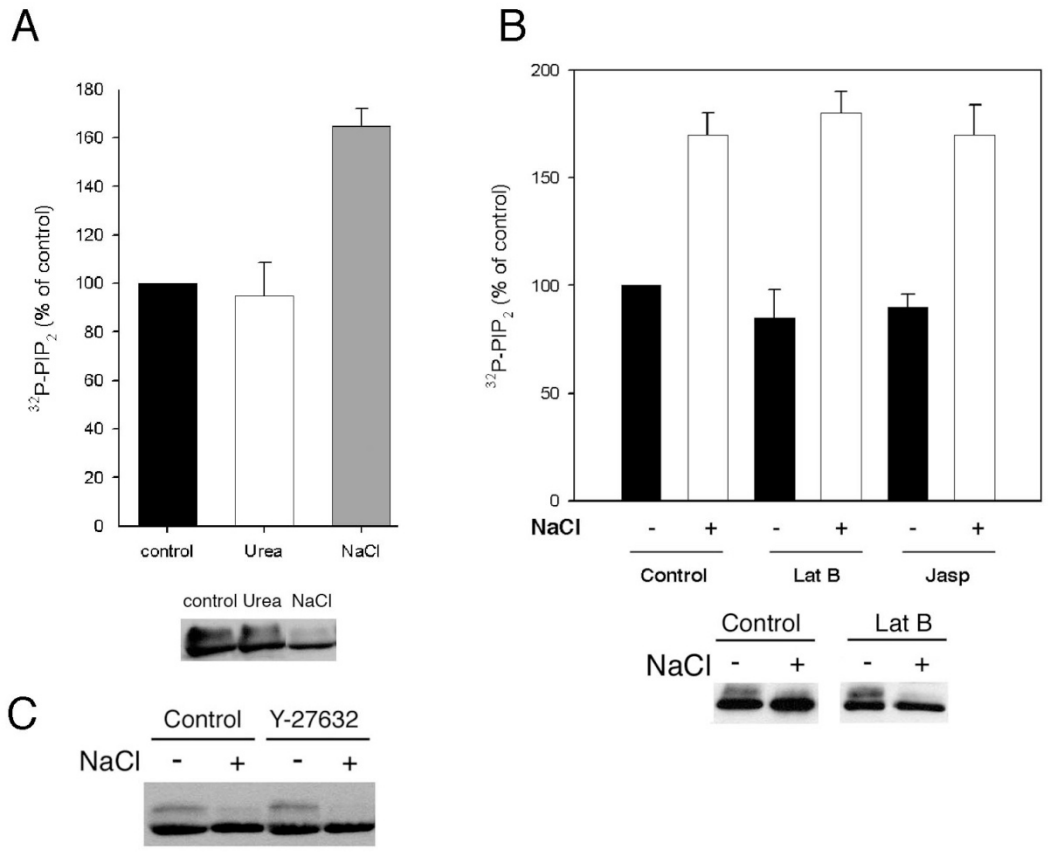


Figure 2.6

Effects of urea, actin poisons and Rho kinase inhibition. (A). Effect of urea on the PIP_2 response. HeLa cells were exposed to either 100 mM NaCl or 200 mM urea for 10 min. Top panel, ^{32}P-PIP_2 expressed as a percent of control. Data shown is mean±SEM of two independent experiments. Bottom panel, western blot of HA-PIP5Kβ. (B). Effects of actin depolymerization or stabilization. Cells were incubated with 100 nM latrunculin B (lat B) or 500 nM jasplakinolide for 30 min. prior to exposure to 100 mM NaCl. Top panel, ^{32}P-PIP_2, mean ± SEM of three independent experiments. Bottom panel, western blotting of HA-PIP5Kβ. (C). Effect of Rho kinase inhibition. Cells were incubated with 10 μM Y23187 for 30 min. prior to exposure to NaCl. HA-PIP5Kβ was detected by western blotting.

2.9 DISCUSSION

This section establishes hypertonic stress as an activator of PIP5Kβ to promote membrane translocation and site-specific PIP_2 generation. The increase in PIP_2 levels is a direct result of PIP5Kβ activation by ser/thr dephosphorylation.

PIP5Kβ involvement in the hypertonic response was established by RNAi. In contrast, PIP5Kβ RNAi has surprisingly little effect on IP_3 generation in the same type of cells (Wang et al. 2004). Surprisingly, RNAi of the the other PIP5Ks did not block the hypertonicity induced increase in PIP_2, although other functions were altered (Wang et al. 2004). Taken together, these results support the growing realization that the PIP5Ks are functionally specialized, and by extension, the cell's PIP_2 pools may be functionally and perhaps even physically segregated.

The phosphorylation status of PIP5Kβ is likely to be maintained by a balance between protein kinases and phosphatases. Theoretically, the hypertonicity-induced PIP5Kβ dephosphorylation can be achieved either by inhibiting a PIP5Kβ protein kinase, or activating its phosphatase. However, although hypertonicity activates many kinases (Sheikh-Hamad and Gustin 2004; Strange et al. 2006), there are relatively few examples of hypertonicity-inactivated kinases. Therefore, the current working hypothesis is that PIP5Kβ is dephosphorylated primarily by activation of a caly A-sensitive PP1. The involvement of PP1 is supported by the ability of calyA to inhibit PIP5Kβ dephosphorylation and activation, and PIP_2 increase in cells. This is further supported by the finding that PP1 dephosphorylates and activates PIP5Kβ *in vitro* (Park et al. 2001a). PP1 activity has been implicated in response to osmotic swelling in yeast (Andrews pd,

stark). While no studies have found a direct link between osmotic shrinkage and PP1 activity, it is conceivable that PP1 plays an essential role in regulating the cell wall remodeling process.

The data show for the first time that PIP5Kβ depletion decreases the amount of actin filaments under isotonic conditions and blocks hypertonicity induced actin polymerization/reorganization. These results place PIP5Kβ activation and PIP_2 increase upstream of actin remodeling. This is supported by additional lines of evidence. Urea, which increases osmolarity without causing cell shrinkage, does not promote PIP5Kβ dephosphorylation nor increase PIP_2. Since urea also does not promote actin assembly (Lewis et al. 2002), these events are inter-related. Results with actin poisons and Rho kinase inhibitor clearly establish that PIP5Kβ dephosphorylation is not dependent on mechanical transduction by the actin cytoskeleton.

Rho is activated by hypertonic stress, and Rho recruits PIP5Kβ to the plasma membrane under isotonic conditions (Chong et al. 1994a; Yang et al. 2004). However, we find that PIP5Kβ is dephosphorylated normally in the presence of the Rho kinase inhibitor and dephosphorylated PIP5KIβ recruitment to membranes is not inhibited. It is possible that the current result can be explained by hypothesizing that PIP5Kβ is subject to regulation at multiple levels. Exposure to hypertonicity immediately triggers PIP5Kβ dephosphorylation by a volume sensing, Rho kinase independent protein phosphatase. In addition, Rho kinase is also activated either independently or downstream of PIP5Kβ dephosphorylation. The latter possibility is suggested by the finding that PIP_2 activates a Rho GEF in yeast (Audhya and Emr 2002b). Rho and Rho kinase can then stimulate

PIP5Kβ further by direct binding and targeting. Thus, PIP5Kβ activation by dephosphorylation is an apical signal, which can be further modulated or propagated by other downstream regulators and crosstalk at multiple levels. The actin cytoskeleton may be remodeled initially by the PIP_2 generated through PIP5Kβ activation, and in conert with Rho activation.

It is hypothesized that a stronger cortical actin network tempers the inflammatory cascade during traumatic injury (Rizoli et al. 2000; Di Ciano et al. 2002b) by blocking leukocyte exocytosis (Rizoli et al. 2000; Cuschieri et al. 2002; Di Ciano et al. 2002b). In addition, the reorganized cytoskeleton reinforces endothelial cell:cell and cell:matrix adhesion, to minimize the monolayer disruption that exacerbates the injury response (Victorino et al. 2003). The type of actin remodeling appears to be cell specific. For example, hypertonicity induces cortical actin assembly in CHO cells through an Arp2/3:cortactin pathway that is activated by Rac and Cdc42 , but it induces stress fiber assembly in macrophages and epithelial cells by activating Rho and Rho kinase (Cuschieri et al. 2002). These experiments find that hypertonicity increases actin assembly as well as actin filament crosslinking into stress fiber in HeLa cells, suggesting that PIP_2 activates regulatory proteins that favor actin nucleation and assembly, and inhibits those that promote filament depolymerization or severing (Yin and Janmey 2003). These results are consistent with our lab's previous finding that overexpression of PIP5Kβ induces NWASP:Arp2/3 dependent actin polymerization (Rozelle et al. 2000) and actin stress fiber formation in CV1 cells (Yamamoto et al. 2001).

Our study suggests that the increase in PIP_2 levels may explain how hypertonic resuscitation protects against the inappropriate inflammatory responses in burn and

trauma patients. This model is supported by the finding that knockout mice that do not express the mouse equivalent of human PIP5Kβ exhibit enhanced passive cutaneous and systemic anaphylaxis (Sasaki et al. 2005). Like the PIP5Kβ RNAi HeLa cells, the mast cells of these null animals have decreased actin filaments, and they are hyperactive in degranulation and cytokine production. We predict that these mice will be more susceptible to burn induced complications, and hypertonic resuscitation might be less effective in protecting against these injuries because of the lack of a PIP_2 response.

In conclusion, PIP5Kβ is the major source of the PIP_2 generated during hypertonic stress, and this PIP_2 is necessary for the hypertonicity-induced reorganization of the actin cytoskeleton. This study provides mechanistic understanding of how hypertonicity induces PIP5Kβ activation, increases PIP_2 and reorganizes the actin cytoskeleton. PIP5Kβ is dephosphorylated in response to volume changes upstream of cytoskeletal reorganization and generates an apical signal that has a central role in regulating the actin cytoskeleton and most likely a host of other hypertonic responses.

CHAPTER THREE

OXIDATIVE STRESS DECREASES PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE LEVELS BY DEACTIVATING PIP5KIβ IN A SYK DEPENDENT MANNER

3.1 INTRODUCTION

Several observations have found that oxidative stress leads to a profound change in PPI homeostasis and the organization of the actin cytoskeleton (Rokutan et al. 1994). The actin stress fiber network breaks down while a concomitant increase in actin polymerization in association with contraction of the cell occurs. The breakdown of the cytoskeleton can be reversed within a matter of minutes. The underlying mechanism of this event though remains unclear.

The previous chapter demonstrated that hypertonic stress specifically increases PIP5Kβ activity through ser/thr dephosphorylation. This post-translational modification leads to plasma membrane localization of PIP5Kβ. The PIP_2 generated by this kinase ultimately leads to actin polymerization (Yamamoto et al. 2006). Conversely, Divecha and colleagues have reported a role for mPIP5Kα (hPIP5Kβ) in regulating UV and oxidant dependent apoptosis. These stresses lead to a depletion of PIP_2 which could be rescued by overexpression of mPIP5Kα and ultimately attenuation of the apoptotic response (Halstead et al. 2006).

A proximal event in the oxidative stress response pathway is the activation of protein-tyrosine kinases (PTK) such as Lck, Fyn, and Syk (Aslan and Ozben 2003). While their activities are greatly enhanced, the underlying physiological role of PTK

activation has yet to be completely understood. Syk in particular is a PTK that was first identified in the spleen but has been the subject of intense scrutiny for its role in mast cell and B-cell signaling (Takano et al. 2002). Syk has been more recently identified in non-hematopoietic cells as well including, epithelial cells, fibroblasts, hepatocytes, neuronal cells and breast tissue(Renedo et al. 2001; Yanagi et al. 2001b). Syk has been implicated in a variety of processes such as calcium signaling, phagocytosis, and motility(Siraganian et al. 2002; Tohyama and Yamamura 2006). Oxidative stress has been identified as a major regulator of Syk activation but its role in the actin response is not known.

In this chapter I identify a concomitant decrease of actin stress fibers and PIP_2 in epithelial cells upon oxidative stress induced by hydrogen peroxide. I performed lipid kinase assays on each PIP5K isoform isolated from oxidataively stressed cells and found that PIP5Kβ is selectively deactivated. Oxidative stress leads to tyrosine phosphorylation of PIP5Kβ. Using tyrosine kinase inhibitors and co-immunoprecipitation experiments, I showed that Syk is involved in this process. The site of tyrosine phosphorylation was mapped to residue Y105. Through Y105 point mutation to generate nonphosphorylatable or phosphorylated , I establish that Y105 tyrosine phosphorylation decreases PIP5Kβ lipid kinase activity and membrane localization during oxidative stress.

The data in this section were a collaborative effort between myself, Xiaohui Zhu, and several former lab members (Masaya Yamamoto, Liying Chen and Yongjie Wei).

3.2 H_2O_2 DECREASES PIP_2

The previous chapter found that hypertonic stress leads to PIP_2 increase and subsequent actin polymerization (Yamamoto et al. 2006). In testing a variety of stimuli, it was found that oxidative stress had the opposite effect in that it lead to a decrease in total PIP_2 levels in a variety of cell types (Figure 3.1A). Endothelial cells were initially tested which are known to be exposed to high levels of ROS during vascular disease and sensitive to oxidative stress (Pantaloni et al. 2001). To see if this effect was applicable to epithelial cells as well, I labeled phospholipids in HeLa cells with ^{32}P-orthophosphate and exposed them to 1mM H_2O_2 As early as 15 min, I could see a remarkable decrease in PIP_2 levels as assayed by both Thin Layer Chromatography (TLC) and HPLC. Masaya Yammamoto's HPLC results confirmed that the major PIP_2 peak, $PI(4,5)P_2$ (hereafter referred to as PIP_2) was the species being decreased (Figure 3.1B). To see if this effect was also relevant in other cell types, notably epithelial and macrophage cells, I tested HeLa, 293, CHO, COS and RAW 264.7. All cell types tested showed the same marked decrease in PIP_2 levels albeit, at higher doses of H_2O_2, 600μM to 1mM compared to 100μM for HUVEC cells (data not shown). These results suggest PIP_2 decrease is either a result of lipid phosphatase activity or a result of deactivation of the PIP5 kinases that synthesize PIP_2.

The cell maintains a proper redox state by expressing proteins that act as reducing agents. Liying Chen examined if free radical scavengers, GSH, GSSG and catalase in addition to the chemical DTT ablated the decrease in PIP_2 (Figure 3.1C). Cells pretreated for 15 min with catalase prevented a decrease in PIP_2 levels upon

addition of H_2O_2. Similarly, addition of sulfhydryls for 15 minutes prior to exposure to H_2O_2 blocked the decrease in PIP_2. Of the three free radical scavengers tested, all but oxidized glutathione (GSSG), which is an inactive form of GSH, were able to block PIP_2 decrease. These results indicate that PIP_2 decrease is caused either by H_2O_2 reactivity or activation of oxidative stress sensitive signaling pathway within the cell.

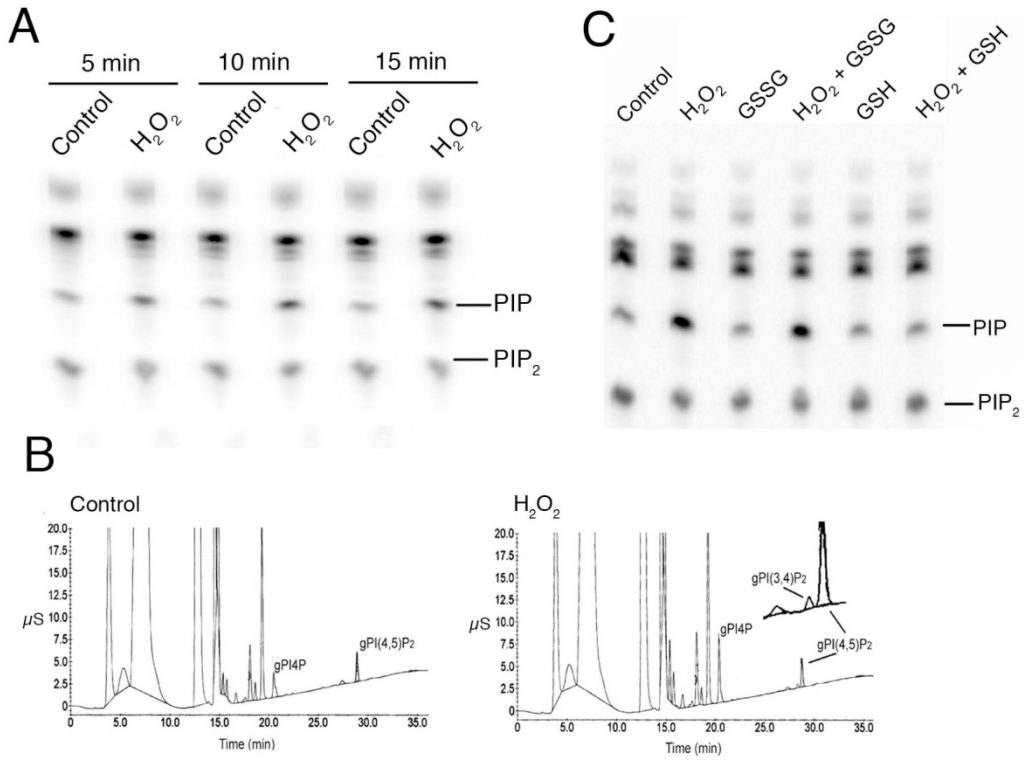


Figure 3.1

H_2O_2 disrupts phosphoinositide homeostasis. A. HeLa cells were exposed to 1 mM H_2O_2 for 15 min. A. TLC. HeLa were labeled with ^{32}P-orthophosphate and exposed to H_2O_2. Lipids were analyzed by TLC. B. HPLC quantification. Extracted phospholipids were deacylated and negatively charged glycerol head groups were eluted and detected with suppressed conductivity (μS, microsiemen units). Inset in the H_2O_2 treated sample shows expanded view of peaks that co migrate with authentic gPI(3,4)P_2 and gPI(4,5P)$_2$. C. Anti-oxidant treatment. Cells were labeled with ^{32}P-orthophosphate and pre-incubated for 15 min with 25 mM GSH or GSSG prior to exposure to H_2O_2. Phospholipids were analyzed by TLC. D. Actin cytoskeleton

staining. Cells were fixed and stained with FITC-phalloidin to stain polymerized actin fibers. E. Partitioning of actin in Triton-soluble and -insoluble fractions.

3.3 H_2O_2 DEPOLYMERIZES ACTIN

PIP_2 is a major regulator of the actin cytoskeleton (Mao and Yin 2007). To further examine the consequences of decreased PIP_2 and to see if this decrease had a negative effect on actin polymerization since the previous chapter showed that increased PIP_2 synthesis during hypertonic stress lead to increased levels polymerized actin. Therefore I reasoned that a drop in PIP_2 might cause a loss of actin stress fibers. HeLa cells were treated with 1mM H_2O_2 for 15 minutes, fixed and stained with FITC labeled phalloidin, which binds to polymerized F-actin. (Figure 3.2A) By visual inspection, actin stress fibers were decreased in relation to control PBS treated cells. Yongjie Wei biochemically confirmed this observation by partitioning actin by differential centrifugation (Figure 3.2B). The high speed supernatant (HSS), which contains the actin monomers and small oligomers, was increased in the H_2O_2 treated sample suggesting a depolymerization of the actin cytoskeleton. This data suggests a link between decreased PIP_2 and loss of actin stress fibers.

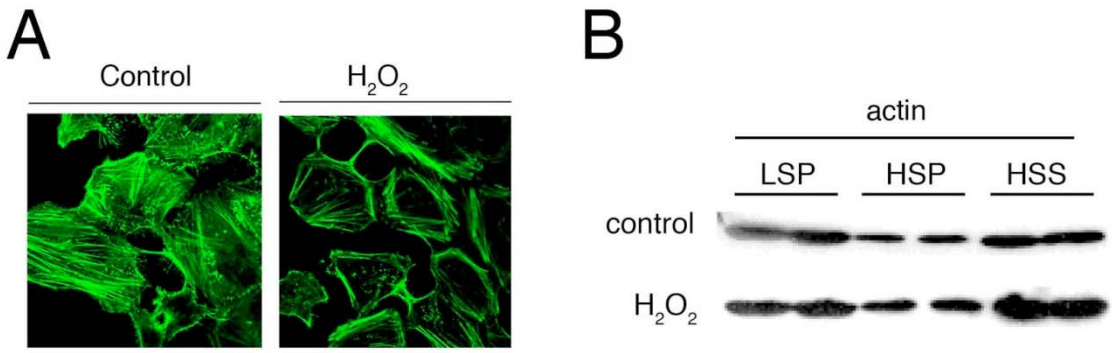


Figure 3.2

H_2O_2 Depolymerizes Actin. A. Actin cytoskeleton staining. Cells were fixed and stained with FITC-phalloidin to stain polymerized actin fibers. B. Partitioning of actin in Triton-soluble and -insoluble fractions. Actin in the low speed pellet (LSP), high speed pellet (HSP) and high speed supernatant (HSS).

3.4 PIP5Kβ IS DEACTIVATED BY TYROSINE PHOSPHORYLATION DURING OXIDATIVE STRESS

The drop in PIP_2 prompted examination into the mechanism underlying this change. Previous studies point to tyrosine phosphorylation as a regulator of PIP_2 homeostasis (Rumenapp et al. 1998). Yongjie Wei found that the tyrosine phosphatase inhibitor pervanadate mimicked the effects of H_2O_2. (Figure 3.3A.) H_2O_2 at higher levels on the order of 1 mM causes the inactivation of tyrosine phosphatases in addition to activation of ROS signaling pathways (Knebel et al. 1996). HeLa cells treated with 500 μM Pervanadate or 1 mM H_2O_2 both showed roughly a 50% drop after 15 minutes. In addition, PIP levels increased for both conditions as well. HPLC results identified this as PI4P.

PIP_2 levels can be regulated in three different ways. PIP_2 can be dephosphorylated at one of the phosphates of the inositol ring to form PIP, phosphorylated to form PIP_3 or a drop in the activity of the PI(4)P5 Kinase that synthesize PIP_2 (Balla 2006). Because PIP_2 turnover occurs very quickly, we decided to first look at the change in activity of the different isoforms of PIP5K. The different PIP5K isoforms were immunoprecipitated and subjected to *in vitro* lipid kinase activity assay (Figure 3.3B). Of the three PIP5Ks, PIP5Kβ was the only kinase that showed a significant drop in activity. This is in accordance with the previous finding that overexpression of PIP5Kβ blocks the drop in PIP_2 during H_2O_2 treatment and ultimately apoptosis in HeLa cells (Halstead et al. 2006). Interestingly, PIP5Kγ's activity was increased during the treatment. Because PIP5Kγ's relative contribution of PIP_2 in HeLa cells is significantly lower than PIP5Kβ (Padron et al. 2003b), we focused on the behavior of PIP5Kβ.

The next step was to understand the mechanism of deactivation. Protein levels remained constant during H_2O_2 treatment indicating the change in PIP_2 production was a post-translational event rather than a transcriptional regulatory event (Figure 3.3C). Pervanadate is a tyrosine phosphatase inhibitor, therefore we looked at tyrosine phosphorylation as a possible post-translational modification regulating the drop in kinase activity. Different PIP5K isoforms were expressed in HeLa cells and exposed to oxidative stress. PIP5Kβ and interestingly, PIP5Kγ were both tyrosine phosphorylated as assessed by Western blot. (Fig 3.3C) PIP5Kγ has been shown to be phosphorylated by Src kinase albeit with no stimulus (Ling et al. 2003). At resting state, PIP5Kβ had low

levels of tyrosine phosphorylation but phosphorylation was greatly increased during oxidative stress. Hyperosmotic stress was also tested to see if it was able to cause tyrosine phosphorylation of PIP5Kβ. No increase in tyrosine phosphorylation was detected (data not shown).

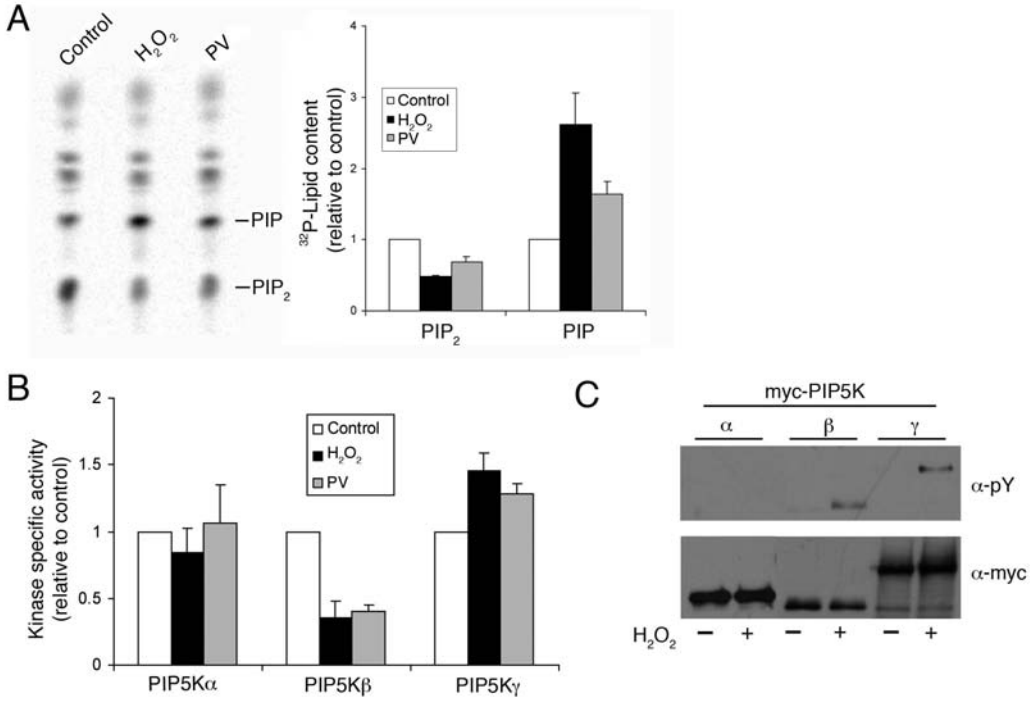


Figure 3.3

H_2O_2 disrupts PPI homeostasis by selective PIP5K tyrosine phosphorylation.

HeLa cells were treated with either 1 mM H_2O_2 or 10 μM pervanadate (PV) for 15 min. A. PV and H_2O_2 have similar effects on PPI homeostasis Cells were labeled with ^{32}P-orthophosphate and lipids were resolved by TLC. Left panel, a typical fluorogram of the lipids after TLC; *right panel*, quantification of ^{32}P-lipids (mean±s.e.m, n=3). B. PV and H_2O_2 have similar effects on PIP5K lipid kinase activity. *In Vitro* Lipid Kinase activity assay. HeLa cells transiently transfected with myc-PIP5K isoforms were exposed to either H_2O_2, or PV. C. H_2O_2 induces tyrosine phosphorylation of PIP5β, γ87 but not α. HeLa cells transiently overexpressing myc-PIP5K isoforms were exposed to H_2O_2 and myc-PIP5K was immunoprecipitated from the cell lysate with an anti-myc antibody.

3.5 OXIDATIVE STRESS LEADS TO PLASMA MEMBRANE DELOCALIZATION OF PIP5Kβ

PIP5Kβ is localized to the cytoplasm and plasma membrane where it plays a role in receptor-mediated endocytosis and actin polymerization (Yamamoto et al. 2001; Padron et al. 2003b). Previous reports by Divecha and colleagues have shown a loss of plasma membrane localization of PIP5Kβ during H_2O_2 treatment. I checked to see if this phenotype was present in our cells. Cos cells expressing PIP5Kβ were treated with H_2O_2 and examined by confocal microscopy to check the cellular localization (Figure 3.4A). In accordance with previous reports that PIP5Kβ is located at both the plasma membrane and cytosol, this localization was present as well. Cells subjected to oxidative stress showed a loss of plasma membrane PIP5Kβ staining indicating a shift to the cytosol. HeLa cells were checked to see if this occurred in this cell type as well. Like the Cos cells, HeLa cells showed a shift to the cytosolic area of the cell (Figure 3.7A).

PIP5Kβ is a peripheral protein that is both plasma membrane (PM)-associated and cytosolic (Yamamoto et al. 2001; Padron et al. 2003b). Immunofluorescence studies showed that H_2O_2 decreased PIP5Kβ at the cell periphery and increased accumulation in the perinuclear region. Wei's results are consistent with a previous study (Halstead et al. 2006).

PIP5Kβ translocation was confirmed by two different membrane fractionation protocols. Using a single step 100,000 x g fractionation, we found that H_2O_2 induced an increase in cytosolic PIP5Kβ and a decrease in microsome membrane as well as PM associated PIP5Kβ (Figure 3.4B,D). Western blotting with anti-pY antibody showed that there is a preferential enrichment of tyrosine phosphorylated PIP5Kβ in the cytosol (Cyt) fraction after PV treatment (Figure 3.4C). A .5 fold drop in PIP5Kβ on the plasma membrane was found when the cells were treated with H_2O_2. Therefore, it was concluded that tyrosine phosphorylation leads to dissociation from the plasma membrane as well as internal membranes.

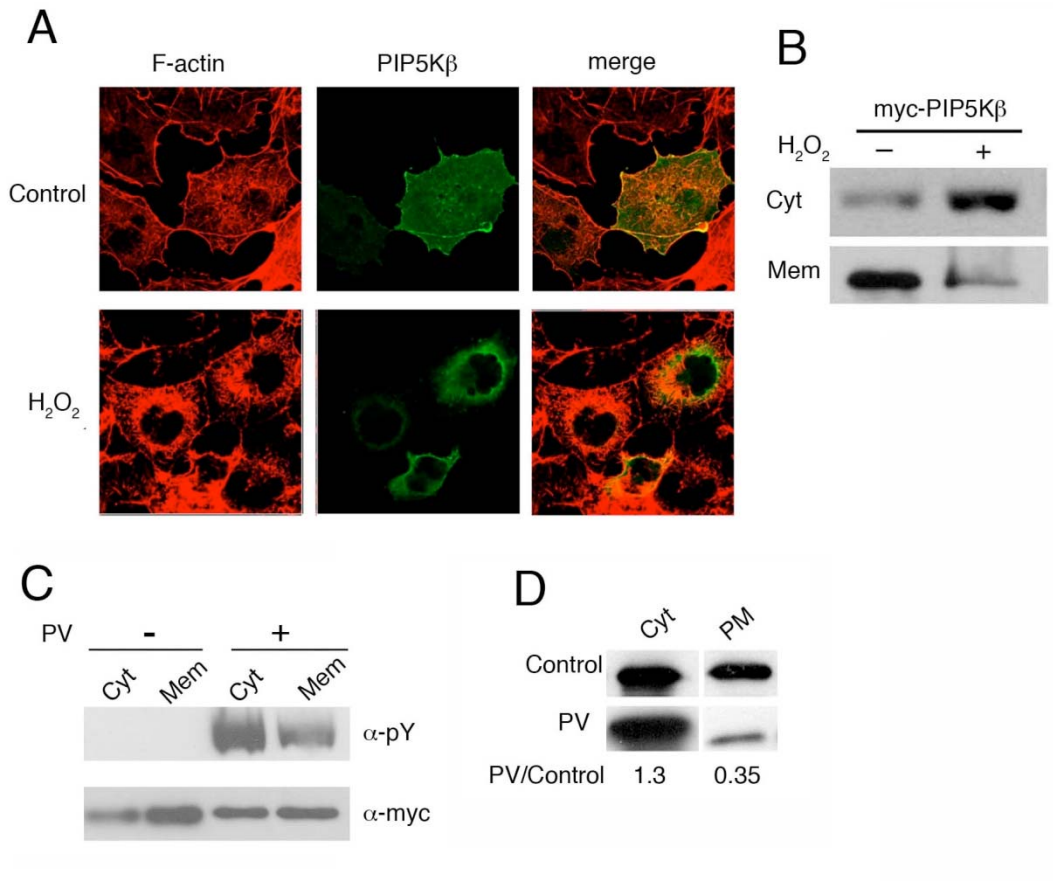


Figure 3.4

Tyrosine phosphorylation decreases PIP5Kβ association with membranes.

COS-7 cells overexpressing myc-PIP5Kβ were exposed to 1 mM H_2O_2 or 10 μM PV for 15 min. A. Immunofluoresence localization. Cos cells treated with or without H_2O_2 were stained with anti-myc/FITC and TRITC-phalloidin. B. Dissociation from microsome membranes. Cell lysates from H_2O_2 or control cells were centrifuged at 100,000 X g for 20 min. to separate microsome membranes (mem) from the cytosol (Cyt). Fractions were analyzed by Western blotting with anti-myc. The membrane fraction loaded was 10 times more than the Cyt fraction. C. Preferential dissociation of tyrosine phosphorylated myc-PIP5Kβ from membranes. Cells were stimulated with PV, homogenized and centrifuged at 100,000 X g for 20 min. D. Dissociation from the plasma membrane (PM). Cells expressing myc-PIP5Kb were treated with PV were homogenized and subjected to sequential centrifugation as described above. Fractions were run on SDS-PAGE and blotted with α-myc antibody.

3.6 TYROSINE KINASE SYK PHOSPHORYLATES PIP5Kβ

To identify the tyrosine kinase that phosophorylates PIP5Kβ during oxidative stress, we did a preliminary screen with a panel of tyrosine kinase inhibitors. These include AG957 (a Bcr/Abl inhibitor), AG1296 (a PDGF inhibitor), AG1478 (an EGFR inhibitor), PP2 (a Src family inhibitor) and piceatannol (a spleen tyrosine kinase (Syk)) inhibitor (data not shown). Among these, only PP2 and piceatannol, completely inhibited PIP5Kβ tyrosine phosphorylation at 80 and 60 μM, respectively (Figure 3.5A, top panel). Since Syk is activated downstream of Src family kinase, Lyn during oxidative stress (Qin et al. 1996) and is inhibited by piceatannol at an IC50 of approximately 25 μM (Yamamoto et al. 2003), we focused on the possibility that Syk directly phosphorylates PIP5Kβ. Although Syk was first identified in hematopoietic cells, it is also found in nonhematopoietic cells such as HeLa (Renedo et al. 2001), We performed a series of experiment to evaluate the role of Syk in oxidant induced PIP5Kβ phosphorylation.

First, we determined if piceatannol, which inhibits PIP5Kβ tyrosine phosphorylation, also prevented the H_2O_2-dependent decrease in cellular PIP_2 (Figure 3.5B). This is indeed the case, strongly suggesting that Syk-mediated PIP5Kβ tyrosine phosphorylation can account for the oxidant induced PIP_2 decrease in cells. Significantly, piceatannol had no effect on the H_2O_2-induced increase in PI4P. Therefore, although PI4P is an obligatory substrate for PIP_2 synthesis by the PIP5Ks, the oxidant induced PI4P increase was not secondary to a decrease in its utilization for PIP_2 synthesis.

Second, to establish that Syk is a *bona fide* PIP5Kβ kinase, we determined if overexpressed Syk phosphorylates PIP5Kβ in the cellular context. Cos cells were

transfected with a constitutively active form of human Syk (hSyk) and myc-PIP5Kβ (Figure 3.5C). PIP5Kβ was tyrosine phosphorylated in a Syk dependent manner, establishing that Syk can phosphorylate PIP5Kβ in cells.

Third, we determined if Syk associates with PIP5Kβ in a H_2O_2-dependent manner. To reduce basal tyrosine phosphorylation of myc-PIP5Kβ in cells also overexpressing Syk, we switched to a HA-tagged mouse Syk, which is not constitutively active. We found that HA-mSyk coimmunoprecipitated with myc- PIP5Kβ in a H_2O_2 dependent manner (Figure 3.5D). Taken together, our results suggest that activated Syk binds and phosphorylates PIP5Kβ in cells.

Fourth, we showed that Syk phosphorylates PIP5Kβ *in vitro*. Immunoprecipitated hSyk was added to independently immunoprecipitated myc-PIP5Kβ and phosphorylation was detected by Western blotting with anti-pY (Figure 3.5E). As expected, hSyk as well as myc-PIP5Kβ were both tyrosine phosphorylated and their phosphorylation was blocked by piceatannol (Figure 3.5E). Tyrosine phosphorylation of PIP5Kβ was further increased by activating Syk with H_2O_2 prior to immunoprecipitation (Figure 3.5E, asterisk).

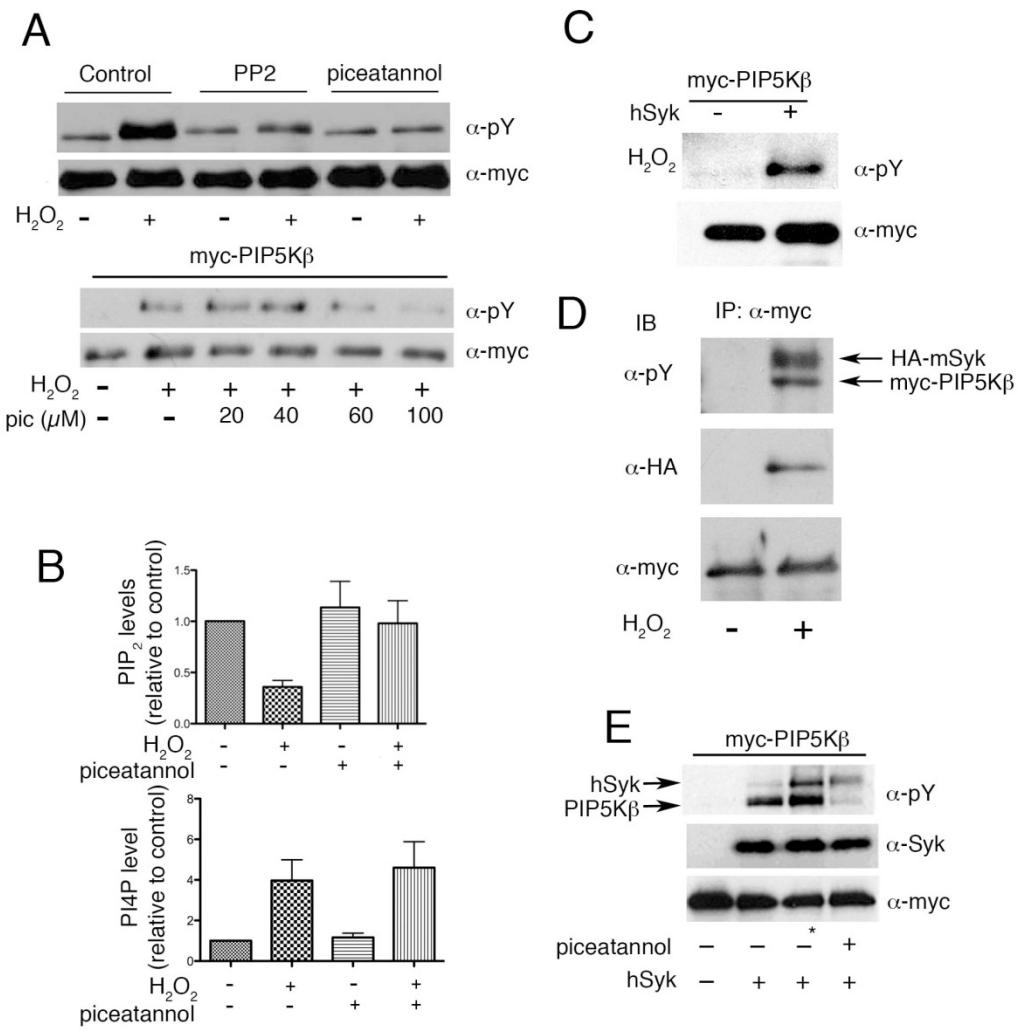


Figure 3.5

H_2O_2 induces PIP5Kβ tyrosine phosphorylation by activating Syk. A. H_2O_2 induced PIP5Kβ tyrosine phosphorylation was blocked by selected tyrosine kinase inhibitors. *Top panel*, Cos cells transfected with myc-PIP5Kβ were treated with 80 μM PP2 or 60 μM piceatannol for 30 min prior to stimulation with 1 mM H_2O_2 for 15 min. Bottom panel, dose response of piceatannol inhibition. B. Piceatannol blocks H_2O_2 induced PIP2 decrease but not PI4P increase. ^{32}P-labeled HeLa cells were treated with 60 μM picceatannol or vehicle for 30 min prior to H_2O_2 stimulation. Lipids were run subjected to TLC and quantitated. Results are mean±s.e.m of 4 independent experiments. C. Overexpressed constitutively active HA tagged human Syk (hSyk) phosphorylates coexpressed myc-PIP5Kβ in COS cells. myc-PIP5Kβ was immunnoprecipitated and blotted with anti-pY or anti-myc antibody. D. H_2O_2 induces Syk association with PIP5Kβ. COS cells were

co-transfected with HA tagged mouse Syk (mSyk), not constitutively active) and myc-PIP5Kβ and treated with H_2O_2. Myc-PIP5Kβ was immunoprecipitated with anti-myc and the immune complexes were probed with anti-myc, anti-HA and anti-pY. E. Syk phosphorylates PIP5Kβ. hSyk and PIP5Kβ were transfected separately in Cos cells and immunoprecipitated. Protein G beads containing immunoprecipitates were mixed and incubated together in the presence or absence of picceatannol. Asterisk denotes pretreatment of Syk expressing Cos cells with H_2O_2.

3.7 MAPPING OF PIP5Kβ TYROSINE PHOSPHORYLATION SITE

While PIP5Kβ has been shown to be tyrosine phosphorylated, a detailed map of which tyrosine residues involved in this regulation has yet to be shown. I used the PIP5Kβ sequence to search for sites with high likelihood of phosphorylation using the NetPhos prediction software *(http://www.cbs.dtu.dk/services/NetPhos/)* (Figure 3.6A).

Tyrosines at positions, 105, 209, 239, 285, 498 518, and 539 scored the highest and were chosen for further investigation. These tyrosines at these positions were mutated to alanines (Figure 3.6B). In addition, truncation mutants from 518 to the end of the C terminus were generated. Mutants were co-expressed with hSyk in Cos cells to determine if any of these sites were phosphorylated by Syk. Of the mutants tested, I found that the Y105 showed the most significant drop in phosphorylation while the other point mutants showed less significant decreases in phosphorylation. Y518 also showed a slight drop in tyrosine phosphorylation as well, which prompted me to see if these mutants responded alike under oxidative stress. To confirm the site phosphorylated by Syk was the same site that was phosphorylated during oxidative stress, the mutants were expressed in Cos cells and stimulated with H_2O_2 (Figure 3.6C). Y105A was still unable to be tyrosine phosphorylated while Y518A was slightly increased, therefore I chose to focus on position 105 for the rest of the experiments.

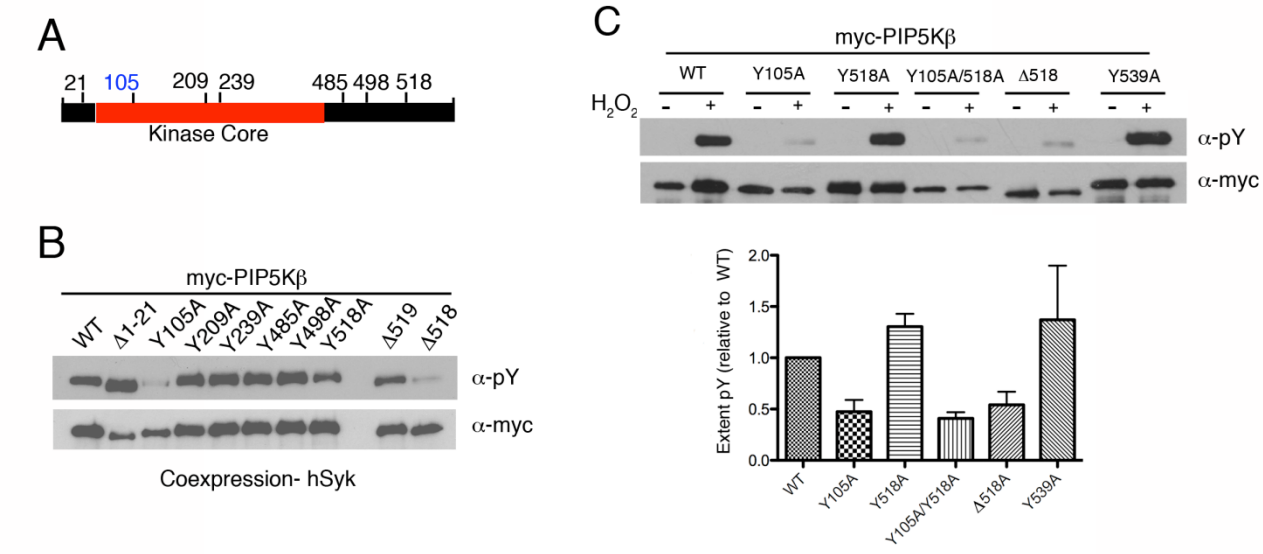


Figure 3.6

Identification of the H_2O_2/Syk dependent PIP5Kβ tyrosine phosphorylation site(s). A. Map of potential tyrosine phosphorylation sites as predicted by the NetPhos program. B. Tyrosine phosphorylation of PIP5Kβ mutants by the constitutively active hSyk. Point or truncated mutants were co-expressed with hSyk in COS cells and immunoprecipitated. *Top panel*, immunoprecipitates were blotted with anti-pY and anti-myc. *Bottom panel*; the ratio of tyrosine phosphorylated vs. total myc-PIP5Kβ was plotted. Data shown are mean±s.e.m (n=4). C. Effect of mutation on H_2O_2-mediated tyrosine phosphorylation. COS cells expressing PIP5Kβ mutants were treated with or without H_2O_2 for 15 min. myc-PIP5Kβ was immunoprecipitated and blotted with anti-pY or anti-myc. Phosphorylation was quantified by comparing the ratio of H_2O_2 induced tyrosine phosphorylation to untreated immunoprecipitates.

3.8 EFFECT OF Y105 PHOSPHORYLATION ON LOCALIZATION OF PIP5Kβ

The ability of oxidative stress to cause tyrosine phosphorylation of PIP5Kβ and simultaneously lead to PM delocalization suggests that the 105Y phosphorylation site may influence the cellular localization of PIP5Kβ as well. To further assess the role of the

Y105 residue in controlling PIP5Kβ localization, amino acid mutants mimicking tyrosine phosphorylation, glutamic Acid (E) and nonphosphorylatable tyrosine, phenylalanine (F), were generated (Figure 3.7A). These mutants could not be tyrosine phosphorylated during oxidative stress in agreement with the previous results (Figure 3.6C).

I next expressed mutants in HeLa cells and used confocal immunoflourescence to assess the point mutants' localization (Figure 3.7B). While WT PIP5Kβ is both cytosolic and plasma membrane bound, the Y105E mutant staining was completely cytosolic, similar to WT PIP5Kβ during oxidative stress. Furthermore, the Y105E did not change localization during oxidative stress. The Y105F mutant localization significantly contrasted with both the WT and Y105E mutant. Y105F clearly had a higher level of localization at the plasma membrane and despite treatment with H_2O_2, did not become cytosolic like the WT and 105E mutant. To support these observations, biochemical partitioning was used to look at the levels of PIP5Kβ and mutants localized at the plasma membrane versus the cytosol (Figure 3.7C). In accordance with the immunoflouresence data, Y105E was contained exclusively in the cytosolic fraction while Y105F localization was located more in the plasma membrane fraction. Taken together, these results suggest Y105 is the key residue influencing PIP5Kβ's localization.

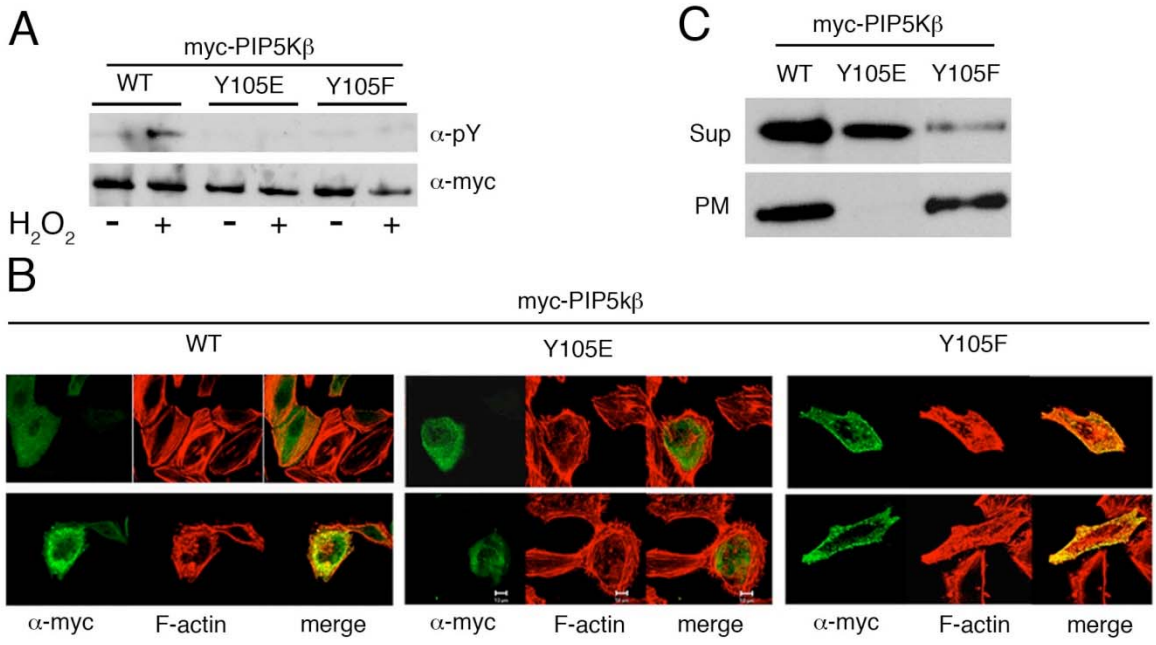


Figure 3.7

PIP5Kβ association with the plasma membrane is regulated by Y105 phosphorylation. A. myc-PIP5Kβ was mutated to either glutamic acid (E) or phenylalanine (F), to simulate a pY-phosphomimetic or non-tyrosine phosphorylatable PIP5Kβ, respectively. Cells were transfected with myc-PIP5Kβ WT or mutant constructs were exposed to 1 mM H_2O_2 for 15 min. B. Altered intracellular distribution as determined by immunoflouresence microscopy. HeLa cells were fixed and stained with anti-myc/ secondary mouse-FITC and TRITC-phalloidin. Images were examined by confocal microscopy. Cells were treated with water (top row) or 1 mM H_2O_2 (bottom row). C. Altered association with plasma membrane. COS cells were lysed by dounce homogenization and centrifuged to obtain a PM enriched fraction and Sup. Fractions were subjected to SDS PAGE and Western blotting with anti-myc. E. PIP5Kβ Y105F association with membranes is increased by H_2O_2. Cell lysates were centrifuged at 100,000xg to separate microsomes (Mem) from Cyt. The PM fraction loaded was 10 times more than the Cyt fraction. Fractions were Western blotted with anti-myc antibody.

3.9 TYROSINE 105 CONTROLS KINASE ACTIVITY IN VITRO

The involvement of Y105 in controlling PIP5Kβ localization raised the possibility that this residue also played a role in modulating kinase activity. PIP5Kβ and

Y105 mutants treated under control or oxidative stress conditions were subjected to *in vitro* lipid kinase assay (Figure 3.8A). The glutamic acid substitution, Y105E, which mimics a phosphorylated state, had no detectable activity (data not shown). Conversely, the basal activity of the Y105F mutant was markedly higher than the WT PIP5Kβ. I initially hypothesized that the activity of Y105F would not change during oxidative stress because the tyrosine on this position could not be phosphorylated and hence resistant to decrease in activity. If Y105 was the sole posttranslational modification position governing the activity of PIP5Kβ, the activity should remain constant despite the oxidative stress. Unexpectedly, treatment with H_2O_2 actually increased the activity of Y105F. In addition, basal activity of the Y105F kinase was 15 times higher than the WT kinase.

The previous chapter showed that PIP5Kβ activity increases under hypertonic stress through ser/thr dephosphorylation. Conceivably, PIP5Kβ could be ser/thr dephosphorylated by oxidative stress as well as being simultaneously tyrosine phosphorylated. I labeled mutants with ^{32}P to determine if the phosphorylation level of the mutants is changed under oxidative stress (Figure 3.8B). Similar to hypertonic stress, PIP5Kβ was dephosphorylated. Y105E did not show any ser/thr phosphorylation in neither basal nor stimulated conditions while Y105F was dephosphorylated. In this section, I have used a myc tagged PIP5Kβ which does not show the phosphorylation doublet as well as the HA tagged PIP5Kβ used in the previous work. To see if there was a collapse of the phosphorylation band, HA-PIP5Kβ was expressed in Cos cells and treated the cells with oxidative and hypertonic stress (Figure 3.8C). HA-PIP5Kβ collapsed into a singlet as we had seen before during the ser/thr dephosphorylation.

During hypertonic stress, there was a consequent increase in membrane association of PIP5Kβ. If Y105F was ser/thr dephosphorylated, it should also follow this pattern. To examine this, biochemically purified unstimulated and H_2O_2 treated Y105F was used to see if there was a shift in membrane localization (Figure 3.8D). While WT shifted to the soluble cytoslic portion during oxidative stress (Figure 3.4B), Y105F had the opposite pattern in that its localization was shifted towards the membrane fraction. In summary, this data suggests residue at Y105 is the dominant modification during stress response and governs both membrane trafficking and enzyme activity.

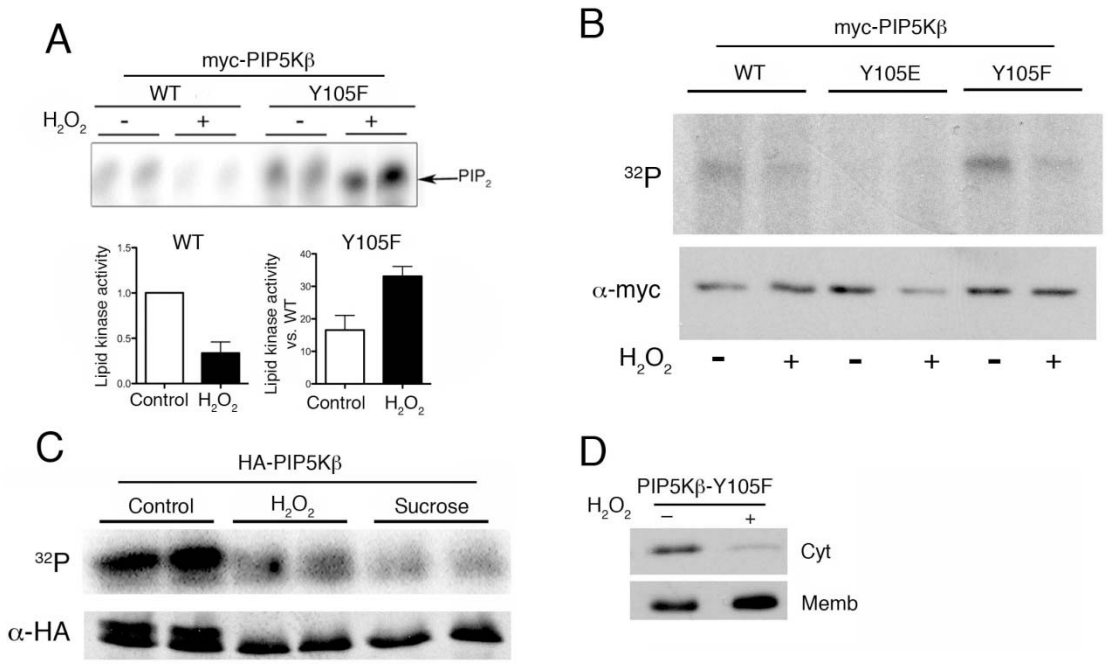


Figure 3.8

H_2O_2 induces concomitant PIP5Kβ tyrosine phosphorylation and ser/thr dephosphorylation. A. myc-PIP5Kβ Y105 mutants have altered lipid kinase activity. COS cells and stimulated with 1 mM H_2O_2 for 15 min. The cell lysates were immunoprecipitated and used for *in vitro* lipid kinase activity assay. Products were quantified by TLC. *Top panel*, autoradiogram. Bottom panel, Quantitation of PIP_2 generated. Values shown are mean±s.e.m (n=4). B. myc-PIP5Kβ Y105 mutants have altered ^{32}P labeling. HeLa? Labeled with ^{32}P were exposed to 1 mM H_2O_2 for 15 min. C. PIP5Kβ phosphorylation/dephosphorylation. HeLa cells expressing HA-PIP5Kβ were labeled with ^{32}P and exposed to isotonic buffer with and without 1 mM H_2O_2 or with 250 mM sucrose. HA-PIP5Kβ was immunoprecipitated with anti-HA and subjected to SDS-PAGE electrophoresis. ^{32}P-labeled proteins were detected by phosphorimager analysis and HA-PIP5Kβ or by Western blot with anti-HA.

3.10 DISCUSSION

While the actin response to oxidative stress has been characterized, the actual mechanism underlying this response remains unclear. A previous report demonstrated overexpression of PIP5Kβ attenuates apoptosis during oxidative and UV stress (Halstead et al. 2006). While PIP_2 has been implicated as an essential regulator of the actin cytoskeleton, my results here show for the first time that PIP5Kβ has a role in actin depolymerization during oxidative stress. PIP_2 is decreased by oxidative stress in several cell types. Of the three isoforms of PIP5 kinases, PIP5Kβ is the only kinase deactivated. Oxidative stress leads to activation of several tyrosine kinase signaling cascades resulting in phosphorylation of PIP5Kβ. Using tyrosine kinase inhibitors, I find that PIP5Kβ is phosphorylated by the kinase, Syk, either directly or in a complex. Employing site-directed mutagenesis, I mapped the tyrosine phosphorylation site to amino acid 105, and showed that its tyrosine phosphorylation regulates both cellular localization and kinase activity.

Interestingly, the ^{32}P labeling experiments suggest that while oxidative stress leads to tyrosine phosphorylation, PIP5Kβ is still ser/thr dephosphorylated. Data from the last chapter showed that hypertonic stress induces ser/thr dephosphorylation and activates lipid kinase activity. During this event, PIP5Kβ shifted localization from the cytoplasm to the plasma membrane, suggesting that ser/thr dephosphorylation is tied to the change in localization. The tyrosine mutants suggest that while ser/thr dephosphorylation still occurs, the modification at Y105 is the dominant signal in determining PIP5Kβ localization and activity. The membrane partitioning of the tyrosine phosphorylated species into the soluble fraction further illustrates this point. Y105E, which mimics constitutive tyrosine phosphorylation, had no detectable lipid kinase activity and was unable to traffic to the plasma membrane (data not shown, Figure 6). Conversely, the Y105F mutant which mimics a nonphosphorylatable tyrosine residue had a more even distribution between the plasma membrane and the cytosol. Y105F also had a higher level of basal activity. This suggests that under normal conditions, PIP5Kβ exists in an equilibrium between a tyrosine phosphorylated and non phosphorylated form at steady state. ser/thr dephosphorylation increases activity but ultimately, the tyrosine at 105 is the dominant modification.

Paradoxically, PP1 phosphatase family member, calcineurin is reported to be deactivated by oxidative stress (Sommer et al. 2002). Interestingly though, while calceneurin is deactivated by H_2O_2, PP1 activity is increased during treatment with 1 mM H_2O_2 *in vitro*. PP2A is also increased, but at higher concentrations, on the order of 10 mM. This along with the finding that many tyrosine phosphatases are inactivated by oxidative stress due to oxidation of the catalytic thiol group fits this model. The tyrosine

phosphatase of PIP5Kβ is inactivated while the ser/thr phosphatase is simultaneously activated. This leads to both activation of Syk and inactivation of the phosphatase leading to the net tyrosine phosphorylation and deactivation of PIP5Kβ.

The non-receptor tyrosine kinase Syk was first identified in porcine spleen and has been intensively studied in hematopoietic derived cells where it has been implicated in immune synapse signaling (Wilson et al. 2002). Recently, Syk has been identified in a variety of non-hematopoietic cell types such as fibroblasts and endothelial cells, suggesting a more general role for Syk in signal transduction (Yanagi et al. 2001b). Syk is reported to be expressed in HeLa cells (Renedo et al. 2001). Syk has been implicated in a variety of cellular processes such as cell proliferation, differentation, survival and phagocytosis (Tohyama and Yamamura 2006). Oxidative stress in B cells activates where it signals downstream to the MAP Kinase pathway and to ultimately to p38. We find that Syk inhibitor, piceatannol, blocks tyrosine phosphorylation of PIP5Kβ during oxidative stress which leads to a decrease in PIP_2 drop in Hela cells. While Syk activation is crucial for p38 activation, treatment with SB205380, a p38 inhibitor did not block PIP5Kβ phosphorylation suggesting that PIP5Kβ is upstream of p38 activation or independent of p38 signaling (data not shown). While I found that Syk and PIP5Kβ were able to associate especially when treated with oxidative stress, I cannot say for certain if this is a direct or indirect interaction because this was found using co-immunoprecipitation. In addition, the *in vitro* kinase assay used immunoprecipitated proteins and not purified proteins. Despite mutating PIP5Kβ's Y105 residue, this does not prevent Syk from associating with PIP5Kβ during H_2O_2 treatment. This may be due

to Syk being able to bind PIP5Kβ on a site distal to 105 or by binding in a complex that is not affected by the mutation.

The actin response to H_2O_2 is complicated, often with opposite observations in different cell types (Rokutan et al. 1994; Huot et al. 1997) (Rokutan et al. 1994; Huot et al. 1997). Huot and colleges have observed H_2O_2 increases stress fiber formation in endothelial cells while other reports describe a decrease of stress fibers in fibroblast cells (Mocali et al. 1995; Houle et al. 2007). In this system, I find H_2O_2 at concentrations of 100μM and 1mM lead to a loss in F-actin stress fibers in both HUVEC and endothelial cells, respectively. In accordance with previous observations, HUVECs are more sensitive to oxidative stress, due to the loss of stress fibers occurring with treatment of 250μM H_2O_2. HeLa cells required a higher level of H_2O_2 treatment, 1mM H_2O_2. The typical time it takes for us to see a loss of stress fibers occurs at 10 to 15 minutes. The phenotype is most pronounced at 30 minutes. The connection between actin and PIP_2 is well documented (Yin and Janmey 2003). PIP_2 has been shown to positively regulate several actin polymerizing proteins such as N-WASP family of proteins, cofilin, and profilin, while decrease in PIP_2 levels by overexpression of PIP_2 phosphatase, synaptojanin, leads to a loss of stress fibers (Pantaloni and Carlier 1993; Sakisaka et al. 1997; Pantaloni et al. 2001). Furthermore, PIP5Kβ overexpression induces actin polymerization in a Rho-A dependent manner so deactivation of PIP5Kβ fits the model that this kinase is a major contributor to actin assembly (Yamamoto et al. 2001). It is conceivable that the decrease in PIP_2 during oxidative stress leads to the eventual loss of the above-mentioned proteins activities which leads to the phenotype observed.

Interestingly, during oxidative stress, PIP5Kγ increased activity while PIP5Kβ decreased. PIP5Kγ has been implicated in neuronal and cardiac development in addition to regulation of ion channels (Li et al. 2005; Wang et al. 2007). Yet the overall contribution to total cellular PIP_2 levels is very low compared to PIP5Kβ (Padron et al. 2003b). Notably though, the phenotype of the PIP5Kγ knock out mouse is lethal, dying within a day of birth while PIP5Kβ knock out mice are viable (Di Paolo et al. 2004; Sasaki et al. 2005). This suggests an adaptive compensatory mechanism for PIP_2 production in lieu of PIP5Kβ, which is the most abundant PIP5K in some types of cells.

Burn trauma induces a massive oxidative stress (Horton 2003). After burn occurs, levels of ATP in the afflicted areas decrease, leading to an increase in AMP levels. The AMP produced is converted into hypoxanthine. Hypoxanthine is a substrate for xanthine oxidase. Through a series of reactions, free radicals and oxidative stress agents are produced from the product of the xanthine oxidase reaction. Patients experiencing severe burn injury typically develop generalized edema. The edema is most pronounced at the lung area where the lung microvascular seems to be the most susceptible to free radical damage. The mechanism by which the cellular junctions break down and allow a large fluid movement from the blood system through the endothelial vasculature is not completely understood (Turnage et al. 2002). The inflammatory cytokine, Tumor Necrosis Factor-α (TNF-α) has been shown to be upregulated during burn injury. TNF-α has been shown to alter endothelial vasculature by altering the cellular morphology, intracellular junctions and remodeling the cytoskeleton (Wright et al. 2000). It is conceivable that burn injury and the associated fluid movement across the

permeabilized endothelial barrier is from a combination of TNF-α and oxidative radical exposure. Hypertonic fluid resuscitation is a common treatment for treating acute burn trauma (Kreimeier and Messmer 2002). We have previously demonstrated that hypertonic stress increases PIP_2 levels and actin polymerization. Hypertonic stress modulating levels of PIP_2 is a possible mechanism by which this treatment can dampen the acute effects of burn trauma by fortifying the cell structure and intracellular junctions in addition to dampening the inflammatory response by preventing neutrophil extravasation.

In conclusion, these findings suggest that PIP5Kβ is active at the plasma membrane while inactive in the cytosol. This activity and localization is presumably important for actin polymerization and depolymerization and dependent upon ser/thr phosphorylation and mainly tyrosine phosphorylation at amino acid position Y105. These data provide further evidence for specific pools of PIP_2 that are spatially and temporally regulated.

CHAPTER 4

REGULATION OF PHOSPHATIDYLINOSITOL 4 KINASES

4.1 INRODUCTION

At the moment, much less is known about how PI4Ks are regulated. The one exception is NCS-1, a calcium binding protein that activates PI4KIIIβ and increases secretion of insulin when overexpressed in pancreatic beta cells (Gromada et al. 2005). NCS-1 has four EF-Hand domains that bind calcium with high affinity. The binding studies done in vitro suggest that NCS-1 is able to sense and transduce small changes in intracellular calcium levels (Hilfiker 2003).

Ying Jie Wang in our lab previously found that PIP5Kγ87 specifically contributes a pool of PIP_2 that is used by PLCβ for IP_3 generation and calcium signaling (Wang et al. 2004). This finding supports the idea that specific PIP5Ks produce specialized and non-redundant pools of PIP_2. Similar to the PIP5Ks, the PI4Ks are differentially localized within the cell. I wanted to see if a specific PI4K contributed a specialized pool of PI4P that is the precursor for PIP5Kγ87 generated PIP_2. The first part of this chapter will be devoted to characterizing the role of NCS-1 in regulating PI4P levels and its role in calcium signaling. The second part of this chapter will discuss the observation that PI4P increases during oxidative stress and pervanadate (PV) treatment. My goal was to identify which PI4K was responsible for the oxidative stress stimulated increase in PI4P. Pharmacological inhibition and RNAi will be used to determine effect

on oxidative stress dependent PI4P increase. The preliminary results presented here have not been submitted for publication.

4.2 LOCALIZATION OF NCS-1 IN HELA CELLS

Early studies of NCS-1 undertaken in yeast cells found that Frq1p, the yeast homolog was able to interact Pik1p, the mammalian homolog of PI4KIIIβ where it was found that Frq1p was essential for Golgi localization of Pik1p (Strahl et al. 2005). Although this finding has not been confirmed in mammalian cells, NCS-1 is nevertheless a *bonafide* regulator of PI4KIIIβ activity. In collaboration with Andreas Jeromin who kindly provided me with his antibody against NCS-1, I decided to test the contribution of NCS-1 mediated PI4P to hormone sensitive pools of PIP_2.

I first checked the localization of NCS-1 in our HeLa cells (Figure 4.1A). In accordance with other groups, NCS-1 was localized in the cytoplasm with a punctate staining, indicating that some of it was localized to vesicles. NCS-1 is also colocalized with the *trans* Golgi marker, TGN-46, further strengthening its role in secretion. When NCS-1 localization was compared with that of exogenously expressed PI4KIIIβ, both proteins had a high level of colocalization in perinculear region and in some cells at the plasma membrane (Figure 4.1B).

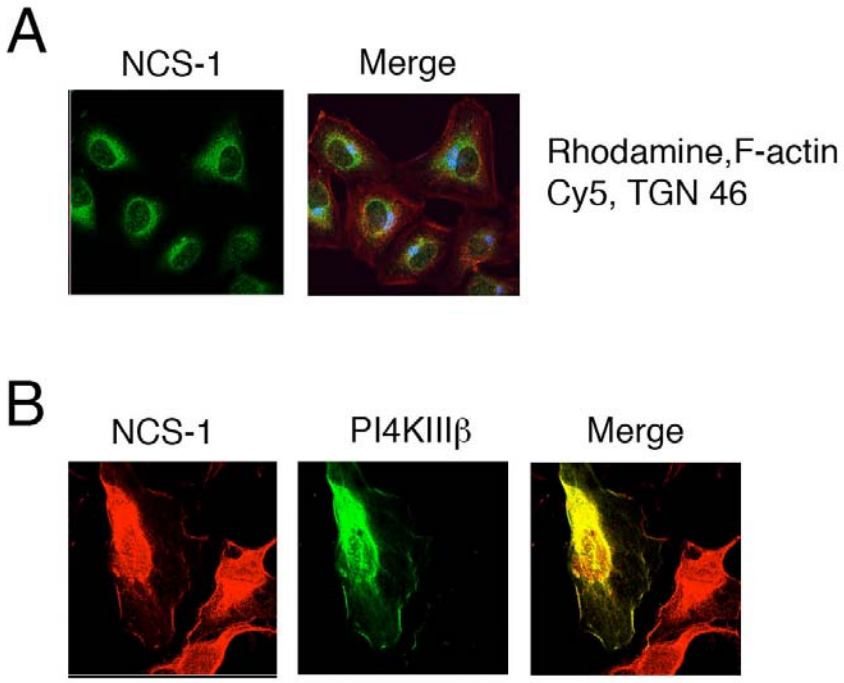


Figure 4.1

Localization of NCS-1. A. HeLa cells were fixed with paraformaldehyde and stained with αNCS-1 (1:500) and FITC conjugated anti –rabbit. F-actin was stained with rhodamine conjugated phalloidin and α-TGN-46 (1:1000) was used to visual the Golgi. B. HeLa cells were transfected with pCMV5 myc-PI4KIIIβ. Endogenous NCS-1 was stained with α-NCS-1. Monoclonal myc antibody was used to stain PI4KIIIβ. All images were examined using LSM 5 (Carl Zeiss) confocal microscopy software.

4.3 EFFECT OF NCS-1 KNOCKDOWN ON PI4P LEVELS

To further examine the interplay between NCS-1 and PI4Ks, I decided to knock down NCS-1 to determine the effect in total PI4P levels in non-professional secretory cells. I first validated NCS-1 KD by Western blotting for the endogenous NCS-1 (Figure 4.2A). Actin was used as an internal control. NCS-1 levels were clearly

decreased when compared with cells treated with the control scramble siRNA. NCS-1 depletion also decreased PI4P levels modestly (Figure 4.2B). This decrease was similar to that obtained with PI4KIIIβ knockdown and was much less than that observed with PI4KIIα knockdown.

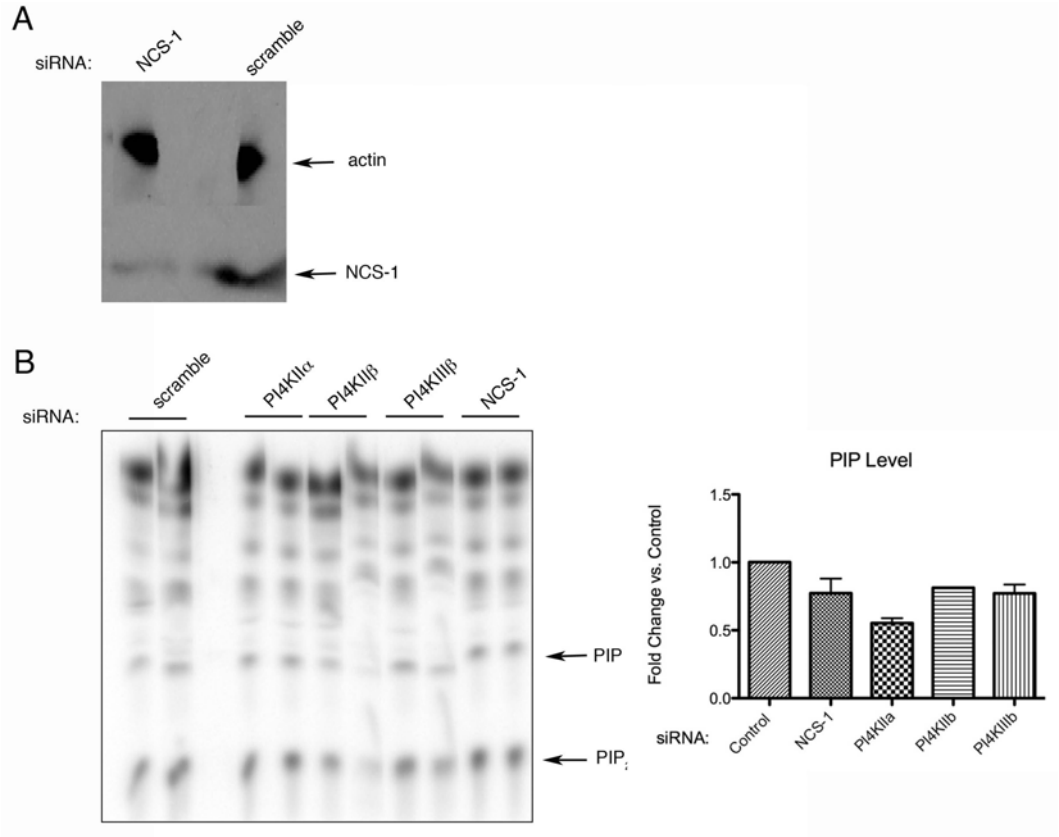


Figure 4.2

NCS-1 knockdown decreases PIP levels. A. Knockdown of NCS-1. HeLa cells were transfected with siRNA oligos against NCS-1. RNAi treatment was allowed to go 72 hours before cells were lysates were harvested. Lysates were run on SDS-PAGE and blotted with α–NCS-1. α-actin was used as an internal control. B. Effect of knockdown on PIP levels. HeLa cells were treated with siRNA oligos for 72 hours. Cells were labeled with ^{32}P-orthophosphate for 4 hours. Lipids were extracted with chloroform:methanol

mixture and run on TLC. Lipid levels were assessed by phosphorimager densitometry and normalized to the total lipid levels in respective lane. n=3.

4.4 EFFECT OF NCS-1 KNOCKDOWN ON IP_3 PRODUCTION

Since NCS-1 caused a decrease in PI4P levels, I next wanted to see if this decrease in PIP affected PIP_2 generation and subsequent IP_3 production. I treated HeLa cells with siRNA directed against NCS-1 and then stimulated IP_3 production with histamine. Histamine activates the H1 type GPCR to activate PLCβ which cleaves PIP_2 to form IP_3. Cells were lysed and IP_3 was quantitated using a microsome binding assay (Sun et al. 1995). Microsome IP_3 receptors from bovine brains were used to titrate tritium labeled IP_3. Activity was quantified and the level of IP_3 production was extrapolated from a standard curve (Figure 4.3). Unfortunately, I was not able to obtain meaningful results due to large variability in the IP_3 response. Whereas Ying Jie's results yielded a biphasic response with a major peak occurring at 5 seconds and a second smaller peak at 20 seconds (Wang et al. 2004), my results did not consistently show these peaks. In the end, no conclusion could be drawn from this series of experiments.

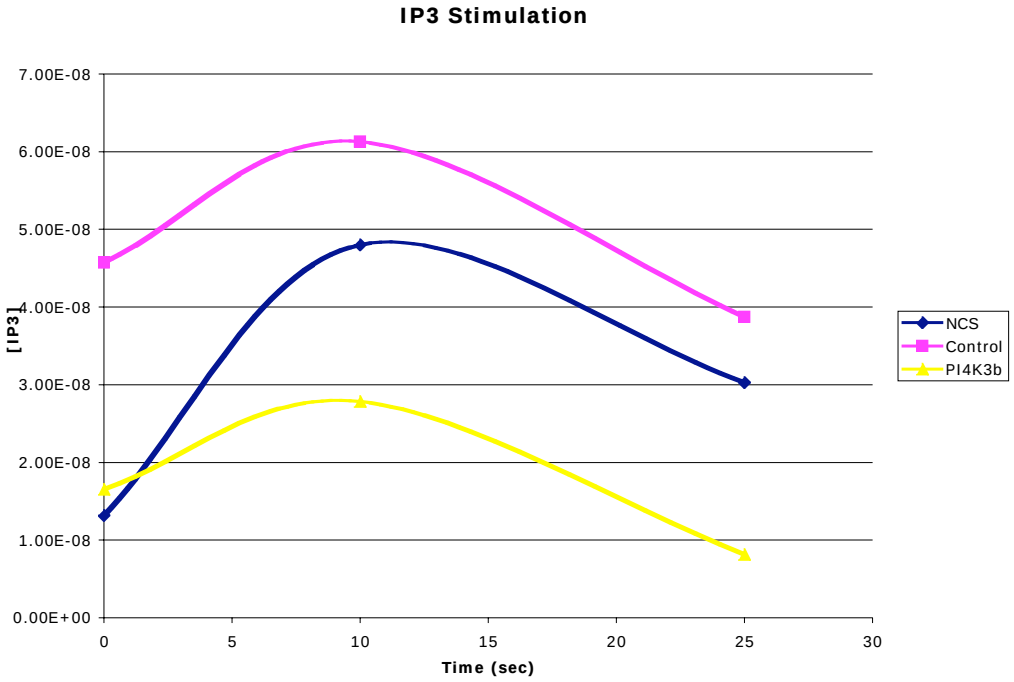


Figure 4.3

NCS-1 RNAi and IP_3 Stimulation. HeLa cells were treated with siRNA oligos for 72 hours. After 72 hours, cells were washed with serum-free DMEM. Cells were put at room temperature and allowed to equilibriate for 30 minutes. Result from one set of experiments is shown.

4.5 PV DOES NOT PROMOTE PI4K TYROSINE PHOSPHORYLATION

In the previous chapter, I found that both H_2O_2 and the tyrosine phosphatase inhibitor, PV, decreased PIP_2 but increase PI4P. PV caused tyrosine phosphorylation of PIP5Kβ and PIP5Kγ. Therefore, it may promote PI4K tyrosine phosphorylation to increase lipid kinase activity. HeLa cells transfected with epitope-tagged PI4Ks or PIP5K were treated with 50 μM PV. Immunoprecipitated epitope-tagged proteins were checked for tyrosine phosphorylation using a phospho-tyrosine antibody (Figure 4.4). As

described previously, PIP5Kβ is tyrosine phosphorylated after PV treatment. By contrast, the three PI4Ks tested were not tyrosine phosphorylated. The PI4K family also has a 4th isoform, PI4KIIIα. PI4KIIIα is a large protein (over 220KDa) which makes it both difficult to express and detect. I attempted to express PI4KIIIα in both HeLa and Cos cells but had no success in detecting this isoform by Western blot.

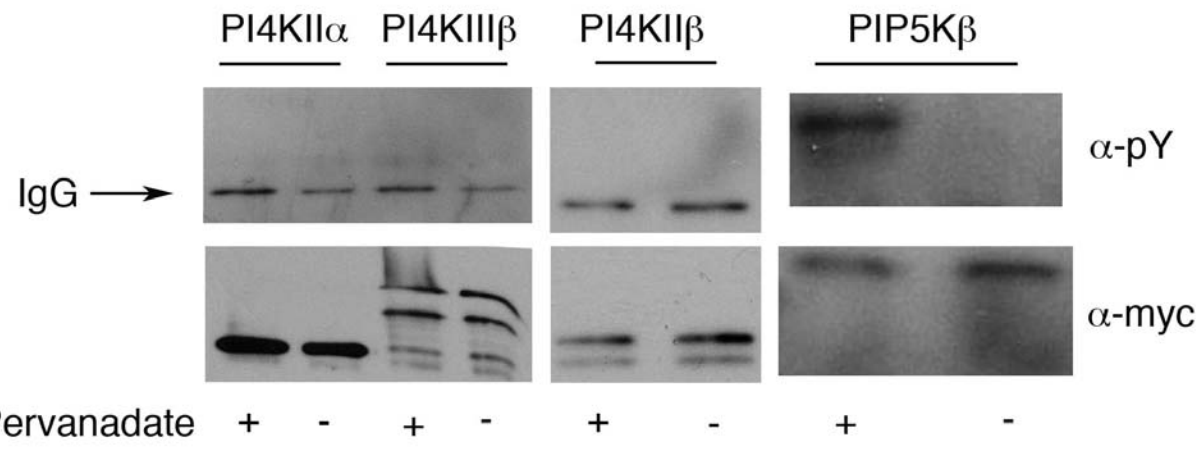


Figure 4.4

Effect of PV on PI4K tyrosine phosphorylation. Cos-1 cells were transfected for 16 hours with myc-PI4K and PIP5K constructs. Cells were washed with serum-free DMEM three times before stimulation. 50μM PV was used to stimulate the cells for 15 minutes. After 15 minutes, cells were lysed and immunoprecipitated with monoclonal myc antibody and Protein G sepharose. Proteins were run on SDS-PAGE.

4.6 USE OF WORTMANNIN AND RNAi TO IDENTIFY THE PI4K FAMILY THAT CONTRIBUTES TO THE PV INDUCED PI4P INCREASE

The type II and III PI4Ks can be distinguished by the pharmacological inhibitor wortmannin. Cells were treated with 10μM wortmannin to inhibit PI4KIII enzymes prior to PV stimulation. TLC analysis showed that basal levels of PI4P were decreased 40% with wortmannin treatment (Figure 4.5A). Nevertheless, this did not block the increase in PI4P during PV treatment. Since PI4P increased to a similar extent with or without wortmannin treatment, I conclude that type III PI4Ks are not primarily responsible for the PV induced PI4P increase.

I next focused on the Type II PI4Ks. Because there is no *in vivo* pharmacological inhibitor of the Type II family, I used RNAi to knockdown the PI4Ks to see if this blocked PI4P increase. siRNA against PI4KIIα and PI4KIIβ were used to decrease protein levels. Knocking these two kinases decreased basal PIP levels also (Figure 4.5B), however this did not block the increase in PIP levels upon stimulation with PV. PIP levels increased the same fold level as mock siRNA treated cells. Thus it appears that neither Type II nor Type III PI4K is involved in the PV stimulated increase in PI4P.

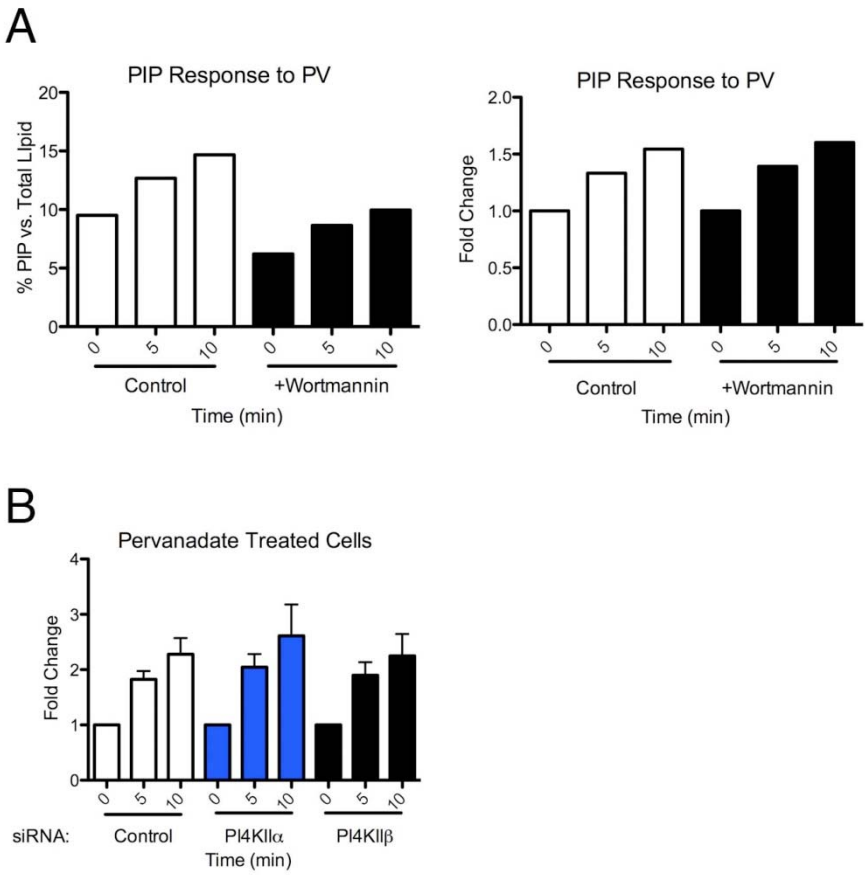


Figure 4.5

Inhibition of PI4K during PV Treatment. A. Wortmannin does not block the PV response. HeLa cells were labeled with ^{32}P Orthophosphate for 4 hours. Cells were incubated with 10μM wortmannin for 45 minutes prior to stimulation with 50μM PV. Cells were then lysed and lipids extracted. Lipids were run on TLC plate and quantitated by phosphorimaging software (n=2). B. Inhibition of Type II PI4K does not dampen PI4P increase. HeLa cells were treated with siRNA oligos for 72 hours. Cells were then labeled with ^{32}P orthophosphate as described above. Cells were stimulated with PV and lipids were extracted.

4.7 DISCUSSION

The establishment of PI4P as a *bona fide* signaling molecule has given hints as to the possible regulation of these kinases, yet the overall picture remains unclear. Utilizing data from previous findings that NCS-1 increases PI4P levels (Zhao et al. 2001), I examined the role of NCS-1 influencing the pool of PI4P produced for IP_3 signaling. Knockdown of NCS-1 lead to a decrease in PI4P levels as assessed by TLC. However, the overall effect on IP_3 signaling was inconclusive. The inconsistency between experiments made it difficult for me to make a conclusion about the contribution of NCS-1 stimulated PI4P to PLCβ dependent generation of IP_3.

The variability between experiments may arise from several different sources. First, the HeLa cells sensitivity to histamine is a very high. The spike in IP_3 production occurs relatively quickly during histamine stimulation. Once IP_3 is produced, it is quickly phosphorylated by IP_3 kinases (Michell 2008). In order to slow this reaction down, I incubated the cells at room temperature for 30 minutes prior to stimulation. According to our established protocols, this should delay the spike in production of IP_3 to about 5-10 seconds post-stimulation (Wang et al. 2004). Despite this delay, it was still difficult to capture the spike in IP_3 production. Another source of variability were the microsomes containing the IP_3 receptors. The microsomes were frozen and thawed no more than 3 times in order to perform the experiment. It is possible that even one freeze-thaw cycle may adversely destabilize the IP_3 receptor and its ability to bind the substrate. Furthermore the microsomes were contained in a viscous and heterogeneous solution that was difficult to evenly distribute into the microcentrifuge tubes. The variability in

distribution between samples could cause an inaccurate reading for the IP_3 produced. The amount of IP_3 produced was calculated by generating a standard curve with increasing amounts of cold IP_3 to titrate the tritium-labeled IP_3. During the generation of the standard curves, there were often inconsistencies with the final curve that did not follow the proper trend, suggesting experimental error.

I believe that while NCS-1 and PI4KIIIβ both decreased PI4P and PIP_2 levels, this may not necessarily be the specific kinase that generates the pool of PI4P for IP_3 generation. First, cells have a compensatory mechanism for maintaining PPI levels. If the pool of PI4P for PIP5Kγ is lost, the other 3 PI4K may be able to generate the necessary PI4P for phosphorylation by PIP5Kγ87. Because PI4P is an indirect substrate of PLC while PIP_2 is the direct substrate of PLC, it is more likely that PIP5Kγ produces a specific pool of PIP_2 for IP_3 production rather than a specific PI4K. Furthermore, biochemical studies have found PI4KIIIα as well as PI4KIIβ localized at the plasma membrane. Therefore, it is possible that PI4KIIIα or PI4KIIβ generates the PI4P essential for PIP5Ks at the PM for various PIP_2 dependent processes including IP_3 signaling.

The second set of experiments trying to understand the mechanism of PV-induced PI4P increase presented me with interesting results. Using wortmannin to inhibit the activity of the type III PI4K's, I found this decreased basal levels of PI4P but did not block the overall increase in PI4P. The fold change between the control and wortmannin treated samples were similar, indicating the type III PI4Ks were not activated during PV treatment. Wortmannin does not block the type II PI4Ks. The type II PI4Ks can only be

inhibited by adenosine *in vitro*. In order to evaluate if PI4KIIα or PI4KIIβ was involved in this response, I used RNAi to knockdown each isoform. Knockdown of either isoform did not block PV increased PI4P. While basal level of PI4P decreased, the fold change in PI4P production did not differ from the control. This seems to rule out both the type II and type III PI4ks from increasing PI4P level during PV treatment. A caveat to these observations is that there are no high quality commercial antibodies against any of the PI4Ks. Therefore, it difficult to determine the level of knockdown in these cells because I could not normalize the decrease in PI4P to actual protein levels of PI4K. Our only standard of comparison was the amount of PI4P produced versus the total lipids as judged by TLC. It is possible that our siRNA only partially decreased the PI4Ks and this may have masked the true extent of each kinase's respective contribution of PI4P. Another way to test if one of the kinases were activated during PV treatment would be to do an *in vitro* lipid kinase assay to see if the activity changed. Even though pharmacological and RNAi inhibition of the PI4Ks do not show a block in PI4P production, this does not completely rule out that these kinases are not involved in this process. Although tyrosine phosphorylation does not seem to be a direct post-translational modification governing the activity of the PI4Ks, it is possible that ser/thr phosphorylation may regulate the activity of PI4K. It has been found that PI4KIIα is ser/thr phosphorylated but how this regulates the kinase activity remains unclear (Barylko et al. 2001). An alternate explanation for the increase in PI4P is through an alternative way of generating PI4P. PI4P is synthesized through phosphorylation of the D4 position by the PI4Ks. Alternatively, PIP can be generated by dephosphorylation of $PI(4,5)P_2$ by the SHIP

family of inositol phosphatases *in vitro* (Pesesse et al. 2006). tyrosine phosphorylation leads to SHIP PM localization (Lioubin et al. 1996) Therefore, it is possible that increasing tyrosine phosphorylation with PV or H_2O_2 leads to activation of SHIP which in turn increases PIP levels.

While the data in this section is inconclusive, the mechanism underlying these changes may present an interesting and novel mode of regulation of the PI4Ks. Different approaches as well as better controls in the future may be able to answer these questions.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 CONCLUSIONS

PPIs play a crucial role in maintaining cellular homeostasis. Preturbations in PPI homeostasis have been linked to several human disease phenotypes. In the 1980's, the discovery that hydrolysis of PIP_2 by PLC generates IP_3 and DAG led to a renewed interest of these lipids as integral signaling molecules (Berridge 1993). Subsequently, many lipid kinases that generate the PPI derivatives were identified. The surprising finding that multiple kinases, such as PIP5Ks, generating the same PPI species led many to examine the underlying reasons for having multiple kinases performing the identical enzymatic reaction. Since PPIs regulate many different cellular functions, it is proposed that multiple kinase isoforms exist to perform specific non-redundant functions. Currently, there is increasing understanding of the specific role for each PIP5K isoform.. The advent of RNAi technology has further provided a boon to the understanding of specific roles of these lipid kinases.

The aim of my research has been to understand specific roles for PIP5Ks and PI4Ks. I began by making the observation that two different cellular stresses lead to opposite changes in PPI homeostasis. Finding that hypertonic stress leads to an increase in $PI(4,5)P_2$ levels, I set out to determine if a PIP5K isoform was activated during this process. I found that PIP5Kβ was preferentially activated and it contributed to the increase in PIP_2 (Yamamoto et al. 2006). Conversely, oxidative stress leads to a decrease

in PIP_2. I examined the possibility that one of the PIP5Ks was specifically deactivated during this process and identified PIP5Kβ once again as the relevant isoform. The opposite effects of hypertonic and oxidative stress on PIP5Kβ, PIP_2 levels and the actin cytoskeleton suggest that PIP5Kβ is an important stress mediator and that it acts through regulation of the actin cytoskeleton.

5.2 DIRECT AND INDIRECT REGULATORS OF PIP5Kβ

My studies further identify a novel mode of regulation of PIP5Kβ by ser/thr dephosphorylation. While it has been shown previously that PIP5Kβ is phosphorylated on a serine residue (Park et al. 2001b) and that dephosphorylation leads to increased activity, the physiological regulation of PIP5Kβ was not understood. An *in vitro* study suggested that PIP5Kβ was phosphorylated on ser214 by PKA, but there appears to be multiple other phosphorylation sites. The kinases that phosphorylate these other sites have not been identified. My phosphatase inhibitor studies suggest that the hypertonicity induced PIP5Kβ ser/thr dephosphorylation is mediated by the protein phosphatase type I (PP1) family (Yamamoto et al. 2006). Additional studies will be required to identify the relevant protein kinases and phosphatases that regulate PIP5Kβ ser/thr phosphorylation during hypertonic stress. One approach that may be used in the future, is to examine *in vitro* ser/thr phosphorylation/dephosphorylation.

My results suggest that PIP_2 produced by the increased activity of PIP5Kβ during hypertonic stress induces actin polymerization. The identities of the proteins that are regulated by PIP_2 during this process are unknown, although there are many candidates.

For example, osmotic shrinkage recruits the ERM proteins to the PM (Rasmussen et al. 2008). Since it is known that PIP_2 changes ERM conformation to expose its PM binding domains and attachment to the actin cytoskeleton (Niggli and Rossy 2008), it is possible that the changes I have observed with the different stresses modulates these proteins, ERM may account at least in part for the increased actin polymerization response during hypertonic stress. ERM may not be the entire story though, because RNAi of the ezrin did not have a significant effect on the formation of F-actin bundles *per se* during hypertonic stress. Since oxidative stress induces actin depolymerization and a decrease in PIP_2, it would be attractive to hypothesize that oxidative stress inhibits the ERM proteins by decreasing PIP_2. Additional studies will be required to determine if this is indeed the case.

5.3 STRUCTURE BASED STUDIES OF TYPE I PIP5Kβ

The experiments in Chapter 3 show that during oxidative stress, PIP5Kβ is deactivated by tyrosine phosphorylation by Syk. Studies with immune cells have clearly established that Syk itself is activated by oxidative stress (Takano et al. 2002). My results show for the first time that Syk plays a key role in the oxidative stress response in epithelial cells as well. This implicates Syk as a regulator of actin through modulation of PIP5Kβ tyrosine phosphorylation/inactivation. In addition, oxidative stress may further increase PIP5Kβ tyrosine phosphorylation by inhibiting the tyrosine phosphatase that dephosphorylates PIP5Kβ. The physiologically relevant tyrosine phosphatase has not been identified, but the cysteine-based tyrosine phosphatases (CBPs) that are inhibited by

ROS are attractive candidates (Genestra 2007). ROS oxidize the reactive cysteine on CBPs, rendering them unable to carry a nucleophilic attack and thus dephosphorylate their targets. For example, SHP-2 is a cytosolic phosphatase that is deactivated during oxidative signaling in response to PDGF stimulation (Meng et al. 2002). It is conceivable that PIP5Kβ is maintained in a dephosphorylated state by SHP-2 but upon oxidative stress, SHP-2 is inactivated leading to net tyrosine phosphorylation of PIP5Kβ by Syk.

My data suggest that PIP5Kβ is maintained in multiple combinatorial states of phosphorylation/dephosphorylation. It is inactive when either tyrosine phosphorylated, or ser/thr phosphorylated and more active when tyrosine dephosphorylated or ser/thr dephosphorylated. Upon hypertonic stress, PIP5Kβ is dephosphorylated on ser/thr residues with no tyrosine phosphorylation. I speculate that these posttranslational modifications alter the conformation of either the membrane docking site and/or the catalytic site. Since the three dimensional structure of type I PIP5Kβ has not been solved, I speculate about the nature of the changes based on the 3D structure of the type II PIP5Kβ (Rao et al. 1998). This is a reasonable approach because it has been suggested that much of the secondary and tertiary structures in the large lipid kinase families are conserved. Significantly, the tyrosine at position 105 in PIP5Kβ, which is phosphorylated during oxidative stress, is also found in an equivalent position in type II PIP5Kβ. The structure of PIP5KIIβ reveals a large acidic patch on a massive area that contains a flat surface suggesting a plasma membrane docking site. I find that type I PIP5Kβ ser/thr dephosphorylation increases PM binding while tyr phosphorylation decreases PM assosciation. It is possible that ser/thr dephosphorylation leads to a loss of

net negative charge thus allowing for the net charge of the surface to become more positive, subsequently leading to plasma membrane binding becoming more favorable. In addition, ser/thr dephosphorylation may induce a change in tertiary structure to allow for the flat surface to become more accessible to the negative charge of the PM. By contrast, PIP5Kβ tyrosine phosphorylation decreases membrane binding. An intriguing question is how does a single tyrosine phosphorylation lead to a loss of membrane binding? Is this tyrosine affecting the tertiary structure to conceal the flat surface? Is the structure changed such that the positive residues are rendered inaccessible to the plasma membrane thus causing detachment and a shift to the cytosolic fraction of the cell?

Solving the structure of PIP5KIβ would be an important step forward in understanding the mode of regulation. The structures of tyrosine phosphorylated and ser/thr phosphorylated PIP5KIβ would provide even more information as to how the PIP5K family functions and is regulated. Furthermore, it would be interesting to see how tyrosine phosphorylation affects the ser/thr residues that are dephosphorylated during oxidative stress.

5.4 CLINICAL IMPLICATIONS

The use of H_2O_2 to induce oxidative stress in our system mimics exposure of cells to injury and inflammation. Our lab has focused on dermal burn trauma, and I will therefore concentrate on this injury. Massive dermal burn induces not only dermal injury, but also massive injury response in multiple organs at a distance from the burn sites. This is attributed to the generation of ROS by the initial burn injury and subsequent

activation of inflammatory cells that is further exacerbated by secondary bacterial infection. Although many organs are affected, the lungs are particularly sensitive. In many cases, the lung microvasculature becomes permeable to fluid and small molecules leading to edema (Horton 2003). This is because the cell:cell junctions are disrupted as a result of massive actin reorganization. The PIP_2 decrease may also affect the cell to cell adhesions by causing a loss of integrin adhesion to the ECM and conjugate integrins. FA and its associated ECM proteins are implicated in the regulation of vascular permeability (Partridge et al. 1992; Dudek and Garcia 2001). Disruption of FA components using antibodies that alter cell attachment and spreading increases permeability (Lampugnani et al. 1991). PIP5Kγ90 has been implicated in regulating the formation of FA. PIP5Kγ90 was shown to be phosphorylated by Src (Ling et al. 2003). This association was regulated by focal adhesion kinase (FAK). PIP_2 has also been shown to activate vinculin which binds FA components such as talin, α-actinin, and F-actin. It is possible that loss of PIP_2 through oxidative stress disrupts the structure of FA leading to the permeability of macromolecules through these junctions.

To test the involvement of PIP5K contribution to endothelial permeability, one would need to isolate microvascular endothelial cells from PIP5K KO mice. This was previously an extremely difficult undertaking, but which as recently been optimized (Cha et al. 2005). Once isolated, the endothelial cells would be grown as a monolayer and tested for their permeability by transendothelial electrical resistance measurement (Verin et al. 2001).

5.5 ENDOGENOUS ROS REGULATION OF PIP5Ks

Neutrophils are immunological cells that are activated upon pathogenic stimuli. One aspect of neutrophil activation that may be pertinent to the previous data is the process of cell spreading. Neutrophil "rolling" is the process by which neutrophils travel through the bloodstream searching for a signals that trigger it to adhere to an area in order to fight an infection (Ley 2002). Once the neutrophil is signaled through cytokines or engagement of the β2 integrin, the neutrophil adheres to a specific area where the cell goes from a compacted state to a "spreaded" state in which the surface area of the membrane increases dramatically (Dewitt and Hallett 2007). The engagement of the β2 integrin has been found to lead to the activation of NADPH oxidase (Mayadas and Cullere 2005). NADPH oxidase activity leads to the generation of ROS. Once the neutrophil decides it must spread, it is believed that the membranes that are sequestered in folded stacks in order to allow for efficient "rolling" are then unleashed by detachment of proteins that are tethered to the actin cytoskeleton. This event requires a remodeling of the actin cytoskeleton. Therefore, it is tempting to speculate that the ROS produced by β2 integrin signaling leads to activation of tyrosine kinases including Syk which then phosphorylates PIP5Kβ and allows for efficient remodeling of the cytoskeleton and reorganization of the neutrophil plasma membrane.

Monocytes undergo a similar morphological change in that it undergoes cell spreading as well during engagement with a substrate. Ligation of the monocyte's β1 integrin leads to cytokine production (Nishibori et al. 2003). A signaling event activated

by β1 engagement is the activation of several PTKs, including Syk (Lin et al. 1995). Using tyrosine kinase inhibitors, genistein and herbimycin A, Syk's activity was decreased in response to β1 integrin engagement. It is possible that in monocytes, β1 integrin similarly leads to activation of Syk, which regulates PIP5Kβ activity and ultimately the ability of monocytes to undergo cell spreading.

While NADPH is known as a source of ROS in immune cells, it has also been identified in non-phagocytic cells TNF-α treatment of endothelial cells leads to the activation of NADPH and generation of ROS. TNF-α leads to morphological changes in the intercellular junctions (Turnage et al. 2002). It is believed that TNF-α is one of the main factors leading to permeability of cell-cell junctions in the endothelial vasculature. Several observations find that the actin and VE-cadherin structure is greatly altered during exposure to TNF-α (Wojciak-Stothard et al. 1998; Turnage et al. 2002; Wojciak-Stothard et al. 2005; Wojciak-Stothard et al. 2006). A mechanism that may be regulated by these events is the activity of PIP5Kβ which in turn leads to the remodeling of the cell junctions. It is possible that TNF-α activates an alternate pathway that regulates PIP5Kβ activity, perhaps through a PP1 phosphatase, which leads to increased PIP_2 and polymerization of actin

Pulmonary artery endothelial cells that have been kept in hypoxic conditions and then reoxygenated leads to a loss of F-actin stress fibers (Wojciak-Stothard et al. 2005; Wojciak-Stothard et al. 2006). The reoxygenation step leads to a production of ROS through the activation of NADPH, therefore it is possible the ROS in endothelial cells

leads to the deactivation of PIP5Kβ. This suggests an alternate pathway of NADPH activation and downstream signaling by TNF-α and hypoxic to normoxic transitioning.

5.6 REGULATION OF PIP5Kβ BY VEGF

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that stimulates endothelial cell growth and motility (Tabernero 2007). VEGF activates NADPH oxidase in the endothelial cells leading to a burst of ROS that acts as a signaling molecule. Interestingly, lower levels of VEGF treatment in endothelial cells induces actin polymerization, stress fiber formation and migration while higher levels leads to the opposite effects, actin depolymerization, loss of stress fibers, and inhibition of migration (Waltenberger et al. 1994; Rousseau et al. 1997).

Another target of VEGF signaling is the activation of the Src kinases (Eliceiri et al. 1999). Src activity induced by VEGF has been shown to be essential for angiogenesis during tumor progression. Furthermore, Syk has been shown to be a critical mediator in the development of the lymphatic vascular system. $Syk^{-/-}$ mice day shortly after birth (Yanagi et al. 2001a). This was shown to be as a consequence of decreased number and abnormal morphology of endothelial cells which leads to vascular permeability in the blood vessels of mice.

VEGFR activation also leads to tyrosine phosphorylation of several substrates. Tyrosine phosphorylation of PLC-γ through Src occurs during VEGF signaling although it is unclear exactly how this occurs (He et al. 1999). It is possible that Src activation during this event activates Syk. Syk has been shown to phosphorylate PLC-γ, thus

activating its catalytic activity. At the same time, Syk may be phosphorylating PIP5Kγ as we have shown in Chapter 3. This phosphorylation may be activating PIP5Kγ which contributes a specific pool of PIP_2 for PLCγ leading to Ca^{2+} signaling and cell motility. Thus, VEGF may be stimulating EC would repair through the generation of ROS and activation of Syk which leads to PIP5Kγ activation and Ca^{2+} signaling, while at the same time regulating the function of PIP5Kβ leading to regulation of the actin cytoskeleton. This may be dependent of a delicate balance of ROS production and signaling.

An experiment that might give us clues as to a connection between VEGF signaling and PIP5K regulation would be to test VEGF's ability to induce migration. Using endothelial cells from WT, PIP5K$\gamma^{-/-}$ and PIP5K$\beta^{-/-}$ mice, we could test if there is a differential rate of cell migration induced in chemotaxis chambers between the different types of cells. If VEGFR signaling is regulating PIP5Ks, we may be able to see a tyrosine phosphorylation of one of the isoforms in WT cells as well as a difference in migration rate in the KO cells. This could suggest that one of the PIP5Ks may play a role in enhancing or inhibiting VEGFR signaling.

5.7 REGULATION OF PI4K

The results of Chapter 4 present an inconclusive picture as to the regulation of the PI4K family. Yet this is not to say that the observations I have made leave us with a null hypothesis. Many of the experiments performed were difficult to control due to lack of reagents available at the time. While NCS-1 knockdown could be appropriately quantified due to an endogenous antibody for NCS-1, the majority of PI4Ks currently

lack a high specificity antibody that we can use to quantify the knockdown of each isoform. This led us to speculate at the level of knockdown by drawing conclusions from the level of PIP decrease. This however presents a problem due to the fact that we cannot determine effectively the true relative contribution of PI4P by each kinase to the total cellular PI4P levels. One way to get around this problem is to use RT-PCR to measure the levels of PI4K isoform mRNA decrease during RNAi. While this may not be as direct as measuring the protein level, this would still give us an idea as to how effect our siRNA constructs worked. RT-PCR was unavailable at the time of these experiments but if we were to revisit these experiments, this method could be utilized as a tool to quantify our knockdown.

Quantification of PI4P using visualization methods has been largely ineffective. The PI4P antibodies used have had a high degree of non-specificity while lipid binding domains fused to GFP, while allowing us to visulize internal membrane pools of PI4P have still given questionable results. This stems from the belief that PI4P exists at the PM so that it can be phosphorylated by PIP5Ks to form $PI(4,5)P_2$. Furthermore, localization studies find PI4KIIIα localized at the PM (Balla et al. 2008). Recently, Balla and colleagues provided a breakthrough by generating a PI4P GFP sensor more sensitive than the previously used PI4P sensors. Using a tandem OSH2-PH domain, they were able to finally visualize pools of PI4P located at PM. Using this same technology, it may allow us to monitor the pools of PI4P at the PM and the relative contribution of each kinase to steady state PI4P levels at the PM. In addition, this may allow us to monitor how each PI4P levels respond to agonist stimulation such as oxidative stress. This would allow us to see the appearance or disappearance of PI4P at particular locations within the

cell during agonist stimulation. In addition, we may be able to see how knockdown of each kinase affects PI4P levels at distinct organelles as well.

CHAPTER 6

MATERIALS AND METHODS

6.1 Isoform Designation

The nomenclature of the PIP5KI field is unfortunately reversed between the human and mouse isoform designation between PIP5KIα and PIP5KIβ. Therefore, throughout this document, I have used the human designation to describe all PIP5Ks.

PIP5KI

PIP5KI Kinase	GenBank Accession Number
PIP5Kα	NM_003557
PIP5Kβ	NP_003549
PIP5Kγ87	NP_036530
PIP5Kγ90	NM_012398

PI4K

PI4K Kinase	GenBank Accession Number
PI4KIIα	NM_018425
PI4KIIβ	NM_018323
PI4KIIIα	NM_058004.2
PI4KIIIβ	NP_002642.1

HA-tagged PIP5KIα, β, γ87 and γ90 were cloned from cDNA provided by other laboratories.

6.2 Cell Culture

Hela, Cos1, 293 were grown in DMEM (Invitrogen) with 10% Fetal Bovine Serum (Atlanta). Media was supplemented with 15mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 1mM Sodium Pyruvate and 25mM Sodium Bicarbonate. Cells were grown at 37°C in 5% CO_2.

6.3 Transfection

For 30mM plates, plasmids were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) 1μg DNA/well. Cells were transfected overnight, 16 hours. In some experiments, recombinant adenovirus vectors expressing β-gal, hemagglutinin (HA)-tagged human PIP5KIβ or myc-tagged human PIP5KIα were used. These were generated using the AdEasyTM Adenoviral Vector System (Stratagene)(Yamamoto et al. 2001).

6.4 Antibodies Used

α-HA monoclonal antibody was purchased from Covance. α-myc was purchased from Santa Cruz Biotech. α-actin was purchased from sigma. α-PIP2 was purchased from BioAssay Systems. α-NCS-1 was kindly provided by Dr. Andreas Jeromin (University of Texas, Austin).

6.5 RNAi

siRNA oligonucleotides were designed according to the protocol provided by Dharmacon Research, Inc. In brief, sequences of the type AA(N19) (N = any nucleotide) from the ORF of the targeted mRNA were selected and subjected to a BLAST® search (National Center for Biotechnology Information database) against the human genome sequence to ensure the specificity of targeting. RNA oligonucleotides encoding both the sense and antisense of the target were synthesized by the Center for Biomedical Inventions (University of Texas Southwestern Medical Center at Dallas, Dallas, TX), and annealed after a protocol from Dharmacon Research, Inc. The siRNA sequence targeting PIP5KIα (GenBank/EMBL/DDBJ accession no. U78575) was from position 1923–1943. The siRNA targeting PIP5KIß (GenBank/EMBL/DDBJ accession no. NM_003558) encoded bases 1114–1135. The oligonucleotide targeting PIP5KIγ (GenBank/EMBL/DDBJ accession no. XM_047620) encoded bases 619–639. An oligonucleotide corresponding to nucleotides 695–715 of the firefly luciferase (GenBank EMBL/ DDBJ accession no. U31240) was used as a negative control. On d 1, HeLa cells were plated in 6-well plates at 30–40% confluency in antibiotic-free DME supplemented with 10% (vol/vol) FCS, 10 mM Hepes, and 1 mM sodium pyruvate. On d 2, siRNA was introduced into cells using OligofectAMINE™ reagent according to the manufacturer's instructions (Life Technologies), with 10 µl of 20 µM siRNA and 4 µl transfection reagent/well. On d 4, cells were lysed and analyzed by Western blotting.

6.6 Immunofluoresence

Cells were grown on glass coverslips. In most cases, cells were fixed in 3.7% formaldehyde, permeabilized with 0.1% Triton X-100 on ice, and labeled with antibodies in blocking buffer (1% BSA, 3% donkey serum in PBS). Cells were examined by a Zeiss 510 Laser Scanning Confocal Microscope using a 63 × 1.3 NA PlanApo objective. Captured images were analyzed using Metamorph Image software.

6.7 HPLC

Lipids were extracted, deacylated and analyzed on anion exchange HPLC columns. Negatively charged glycerol head groups were eluted with a NaOH gradient and detected online by suppressed conductivity(Nasuhoglu et al. 2002a; Nasuhoglu et al. 2002c). Individual peaks were identified with glycerophosphoryl inositol standards. Peak assignment was validated by spiking some cell samples with purified phospholipids as standards.

6.8 Immunoprecipitation

HeLa cells overexpressing the epitope tagged PIP5KI isoforms were lysed in buffer containing 25 mM-HCl, pH 7.5, 0.15 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1 mM DTT, 1 mM sodium vanadate, 0.5% NP40 and protease inhibitors. The overexpressed PIP5KIs were immunoprecipitated with monoclonal anti-epitope tag antibody bound to Protein G-Sepharose. Beads were washed 3 times with PBS or lysis buffer, and boiled with Sample buffer.

6.9 Isolation of the Triton X-100-insoluble Actin Cytoskeleton

Cells were lysed in Triton X-100, and centrifuged sequentially at low speed (15,900xg) to collect crosslinked actin stress fibers and networks (LSP), and at high speed (336,000 x g) to sediment long filaments that were not crosslinked (HSP).

6.10 Membrane Fractionation

Cells were lysed by Dounce homoginezation. Unbroken cells and nuclei were removed by centrifugation at 1,000 X *g* for 5 min to obtain a postnuclear fraction. The supernatant was centrifuged at 19,000 X *g* for 20 min at 4 °C. The pellet was resuspended in 100 μL of lysis buffer and overlaid onto a 0.8-mL cushion of 1.12 M sucrose. After centrifugation at 100,000 X *g* for 1 h, the membrane layer at the top of the sucrose cushion was collected with a long needle and sedimented at 40,000 X *g* for 20 min. The 190,000 X *g* pellets were washed once in the lysis buffer and recentrifuged. The pellets were boiled and analyzed on SDS-polyacrylamide gels.

6.11 Hypertonic Treatments

250 mM sucrose or 100mM or 150 mM NaCl was added to the isotonic serum-free DMEM media during hypertonic stimulation. Cells were incubated with hypertonic medium for 15 minutes.

6.12 Oxidative Stress Treatment, PV

Cells were treated with H_2O_2 (Sigma) 1mM for 15 minutes. Cells were pre-incubated with 60 μM Picceatannol or 80 μM (CalBiochem 527948) for 30 minutes before stimulation. Pervanadate was activated by incubating Sodium Vanadate with 1μM H_2O_2 for 30 minutes and then 1mM catalase for 5 minutes.

6.13 In Vitro Lipid Kinase Assay

Lipid kinase activity was measured by phosphorylation of PI4P using [γ-^{32}P] ATP as a phosphate donor. Sepharose G beads containing immunoprecipitated epitope-tagged PIP5KI were suspended in a solution containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 10 mM EDTA, 0.1 mM EGTA, 0.4% NP40, 10% glycerol, 1 mM DTT, 1 mM sodium vanadate, 70 μM PI4P (Biomol Inc.) and 35 μM phosphatidylserine (Avanti). Kinase assay was initiated by adding [γ-^{32}P] ATP (1 μCi/50 μl reaction with a final concentration of .2 mM ATP, NEN), and reaction proceeded at room temperature for 15 min. The reactions were stopped by adding $CHCl_3$:MeOH:HCl, and lipids were extracted, and separated by TLC. Radioactivity associated with the PIP band that comigrates with a PI4P standard was quantitated by Phosphorimager analysis and the amount of PIP5KI protein in the equivalent immunoprecipitate was determined by western blotting. Kinase activity was normalized to the amount of immunoprecipitated protein, and expressed as percent of the activity without sucrose stimulation.

6.14 *In Vitro* Kinase Assay

COS cells were transfected with myc-PIP5Ks or Syk. Cells were lysed by RIPA buffer containing protease and phosphatase inhibitors and PIP5Ks or Syk were immunoprecipitated with anti-myc or -Syk antibody at 4ºC overnight, then incubated with protein G sepharose beads (GE Healthcare) for 2 hr at 4ºC. The *in vitro* protein kinase reaction was started by the addition of ATP (with final concentration of 2 μM)

with or without 100 mM piceatannol (Calbiochem). The mixture was placed at room temperature for 20 min and the reaction was stopped by centrifugation for 20 s. The supernatant was discarded and the beads were boiled and subjected to Western blot.

6.15 Point Mutant Generation

Point mutations to PIP5Kβ were generated by use of QuickChange Site-Directed Mutagenesis Kit (Stratagene). Sites were identified using prediction program, NetPhos, http://www.cbs.dtu.dk/services/NetPhos/.

6.16 ^{32}P labeling

Cells were washed 3X with phosphate free DMEM and labeled for 4 h with 40 μCi/ml ^{32}P-PO_4. Lipids were extracted, resolved by thin layer chromatography (TLC), and detected by autoradiography.

6.17 IP_3 Assay

Hela cells incubated in Ca^{2+}-free Hank's buffer supplemented with 0.1% BSA at room temperature for 30 min were stimulated with 100 μM histamine (Sigma-Aldrich) for 0–25 s at RT and the reaction was stopped with Perchloric acid. IP_3 content was assayed by competition with exogenous [^{3}H]IP_3 to bind calf cerebellar microsomes. Assay was performed as previously described (Sun et al. 1995).

BIBLIOGRAPHY

Aikawa, Y. and Martin, T.F. 2003. ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5)bisphosphate required for regulated exocytosis. *J Cell Biol* **162**(4): 647-659.

Aslan, M. and Ozben, T. 2003. Oxidants in receptor tyrosine kinase signal transduction pathways. *Antioxid Redox Signal* **5**(6): 781-788.

Audhya, A. and Emr, S.D. 2002a. Stt4 PI 4-kinase localizes to the plasma membrane and functions in the Pkc1-mediated MAP kinase cascade. *Dev Cell* **2**(5): 593-605.

Audhya, A., Foti, M., and Emr, S.D. 2000. Distinct roles for the yeast phosphatidylinositol 4-kinases, Stt4p and Pik1p, in secretion, cell growth, and organelle membrane dynamics. *Mol Biol Cell* **11**(8): 2673-2689.

Auvinen, E., Kivi, N., and Vaheri, A. 2007. Regulation of ezrin localization by Rac1 and PIPK in human epithelial cells. *Exp Cell Res* **313**(4): 824-833.

Balla, A. and Balla, T. 2006. Phosphatidylinositol 4-kinases: old enzymes with emerging functions. *Trends Cell Biol* **16**(7): 351-361.

Balla, A., Kim, Y.J., Varnai, P., Szentpetery, Z., Knight, Z., Shokat, K.M., and Balla, T. 2008. Maintenance of Hormone-sensitive Phosphoinositide Pools in the Plasma Membrane Requires Phosphatidylinositol 4-Kinase III{alpha}. *Mol Biol Cell* **19**(2): 711-721.

Balla, A., Tuymetova, G., Barshishat, M., Geiszt, M., and Balla, T. 2002. Characterization of type II phosphatidylinositol 4-kinase isoforms reveals association of the enzymes with endosomal vesicular compartments. *J Biol Chem* **277**(22): 20041-20050.

Balla, T. 2006. Phosphoinositide-derived messengers in endocrine signaling. *J Endocrinol* **188**(2): 135-153.

Barylko, B., Gerber, S.H., Binns, D.D., Grichine, N., Khvotchev, M., Sudhof, T.C., and Albanesi, J.P. 2001. A novel family of phosphatidylinositol 4-

kinases conserved from yeast to humans. *J Biol Chem* **276**(11): 7705-7708.

Berridge, M.J. 1993. Inositol trisphosphate and calcium signalling. *Nature* **361**(6410): 315-325.

Brown, F.D., Rozelle, A.L., Yin, H.L., Balla, T., and Donaldson, J.G. 2001. Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J Cell Biol* **154**(5): 1007-1017.

Bruns, J.R., Ellis, M.A., Jeromin, A., and Weisz, O.A. 2002. Multiple roles for phosphatidylinositol 4-kinase in biosynthetic transport in polarized Madin-Darby canine kidney cells. *J Biol Chem* **277**(3): 2012-2018.

Bunce, M.W., Bergendahl, K., and Anderson, R.A. 2006. Nuclear PI(4,5)P(2): a new place for an old signal. *Biochim Biophys Acta* **1761**(5-6): 560-569.

Burg, M.B., Ferraris, J.D., and Dmitrieva, N.I. 2007. Cellular response to hyperosmotic stresses. *Physiol Rev* **87**(4): 1441-1474.

Bustamante, M., Roger, F., Bochaton-Piallat, M.L., Gabbiani, G., Martin, P.Y., and Feraille, E. 2003. Regulatory volume increase is associated with p38 kinase-dependent actin cytoskeleton remodeling in rat kidney MTAL. *Am J Physiol Renal Physiol* **285**(2): F336-347.

Cao, G., Sofic, E., and Prior, R.L. 1997. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic Biol Med* **22**(5): 749-760.

Carlton, J.G. and Cullen, P.J. 2005. Coincidence detection in phosphoinositide signaling. *Trends Cell Biol* **15**(10): 540-547.

Carpenter, C.L. 2004. Btk-dependent regulation of phosphoinositide synthesis. *Biochem Soc Trans* **32**(Pt 2): 326-329.

Cha, S.T., Talavera, D., Demir, E., Nath, A.K., and Sierra-Honigmann, M.R. 2005. A method of isolation and culture of microvascular endothelial cells from mouse skin. *Microvasc Res* **70**(3): 198-204.

Chang, F.S., Han, G.S., Carman, G.M., and Blumer, K.J. 2005. A WASp-binding type II phosphatidylinositol 4-kinase required for actin polymerization-driven endosome motility. *J Cell Biol* **171**(1): 133-142.

Chatah, N.E. and Abrams, C.S. 2001. G-protein-coupled receptor activation induces the membrane translocation and activation of phosphatidylinositol-4-phosphate 5-kinase I alpha by a Rac- and Rho-dependent pathway. *J Biol Chem* **276**(36): 34059-34065.

Chong, L.D., Traynor-Kaplan, A., Bokoch, G.M., and Schwartz, M.A. 1994a. The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. *Cell* **79**(3): 507-513.

Choudhury, R., Diao, A., Zhang, F., Eisenberg, E., Saint-Pol, A., Williams, C., Konstantakopoulos, A., Lucocq, J., Johannes, L., Rabouille, C., Greene, L.E., and Lowe, M. 2005. Lowe syndrome protein OCRL1 interacts with clathrin and regulates protein trafficking between endosomes and the trans-Golgi network. *Mol Biol Cell* **16**(8): 3467-3479.

Ciano-Oliveira, C.D., Sirokmany, G., Szaszi, K., Arthur, W.T., Masszi, A., Peterson, M., Rotstein, O.D., and Kapus, A. 2003. Hyperosmotic stress activates Rho: differential involvement in Rho kinase-dependent MLC phosphorylation and NKCC activation. *Am J Physiol Cell Physiol* **285**(3): C555-566.

Cidlowski, J.A., King, K.L., Evans-Storms, R.B., Montague, J.W., Bortner, C.D., and Hughes, F.M., Jr. 1996. The biochemistry and molecular biology of glucocorticoid-induced apoptosis in the immune system. *Recent Prog Horm Res* **51**: 457-490; discussion 490-451.

Cockroft, S. 2000. *Biology of Phosphoinositides*. Oxford University Press, Oxford.

Cuschieri, J., Gourlay, D., Garcia, I., Jelacic, S., and Maier, R.V. 2002. Hypertonic Preconditioning Inhibits Macrophage Responsiveness to Endotoxin. *J Immunol* **168**(3): 1389-1396.

Dahl, S.v., Schliess, F., Reissmann, R., Gorg, B., Weiergraber, O., Kocalkova, M., Dombrowski, F., and Haussinger, D. 2003. Involvement of Integrins in Osmosensing and Signaling toward Autophagic Proteolysis in Rat Liver. *J Biol Chem* **278**(29): 27088-27095.

Dalle-Donne, I., Rossi, R., Milzani, A., Di Simplicio, P., and Colombo, R. 2001. The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. *Free Radic Biol Med* **31**(12): 1624-1632.

De Matteis, M.A. and Godi, A. 2004. PI-loting membrane traffic. *Nat Cell Biol* **6**(6): 487-492.

Denu, J.M. and Tanner, K.G. 1998. Specific and reversible inactivation of protein tyrosine phosphatases by hydrogen peroxide: evidence for a sulfenic acid intermediate and implications for redox regulation. *Biochemistry* **37**(16): 5633-5642.

DesMarais, V., Ghosh, M., Eddy, R., and Condeelis, J. 2005. Cofilin takes the lead. *J Cell Sci* **118**(Pt 1): 19-26.

Desrivieres, S., Cooke, F.T., Parker, P.J., and Hall, M.N. 1998. MSS4, a phosphatidylinositol-4-phosphate 5-kinase required for organization of the actin cytoskeleton in Saccharomyces cerevisiae. *J Biol Chem* **273**(25): 15787-15793.

Dewitt, S. and Hallett, M. 2007. Leukocyte membrane "expansion": a central mechanism for leukocyte extravasation. *J Leukoc Biol* **81**(5): 1160-1164.

Di Ciano, C., Nie, Z., Szaszi, K., Lewis, A., Uruno, T., Zhan, X., Rotstein, O.D., Mak, A., and Kapus, A. 2002a. Osmotic stress-induced remodeling of the cortical cytoskeleton. *Am J Physiol Cell Physiol* **283**(3): C850-865.

Di Paolo, G., Moskowitz, H.S., Gipson, K., Wenk, M.R., Voronov, S., Obayashi, M., Flavell, R., Fitzsimonds, R.M., Ryan, T.A., and De Camilli, P. 2004. Impaired PtdIns(4,5)P2 synthesis in nerve terminals produces defects in synaptic vesicle trafficking. *Nature* **431**(7007): 415-422.

Divecha, N., Roefs, M., Halstead, J.R., D'Andrea, S., Fernandez-Borga, M., Oomen, L., Saqib, K.M., Wakelam, M.J., and D'Santos, C. 2000. Interaction of the type Ialpha PIPkinase with phospholipase D: a role for the local generation of phosphatidylinositol 4, 5-bisphosphate in the regulation of PLD2 activity. *Embo J* **19**(20): 5440-5449.

Dmitrieva, N.I., Bulavin, D.V., and Burg, M.B. 2003. High NaCl causes Mre11 to leave the nucleus, disrupting DNA damage signaling and repair. *Am J Physiol Renal Physiol* **285**(2): F266-274.

Dmitrieva, N.I. and Burg, M.B. 2007. Osmotic stress and DNA damage. *Methods Enzymol* **428**: 241-252.

Donaldson, J.G. and Honda, A. 2005. Localization and function of Arf family GTPases. *Biochem Soc Trans* **33**(Pt 4): 639-642.

Dressman, M.A., Olivos-Glander, I.M., Nussbaum, R.L., and Suchy, S.F. 2000. Ocrl1, a PtdIns(4,5)P(2) 5-phosphatase, is localized to the trans-Golgi network of fibroblasts and epithelial cells. *J Histochem Cytochem* **48**(2): 179-190.

Dudek, S.M. and Garcia, J.G. 2001. Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol* **91**(4): 1487-1500.

Eggermont, J. 2003a. Rho's role in cell volume: sensing, strutting, or signaling? Focus on "Hyperosmotic stress activates Rho: differential involvement in Rho kinase-dependent MLC phosphorylation and NKCC activation". *Am J Physiol Cell Physiol* **285**(3): C509-511.

Ekblad, L. and Jergil, B. 2001. Localization of phosphatidylinositol 4-kinase isoenzymes in rat liver plasma membrane domains. *Biochim Biophys Acta* **1531**(3): 209-221.

Eliceiri, B.P., Paul, R., Schwartzberg, P.L., Hood, J.D., Leng, J., and Cheresh, D.A. 1999. Selective requirement for Src kinases during VEGF-induced angiogenesis and vascular permeability. *Mol Cell* **4**(6): 915-924.

Engelman, J.A., Luo, J., and Cantley, L.C. 2006. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat Rev Genet* **7**(8): 606-619.

Erdmann, K.S., Mao, Y., McCrea, H.J., Zoncu, R., Lee, S., Paradise, S., Modregger, J., Biemesderfer, D., Toomre, D., and De Camilli, P. 2007. A role of the Lowe syndrome protein OCRL in early steps of the endocytic pathway. *Dev Cell* **13**(3): 377-390.

Esper, R.J., Vilarino, J.O., Machado, R.A., and Paragano, A. 2008. Endothelial dysfunction in normal and abnormal glucose metabolism. *Adv Cardiol* **45**: 17-43.

Faraci, F.M. and Didion, S.P. 2004. Vascular protection: superoxide dismutase isoforms in the vessel wall. *Arterioscler Thromb Vasc Biol* **24**(8): 1367-1373.

Fievet, B., Louvard, D., and Arpin, M. 2007. ERM proteins in epithelial cell organization and functions. *Biochim Biophys Acta* **1773**(5): 653-660.

Garner, M.M. and Burg, M.B. 1994. Macromolecular crowding and confinement in cells exposed to hypertonicity. *Am J Physiol* **266**(4 Pt 1): C877-892.

Gaut, J.P., Yeh, G.C., Tran, H.D., Byun, J., Henderson, J.P., Richter, G.M., Brennan, M.L., Lusis, A.J., Belaaouaj, A., Hotchkiss, R.S., and Heinecke, J.W. 2001. Neutrophils employ the myeloperoxidase system to generate antimicrobial brominating and chlorinating oxidants during sepsis. *Proc Natl Acad Sci U S A* **98**(21): 11961-11966.

Genestra, M. 2007. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cell Signal* **19**(9): 1807-1819.

Gillis, D., Shrode, L.D., Krump, E., Howard, C.M., Rubie, E.A., Tibbles, L.A., Woodgett, J., and Grinstein, S. 2001. Osmotic stimulation of the Na+/H+ exchanger NHE1: relationship to the activation of three MAPK pathways. *J Membr Biol* **181**(3): 205-214.

Gilmore, A.P. and Burridge, K. 1996. Regulation of vinculin binding to talin and actin by phosphatidyl-inositol-4-5-bisphosphate. *Nature* **381**(6582): 531-535.

Godi, A., Pertile, P., Meyers, R., Marra, P., Di Tullio, G., Iurisci, C., Luini, A., Corda, D., and De Matteis, M.A. 1999. ARF mediates recruitment of PtdIns-4-OH kinase-beta and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex. *Nat Cell Biol* **1**(5): 280-287.

Golub, T. and Caroni, P. 2005. PI(4,5)P2-dependent microdomain assemblies capture microtubules to promote and control leading edge motility. *J Cell Biol* **169**(1): 151-165.

Gromada, J., Bark, C., Smidt, K., Efanov, A.M., Janson, J., Mandic, S.A., Webb, D.L., Zhang, W., Meister, B., Jeromin, A., and Berggren, P.O. 2005. Neuronal calcium sensor-1 potentiates glucose-dependent exocytosis in pancreatic beta cells through activation of phosphatidylinositol 4-kinase beta. *Proc Natl Acad Sci U S A* **102**(29): 10303-10308.

Gupte, S.A. and Wolin, M.S. 2008. Oxidant and redox signaling in vascular oxygen sensing: implications for systemic and pulmonary hypertension. *Antioxid Redox Signal* **10**(6): 1137-1152.

Halet, G. 2005. Imaging phosphoinositide dynamics using GFP-tagged protein domains. *Biol Cell* **97**(7): 501-518.

Halstead, J.R., Jalink, K., and Divecha, N. 2005. An emerging role for PtdIns(4,5)P2-mediated signalling in human disease. *Trends Pharmacol Sci* **26**(12): 654-660.

Halstead, J.R., van Rheenen, J., Snel, M.H., Meeuws, S., Mohammed, S., D'Santos, C.S., Heck, A.J., Jalink, K., and Divecha, N. 2006. A role for PtdIns(4,5)P2 and PIP5Kalpha in regulating stress-induced apoptosis. *Curr Biol* **16**(18): 1850-1856.

Hartwig, J.H., Bokoch, G.M., Carpenter, C.L., Janmey, P.A., Taylor, L.A., Toker, A., and Stossel, T.P. 1995. Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. *Cell* **82**(4): 643-653.

Hausser, A., Storz, P., Martens, S., Link, G., Toker, A., and Pfizenmaier, K. 2005. Protein kinase D regulates vesicular transport by phosphorylating and activating phosphatidylinositol-4 kinase IIIbeta at the Golgi complex. *Nat Cell Biol* **7**(9): 880-886.

He, H., Venema, V.J., Gu, X., Venema, R.C., Marrero, M.B., and Caldwell, R.B. 1999. Vascular endothelial growth factor signals endothelial cell production of nitric oxide and prostacyclin through flk-1/KDR activation of c-Src. *J Biol Chem* **274**(35): 25130-25135.

Heilmeyer, L.M., Jr., Vereb, G., Jr., Vereb, G., Kakuk, A., and Szivak, I. 2003. Mammalian phosphatidylinositol 4-kinases. *IUBMB Life* **55**(2): 59-65.

Hilfiker, S. 2003. Neuronal calcium sensor-1: a multifunctional regulator of secretion. *Biochem Soc Trans* **31**(Pt 4): 828-832.

Hokin, M.R. and Hokin, L.E. 1953. Enzyme secretion and the incorporation of P32 into phospholipides of pancreas slices. *J Biol Chem* **203**(2): 967-977.

Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A.J., Frohman, M.A., and Kanaho, Y. 1999. Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99**(5): 521-532.

Horton, J.W. 2003. Free radicals and lipid peroxidation mediated injury in burn trauma: the role of antioxidant therapy. *Toxicology* **189**(1-2): 75-88.

Houle, F., Poirier, A., Dumaresq, J., and Huot, J. 2007. DAP kinase mediates the phosphorylation of tropomyosin-1 downstream of the ERK pathway, which regulates the formation of stress fibers in response to oxidative stress. *J Cell Sci* **120**(Pt 20): 3666-3677.

Huang, T.Y., DerMardirossian, C., and Bokoch, G.M. 2006. Cofilin phosphatases and regulation of actin dynamics. *Curr Opin Cell Biol* **18**(1): 26-31.

Huot, J., Houle, F., Marceau, F., and Landry, J. 1997. Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. *Circ Res* **80**(3): 383-392.

Hyvola, N., Diao, A., McKenzie, E., Skippen, A., Cockcroft, S., and Lowe, M. 2006. Membrane targeting and activation of the Lowe syndrome protein OCRL1 by rab GTPases. *Embo J* **25**(16): 3750-3761.

Irvine, R.F. 2003. 20 years of Ins(1,4,5)P3, and 40 years before. *Nat Rev Mol Cell Biol* **4**(7): 586-590.

Itoh, T., Ishihara, H., Shibasaki, Y., Oka, Y., and Takenawa, T. 2000. Autophosphorylation of type I phosphatidylinositol phosphate kinase regulates its lipid kinase activity. *J Biol Chem* **275**(25): 19389-19394.

Jenkins, G.H., Fisette, P.L., and Anderson, R.A. 1994. Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J Biol Chem* **269**(15): 11547-11554.

Jenkins, G.M. and Frohman, M.A. 2005. Phospholipase D: a lipid centric review. *Cell Mol Life Sci* **62**(19-20): 2305-2316.

Jin, Z. and El-Deiry, W.S. 2005. Overview of cell death signaling pathways. *Cancer Biol Ther* **4**(2): 139-163.

Jones, D.H., Morris, J.B., Morgan, C.P., Kondo, H., Irvine, R.F., and Cockcroft, S. 2000. Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the golgi compartment. *J Biol Chem* **275**(18): 13962-13966.

Jung, G., Wang, J., Wlodarski, P., Barylko, B., Binns, D.D., Shu, H., Yin, H.L., and Albanesi, J.P. 2008. Molecular determinants of activation and membrane targeting of phosphoinositol 4-kinase IIbeta. *Biochem J* **409**(2): 501-509.

Kanzaki, M., Furukawa, M., Raab, W., and Pessin, J.E. 2004. Phosphatidylinositol 4,5-bisphosphate regulates adipocyte actin dynamics and GLUT4 vesicle recycling. *J Biol Chem* **279**(29): 30622-30633.

Kashani, A.H., Chen, B.-M., and Grinnell, A.D. 2001. Hypertonic enhancement of transmitter release from frog motor nerve terminals: Ca2+ independence and role of integrins. *J Physiol (Lond)* **530**(2): 243-252.

King, C.E., Hawkins, P.T., Stephens, L.R., and Michell, R.H. 1989. Determination of the steady-state turnover rates of the metabolically active pools of phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in human erythrocytes. *Biochem J* **259**(3): 893-896.

Knebel, A., Rahmsdorf, H.J., Ullrich, A., and Herrlich, P. 1996. Dephosphorylation of receptor tyrosine kinases as target of regulation by radiation, oxidants or alkylating agents. *Embo J* **15**(19): 5314-5325.

Kozma, R., Ahmed, S., Best, A., and Lim, L. 1995. The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin

microspikes and filopodia in Swiss 3T3 fibroblasts. *Mol Cell Biol* **15**(4): 1942-1952.

Krauss, M., Kinuta, M., Wenk, M.R., De Camilli, P., Takei, K., and Haucke, V. 2003. ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type Igamma. *J Cell Biol* **162**(1): 113-124.

Kreimeier, U. and Messmer, K. 2002. Small-volume resuscitation: from experimental evidence to clinical routine. Advantages and disadvantages of hypertonic solutions. *Acta Anaesthesiol Scand* **46**(6): 625-638.

Kuro-o, M. 2008. Klotho as a regulator of oxidative stress and senescence. *Biol Chem* **389**(3): 233-241.

Lampugnani, M.G., Resnati, M., Dejana, E., and Marchisio, P.C. 1991. The role of integrins in the maintenance of endothelial monolayer integrity. *J Cell Biol* **112**(3): 479-490.

Lassing, I. and Lindberg, U. 1985. Specific interaction between phosphatidylinositol 4,5-bisphosphate and profilactin. *Nature* **314**(6010): 472-474.

Laux, T., Fukami, K., Thelen, M., Golub, T., Frey, D., and Caroni, P. 2000. GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. *J Cell Biol* **149**(7): 1455-1472.

Lee, M.Y. and Griendling, K.K. 2008. Redox signaling, vascular function, and hypertension. *Antioxid Redox Signal* **10**(6): 1045-1059.

Lemmon, M.A. 2008. Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol* **9**(2): 99-111.

Lewis, A., Di Ciano, C., Rotstein, O.D., and Kapus, A. 2002. Osmotic stress activates Rac and Cdc42 in neutrophils: role in hypertonicity-induced actin polymerization. *Am J Physiol Cell Physiol* **282**(2): C271-279.

Ley, K. 2002. Integration of inflammatory signals by rolling neutrophils. *Immunol Rev* **186**: 8-18.

Li, Y., Gamper, N., Hilgemann, D.W., and Shapiro, M.S. 2005. Regulation of Kv7 (KCNQ) K+ channel open probability by phosphatidylinositol 4,5-bisphosphate. *J Neurosci* **25**(43): 9825-9835.

Lin, K.M., Wenegieme, E., Lu, P.J., Chen, C.S., and Yin, H.L. 1997. Gelsolin binding to phosphatidylinositol 4,5-bisphosphate is modulated by calcium and pH. *J Biol Chem* **272**(33): 20443-20450.

Lin, T.H., Rosales, C., Mondal, K., Bolen, J.B., Haskill, S., and Juliano, R.L. 1995. Integrin-mediated tyrosine phosphorylation and cytokine message induction in monocytic cells. A possible signaling role for the Syk tyrosine kinase. *J Biol Chem* **270**(27): 16189-16197.

Ling, K., Doughman, R.L., Iyer, V.V., Firestone, A.J., Bairstow, S.F., Mosher, D.F., Schaller, M.D., and Anderson, R.A. 2003. Tyrosine phosphorylation of type Igamma phosphatidylinositol phosphate kinase by Src regulates an integrin-talin switch. *J Cell Biol* **163**(6): 1339-1349.

Ling, K., Schill, N.J., Wagoner, M.P., Sun, Y., and Anderson, R.A. 2006. Movin' on up: the role of PtdIns(4,5)P(2) in cell migration. *Trends Cell Biol* **16**(6): 276-284.

Lioubin, M.N., Algate, P.A., Tsai, S., Carlberg, K., Aebersold, A., and Rohrschneider, L.R. 1996. p150Ship, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev* **10**(9): 1084-1095.

Lowe, M. 2005. Structure and function of the Lowe syndrome protein OCRL1. *Traffic* **6**(9): 711-719.

Mao, Y.S. and Yin, H.L. 2007. Regulation of the actin cytoskeleton by phosphatidylinositol 4-phosphate 5 kinases. *Pflugers Arch* **455**(1): 5-18.

Marsh, D.J. and Azen, S.P. 1975. Mechanism of NaCl reabsorption by hamster thin ascending limbs of Henle's loop. *Am J Physiol* **228**(1): 71-79.

Martin, T.F. 2001. PI(4,5)P(2) regulation of surface membrane traffic. *Curr Opin Cell Biol* **13**(4): 493-499.

Martin-Belmonte, F., Gassama, A., Datta, A., Yu, W., Rescher, U., Gerke, V., and Mostov, K. 2007. PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. *Cell* **128**(2): 383-397.

Martin-Belmonte, F. and Mostov, K. 2007. Phosphoinositides control epithelial development. *Cell Cycle* **6**(16): 1957-1961.

Mayadas, T.N. and Cullere, X. 2005. Neutrophil beta2 integrins: moderators of life or death decisions. *Trends Immunol* **26**(7): 388-395.

McGough, A.M., Staiger, C.J., Min, J.K., and Simonetti, K.D. 2003. The gelsolin family of actin regulatory proteins: modular structures, versatile functions. *FEBS Lett* **552**(2-3): 75-81.

McLaughlin, S. and Murray, D. 2005. Plasma membrane phosphoinositide organization by protein electrostatics. *Nature* **438**(7068): 605-611.

McPherson, P.S., Garcia, E.P., Slepnev, V.I., David, C., Zhang, X., Grabs, D., Sossin, W.S., Bauerfeind, R., Nemoto, Y., and De Camilli, P. 1996. A presynaptic inositol-5-phosphatase. *Nature* **379**(6563): 353-357.

Meng, T.C., Fukada, T., and Tonks, N.K. 2002. Reversible oxidation and inactivation of protein tyrosine phosphatases in vivo. *Mol Cell* **9**(2): 387-399.

Mesaeli, N., Tappia, P.S., Suzuki, S., Dhalla, N.S., and Panagia, V. 2000. Oxidants depress the synthesis of phosphatidylinositol 4,5-bisphosphate in heart sarcolemma. *Arch Biochem Biophys* **382**(1): 48-56.

Michea, L., Ferguson, D.R., Peters, E.M., Andrews, P.M., Kirby, M.R., and Burg, M.B. 2000. Cell cycle delay and apoptosis are induced by high salt and urea in renal medullary cells. *Am J Physiol Renal Physiol* **278**(2): F209-218.

Michell, R.H. 2008. Inositol derivatives: evolution and functions. *Nat Rev Mol Cell Biol* **9**(2): 151-161.

Mocali, A., Caldini, R., Chevanne, M., and Paoletti, F. 1995. Induction, effects, and quantification of sublethal oxidative stress by hydrogen peroxide on cultured human fibroblasts. *Exp Cell Res* **216**(2): 388-395.

Morris, J.B., Huynh, H., Vasilevski, O., and Woodcock, E.A. 2006. Alpha1-adrenergic receptor signaling is localized to caveolae in neonatal rat cardiomyocytes. *J Mol Cell Cardiol* **41**(1): 17-25.

Munro, S. 2005. The Arf-like GTPase Arl1 and its role in membrane traffic. *Biochem Soc Trans* **33**(Pt 4): 601-605.

Nakagawa, T., Goto, K., and Kondo, H. 1996. Cloning, expression, and localization of 230-kDa phosphatidylinositol 4-kinase. *J Biol Chem* **271**(20): 12088-12094.

Nakanishi, S., Catt, K.J., and Balla, T. 1995. A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. *Proc Natl Acad Sci U S A* **92**(12): 5317-5321.

Nakano-Kobayashi, A., Yamazaki, M., Unoki, T., Hongu, T., Murata, C., Taguchi, R., Katada, T., Frohman, M.A., Yokozeki, T., and Kanaho, Y. 2007. Role of activation of PIP5Kgamma661 by AP-2 complex in synaptic vesicle endocytosis. *Embo J* **26**(4): 1105-1116.

Nasuhoglu, C., Feng, S., Mao, J., Yamamoto, M., Yin, H.L., Earnest, S., Barylko, B., Albanesi, J.P., and Hilgemann, D.W. 2002a. Nonradioactive analysis of phosphatidylinositides and other anionic phospholipids by anion-exchange high-performance liquid chromatography with suppressed conductivity detection. *Anal Biochem* **301**(2): 243-254.

Nasuhoglu, C., Feng, S., Mao, Y., Shammat, I., Yamamato, M., Earnest, S., Lemmon, M., and Hilgemann, D.W. 2002b. Modulation of cardiac PIP2 by cardioactive hormones and other physiologically relevant interventions. *Am J Physiol Cell Physiol* **283**(1): C223-234.

Nauseef, W.M. 2007. How human neutrophils kill and degrade microbes: an integrated view. *Immunol Rev* **219**: 88-102.

Niggli, V. and Rossy, J. 2008. Ezrin/radixin/moesin: versatile controllers of signaling molecules and of the cortical cytoskeleton. *Int J Biochem Cell Biol* **40**(3): 344-349.

Nishibori, M., Takahashi, H.K., and Mori, S. 2003. The regulation of ICAM-1 and LFA-1 interaction by autacoids and statins: a novel strategy for

controlling inflammation and immune responses. *J Pharmacol Sci* **92**(1): 7-12.

Oude Weernink, P.A., Schmidt, M., and Jakobs, K.H. 2004. Regulation and cellular roles of phosphoinositide 5-kinases. *Eur J Pharmacol* **500**(1-3): 87-99.

Padron, D., Wang, Y.J., Yamamoto, M., Yin, H., and Roth, M.G. 2003a. Phosphatidylinositol phosphate 5-kinase I{beta} recruits AP-2 to the plasma membrane and regulates rates of constitutive endocytosis. *J Cell Biol* **162**(4): 693-

Pantaloni, D. and Carlier, M.F. 1993. How profilin promotes actin filament assembly in the presence of thymosin beta 4. *Cell* **75**(5): 1007-1014.

Pantaloni, D., Le Clainche, C., and Carlier, M.F. 2001. Mechanism of actin-based motility. *Science* **292**(5521): 1502-1506.

Paris, S., Beraud-Dufour, S., Robineau, S., Bigay, J., Antonny, B., Chabre, M., and Chardin, P. 1997. Role of protein-phospholipid interactions in the activation of ARF1 by the guanine nucleotide exchange factor Arno. *J Biol Chem* **272**(35): 22221-22226.

Park, S.J., Itoh, T., and Takenawa, T. 2001a. Phosphatidylinositol 4-Phosphate 5-Kinase Type I Is Regulated through Phosphorylation Response by Extracellular Stimuli. *J Biol Chem* **276**(7): 4781-4787.

Partridge, C.A., Horvath, C.J., Del Vecchio, P.J., Phillips, P.G., and Malik, A.B. 1992. Influence of extracellular matrix in tumor necrosis factor-induced increase in endothelial permeability. *Am J Physiol* **263**(6 Pt 1): L627-633.

Pedersen, S.F., Hoffmann, E.K., and Mills, J.W. 2001. The cytoskeleton and cell volume regulation. *Comp Biochem Physiol A Mol Integr Physiol* **130**(3): 385-399.

Perera, N.M., Michell, R.H., and Dove, S.K. 2004. Hypo-osmotic stress activates Plc1p-dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and inositol Hexakisphosphate accumulation in yeast. *J Biol Chem* **279**(7): 5216-5226.

Pesesse, X., Backers, K., Moreau, C., Zhang, J., Blero, D., Paternotte, N., and Erneux, C. 2006. SHIP1/2 interaction with tyrosine phosphorylated peptides mimicking an immunoreceptor signalling motif. *Adv Enzyme Regul* **46**: 142-153.

Pike, L.J. 1992. Phosphatidylinositol 4-kinases and the role of polyphosphoinositides in cellular regulation. *Endocr Rev* **13**(4): 692-706.

Qin, S., Inazu, T., Takata, M., Kurosaki, T., Homma, Y., and Yamamura, H. 1996. Cooperation of tyrosine kinases p72syk and p53/56lyn regulates calcium mobilization in chicken B cell oxidant stress signaling. *Eur J Biochem* **236**(2): 443-449.

Rajebhosale, M., Greenwood, S., Vidugiriene, J., Jeromin, A., and Hilfiker, S. 2003. Phosphatidylinositol 4-OH kinase is a downstream target of neuronal calcium sensor-1 in enhancing exocytosis in neuroendocrine cells. *J Biol Chem* **278**(8): 6075-6084.

Rao, V.D., Misra, S., Boronenkov, I.V., Anderson, R.A., and Hurley, J.H. 1998. Structure of type IIbeta phosphatidylinositol phosphate kinase: a protein kinase fold flattened for interfacial phosphorylation. *Cell* **94**(6): 829-839.

Rasmussen, M., Alexander, R.T., Darborg, B.V., Mobjerg, N., Hoffmann, E.K., Kapus, A., and Pedersen, S.F. 2008. Osmotic cell shrinkage activates ezrin/radixin/moesin (ERM) proteins: activation mechanisms and physiological implications. *Am J Physiol Cell Physiol* **294**(1): C197-212.

Reimann, B., Schwarzer, E., Standera, S., and Maretzki, D. 1990. Metabolism of phosphatidylinositides in rabbit red blood cells. *Biomed Biochim Acta* **49**(2-3): S147-148.

Renedo, M.A., Fernandez, N., and Crespo, M.S. 2001. FcgammaRIIA exogenously expressed in HeLa cells activates the mitogen-activated protein kinase cascade by a mechanism dependent on the endogenous expression of the protein tyrosine kinase Syk. *Eur J Immunol* **31**(5): 1361-1369.

Ridley, A.J. and Hall, A. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**(3): 389-399.

Rizoli, S.B., Rotstein, O.D., Parodo, J., Phillips, M.J., and Kapus, A. 2000. Hypertonic inhibition of exocytosis in neutrophils: central role for osmotic actin skeleton remodeling. *Am J Physiol Cell Physiol* **279**(3): C619-633.

Rohatgi, R., Ho, H.Y., and Kirschner, M.W. 2000. Mechanism of N-WASP activation by CDC42 and phosphatidylinositol 4, 5-bisphosphate. *J Cell Biol* **150**(6): 1299-1310.

Rokutan, K., Johnston, R.B., Jr., and Kawai, K. 1994. Oxidative stress induces S-thiolation of specific proteins in cultured gastric mucosal cells. *Am J Physiol* **266**(2 Pt 1): G247-254.

Rousseau, S., Houle, F., Landry, J., and Huot, J. 1997. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene* **15**(18): 2169-2177.

Routt, S.M., Ryan, M.M., Tyeryar, K., Rizzieri, K.E., Mousley, C., Roumanie, O., Brennwald, P.J., and Bankaitis, V.A. 2005. Nonclassical PITPs activate PLD via the Stt4p PtdIns-4-kinase and modulate function of late stages of exocytosis in vegetative yeast. *Traffic* **6**(12): 1157-1172.

Rozelle, A.L., Machesky, L.M., Yamamoto, M., Driessens, M.H., Insall, R.H., Roth, M.G., Luby-Phelps, K., Marriott, G., Hall, A., and Yin, H.L. 2000. Phosphatidylinositol 4,5-bisphosphate induces actin-based movement of raft-enriched vesicles through WASP-Arp2/3. *Curr Biol* **10**(6): 311-320.

Rumenapp, U., Schmidt, M., Olesch, S., Ott, S., Eichel-Streiber, C.V., and Jakobs, K.H. 1998. Tyrosine-phosphorylation-dependent and rho-protein-mediated control of cellular phosphatidylinositol 4,5-bisphosphate levels. *Biochem J* **334 (Pt 3)**: 625-631.

Sahnoun, Z., Jamoussi, K., and Zeghal, K.M. 1997. [Free radicals and antioxidants: human physiology, pathology and therapeutic aspects]. *Therapie* **52**(4): 251-270.

Sakisaka, T., Itoh, T., Miura, K., and Takenawa, T. 1997. Phosphatidylinositol 4,5-bisphosphate phosphatase regulates the rearrangement of actin filaments. *Mol Cell Biol* **17**(7): 3841-3849.

Salazar, G., Craige, B., Wainer, B.H., Guo, J., De Camilli, P., and Faundez, V. 2005. Phosphatidylinositol-4-kinase type II alpha is a component of adaptor protein-3-derived vesicles. *Mol Biol Cell* **16**(8): 3692-3704.

Santarius, M., Lee, C.H., and Anderson, R.A. 2006. Supervised membrane swimming: small G-protein lifeguards regulate PIPK signalling and monitor intracellular PtdIns(4,5)P2 pools. *Biochem J* **398**(1): 1-13.

Sasaki, J., Sasaki, T., Yamazaki, M., Matsuoka, K., Taya, C., Shitara, H., Takasuga, S., Nishio, M., Mizuno, K., Wada, T., Miyazaki, H., Watanabe, H., Iizuka, R., Kubo, S., Murata, S., Chiba, T., Maehama, T., Hamada, K., Kishimoto, H., Frohman, M.A., Tanaka, K., Penninger, J.M., Yonekawa, H., Suzuki, A., and Kanaho, Y. 2005. Regulation of anaphylactic responses by phosphatidylinositol phosphate kinase type I {alpha}. *J Exp Med* **201**(6): 859-870.

Sciorra, V.A., Audhya, A., Parsons, A.B., Segev, N., Boone, C., and Emr, S.D. 2005. Synthetic genetic array analysis of the PtdIns 4-kinase Pik1p identifies components in a Golgi-specific Ypt31/rab-GTPase signaling pathway. *Mol Biol Cell* **16**(2): 776-793.

Scott, C.C., Dobson, W., Botelho, R.J., Coady-Osberg, N., Chavrier, P., Knecht, D.A., Heath, C., Stahl, P., and Grinstein, S. 2005. Phosphatidylinositol-4,5-bisphosphate hydrolysis directs actin remodeling during phagocytosis. *J Cell Biol* **169**(1): 139-149.

Sheetz, M.P., Sable, J.E., and Dobereiner, H.G. 2006. Continuous membrane-cytoskeleton adhesion requires continuous accommodation to lipid and cytoskeleton dynamics. *Annu Rev Biophys Biomol Struct* **35**: 417-434.

Sheikh-Hamad, D. and Gustin, M.C. 2004. MAP kinases and the adaptive response to hypertonicity: functional preservation from yeast to mammals. *Am J Physiol Renal Physiol* **287**(6): F1102-1110.

Shelton, S.N., Barylko, B., Binns, D.D., Horazdovsky, B.F., Albanesi, J.P., and Goodman, J.M. 2003. Saccharomyces cerevisiae contains a Type II phosphoinositide 4-kinase. *Biochem J* **371**(Pt 2): 533-540.

Shibasaki, Y., Ishihara, H., Kizuki, N., Asano, T., Oka, Y., and Yazaki, Y. 1997. Massive actin polymerization induced by phosphatidylinositol-4-phosphate 5-kinase in vivo. *J Biol Chem* **272**(12): 7578-7581.

Siraganian, R.P., Zhang, J., Suzuki, K., and Sada, K. 2002. Protein tyrosine kinase Syk in mast cell signaling. *Mol Immunol* **38**(16-18): 1229-1233.

Skippen, A., Jones, D.H., Morgan, C.P., Li, M., and Cockcroft, S. 2002. Mechanism of ADP ribosylation factor-stimulated phosphatidylinositol 4,5-bisphosphate synthesis in HL60 cells. *J Biol Chem* **277**(8): 5823-5831.

Smith, D.G., Cappai, R., and Barnham, K.J. 2007. The redox chemistry of the Alzheimer's disease amyloid beta peptide. *Biochim Biophys Acta* **1768**(8): 1976-1990.

Sommer, D., Coleman, S., Swanson, S.A., and Stemmer, P.M. 2002. Differential susceptibilities of serine/threonine phosphatases to oxidative and nitrosative stress. *Arch Biochem Biophys* **404**(2): 271-278.

Strahl, T., Hama, H., DeWald, D.B., and Thorner, J. 2005. Yeast phosphatidylinositol 4-kinase, Pik1, has essential roles at the Golgi and in the nucleus. *J Cell Biol* **171**(6): 967-979.

Strange, K., Denton, J., and Nehrke, K. 2006. Ste20-Type Kinases: Evolutionarily Conserved Regulators of Ion Transport and Cell Volume. *Physiology* **21**(1): 61-68.

Sun, H.Q., Kwiatkowska, K., Wooten, D.C., and Yin, H.L. 1995. Effects of CapG overexpression on agonist-induced motility and second messenger generation. *J Cell Biol* **129**(1): 147-156.

Tabernero, J. 2007. The role of VEGF and EGFR inhibition: implications for combining anti-VEGF and anti-EGFR agents. *Mol Cancer Res* **5**(3): 203-220.

Takano, T., Sada, K., and Yamamura, H. 2002. Role of protein-tyrosine kinase syk in oxidative stress signaling in B cells. *Antioxid Redox Signal* **4**(3): 533-541.

Tohyama, Y. and Yamamura, H. 2006. Complement-mediated phagocytosis--the role of Syk. *IUBMB Life* **58**(5-6): 304-308.

Tolias, K.F., Hartwig, J.H., Ishihara, H., Shibasaki, Y., Cantley, L.C., and Carpenter, C.L. 2000. Type Ialpha phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. *Curr Biol* **10**(3): 153-156.

Turnage, R.H., Nwariaku, F., Murphy, J., Schulman, C., Wright, K., and Yin, H. 2002. Mechanisms of pulmonary microvascular dysfunction during severe burn injury. *World J Surg* **26**(7): 848-853.

van Horck, F.P., Lavazais, E., Eickholt, B.J., Moolenaar, W.H., and Divecha, N. 2002. Essential role of type I(alpha) phosphatidylinositol 4-phosphate 5-kinase in neurite remodeling. *Curr Biol* **12**(3): 241-245.

Verin, A.D., Birukova, A., Wang, P., Liu, F., Becker, P., Birukov, K., and Garcia, J.G. 2001. Microtubule disassembly increases endothelial cell barrier dysfunction: role of MLC phosphorylation. *Am J Physiol Lung Cell Mol Physiol* **281**(3): L565-574.

Victorino, G.P., Newton, C.R., and Curran, B. 2003. Effect of hypertonic saline on microvascular permeability in the activated endothelium. *Journal of Surgical Research* **112**(1): 79-83.

Voronov, S.V., Frere, S.G., Giovedi, S., Pollina, E.A., Borel, C., Zhang, H., Schmidt, C., Akeson, E.C., Wenk, M.R., Cimasoni, L., Arancio, O., Davisson, M.T., Antonarakis, S.E., Gardiner, K., De Camilli, P., and Di Paolo, G. 2008. Synaptojanin 1-linked phosphoinositide dyshomeostasis and cognitive deficits in mouse models of Down's syndrome. *Proc Natl Acad Sci U S A* **105**(27): 9415-9420.

Walch-Solimena, C. and Novick, P. 1999. The yeast phosphatidylinositol-4-OH kinase pik1 regulates secretion at the Golgi. *Nat Cell Biol* **1**(8): 523-525.

Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C.H. 1994. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* **269**(43): 26988-26995.

Wang, Y., Lian, L., Golden, J.A., Morrisey, E.E., and Abrams, C.S. 2007. PIP5KI gamma is required for cardiovascular and neuronal development. *Proc Natl Acad Sci U S A* **104**(28): 11748-11753.

Wang, Y., Litvinov, R.I., Chen, X., Bach, T.L., Lian, L., Petrich, B.G., Monkley, S.J., Critchley, D.R., Sasaki, T., Birnbaum, M.J., Weisel, J.W., Hartwig, J., and Abrams, C.S. 2008. Loss of PIP5KIgamma, unlike other PIP5KI

isoforms, impairs the integrity of the membrane cytoskeleton in murine megakaryocytes. *J Clin Invest* **118**(2): 812-819.

Wang, Y.J., Li, W.H., Wang, J., Xu, K., Dong, P., Luo, X., and Yin, H.L. 2004. Critical role of PIP5KI{gamma}87 in InsP3-mediated Ca(2+) signaling. *J Cell Biol* **167**(6): 1005-1010.

Wang, Y.J., Wang, J., Sun, H.Q., Martinez, M., Sun, Y.X., Macia, E., Kirchhausen, T., Albanesi, J.P., Roth, M.G., and Yin, H.L. 2003. Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. *Cell* **114**(3): 299-310.

Waugh, M.G., Minogue, S., Anderson, J.S., Balinger, A., Blumenkrantz, D., Calnan, D.P., Cramer, R., and Hsuan, J.J. 2003. Localization of a highly active pool of type II phosphatidylinositol 4-kinase in a p97/valosin-containing-protein-rich fraction of the endoplasmic reticulum. *Biochem J* **373**(Pt 1): 57-63.

Weernink, P.A., Meletiadis, K., Hommeltenberg, S., Hinz, M., Ishihara, H., Schmidt, M., and Jakobs, K.H. 2004a. Activation of type I phosphatidylinositol 4-phosphate 5-kinase isoforms by the Rho GTPases, RhoA, Rac1, and Cdc42. *J Biol Chem* **279**(9): 7840-7849.

Weernink, P.A.O., Meletiadis, K., Hommeltenberg, S., Hinz, M., Ishihara, H., Schmidt, M., and Jakobs, K.H. 2004b. Activation of Type I Phosphatidylinositol 4-Phosphate 5-Kinase Isoforms by the Rho GTPases, RhoA, Rac1, and Cdc42. *J Biol Chem* **279**(9): 7840-7849.

Wehner, F., Olsen, H., Tinel, H., Kinne-Saffran, E., and Kinne, R.K. 2003. Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. *Rev Physiol Biochem Pharmacol* **148**: 1-80.

Wei, Y.J., Sun, H.Q., Yamamoto, M., Wlodarski, P., Kunii, K., Martinez, M., Barylko, B., Albanesi, J.P., and Yin, H.L. 2002. Type II phosphatidylinositol 4-kinase beta is a cytosolic and peripheral membrane protein that is recruited to the plasma membrane and activated by Rac-GTP. *J Biol Chem* **277**(48): 46586-46593.

Weis, S.M. 2008. Vascular permeability in cardiovascular disease and cancer. *Curr Opin Hematol* **15**(3): 243-249.

Wenk, M.R., Pellegrini, L., Klenchin, V.A., Di Paolo, G., Chang, S., Daniell, L., Arioka, M., Martin, T.F., and De Camilli, P. 2001. PIP kinase Igamma is the major PI(4,5)P(2) synthesizing enzyme at the synapse. *Neuron* **32**(1): 79-88.

Wilson, B.S., Pfeiffer, J.R., and Oliver, J.M. 2002. FcepsilonRI signaling observed from the inside of the mast cell membrane. *Mol Immunol* **38**(16-18): 1259-1268.

Wishart, M.J. and Dixon, J.E. 2002. PTEN and myotubularin phosphatases: from 3-phosphoinositide dephosphorylation to disease. *Trends Cell Biol* **12**(12): 579-585.

Wojciak-Stothard, B., Entwistle, A., Garg, R., and Ridley, A.J. 1998. Regulation of TNF-alpha-induced reorganization of the actin cytoskeleton and cell-cell junctions by Rho, Rac, and Cdc42 in human endothelial cells. *J Cell Physiol* **176**(1): 150-165.

Wojciak-Stothard, B., Tsang, L.Y., and Haworth, S.G. 2005. Rac and Rho play opposing roles in the regulation of hypoxia/reoxygenation-induced permeability changes in pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* **288**(4): L749-760.

Wojciak-Stothard, B., Tsang, L.Y., Paleolog, E., Hall, S.M., and Haworth, S.G. 2006. Rac1 and RhoA as regulators of endothelial phenotype and barrier function in hypoxia-induced neonatal pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* **290**(6): L1173-1182.

Wolff, S. and Dillin, A. 2006. The trifecta of aging in Caenorhabditis elegans. *Exp Gerontol* **41**(10): 894-903.

Wong, K., Meyers dd, R., and Cantley, L.C. 1997. Subcellular locations of phosphatidylinositol 4-kinase isoforms. *J Biol Chem* **272**(20): 13236-13241.

Wright, K., Nwariaku, F., Halaihel, N., Schulman, C., Yin, H.L., and Turnage, R.H. 2000. Burn-activated neutrophils and tumor necrosis factor-alpha alter endothelial cell actin cytoskeleton and enhance monolayer permeability. *Surgery* **128**(2): 259-265.

Wymann, M.P. and Schneiter, R. 2008. Lipid signalling in disease. *Nat Rev Mol Cell Biol* **9**(2): 162-176.

Yamamoto, M., Chen, M.Z., Wang, Y.J., Sun, H.Q., Wei, Y., Martinez, M., and Yin, H.L. 2006. Hypertonic stress increases phosphatidylinositol 4,5-bisphosphate levels by activating PIP5KIbeta. *J Biol Chem* **281**(43): 32630-32638.

Yamamoto, M., Hilgemann, D.H., Feng, S., Bito, H., Ishihara, H., Shibasaki, Y., and Yin, H.L. 2001. Phosphatidylinositol 4,5-bisphosphate induces actin stress-fiber formation and inhibits membrane ruffling in CV1 cells. *J Cell Biol* **152**(5): 867-876.

Yamamoto, N., Hasegawa, H., Seki, H., Ziegelbauer, K., and Yasuda, T. 2003. Development of a high-throughput fluoroimmunoassay for Syk kinase and Syk kinase inhibitors. *Anal Biochem* **315**(2): 256-261.

Yanagi, S., Inatome, R., Ding, J., Kitaguchi, H., Tybulewicz, V.L., and Yamamura, H. 2001a. Syk expression in endothelial cells and their morphologic defects in embryonic Syk-deficient mice. *Blood* **98**(9): 2869-2871.

Yanagi, S., Inatome, R., Takano, T., and Yamamura, H. 2001b. Syk expression and novel function in a wide variety of tissues. *Biochem Biophys Res Commun* **288**(3): 495-498.

Yang, S.-A., Carpenter, C.L., and Abrams, C.S. 2004. Rho and Rho-kinase Mediate Thrombin-induced Phosphatidylinositol 4-Phosphate 5-Kinase Trafficking in Platelets. *J Biol Chem* **279**(40): 42331-42336.

Yin, H.L. and Janmey, P.A. 2003. Phosphoinositide regulation of the actin cytoskeleton. *Annu Rev Physiol* **65**: 761-789.

Zhao, X., Varnai, P., Tuymetova, G., Balla, A., Toth, Z.E., Oker-Blom, C., Roder, J.,
Jeromin, A., and Balla, T. 2001. Interaction of neuronal calcium sensor-1 (NCS-1) with phosphatidylinositol 4-kinase beta stimulates lipid kinase activity and affects membrane trafficking in COS-7 cells. *J Biol Chem* **276**(43): 40183-40189.

Zoncu, R., Perera, R.M., Sebastian, R., Nakatsu, F., Chen, H., Balla, T., Ayala, G., Toomre, D., and De Camilli, P.V. 2007. Loss of endocytic clathrin-

coated pits upon acute depletion of phosphatidylinositol 4,5-bisphosphate. *Proc Natl Acad Sci U S A* **104**(10): 3793-379