SURVIVAL SIGNALS IN HUMAN CANCER CELLS MEDIATED BY PHOSPHOLIPASE D AND mTOR

by

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ABSTRACT

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Phospholipase D (PLD), which is commonly elevated in renal and other cancers, provides a survival signal that suppresses apoptosis induced by serum deprivation in renal cancer cells. Hypoxia-Inducible Factors α (HIFα), important effectors of hypoxic response, have been shown to play a pivotal role in the tumorigenesis of renal cancer cells that lack the von Hipple Lindau tumor suppressor gene (VHL), a critical mediator of HIFα proteolytic degradation. We report here a role for PLD as another regulatory component of HIFα expression in renal cancer cells where accumulation of both HIF1α and HIF2α in require functional PLD for efficient translation, independently from pVHL expression.

The expression of HIF1α has been widely shown to be dependent on mTOR, the mammalian target of rapamycin and its sensitivity to rapamycin has been established. In contrast, HIF2α has been reported to be insensitive to rapamycin. mTOR, a critical node for control of cell growth and survival, exists in two complexes, mTORC1 and mTORC2, which are differentially sensitive to rapamycin. We report here that while HIF2α is insensitive to rapamycin in renal

cancer cells, HIF2α expression is still dependent on mTOR expression and we are able to show in the fourth chapter of this work that while HIF1α is dependent on both mTORC1 and mTORC2, HIF2α depends solely on the rapamycin resistant mTORC2.

However, while much is known about the regulation of mTORC1, little is known about the regulation of mTORC2. PLD and its metabolite phosphatidic acid (PA) have been implicated in the regulation mTOR but its role has been controversial. In light of the concomitant regulation by PLD and mTOR of HIFα expression, we investigated the role of PLD in the regulation mTOR activity. We report in the fifth chapter of this work that PA, in competition with rapamycin, is required for functional mTORC1 and mTORC2 complex formation. Suppression of PLD prevented phosphorylation of the mTORC1 substrate S6 kinase at Thr389 and the mTORC2 substrate Akt at Ser473. Suppression of PLD also blocked insulin-stimulated phosphorylation of Akt and the mTORC2- and Akt-dependent phosphorylation of PRAS40 indicating that PA is required for the association of mTOR with Raptor to form mTORC1 and mTOR with Rictor to form mTORC2. The effect of PA was competitive with rapamycin with much higher concentrations of rapamycin needed to compete with the PA-mTORC2 interaction than with the PA-mTORC1 interaction. However, suppressing PA production substantially increased the sensitivity of mTORC2 to rapamycin. The data provided here reveal a PA requirement for the stabilization of both mTORC1 and mTORC2 complexes. The competition between PA and rapamycin for

mTOR suggests a mechanism for the suppression of mTOR by rapamycin and explains the rapamycin resistance of mTORC2 and HIF2α.

The last part of this work implicates PLD in the regulation of another hallmark of cancer cells: aerobic glycolisis. The metabolic shift from oxidative phosphorylation to aerobic glycolysis, also known as the "Warburg effect", is thought to provide a means for cancer cells to survive under conditions where oxygen is limited and to generate metabolites necessary for cell growth. A shift to aerobic glycolysis is also a response to hypoxia, which stimulates the accumulation of HIFα necessary for the expression of proteins involved in glucose uptake and glycolysis. We are able to show here that the metabolic shift from oxidative phosphorylation to aerobic glycolysis in human cancer cells is dependent on the elevated PLD activity in breast and renal cancer cells. Intriguingly, the effect of PLD on the Warburg phenotype was dependent on mTORC1 in breast cancer cells and on mTORC2 in renal cancer cells, consistently with a role for PLD in activating mTOR.

We are able to conclude that elevated PLD signaling, which is common in human cancer cells, is critical for the activation of mTOR complexes and accounts for mTORC2 insensitivity to Rapamycin. Moreover, elevated PLD activity is required for the expression of HIFα and the consequent transcriptional activation of many genes involved in tumorigenesis including genes involved in the metabolic shift to aerobic glycolysis. Taken together, this data provides evidence that targeting PLD could prove therapeutically significant in cancers with elevated PLD activity such as renal and breast cancer.

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The fruit of my graduate work is dedicated to my parents and to my sister. Through the past thirty years I have been supported, encouraged, believed in, rescued and loved beyond measure. They have always been quietly at my side through my laughter and my tears, cheering me up and shining the light that guided me on the road I have been walking on, always wearing a smile even when I had no smiles left in me. Because of them I had the honor to know the essence of unconditional love.

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CHAPTER I

INTRODUCTION

1.1 HYPOXIA INDUCIBLE FACTOS AND ONCOGENESIS

1.1.1 HIF FUNCTION

Availability of molecular oxygen is an absolute requirement for all vertebrate and invertebrate life. Initially the ability to sense oxygen levels were attributed to specialized cells of the carotid and neuroepithelial bodies that are in charge of regulating cardiac and ventilatory rates (Semenza, 2000), but it became soon clear that all cells in the human body have the capability to sense oxygen and are able to elicit a response according to oxygen tension. While oxygen is essential for survival, low oxygen levels are increasingly gaining an important role in tumor biology as hypoxia is frequently found in developing solid tumor (Pugh and Ratcliffe, 2003). Important evens in tumor development such as angiogenesis, invasion , metastasis and tumor growth are all regulated by genes that respond to drop in oxygen tension. The regulation of these genes is orchestrated by the hypoxia-inducible factor (HIF), a heterodimeric transcription factor consisting of one of alpha (α) and one of the beta (β) subunit also called aryl hydrocarbon receptor nuclear translocator (ARNT) that mediates the cellular response to oxygen levels (Wang and Semenza, 1993; 1995). Both α and β subunits belong to the basic helix-loop-helix-Per/Arnt/Sim (bHLH-PAS) superfamily of transcription factors which share a bHLH region for DNA binding and two PAS domains for dimerization and DNA specificity (Wang *et al.,* 1995).

1.1.2 HIF REGULATION

Although the presence of ARNT is an absolute requirement for HIF transcriptional activation (Maltepe *et al.,* 1997), the regulation of HIF primarily at the level of its α subunit (Hunag *et al.,* 1996; Leung and Ohh, 2002). In normal oxygen conditions , HIFα is hydroxylated at two conserved proline residues in the oxygen dependent degradation domain (ODD) by specific prolyl hydroxylase domain-containing proteins (PHD). This hydroxylation in turn mediates the interaction between HIFα and the product of the Von Hipple Lindau gene (pVHL), the recognizing subunit of an E3 Elongin-Cullin ubiquitin ligase complex (VEC) (Ivan *et al.,* 2001; Cockman *et al.,* 2000). Upon recognition by the VEC complex, HIFα is readily ubiquitinated and subsequently targeted for degradation by the proteasome (Ohh *et al.,* 2002; Ivan and Kaelin, 2001).

PHDs have an absolute requirement for molecular oxygen for their hydroxylating activity (Semenza, 1999; 2001), thus upon drops in oxygen tension PHD mediated proline hydroxylation of HIFα is impaired. This will allow *HIFα* to escape ubiquitination by the VEC complex, to heterodimerize with ARNT and to recruit the transcription co-activator p200/CBP (Arnay *et al.,* 1996; Kallio *et al.,*1998; Leung and Ohh, 2002), thus forming the trasncriptionally active HIF complex.

Once active, the α/β heterodimer will migrate to the nucleus and will bind to the hypoxia-responsive element (HRE) in the promoter region of target genes such as vascular endothelial growth factor (VEGF), glucose transporter, glycolitic enzymes and other enzymes that are pivotal for growth and survival (Semenza, 1999).

Mutations of the VHL gene lead to elevated expression of HIFα under normoxic conditions despite the presence of hydroxylated proline residues in the ODD domain (Tanimoto *et al.,* 2000, Leung and Ohh, 2002); the consequent aberrant activation of hypoxia responsive genes results in the insurgence of pathologies such as the Von Hipple Lindau Syndrome, characterized by retinal angiomas, cerebellar and spinal haemangioblastomas and renal clear cell carcinoma (RCC). (Leung and Ohh, 2002).

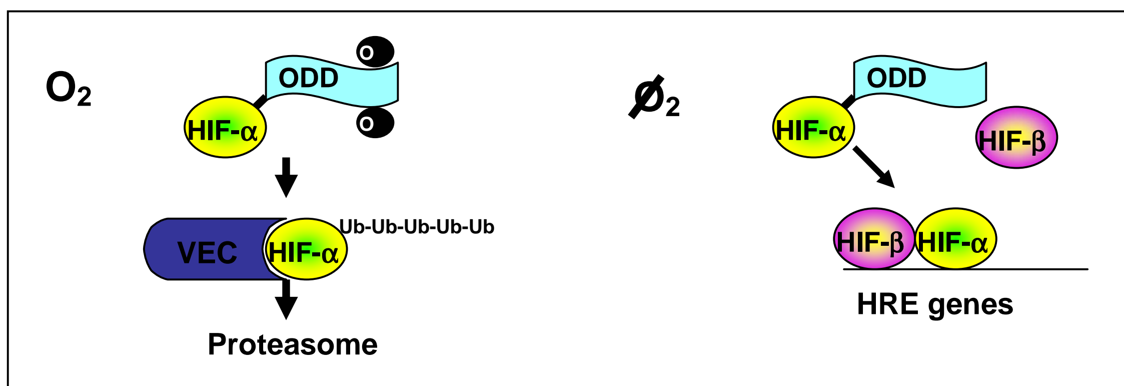

Figure 1.1.1: HIF regulation by VEC. Regulation of HIF is at the levels of the *HIFα* subunit. In the presence of oxygen *HIFα* is hydroxylated at two conserved proline residues located in ODD domain by specific PHDs. Proline hydroxylation will allow *HIFα* to be targeted for ubiquitination and subsequent degradation by the E3 ubiguitin ligase VEC complex, of which VHL is the substrate recognizing subunit. In the absence of oxygen, hydroxylation of ODD proline residues by PHDs is not possible. *HIFα* is then able to escape degradation and is allowed to bind the HIFß subunit to form the active transcription complex.

1.1.3 HIFα ***ISOFORMS***

To date there are three known splice variant isoforms of *HIFα*: *HIF1α*, *HIF2α* and HIF3a. (Maynard *et al.,* 2003; Chun *et.al.,* 2002, 2003)

Among the HIF-α subunits, HIF-1α is the most ubiquitously and abundantly induced in tissues and cells under hypoxic conditions compared to HIF-2α and HIF-3α. *HIF1α* was the first HIF isoform to be identified while investigating the regulation of the erythropoietin (EPO) locus (Wang *et al.,*1995), while the *HIF2α* and HIF3a isoforms were discovered at a later time by homology searches or by screening of interactors with HIFß. The three splice variant of HIF-α share homology in the bHLH and PAS domains but differ in their transactivation domains which may explain the presence of overlapping yet distinct transcriptional target genes (Maynard *et al.* 2003). Moreover most of the HIF-α isoforms share a high degree of homology in their ODD domain including the two critical proline residues (Pro402 and Pro564) that account for O2 tension-associated stabilization of the proteins (Gothie *et al.,* 2000, Lee *et al.,* 2004). In addition, there is a highly conserved sequence of 50 amino acids located near the C-termini of the HIF-1α and HIF-2α isoforms that is important for O2-regulated interaction with the transcriptional coactivator p300 (Hu *et al.,* 2003).

The activity of the three isoforms is , however, non redundant; HIF3a is the most distant related of the *HIFα* isoforms, and in certain splice variants it has been shown that HIF3a actually acts as a negative regulator of the HRE containing hypoxia responsive genes (Maynard *et al.,* 2007).

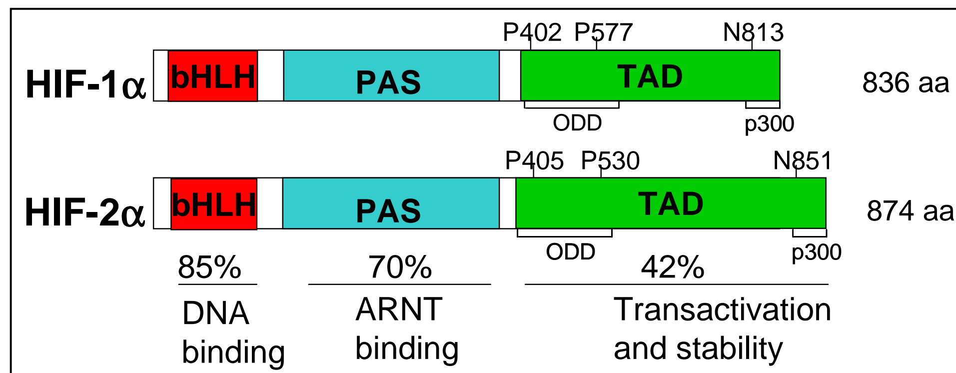

Figure 1.1.2: HIF1α and HIF2α structural comparison

1.1.4 HIF1α AND HIF2α IN CANCER DEVELOPEMENT

HIF1α and *HIF2α* are closely related, sharing an overall 48% amino acid identity (Hu *et al.,* 2003) but while *HIF1α* is expressed in every cell type, *HIF2α* expression is confined predominantly in vascular endothelial cells, kidney fibroblasts, liver hepatocytes, epithelial cells of the intestinal lumen, pancreatic interstitial cells, heart myocites, interstitial cells and lung type II pneumocytes (Wiesener *et al.*, 2003; Rosenberger *et al.*, 2002).

Despite the restricted in vivo localization of *HIF2α*, almost all transformed cell lines have elevated expression of *HIF2α*, indicating that *HIF2α* might have an important role in tumorigenesis (Talks *et al.*, 2000). While HIF1α has been reported to both promote and suppress tumor progression, HIF2α has been implicated only in the promotion of the transformed phenotype (Gordan and Simon, 2007). This somewhat overlapping and antagonistic effects of HIF1α and HIF2α are poorly understood, but it is becoming increasingly clear that in certain tumors, especially following pVHL inactivation, HIF2α is a critical contributor to

the generation of survival signals that protect from apoptosis and facilitate angiogenesis (Kondo *et al* ., 2002, 2003).

1.2 PHOSPHOLIPASE D (PLD) AND CANCER SURVIVAL

1.2.1 PLD FUNCTION

Phospholipase D was first discovered in carrot plants extracts as a distinct phospholipid-specific enzyme that hydrolyses phosphatidylcholine (PC) to phosphatidic acid (PA) and choline (Figure 1.2.1) (Hanahan and Chaikoff, 1947; Hanahan and Chaikoff, 1948). The activation of PLD is believed to play an important role in the regulation of cell function and fate. Multiple PLD activities were characterized in eukaryotic cells, and, more recently, several PLD genes have been cloned (Liscovitch *et al*., 2000; Exton, 2002).

Besides simple hydrolysis, PLD may catalyse a transphosphatidylation reaction utilizing short-chain primary alcohols as phosphatidyl-group acceptors (Yang *et al*., 1967; Ella *et al*., 1967). The resultant phosphatidyl alcohols are produced only by PLD, and are not normally found in biological membranes. Because of their unique origin, their low basal levels and their relative metabolic stability, the formation of phosphatidyl alcohols has served as a convenient and sensitive marker for PLD activation in cultured cells (McDermott *et al*., 2004;; Morris *et al*, 1997, see materials and methods, Chapter II).

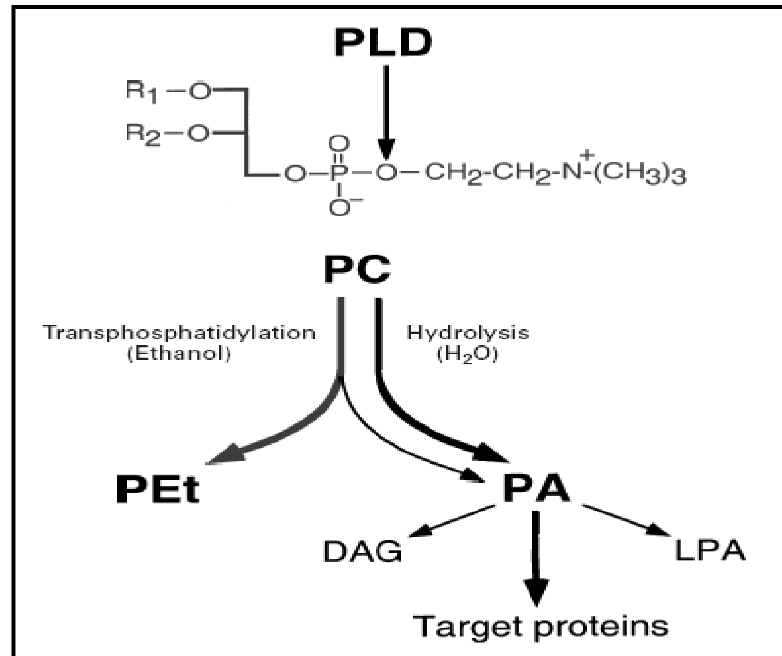

Figure 1.2.1: Phospholipase D – catalyzed reactions (Liscovitch *et al.*, 2000)

PLD hydrolyses the phosphodiester bond in phospholipids such as PC. A phosphatidyl-enzyme intermediate is believed to form transiently which normally is hydrolyzed by water, generating PA. Primary short-chain alcohols (e.g. ethanol) can substitute for water in a competing, transphosphatidylation, reaction. In the presence of ethanol the product of PLD-catalyzed transphosphatidylation is or phosphatidyl ethanol (PEt). This reaction (thick arrow pointing to the left) occurs at the expense of the hydrolytic reaction (thin arrow pointing to the right), decreasing PA formation. PA can also be produced by diacyl glycerol kinase and by acylation of glycerol 3-phosphate. In contrast, phosphatidyl alcohols are uniquely formed by PLD. PA can be further metabolized (thin arrows) to diacyl glycerol (DAG) and lyso-PA (LPA). In contrast, phosphatidyl alcohols are metabolically stable and would accumulate in cells upon PLD activation. (references provided in the text).

PLD has been implicated in membrane trafficking, cytoskeletal reorganization, receptor endocytosis, exocytosis, and cell migration (Exton *et al* 2002). PLD has also been shown to be involved in cell proliferation (Foster and Xu, 2003). The activity of PLD is elevated in response to platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF),

insulin, and insulin-like growth factor 1 (IGF-1) (Plevin *et al.*, 1991; Motoike *et al.*, 1993; Song *et al.*, 1994; Karnam *et al.*, 1997; Sa *et al.*, 1999; Banno *et al.*, 2003). PLD activity is also elevated in cells transformed by a variety of transforming oncogenes including v-Src, v-Ras, v-Fps, and v-Raf (Song *et al.*, 1991; Carnero *et al.*, 1994; Jiang *et al.*, 1994; Jiang *et al.*, 1995; Frankel *et al.*, 1999). Hence, there is a growing body of evidence linking PLD activity with mitogenic signaling and elevated PLD has been shown to contribute to cell transformation and survival (Foster and Xu, 2003). It has been reported that elevated expression of either PLD1 or PLD2, in combination with overexpression of c-Src or EGF receptor, transforms rat fibroblasts (Lu *et al.*, 2000; Joseph *et al.*, 2001). Fibroblasts overexpressing c-Src undergo apoptosis in response to growth factor deprivation, and both PLD1 and PLD2 were able to provide survival signals that prevented apoptosis (Zhong *et al.*., 2003). The capability of PLD to cooperate with a tyrosine kinase such as c-Src or EGFR to transform cells or provide survival signals would implicate PLD as a good candidate oncogene in cancer, especially those cancers that have elevated tyrosine kinase such as c-Src or EGFR (Foster and Xu, 2003).

Elevated PLD expression and activity has been reported in several human cancer tissues including breast cancer (Uchida *et al.*, 1997; Noh *et al.*, 2000), gastric and renal cancers (Uchida *et al.*, 1999; Zhao *et al.*, 2000). Hence, it is of interest whether the elevated PLD in human cancer tissues plays any role in tumorigenesis. Our lab has shown that blocking PLD activity by using either a catalytic inactive mutant of PLD or primary butanol will induce apoptosis in

breast cancer cell line MDA-MB-231 (Zhong *et al.*, 2003) and renal cancer cells (Toschi *et al*, unpublished data) . These cells have very high level of PLD activity, and this finding further implicates PLD in survival signaling in cancer cells. The role of PLD in cancer survival will be discussed more in depth in part 1.2.4.

1.2.2 PLD STRUCTURE

Mammals contain two separate PLD genes PLD1 and PLD2 both with splice variants (Hammond *et al.*, 1995, Colley *et al.*, 1997). Despite homology between the two genes, there are significant differences in the regulation and subcellular distribution of PLD1 and PLD2. The approximately 120 kDa PLD1 is found mainly in intracellular membranes while the approximately 106 kDa PLD2 is localized almost exclusively on the plasma membrane (Liscovitch *et al.*, 2000; Freyberg *et al.*, 2001). The highly conserved domain in the PLD family is the HKD motif, which is used to define the PLD superfamily. It was termed “HKD” because the domain contains the motif HxKxxxxD/E, which is found twice without exception in all cloned PLDs (Hammond *et al.*, 1995), Mammalian PLD1 and PLD2 both contain two HKD motifs, which are critical for enzymatic activity *in vitro* and *in vivo* (Sung *et al.,* 1997).

Other highly conserved regions of the PLD genes are the phox consensus sequence (PX), the plekstrin homology (PH) domain and the PI-4-P 5-kinase binding site (Table 1.2.1). The PH domain is thought to function in the localization of the protein (Sciorra *et al.,* 2002) but is not required for PLD enzymatic activity. The PX domain is thought to mediate protein-protein interactions or to bind

phosphatidylinositol phosphates (PIP) (Xu *et al.,* 2006). Finally, PLD1 has a conserved loop region that is not found in PLD2. The loop region has been proposed to function as a possible negative regulatory element, since deletion of this region from PLD1 increased its basal activity threefold (Sung *et al.,* 1999).

PLD1 and PLD2 both have an absolute requirement for Phosphatidylinositol bisphosphate (PIP2) (Hammond *et al*.,1997), however, reports indicate that PLD1 has low basal activity while PLD2 has high basal activity (Colley *et al* ., 1997). In addition, PLD1 is activated by the ADP-ribosylation factor (ARF)-, Ral- and Rho-family GTPases, as well as by protein kinase Cα (PKCα) while PLD2 is activated by fatty acids (Colley *et al*., 1997). Table 1.2.1 summarizes the distinguishing characteristics of the two PLD isoforms.

Characteristic	PLD1	PLD2
Molecular Weight	~120 kDa	~106 kDa
Substrate Specificity	PC	PC
PIP2-dependent	yes	yes
RalA-associated	yes	no
Basal Activity	low	high
Subcellular localization	Mainly intracellular membranes	Plasma membrane
Activators	ARF-, Ral-, Rho-GTPases, PKCα	fatty acids, ARF

Table 1.2.1: Biochemical properties of Phospholipase D 1 and 2 (Foster and Xu, 2003)

Presented are various characteristics of PLD 1 and 2, corresponding references are presented in the text.

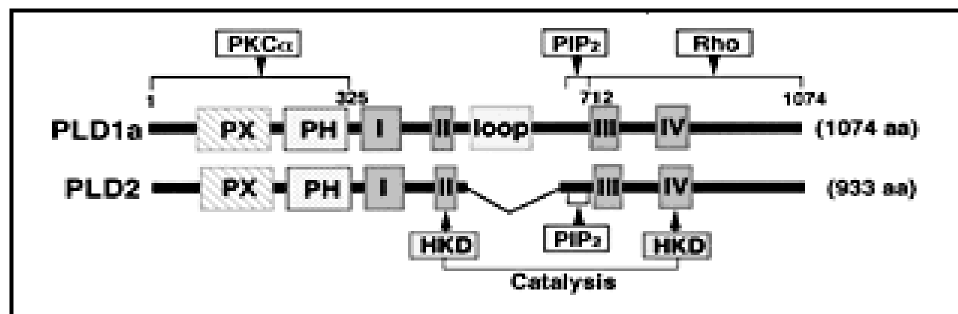

Figure 1.2.2: PLD genes. Basic structure of PLD1 and PLD2. Regions of conserved sequences of PLD1 and PLD2 are shown. PX, phox homology domain; PH, pleckstrin homology domain; motifs I, II, III and IV, regions of sequence conserved among all PLD isozymes. Motifs HKD are found in II and IV. Regions of PLD1a identified as interacting with protein kinase C and Rho, and PIP_2 binding regions of PLD1 and PLD2 are indicated.

1.2.3 PLD SIGNALING PATHWAY

Mammalian PLD activity is highly regulated by a large number of factors. Fatty acids, phosphoinositides, small GTP binding proteins, protein kinase C, Ca^{2+}, phosphorylation, and negative regulators all regulate PLD activity. Many of these factors act in concert to positively or negatively regulate PLD activation (McDermott *et al.*, 2004).

Several small GTPases have been reported to activate PLD1 *in vivo* and *in vitro*, including ARF-, Ral-, and Rho-family members (Exton, 2002). These GTPases are involved in cell membrane trafficking, cytoskeleton regulation, and survival signal pathways, and PLD plays a role in these functions as well.

The ARF family of small GTP-binding proteins plays a central role in membrane trafficking and cytoskeletal remodeling (Liu *et al.*, 2005). ARF proteins were reported to be required for PLD activities that are elevated by various

mitogenic factors, including PDGF, EGF, insulin, phorbol esters, and H-Ras (Foster and Xu, 2003). Among the members of ARF family, ARF6 co-localizes with PLD1 (Powner *et al.*, 2002) in the lipid raft fractions, while ARF4 was recently implicated in the activation of PLD2 (Kim *et al.*, 2003).

Rho-family GTPases regulate cell membrane trafficing and actin dynamics. Rho-family members were implicated in mitogenic signaling through PLD (Hess *et al.*, 1997), and more commonly Rho-family members were shown to mediate PLD responses through agonists that stimulate secretion (Powner *et al.*, 2002).

RalA mediates Ras signaling, and directly interacts with PLD1 (Jiang *et al.*, 1995; Luo *et al.*, 1997). Although RalA alone can not activate PLD1 either *in vitro* or *in vivo*, it is required for the activation of PLD activity by EGF, PDGF, insulin, Src, Ras, Raf, and phorbol esters (Foster and Xu, 2003). These evidence suggest that RalA is critical in PLD response stimulated by mitogenic or oncogenic signals, possibly by working together with Arf6 (Foster and Xu, 2003).

Other than small GTPases like PIP2 is also required for both PLD1 and PLD2 activation (as mentioned above). PIP2 can be generated by various PI kinases including PI-4-P 5-kinase, while PI3-kinase, on the other hand, converts PIP2 to PIP3. PI-4-P 5-kinase is a downstream target of PLD signaling, so by generating PIP2, it may provide a positive feedback loop (Foster and Xu, 2003; Foster, 2006). Since both ARF6 and RalA stimulate PLD activity through interaction with PLD1, but evidences suggest that PLD2 is the major responsible isoform for the mitogenic signal stimulated PLD activity (Xu *et al.*, 2000), and that PLD2 activation was suggested to be dependent on the activation of PLD1

(Mwanjewe *et al.*, 2001), we proposed a model (Figure 1.2.3) in which mitogenic signals stimulate PLD1 activation through the interaction of ARF6 and RalA, which then lead to the activation of PLD2 which mainly reside on the lipid rafts of the plasma membrane (adapted from Foster and Xu, 2003).

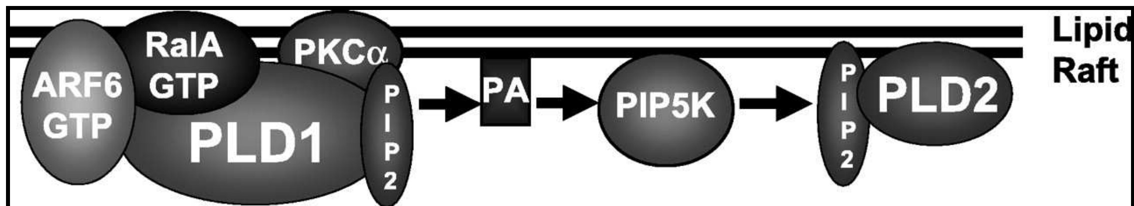

Figure 1.2.3: Mitogenic signaling through PLD1-dependent activation of PLD2 (Foster and Xu, 2003). In this model, it is proposed that PLD1 is activated through the synergistic actions of ARF6 and RalA in lipid rafts. PIP2 is required for both PLD1 and PLD2 activity. On activation of PLD1, all available PIP2 is used by PLD1, which is present in low levels in the lipid rafts. PLD2, which is present at high levels in lipid rafts, is inactive in the absence of sufficient PIP2. The generation of PA by PLD1 would activate PI-4-P 5-kinase (PIP5K), which would then generate sufficient PIP2 to activate the high levels of PLD2.

While lots of evidence indicates PLD involvement in various cell activities, the relevant downstream targets of PLD are still mostly elusive. Raf has a PA binding site and its association with PA was implicated to facilitate the recruitment of Raf to the plasma membrane, where it can participate in the activation of the mitogen-activated protein (MAP) kinase pathway (Foster and Xu, 2003; Rizzo *et al.*, 1999).

The mammalian target of rapamycin (mTOR) has recently emerged as an important downstream target of PLD (Figure 1.2.4). mTOR is a protein kinase directly involved in both cell cycle progression and cell growth by regulating

translation, transcription, membrane traffic, and protein degradation (Kuruvilla and Schreiber., 1999; Schmelzle and Hall., 2000), and mTOR is also a downstream target of PI-3-kinase/Akt survival pathway (Sekulic *et al.*, 2000; Nave *et al.*, 1999). It has recently been reported that PA binds to mTOR competitively with rapamycin and is required for activation of mTOR (Fang *et al.*, 2001; Chen and Fang 2002). Consistently, elevated PLD activity seems to confer resistance to rapamycin in some human cancer cells (Chen *et al.*, 2003). All these evidence suggest the role for mTOR as a particularly important downstream target of PLD signaling pathway.

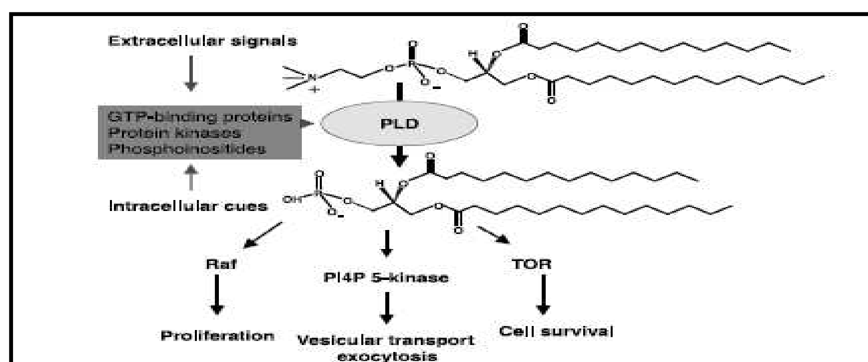

Figure 1.2.4: PLD substrates, products, regulators and targets (McDermott *et al.*, 2004). The structure of the PLD substrate and product phosphatidylcholine and phosphatidic acid are shown, and the figure summarizes the regulation of PLD by cell surface receptors and intracellular signals through actions of intermediate protein and lipid activator

1.2.4 PLD REGULATION IN BREAST CANCER

During the last several years, it has become apparent that PLD is a critical regulator of cell proliferation and survival. PLD has been shown to facilitate cell cycle progression, suppress apoptosis, contribute to the transformation of mice

fibroblasts, and enhance cell migration – all critical steps in tumorigenesis. Moreover, Elevated PLD activity has been reported in several human cancers.

A critical barrier for an emerging tumor is the generation of "survival" signals that suppress default apoptotic programs (Hanahan and Weinberg, 2000). High intensity Raf signals induces cell senescence (Samuels and McMahon, 1994; Kerkhoff and Rapp, 1998) or, in the absence of serum, apoptosis (Joseph *et al*., 2002). Either PLD1 or PLD2 was able to suppress the cell cycle block and apoptosis in cells with high intensity Raf signals (Joseph *et al*., 2002). Similarly, fibroblasts overexpressing c-Src undergo apoptosis in response to growth factor deprivation, and both PLD1 and PLD2 prevented this apoptosis (Zhong *et al*., 2003). More recently, we have shown that in the highly malignant MDA-MB-231 breast cancer cell line, PLD provides a survival signal that suppresses apoptosis induced by the withdrawal of serum (Chen *et al*., 2005). Importantly, this PLD survival signal was dependent upon mTOR, which has been widely implicated in survival signal signals mediated by PI3K (Sawyers, 2003; Foster, 2004). The survival signal generated by PLD in MDA-MB-231 cells could be distinguished from PI3K dependent survival signals in another breast cancer cell line MDA-MB-435s (Chen *et al.,* 2005). These data indicate that PLD generates a distinguishable survival signal in breast cancer cells that overcomes default apoptotic programs.

Tumor invasion is a required step in metastasis and it has been proposed that the ability to invade is actually a deregulated cell migration (Kassis *et al*. 2001). Cytoskeleton networks, which play a central role in cell migration, have

been reported to be regulated by PLD (Aguirre Ghiso *et al.* 1997; Kam and Exton 2002). Interestingly, MDA-MB-231 human breast cancer cells, which have very high levels of PLD activity, migrate and invade matrigel in culture, whereas MCF-7 breast cancer cells, with relatively low PLD activity do not (Zheng *et al.*, 2006). Protease secretion is also a property of invasive cancer cells and PLD activity has also been correlated with elevated protease secretion (Aguirre Ghiso *et al.*, 1999). A dominant negative mutant of RalA, which blocks PLD activity in v-Src- and v-Ras-transformed cells (Jiang *et al.*, 1995), also blocked protease secretion and tumor formation in nude mice (Aguirre-Ghiso *et al.*, 1999).

More recently our lab has found that the ability of MDA-MB-231 cells to migrate and invade Matrigel is dependent upon PLD activity (Zheng *et al.*, 2006). The link between PLD and cytoskeletal organization, protease secretion, and cell migration and invasiveness suggest that PLD plays an important role in metastasis of cancer cells. Our lab has proposed that the link between survival signals and cell migration is part of a "survival program" that suppresses apoptosis during stress and facilitates migration to less stressful sites (Zheng *et al.*, 2006).

A major focus of our lab has been survival signals generated in breast cancer cells by PLD. There are several targets of PA generated by PLD, but perhaps the most significant with regard to cancer is mTOR, which has been widely implicated in survival signals in many cancers (Sawyers, 2003; Foster, 2004). Jie Chen and colleagues reported that mTOR has a PA requirement (Fang *et al.*, 2001), which implicated PLD in mTOR-mediated survival signals.

Our lab has gone on to demonstrate that elevated PLD activity in the human breast cancer cell line MDA-MB-231 cells leads to an mTOR-dependent suppression of both p53 (Hui *et al.*, 2004) and protein phosphatase 2A (PP2A) activity (Hui *et al.*, 2005). PLD activity also led to an mTOR-dependent increase in the expression of Myc (Rodrik *et al.*, 2005). In this thesis, we provide evidence indicating that the elevated PLD in 786-O renal cancer cells accounts for the resistance to Rapamycin in virtue of the PLD dependent production of PA and PA's competition with Rapamycin for mTOR. (see Chapter 5) . This and other studies strongly implicate PLD in many of the critical hallmarks needed for progression to a malignant tumor (Hanahan and Weinberg, 2002). In addition it is also important to mention that suppression of the TGF-β signals that suppress cell cycle progression may be another hallmark needed for tumorigenesis. Moreover, elevated PLD activity in human breast cancer cells is able to alter the expression and activity of key regulators of cell cycle progression and survival in ways that are consistent with a novel PLD/mTOR survival pathway that could be distinguished from the better-characterized PI3K/Akt survival pathway (Cantley, 2002; Luo *et al.*, 2003), which also targets mTOR indirectly.

A model for two alternative strategies for activating mTOR is shown schematically bellow (adapted from Foster, 2006) where mTOR can be activated indirectly by PI3K or directly by PA generated by PLD (Figure 1.2.5). Interestingly, the two mechanisms may be linked by differential dependence on

PIP2 and PIP3, indicating that the two pathways may be integrated. The mTOR signaling pathway and its application to PLD will be further discussed in part 1.3.

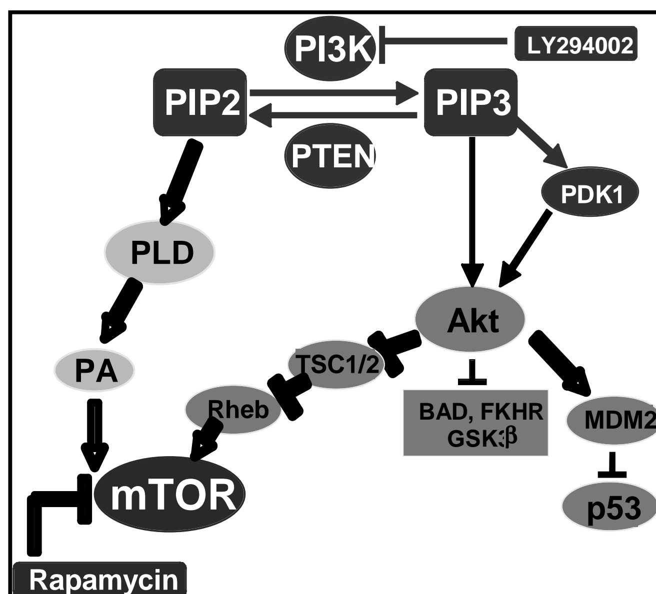

Figure 1.2.5: Alternative PLD and PI3K survival signals (Foster, 2006).
PI3K generates PIP3 from PIP2. The presence of PIP3 leads to the recruitment of PDK1 and Akt. PIP3 levels are also regulated by PTEN, a phosphatase that de-phosphorylates PIP3 to PIP2. PDK1 phosphorylates and inactivates several substrate proteins that negatively regulate cell proliferation or stimulate apoptosis. These include GSK3β, BAD, MDM2 and indirectly, mTOR. PLD generates PA, which also leads to the activation of mTOR. mTOR then phosphorylates several substrate proteins that regulate protein synthesis. The two pathways are also connected by their dependence on PIP2.

Consistent with the model above, PLD suppressed apoptosis in MDA-MB-231 cells that were deprived of serum in a rapamycin-dependent manner (Chen *et al.*, 2005; Gadir *et al.*, 2007).

1.2.5 PLD IN CANCER THERAPY

Hydrolysis of PC by PLD produces the release of PA, a mitogenic second messenger which shown to be involved in cell proliferation and transformation (Foster, 2006; Foster, 2007). Therefore, targeted inhibition of PLD may slow down the process of tumour formation and proliferation and may thus be an interesting target for the development of effective anti-cancer drugs. However, because PLD is considered to play an important role in diverse cellular processes such as nuclear vesicle dynamics during mitosis (Liscovitch *et al.*, 1999), vesicular traffic in the Golgi (Exton *et al* 2002), formation of budding secretory vesicles in the trans- Golgi (Chen *et al.*, 1997), exocytosis (Brown *et al.*, 1998), cytoskeleton and cell motility (Colley *et al.*, 1997), these basic cellular functions may also be affected by PLD inhibition and so appropriate targeting of tumour-related cells would be necessary.

Despite the fact that negative regulation of PLD is still poorly understood, a series of compounds of diverse origin have shown anti-PLD activity during the past few years like APCs (alkylphosphocholines) and certain synthetic 1,3-diacylglycerols (1,3-DAGs) (Unger *et al.*, 1990; Terwogt *et al.*, 1999; Lucas *et al.*, 2001; Rodríguez-González, 2003). Although clear anti-tumoral activity has not as yet been observed with these compounds, a clear correlation is seen in some instances. Such is the case when inhibiting PLD activation showed hopeful anti-

tumour effects in breast cancer patients with skin metastases (Unger *et al*., 1990; Terwogt *et al*., 1999; Lucas *et al*., 2001).

Elevated PLD activity has been implicated in a large number of human cancers including breast, colon, gastric, and kidney (Foster, 2006). PLD activity has been reported to suppress apoptosis in human cancer cells subjected to the stress of serum withdrawal (Foster, 2006) and PLD activity is elevated in several cancer cells in response to the stress of serum withdrawal (Zheng *et al*., 2006). mTOR has been implicated as a key regulator of stress responses by shutting down under conditions of poor nutrition or hypoxia. For a cancer cell to survive and proliferate, it is becoming apparent that it must overcome this stress response (Foster, 2007). The PLD activity elevated in response to serum withdrawal in cancer cells may be a mechanism to activate mTOR that was selected for to overcome the shutdown of mTOR. In this regard, it is significant that the survival signals generated by PLD in the breast cancer cell line MDA-MB-231 cells are dependent upon mTOR (Chen *et al*., 2005). .

Together, the data linking PA production – especially via PLD mediated hydrolysis of phosphatidylcholine – with mTOR activation represents a potentially important mechanism for activating this critical regulator of responses to environmental stress and survival signals in cancer. The significant percentage of cancers with elevated PLD activity strongly suggest that this pathway for activating mTOR be considered – especially since targeting mTOR with rapamycin or rapamycin derivatives in cancer would be strongly influenced by the level of PLD activity in the cancer cells.

Thus, the findings suggest that targeting the signals mediated by PLD will be a promising therapeutic strategy in an apparent large number of cancers where elevated PLD activity is promoting cell cycle progression and suppressing apoptosis. The need for the stressful condition of serum withdrawal indicates that successful strategies for inducing apoptosis may require combination strategies that suppress growth factor signals in addition to suppressing PLD signals; however, cancer cells – especially solid tumors – are subjected to a variety of stressful conditions during tumorigenesis prior to vascularization. Our lab has been working to establish approaches for therapeutic targeting human cancer cells that are dependent on PLD activity for survival.

Rapamycin based therapies: Our previous work has established that rapamycin can induce apoptosis in MDA-MB-231 cells deprived of serum (Chen *et al*., 2005). However, we also demonstrated that elevated PLD activity confers rapamycin resistance in human cancer cells (Chen *et al*., 2003) leading to higher concentrations of rapamycin being required to suppress cell growth and induce apoptosis. We have demonstrated that suppressing PLD activity with a dominant negative PLD2 mutant reduces the concentration of rapamycin to suppress cell proliferation and to inhibit S6 kinase phosphorylation. These preliminary studies indicate that <u>suppressing PLD activity can increase the efficacy of rapamycin</u>.

Honokiol: we have found that a compound isolated from *magnolia grandiflora* known as honokiol suppresses PLD activity in both MDA-MB-231 and T24 cells. This compound also suppresses both tumor growth and angiogenesis (Bai *et al*., *2003*). In the absence of serum, honokiol induces apoptosis in MDA-

MB-231 and T24 cells (our unpublished results). Since suppression of PLD also induces apoptosis in these cells, the data indicate that the effect honokiol is due at least in part to its ability to suppress PLD survival signals. Since honokiol is well tolerated by mice (Bai *et al.*, 2003), honokiol may be an ideal candidate drug to target PLD signals in human cancers were PLD is implicated. Reducing PLD activity in order to increase the efficacy of rapamycin and honokiol should be ideal in this regard. Moreover, the ability to target PLD survival signaling both upstream and downstream with two different drugs (honokiol and rapamycin) may be important therapeutically since single drug approaches usually result in the development of resistant cells (Shah and Sawyers, 2002).

1.3 MAMMALIAN TARGET OF RAPAMYCIN (mTOR)

1.3.1 mTOR FUNCTION

mTOR, the mammalian target of rapamycin (also known as FRAP) is an evolutionary conserved large (~290 kDa) serine/threonine protein kinase. It was originally discovered in the yeast *saccharomyces cerevisiae*, during a screen for resistance to the immunosuppressant drug rapamycin (Kunz *et al.*, 1993; Helliwell *et al.*, 1994). Rapamycin acts by forming an inhibitory complex with its intracellular receptor, the FKBP12 which binds a region in the C terminus of TOR proteins termed FRB (FKBP12-rapamycin binding), and thereby inhibiting TOR activity (Chen *et al.* 1995; Choi *et al.* 1996).

mTOR integrates signals from growth factors, nutrients, and energy to regulate essential cellular processes such as cell growth (increase in cell mass and size) and cell cycle progression (see Figure 1.3.1) (Fingar and Blenis, 2004). The mTOR pathway controls the translation of mRNAs that encode proteins that are required for G1 cell-cycle progression and S-phase initiation, and thus inhibition of mTOR signaling results in a slowing or arrest of cells in G1. mTOR might therefore also be viewed as a gatekeeper, which only allows G1 progression under low nutrients conditions (reviewed by Bjornsti and Houghton, 2004). In the last decade, a role for mTOR in tumorigenesis is emerging as mTOR comprises a critical target of “survival signals” in cancer cells (Sawyers, 2003; Tee and Blenis, 2005).

Most of the work done in mammalian cells focused on the rapamycin-sensitive mTORC1 complex (raptor) and it has been suggested that raptor function as an adaptor for recruiting mTOR substrates. Two well-characterized substrates of the mTORC1 complex that control translation and cell growth are the eukaryotic initiation factor 4E-binding protein (4E-BP1) family of proteins and the S6 protein kinases (S6K1 and S6K2). mTOR regulates protein synthesis through the phosphorylation and inactivation of the repressor of mRNA translation 4E-BP1, and through the phosphorylation and activation of S6K1 (reviewed by Hay and Sonenberg, 2004). Thus, S6K1 or 4E-BP1 phosphorylation is often used as a marker for mTOR activity.

The mTORC2 complex (rictor) has been shown to control cytoskeleton organization (Jacinto *et al.*, 2004). This rapamycin-insensitive complex is

required for phosphorylation of the hydrophobic motif at Ser473 of Akt, a site analogous to the hydrophobic motif site in S6K that is known to be regulated by the mTORC1 complex (Sarbassov *et al.*, 2004). On growth factor stimulation, PI3K activation results in recruitment of Akt to the plasma membrane, in which phosphorylation at Akt Ser473 is mediated by the mTORC2 complex and phosphorylation at Thr308 is mediated by PDK1. The mTORC2-activated Akt then phosphorylates and inactivates tuberin, resulting in increased mTORC1 activity (Sarbassovd *et al.*, 2004; Shaw and Cantley, 2006). The mTOR signaling pathway will be further discussed in part 1.3.3.

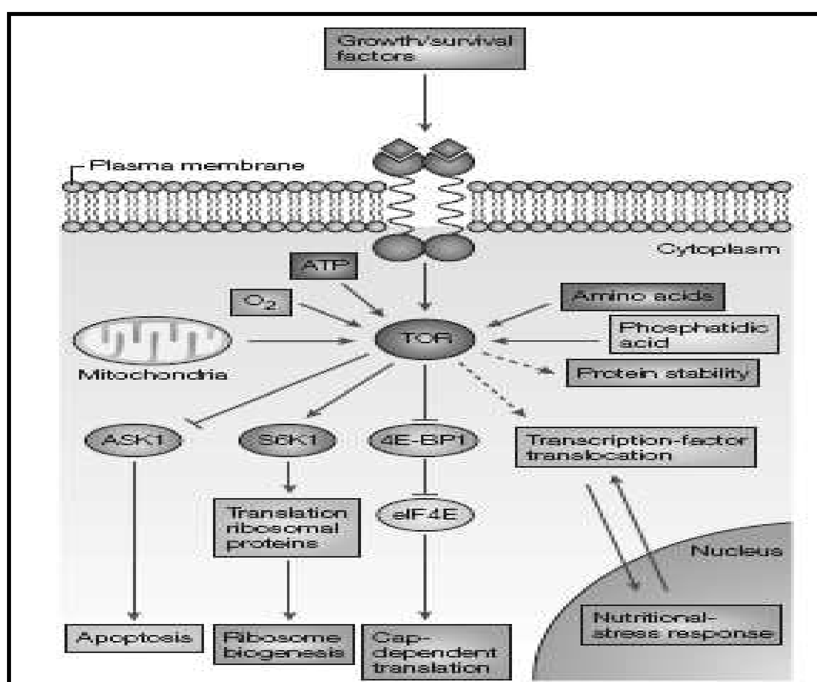

Figure 1.3.1: Target of rapamycin is a central regulator of cell growth and proliferation in response to environmental and nutritional conditions. (Bjornsti and Houghton, 2004). Target of rapamycin (TOR) signalling is regulated by growth factors, amino acids, ATP and Oxygen levels; second messengers (for example, phosphatidic acid); and, possibly, mitochondrial stress. Signalling through TOR seems to regulate several downstream pathways that impinge on cell-cycle progression, translation initiation, transcriptional stress

responses, protein stability and survival. Dashed lines indicate pathways that are best described in yeast.

The role of mTOR in autophagy is conserved from yeast to mammals where it acts to regulate the induction of the autophagic process (Levine and Klionsky, 2004). Autophagy is implicated in the pathology of cancer and although a reduction in autophagy appears to be common in tumor cells, some level of autophagy may be required for the development of cancer (Easton and Houghton, 2006). One mechanism tumor cells may use to cope with nutrient deprivation is to temporally recover nutrients autonomously by activating autophagy (Guertin and Sabatini, 2007). Confirmation for this is provided in breast cancer in which one copy of the essential autophagy gene BECN1, which codes for the protein BECLIN 1, is frequently deleted. If mTOR is inactive autophagy proceeds, and conversely, when mTOR is activated the autophagic process is inhibited. Treatment with rapamycin elevated BECLIN 1 expression in MDA-MB-231 breast cancer cells (our unpublished data).

1.3.2 mTOR STRUCTURE

mTOR is a high molecular-weight protein contains 2549 amino acids and comprises several conserved structural domains. The N terminus possesses 20 tandem HEAT (for Huntignton, EF3, A subunit of PP2A, TOR1) repeats. Tandem HEAT repeats are present in many proteins and are implicated in protein-protein interactions (Andrade and Bork, 1995; Hay and Sonenberg, 2004). The C-

terminal half of mTOR contains the kinase domain, which has sequence similarity with the catalytic domain of PI3K and therefore makes it a member of the phosphoinositide 3-kinase–related kinase (PIKK) family, whose members (ATM, ATR, DNA-PK, hSMG1, mTOR, and TRAPP in mammalian cells) transmit signals related to cell growth, proliferation, and stress responses (Abraham and Gibbons, 2007). In addition, the C-terminal end contains a FAT domain (for FRAP, ATM, TRAP), designated FATC which is necessary for mTOR activity, and the deletion of even a single amino acid from this domain abrogates the activity (Peterson *et al.* 2000; Takahashi *et al.* 2000). mTOR also contains a presumed negative regulatory domain (NRD) between the catalytic and FATC domains (Figure 1.3.2) (Sekulic *et al.* 2000). Figure 1.3.2 below portrays the mTOR structure.

Figure 1.3.2: The primary structure of mTOR (Hay and Soneneberg, 2004). The protein consists of a catalytic kinase domain (CD), an FKBP12-rapamycin-binding (FRB) domain, a putative auto-inhibitory domain near the carboxyl terminus and up to 20 tandemly repeated HEAT motifs at the amino terminus, as well as FAT and FATC (FATC terminus) domains.

In yeast and mammals, there are two distinct TOR complexes, each composed of TOR, a common regulatory subunit called LST8, and at least a third subunit that specifies the downstream substrates (Shaw and Cantley, 2006). Among the PIKK family members, the FRB domain is found only in the TOR proteins, and gives an exclusive specificity of rapamycin and its derivatives for

mTOR in mammalian cells. The drug directly attacks only one subpopulation of mTOR proteins residing in a complex, termed mTORC1 (Sarbassov *et al.*, 2004). In mammals, the substrate-defining subunits are raptor (the mTORC1 complex) and rictor (mTORC2) (Sarbassov *et al.*, 2004; reviewed by Guertin and Sabatini, 2007). Whereas mTORC1 complexes are strongly inhibited by rapamycin, mTORC2 is not affected by the drug (Sarbassov *et al.*, 2004) (discussed also in part 1.3.1).

1.3.3 mTOR SIGNALING PATHWAY

Upstream signaling. The PI3K-AKT pathway is involved in the delivery of growth factor–derived stimulatory signals to the mTORC1 complex. Ligand binding to various growth-factor receptors results in activation of PI3K, which catalyses the conversion of PIP2 to PIP3. The protein and lipid phosphatase PTEN (phosphatase and tensin homolog deleted from chromosome 10), negatively regulates this pathway. Akt, a serine/threonine protein kinase (also known as PKB) is a downstream effector of PI3K. The neighboring target for Akt in this pathway is the tuberous sclerosis 2 (TSC2) protein, which functions in a heterodimeric complex with TSC1. The TSC1/2 complex expresses GTPase-activating protein activity toward the Ras-related GTPase Rheb, and this activity is inhibited by Akt -dependent phosphorylation of TSC2 (Abraham and Gibbons, 2007). When active, TSC1/2 converts the GTP-bound form of Rheb to its inactive, GDP-bound state. When TSC1/2 activity is suppressed, GTP-bound Rheb

stimulates mTORC1 signaling through a poorly understood mechanism that may involve a direct interaction between Rheb and mTORC1 (Long *et al.*, 2005). The location of mTOR as a downstream target in the PI3K- Akt pathway provides a clear link to oncogenesis. Deregulated signaling through the PI3K pathway is a feature of most, if not all, types of cancer cells (Shaw and Cantley, 2006). Second messengers such as phosphatidic acid, ATP levels and polyphosphates might also regulate mTOR signaling in addition to nutrients and mitogens, and will be discusses presently.

Downstream signaling. The mTOR–Raptor complex signals directly to important translational regulators, the translational repressor protein eukaryotic initiation factor 4E-BP1 and S6K1. Binding of 4E-BP1 to eIF-4E is controlled by mTOR dependent phosphorylation of specific serine and threonine residues (Hay and Soneneberg, 2004). Once 4E-BP1 is completely phosphorylated, it dissociates from eIF-4E allowing the formation of an initiation factor complex eIF-4F (Hay and Soneneberg, 2004) which then activate translation of a subset of capped mRNA and encoding proteins involved in G1- to S-phase progression, such as c-Myc (West *et al.*, 1998) and cyclin D1 (Noh *et al.*, 2000; Gera *et al.*, 2004). The second important mTOR target, S6K1, has been implicated in translational regulation of mRNAs such as those that encode ribosomal proteins, proteins involved in translation (Hay and Soneneberg, 2004). Considering the importance of the proteins that are subject to mTOR mediated translational control in cell proliferation and growth, it is expected that cancer cells undergo alterations that impact on mTOR activity.

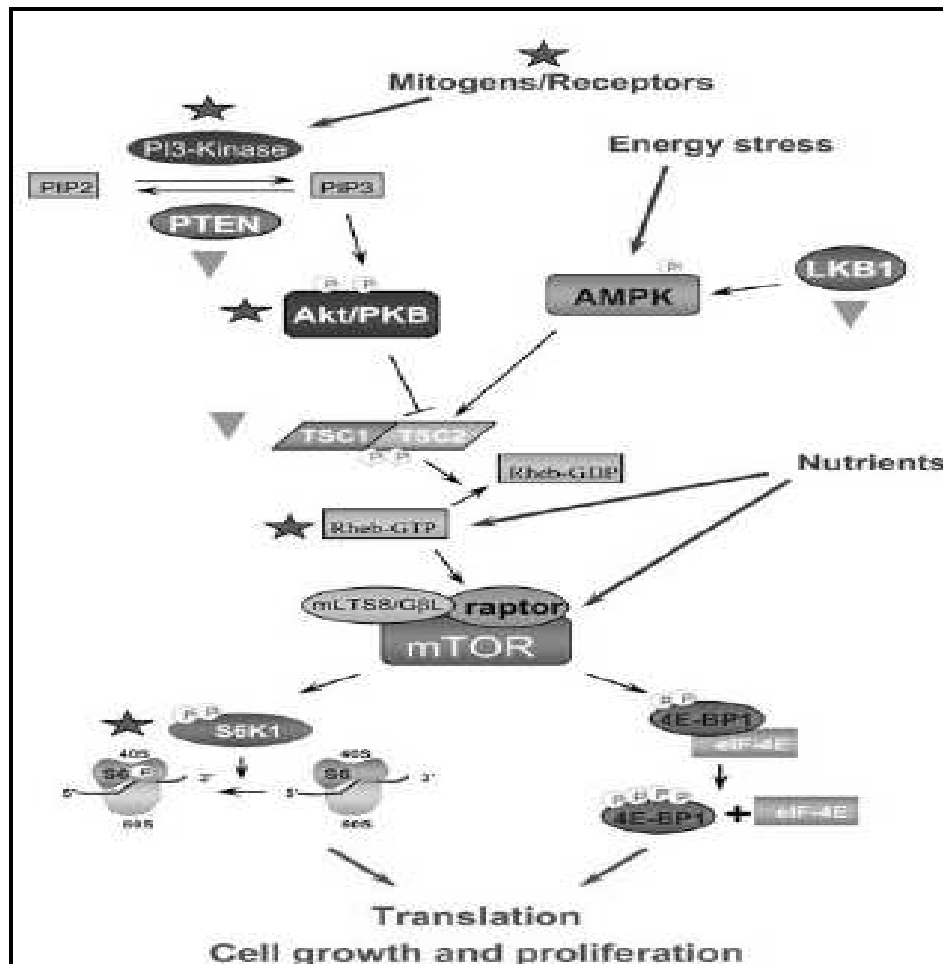

Figure 1.3.3: Model of the mTOR pathway; its regulation and cancer specific alterations. (Hynes and Boulay, 2006) mTOR lies at the border of two major signaling pathways, one initiated by PI3K and the other by AMPK. Mitogen signaling to receptor tyrosine kinases activates PI3K and Akt, which phosphorylates TSC2 leading to activation of Rheb GTPase and mTOR activation. AMPK is a regulator of cellular energy metabolism. In the presence of high AMP, AMPK phosphorylates TSC2, strengthening the ability of the TSC complex to block Rheb GTPase activity and lowering mTOR activity. The asterisk (*) indicates mutations leading to pathway activation in cancer. These include constitutive activation of receptor tyrosine kinases, overexpression or mutational activation of PI3K and Akt as well as overexpression of Rheb and S6K1. The inverted triange (▿) indicates proteins that are lost in cancer cells. These include PTEN, the negative regulator of PI3K, TSC complex proteins hamartin and tuberin and the LKB1 kinase.

Signaling through phosphatidic Acid (PA) – link for PLD in mTOR activation. While the regulation of mTOR through TSC1/2 and Rheb has been widely described and reviewed, another mechanism contributing to the activation of mTOR by phosphatidic acid has been proposed. Jie Chen and colleagues reported a requirement for PA for the activation of mTOR (Fang *et al*., 2001). PA is most commonly generated by the hydrolysis of phosphatidylcholine by PLD (Exton, 2002; see part 1.2). PLD, like PI3K, has been implicated in survival signals in human cancer cells (Foster, 2006). Chen's lab have showed that mTOR bound to PA in a manner that was competitive with rapamycin complexed with the FKBP12 leading them to propose a model for the action of rapamycin whereby rapamycin/FKBP12 binds to mTOR and preventing interaction with PA (see Figure 1.3.4, adapted from Foster, 2007) (Chen and Fang, 2002). Consistent with the proposal that the rapamycin/FKBP12 complex acts competitively with PA, elevated PLD activity in human breast cancer cells increased the concentration of rapamycin required to suppress mTOR (Chen *et al*., 2003).

While the model for the action of rapamycin as a competition with PA for mTOR is consistent with available data, a role for PA and PLD in the regulation of mTOR remains controversial. Both PLD1 and PLD2 have been implicated in regulating mTOR. Exogenously expressed PLD2 was shown to increase S6 kinase phosphorylation in MCF7 cells (Chen *et al*., 2005), and elevated expression of PLD1 was reported to increase S6 kinase phosphorylation in rat fibroblasts (Hui *et al*., 2004). lysophosphatidic acid (LPA) -induced mTOR was

shown to be dependent on PLD1 and Rho (Kam and Exton, 2004). The activation of mTOR by Cdc42 was also dependent on PLD1 (Fang *et al.*, 2003). Interestingly, it was very recently reported that PLD2 forms a functional complex with mTOR and its binding partner Raptor through a TOS (TOR signaling) motif in PLD2 and that interaction with PLD2 was essential for mitogen stimulation of mTOR (Ha *et al.*, 2006).

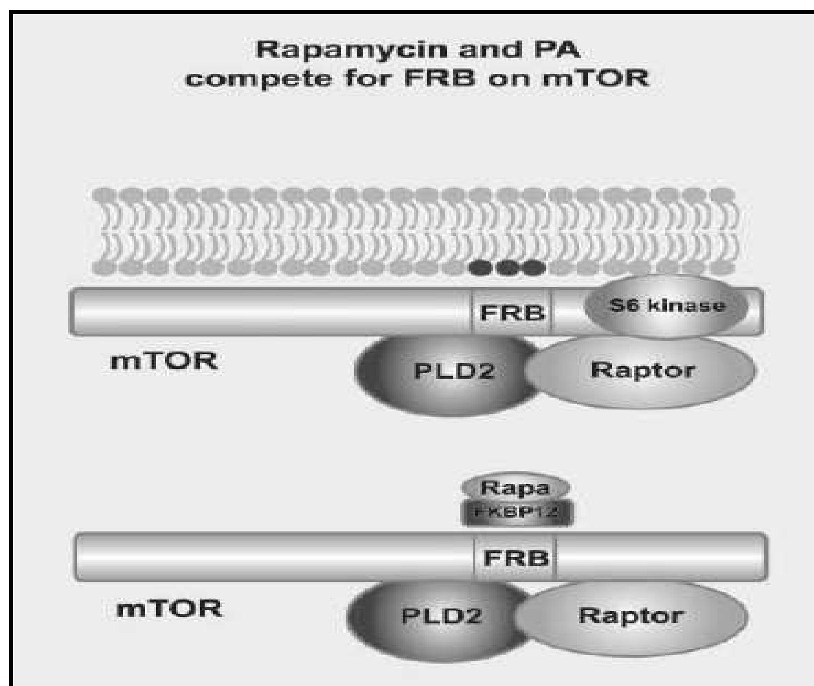

Figure 1.3.4: Regulation of mTOR by phosphatidic acid (PA) (Foster, 2007)
mTOR in complex with raptor and PLD2 associates with PLD2-generated PA through its FRB domain. In the presence of rapamycin (Rapa)/FKBP12 complex, the FRB domain of mTOR is unable to bind PA, and mTOR is unable to access and phosphorylate appropriate substrates, such as S6 kinase. Excess levels of PA on the membrane would provide more sites for binding mTOR and compete with rapamycin/FKBP12 complexes.

1.3.4 <u>mTOR REGULATION IN HUMAN CANCER</u>

The mTOR pathway is abnormally activated in many tumors. Multiple alterations, both upstream and downstream of mTOR, leading to pathway activation have been described indicating that this pathway has an essential role in maintaining a transformed phenotype (see figure 1.3.3).

In view of cancer pathology, elements of the PI3K/Akt pathway have been demonstrated to be activated by membrane receptors, including the HER (or ErbB) family of growth factor receptors in breast cancer, the insulin-like growth factor (IGF) receptor, and the estrogen receptor (ER) (Saunders *et al.*, 2001; Haynes and Boulay, 2006). Stimulation of the PI3K/Akt pathway can also occur through oncogenic Ras. Over-expression of IGF-I receptor and IGF-I commonly occurs in breast cancers and been associated with poor prognosis (Lee *et al.*, 1998). Elevated Akt1 and Akt2 kinase activity have been observed in various human tumors, including breast cancer (Haynes and Boulay, 2006). Rheb, the direct upstream activator of mTOR , has also been found to be overexpressed at the RNA level in many human tumors (Hynes and Boulay, 2006) and it has been shown that overexpression of Rheb increases the activity of the mTOR effector protein S6K1 (Castro *et al.*, 2003). Additionally, the S6K1 gene is amplified in approximately 9% of primary breast cancers (Wu *et al.*, 2000), and elevated levels of S6K1 RNA are found in almost 40% of the tumors (Hynes and Boulay, 2006). An upstream regulator of the PI3K/Akt pathway is the tumor suppressor gene PTEN which inhibits the activity of PI3K. As a result, loss of PTEN

suppressor gene function has been associated with Akt activation and been recorded in breast cancer. Thus, most of the positive regulators of mTOR activity have been found mutated and/or overexpressed in human breast cancer. The most common alteration found is activating mutations in the PI3K pathway (Saunders *et al*., 2001; Haynes and Boulay, 2006).

CHAPTER II

MATERIALS AND METHODS

2.1 CELLS, CELL CULTURE CONDITIONS AND TRANSFECTION

Cells, cell culture conditions and transfectionMCF-7, MDA-MB-231, HEK293, RCC4 and 786-O cells were obtained from the American Type Culture Collection. The 786-VHL and RCC4-VHL cells were generated as described previously (Stickle *et al.*, 2004). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Transfections were carried out using Lipofectamine LTX reagent (Invitrogen, Carlsbad, CA, USA) according to the vendor's instructions. Transfection efficiency was determined by transfection of pEGFP-C1 (Clontech, Mountain View, CA, USA), which expresses green fluorescent protein. The percentage of green cells was determined microscopically and was routinely in excess of 90%.

2.2 MATERIALS

CoCl2 and cyclohexamide were obtained from Sigma-Aldrich (St Louis, MO, USA). Antibodies to PARP (human specific), PLD, Pan-Akt, AKT1, AKT2, P-Akt (Ser473), S6K, P-S6K (Ser389), PDK1 and GLUT1 were obtained from Cell Signaling (Danvers, MA, USA). Antibodies to HIF1α were obtained from BD Biosciences (San Jose, CA, USA). Antibodies against HIF2α, actin, ubiquitin, glyceraldehyde-3-phosphate dehydrogenase (GADPH), Rictor, Raptor, mTOR, GLUT3, GLUT4 and hemagglutinin (HA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [3H]-myristic acid was obtained from PerkinElmer (Waltham, MA, USA). Precoated silica 60A thin-layer chromatography plates were from Whatman (Hillsboro, OR, USA). The

proteasome inhibitors (MG132, lactacystin, PSI and epoxomycin), the lysosome inhibitors (bafilomycin, chloroquine, concanamycin and NH4Cl) and the calpain inhibitors (ALLN, Z-L-L-CHO and ALLM), Rapamycin and FK506 were obtained from EMD Biosciences (San Diego, CA, USA). siRNAs targeting PLD1, PLD2 and GADPH were obtained from Ambion (Austin, TX, USA). siRNAs targeting AKT1, AKT2, mTOR, Rictor and Raptor where from Sigma Aldrich (St. Louis , MO, USA). Phosphatidic acid (1-palmitoyl 2-oleoyl) in chloroform was purchased from Avanti-Polaris Lipids (BIRMINGHAM, AL, USA). Insulin was obtained from Sigma Aldrich (St. Louis, MO, USA).

2.3 PLASMIDS

The pcDNA3.1 control plasmid was obtained from Invitrogen. The plasmid expression vector for catalytically inactive PLD1 and PLD2 mutants (pCGN-PLD1-K898R and pCGN-PLD2-K758R) (Sung *et al.*, 1997, 1999) were generous gifts from Dr Michael Frohman (SUNY, Stony Brook, NY, USA)..

2.4 CELL VIABALITY AND APOPTOSIS ASSAY

Cell viability was determined by Trypan blue exclusion (Sigma-Aldrich). After various treatments, cells were harvested, washed and treated with Trypan blue at a concentration of 0.4% w/v. After 10 min, Trypan blue uptake (dead cells) was determined by counting on a hemocytometer.

2.5 RNA ANALYSIS

Total RNA was purified from cells using the RNeasy RNA isolation Kit (Qiagen, Valencia, CA) using the manufacturer's protocol. RNA (2 mg) was reverse transcribed then amplified for 35 cycles using the Qiagen OneStep RT–PCR Kit according to the manufacturer's instructions. PCR was carried out with the following primers: VEGFa, HIF2α and b-tubulin (Qiagen). PCR products were visualized by electrophoresis using 2% agarose E-gels (Invitrogen).

2.6 WESTERN BLOT ANALYSIS

Samples were adjusted into gel-loading buffer (50 mM Tris-HCl [pH 6.8], 100 mM dithiothreitol, 2% sodium dodecyl sulfate, 0.1% bromophenol blue, 10% glycerol) and then heated for 5 min at 100℃ prior to separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After samples were transferred to nitrocellulose membranes (Osmonics), membrane filters were blocked with 5% nonfat dry milk in phosphate-buffered saline (PBS) with 0.05% Tween 20 and then incubated with the appropriate antibody diluted in 5% nonfat dry milk in PBS with 0.05% Tween 20. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase was used, and the bands were visualized by using an enhanced chemiluminescence detection system (Pierce).

2.7 RNA INTERFERENCE

Cells were plated on 12-well plates at 30% confluence in medium containing 10% serum without antibiotics. After 1 day, cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's directions. After 24 h the media was changed to fresh media containing 10% serum. After 3 days cells were lysed and analysed by western blot.

2.8 MEASUREMENT OF GLUCOSE UPTAKE

Cells were incubated in DMEM containing 0.5% fetal bovine serum in the presence of 200μM 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) (Molecular Probes) for 2 hours. 2-NDBG uptake by live cells was captured using a fluorescent inverted microscope and was quantified using a spectrofluorimeter by using 470 nm as the excitation wavelength and 545 nm as the emission wavelength. Basal fluorescence was subtracted from all measurements.

2.9 LACTATE AND pH MEASUREMENTS

24 hours before the assay, cells were counted and 5.0 × 105 cells were incubated in 3 ml of DMEM containing 0.5% fetal bovine serum. Media samples were collected and pH was measured immediately with a pH meter. Lactate concentration in the same media samples was determined using an EnzyChrom Lactate Assay colorimetric Kit (Bioassays Systems) according to manufacturer's instructions. Optical Density was measured with a spectrophotometer at 565 nm.

3.0 INTRACELLULAR ATP LEVEL MEASUREMENTS

Intracellular ATP levels were quantified using an ATPLite assay kit (Perkin Elmer) according to the manufacturer's instructions. Luminescence from cell lysate was measured with a microplate luminometer (Luminoskan Ascent, Thermo) and normalized to the protein concentration.

3.1 REACTIVE OXYGEN SPECIES H2OH MEASUREMENTS

Intracellular ROS production was measured by staining with dichlorodihydrofluorescein diacetate (H2DCFDA) (Molecular Probes). Cells were loaded with 5 μM H2DCFDA for 1 hr, washed in phosphate-buffered saline, and incubated with fresh media without H2DCFDA for 30 min. The oxidation product of H2DCFDA – 2',7'-dichlorofluorescein (DCF) fluorescence was visualized using inverted fluorescence microscope and quantified with a spectrofluorimeter by using 507 nm as the excitation wavelength and 530 nm as the emission wavelength. Basal fluorescence was subtracted from all measurements. Intracellular hydrogen peroxide levels were measured using a QuantiChrom Peroxide Assay colorimetric Kit (Bioassay Systems) according to the manufacturer's instructions. Optical Density was measured with a spectrophotometer at 585 nm and normalized to the protein concentration.

3.2 IMMUNOPRECIPITATION

Cells were grown in 10 cm diameter plates. Immediately before lysing, culture plates were rinsed once with cold PBS and lysed on ice for 20 min in 500 µl of ice-cold CHAPS IP buffer (40 mM HEPES [pH 7.5], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 0.5 mM orthovanadate, and EDTA-free protease inhibitors [Roche]) containing 0.3% CHAPS. 500 µg of protein was then incubated with appropriate antibodies and the immunoprecipitates were recovered 16 hrs later using protein G sepharose. The immunoprecipitates were then subjected to Western blot analysis along with 40 µg of total cell lysate.

3.3 PREPARATION OF PHOSPHATIDIC ACID

Right before use the appropriate amount of PA was dried under N_2 and resuspended by vortexing for 2 min in 150mM NaCl, 10mM Tris-Cl, pH 8.0. The resulting PA suspension was immediately added to the cell culture to a final concentration of 100 µM. Due to the short half-life of PA, this process was repeated every 60 minutes throughout the experiment.

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CHAPTER III

HIFα EXPRESSION IN VHL-DEFICIENT RENAL CANCER CELLS IS DEPENDENT ON PHOSPHOLIPASE D

3.1 INTRODUCTION

Loss of the von Hippel-Lindau (VHL) gene results in a variety of pathologies—most significantly renal cell carcinoma (Maynard and Ohh, 2004; Kaelin, 2007). The VHL gene product pVHL is part of an E3 ubiquitin ligase complex that targets the a subunits of hypoxia-inducible factor (HIFα) for degradation by the proteasome in the presence of Oxygen (Ohh, 2006). The level of HIFα is regulated by proline hydroxylation, which tags HIFα for recognition by pVHL when Oxygen is present (Kaelin, 2005). In the absence of pVHL, there is an upregulation of HIFα and this elevated expression of HIFα has been strongly implicated in VHL disease and renal cell carcinoma (Maynard and Ohh, 2004; Kaelin, 2007). HIFα dimerizes with HIFβ to form a transcription factor that stimulates the transcription of genes that regulate angiogenesis and other factors important for responding to hypoxic conditions such as vascular endothelial growth factor (VEGF) and glycolytic enzymes (Shaw, 2006). There are several different a subunits—two of which have been implicated in tumorigenesis. The expression of HIF1α and HIF2α is frequently elevated in a variety of cancers (Maynard and Ohh, 2004). HIF1α has been reported to both promote and suppress tumor progression, whereas HIF2α has been implicated only in tumor promotion (Gordan and Simon, 2007). The somewhat overlapping and antagonistic effects of HIF1α and HIF2α are poorly understood, but it is clear that in renal cell carcinoma, HIF2α is a critical factor—in that suppression of HIF2α blocks tumor formation by renal cancer cells (Kondo *et al* ., 2002, 2003). It is believed that the elevated expression of HIF2α contributes to the survival

signals in renal cancer cells that protect from apoptosis and facilitate angiogenesis.

It has become apparent in recent years that a critical hurdle in tumorigenesis is the generation of survival signals that suppress default apoptotic programs (Hanahan and Weinberg, 2000). Phospholipase D (PLD), which has been shown to provide survival signals in human cancer cells (Foster, 2006), has been reported to be elevated in many human cancers including renal cell carcinoma (Zhao *et al* ., 2000). Elevated PLD activity has been reported to contribute to the activation of Raf (Rizzo *et al* ., 1999), mitogen-activated protein (MAP) kinase (Shen *et al* ., 2001), mTOR (Fang *et al* ., 2001; Foster, 2007) and increase Myc expression (Rodrik *et al* ., 2005, 2006)—all of which have been implicated in survival signals in cancer cells. PLD has also been reported to suppress expression of p53 (Hui *et al* ., 2004) and the activity of protein phosphatase 2A (Hui *et al* ., 2005)—both of which have been implicated in tumor suppression. Thus, PLD activity has been implicated in many prosurvival and proliferation signals that have been implicated in many human cancers.

We report here that expression of HIF2α, at the level of translation, in VHL-deficient renal cancer cell lines is dependent on PLD activity. This study provides evidence of a VHL-independent mechanism for HIF2α expression and suggests that targeting PLD signals may represent a novel strategy for treating renal and other cancers where survival is dependent on both PLD and HIF2α.

3.2 RESULTS

3.2.1 PLD ACTIVITY IS ELEVATED IN KIDNEY CANCER CELL LINES

Since elevated PLD activity has been reported in human kidney cancer (Zhao *et al* ., 2000), we examined the level of PLD activity in the well-characterized renal cell carcinoma cell lines 786-O and RCC4. We previously reported that there are highly elevated levels of PLD activity in the MDA-MB-231 human breast cancer cell line relative to MCF-7human breast cancer cells (Zhong *et al* ., 2003; Chen *et al* ., 2005). The level of PLD activity in the 786-O cells was compared with the level of PLD activity in MDA-MB-231 and MCF-7cells. We also examined the PLD activity in the immortalized human embryonic kidney cell line HEK293. As shown in Figure 3.2.1a, the level of PLD activity in both the 786-O and RCC4 cells was comparable to the level of PLD activity in the MDA-MB-231 cells and substantially higher than the PLD activity in the MCF-7and HEK293 cells. These data demonstrate that consistent with the report indicating elevated PLD activity in kidney tumors (Zhao *et al* ., 2000), both the 786-O and RCC4 kidney cancer cell lines also have high levels of PLD activity.

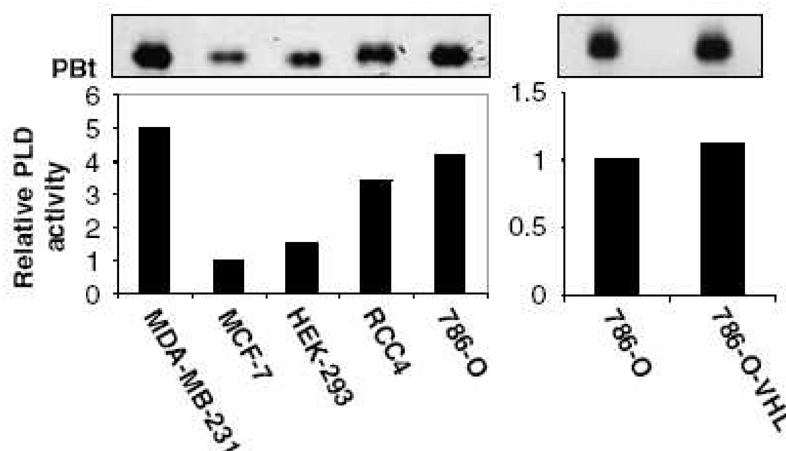

Figure 3.2.1: Elevated Phospholipase D (PLD) activity in renal cancer is due to increased catalytic activity. (a) The relative PLD activity in three human kidney-derived cell lines (HEK293, RCC4 and 786-O) was compared with the PLD activity in two breast cancer cell lines that we have previously characterized (MDA-MB-231 and MCF-7). The PLD activity in 786-O cells with restored pVHL expression was compared to the PLD activity in the parental 786-O cells (right). Cells were put into media containing 0.5% serum overnight prior to performing the assay. PLD activity was determined using the transphosphatidylation reaction and the levels of phosphatidylbutanol (PBt) were determined using thin layer chromatography as described in 'Materials and methods'. The PBt band from a thin-layer chromatograph is shown in the inset. The relative levels of PLD activity were obtained by normalizing to the level of PLD activity in the MCF-7cells, which was given a value of 1. The experiment shown is representative of at least three independent experiments.

We next examined the expression levels of PLD1 and PLD2 protein in the 786-O and RCC4 renal cancer cells relative to the levels observed in HEK293, MDA-MB-231 and MCF-7cells using western blot analysis. As shown in Figure 3.2.1b, there were relatively high levels of PLD1 expression in all three kidney-derived cell lines relative to the breast cancer cell lines. There also appeared to be lower levels of PLD2 expression in the kidney cell lines relative to the breast cancer cell lines. Importantly, there were no significant increases in the

expression in PLD1 or PLD2 in the 786-O and RCC4 cells relative to the HEK293 cells that could explain the increased activity. We also examined the level of PLD activity and protein in 786-O cells where expression of pVHL had been restored (Lonergan *et al* ., 1998; Ohh *et al* ., 1998). As shown in Figure 3.2.1a and Figure 3.2.1b, restoring pVHL expression did not have significant effect upon either the activity expression levels of PLD isoforms. Thus, the increased PLD activity in the 786-O cells was not likely due to the loss of VHL expression.

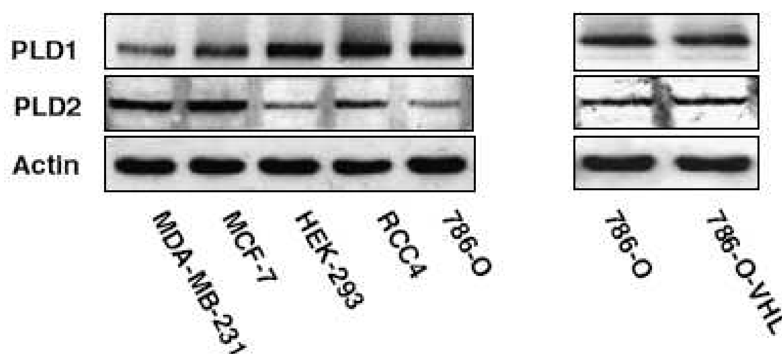

Figure 3.2.1: (Continued). (b) The relative levels of PLD1 and PLD2 protein in the cells evaluated in (a) were determined by western blot. The data shown are representative of an experiment repeated two times.

3.2.2 PLD ACTIVITY IS ELEVATED IN KIDNEY CANCER CELL LINES

We reported previously that PLD activity is critical for the survival of breast cancer cells when subjected to the stress of serum withdrawal (Chen *et al* ., 2005). We therefore examined the effect-suppressing PLD-mediated production of phosphatidic acid (PA) on 786-O cells deprived of serum. To suppress PA production, we treated 786-O cells with the primary alcohol 1-butanol (1-BtOH), which results in the formation of phosphatidylbutanol instead of PA by PLD (Shen

et al ., 2001). As shown in Figure 3.2.2a, in the absence of serum, 1-BtOH caused a loss in cell viability and an increase in cleavage of the caspase-3 substrate poly-(ADP-ribose) polymerase (PARP), indicating that the cells were undergoing apoptosis. PARP cleavage was not observed with the tertiary alcohol t-BtOH, which is not a substrate for PLD (Shen *et al* ., 2001).

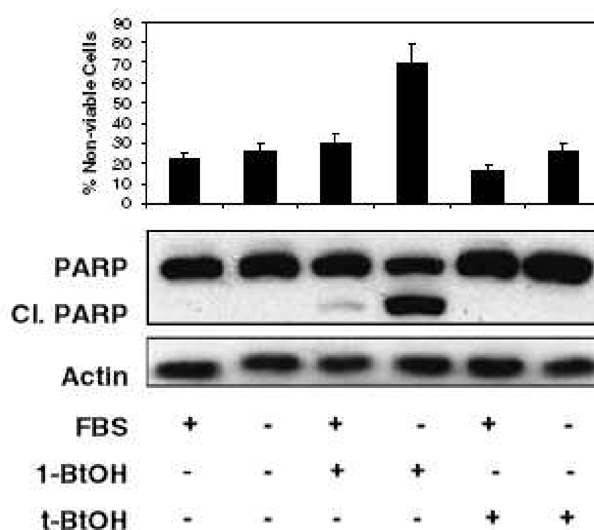

Figure 3.2.2: Phospholipase D (PLD) generates a survival signal in the absence of serum in 786-O cells. (a) Top: 786-O cells were treated with either 1-BtOH or t-BtOH (0.8%) for 4 h in the presence or absence of 10% fetal bovine serum (FBS) as indicated. Cell viability (upper graph) and poly-(ADP-ribose) polymerase (PARP) cleavage (lower blot, Cl PARP) were then determined as described in ‘Materials and methods’. The western blot for PARP was examined for loading by re-probing with an antibody raised against actin. Error bars represent the standard deviation for two independent experiments.

To further establish that the effect observed with 1-BtOH was due to suppressing the production of PLD induced PA, we examined the effect of reducing the expression of PLD1 and PLD2 in 786-O cells with siRNA. The 786-O cells were transfected with siRNA for PLD1 and PLD2 individually and

together. PARP cleavage was then determined in the presence and absence of serum. As shown in Figure 3.2.2b, the PLD1 and PLD2 siRNAs were effective in reducing the levels of PLD1 and PLD2 protein. Also shown is that suppression of either PLD1- or PLD2-enhanced PARP cleavage in the absence of serum, but not in the presence of serum (Figure 3.2.2b). Using both PLD1 and PLD2 siRNAs together increased PARP cleavage. These data indicate that PLD activity is providing a survival signal in the 786-O cells that suppresses apoptosis under conditions of serum withdrawal. Similar data were obtained with RCC4 renal cancer cells (data not shown)—indicating that the elevated PLD activity in these cells also provides a survival signal in these cells as well. The data presented also suggest that both PLD1 and PLD2 are contributing to the survival of 786-O cells under conditions of serum withdrawal.

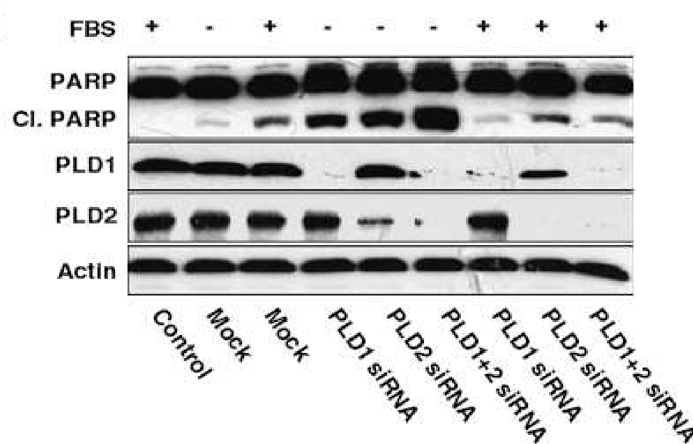

Figure 3.2.2: (Continued). (b) 786-O cells were transfected with the indicated siRNAs as described in 'Materials and methods'. Mock transfections were carried out using nonspecific scrambled siRNA. After 72 h of transfection cells were placed in DMEM containing either 0 or 10% FBS as indicated and PARP cleavage was then determined 16 h later. The levels of PLD1 and PLD2 were examined by western blot analysis. The data shown are representative of two independent experiments.

3.2.3 <u>HIF2α EXPRESSION IN RENAL CANCER CELLS IS DEPENDENT ON PLD</u>

A consistent genetic defect in kidney cancer is the loss of the VHL gene, which results in increased levels of HIF2α (Maynard and Ohh, 2004). HIF2α is a transcription factor that stimulates the expression of genes that contribute to angiogenesis, proliferation and survival (Maynard and Ohh, 2004; Hickey and Simon, 2006). PLD has also been implicated in cell proliferation and survival (Foster and Xu, 2003; Foster, 2006) and the induction of HIF1α in MDA-MB-231 cells (Zheng *et al* ., 2006). Similarly, HIF2α has been shown to be critical for tumor formation by 786- O cells (Kondo *et al* ., 2002, 2003). Since PLD activity was critical for the survival of 786-O cells, we examined whether elevated HIF2α expression in the pVHL-deficient 786-O cells was dependent on PLD activity. As shown in Figure 3.2.3a, 1-BtOH, but not t-BtOH, suppressed HIF2α expression in 786-O cells. We also examined the induction of HIF2α by hypoxia-mimetic conditions generated by CoCl2 in 786-O cells where the VHL gene had been reintroduced (Lonergan *et al* ., 1998; Ohh *et al* ., 1998). As expected, restoration of pVHL expression eliminated HIF2α stabilization under normoxic conditions (Figure 3.2.3b). However, when cells were treated with CoCl2, HIF2α accumulation was observed, and importantly, the induction of HIF2α expression by CoCl2 was prevented by 1-BtOH, but not by t-BtOH.

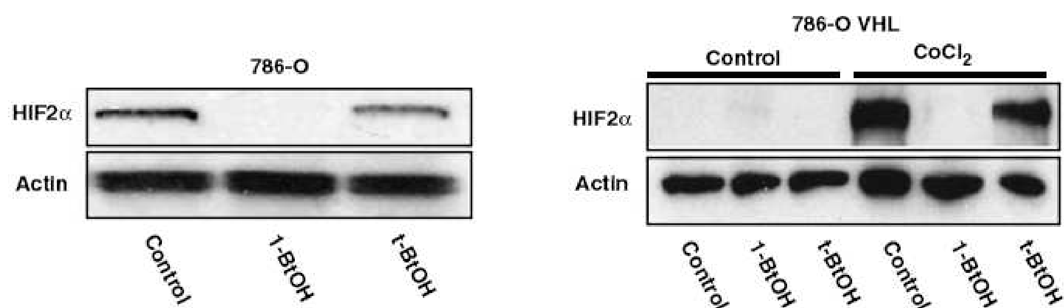

Figure 3.2.3: Active Phospholipase D (PLD) is required for HIF2α **accumulation in 786-O cells.** (a) 786-O cells were plated and shifted to media without serum for 16 h. The cells were then treated with either 1-butanol (1-BtOH) or t-BtOH (0.8%) for 2 h and HIF2α levels were determined 2 h later by western blot analysis. (b) 786-von Hippel-Lindau (VHL) cells prepared as in (a) were examined for HIF2α expression in the presence and absence of CoCl2 (150 mM) which was used to mimic the lack of oxygen.

We also examined the effect of PLD1 and PLD2 siRNA on HIF2α expression, and as shown in Figure 3.2.3c, both PLD1 and PLD2 siRNA suppressed HIF2α in 786-O cells, and PLD1 and PLD2 siRNA together completely abolished HIF2α expression. The effect of the PLD siRNAs on PLD activity is also shown in Figure 3.2.3c, where it can be seen that the effect on PLD activity correlates with the effect on HIF2α expression. Similar data were obtained using catalytically inactive dominant-negative mutants for PLD1 and PLD2 (data not shown). These data reveal that HIF2α expression is dependent on PLD activity in the VHL-deficient kidney cancer cell line 786-O. The data also demonstrate that when the VHL gene is restored and HIF2α is induced by hypoxic mimetic conditions, there is still a dependence on PLD activity for HIF2α expression.

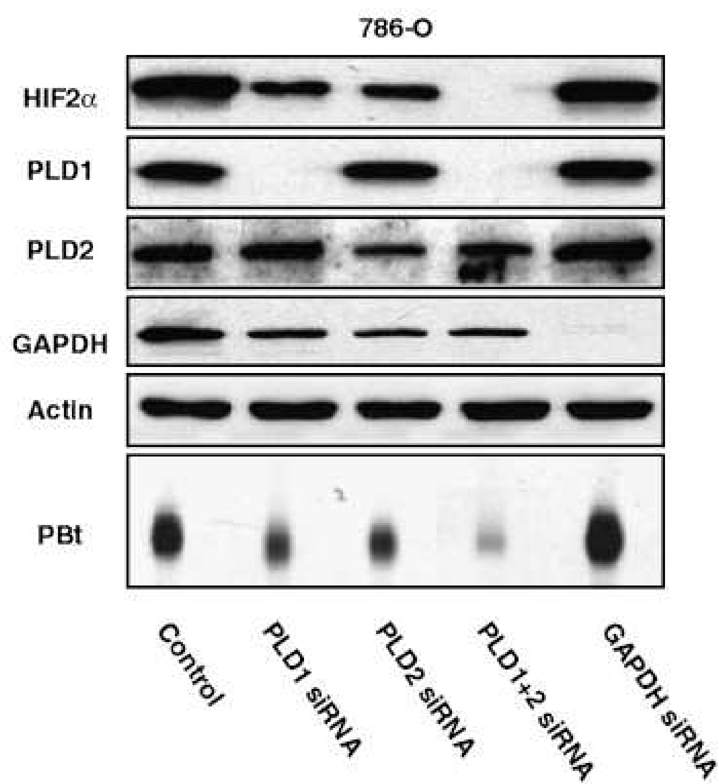

Figure 3.2.3: (Continued). (c) 786-O cells were transfected with PLD1 and PLD2 siRNAs and HIF2α levels were determined 72 h later by western blot analysis. PLD1 and PLD2 levels were also evaluated by western blot. The PLD activity as determined by the generation of phosphatidylbutanol (PBt) in cells transfected with PLD1 and PLD2 siRNAs was evaluated as described in material and methods. Experiments shown are representative of experiments repeated at least two times.

3.2.4 <u>THE DEPENDENCE OF HIF2α ON PLD IS AT THE LEVEL OF TRANSLATION</u>

The data in Figure 3.2.4 indicate that the effect of PLD on HIF2α expression is not due to increased transcription.

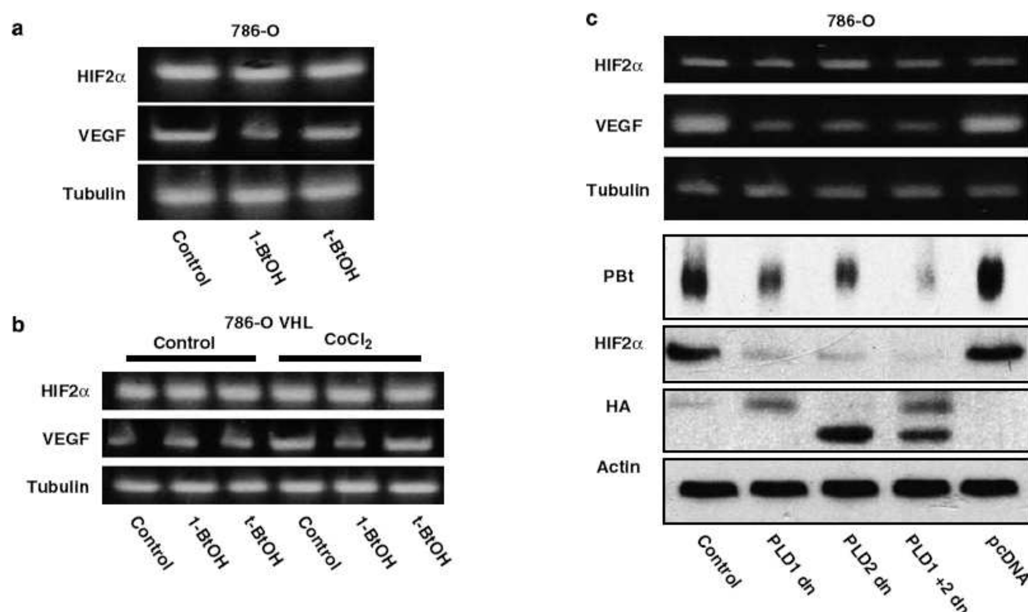

Figure 3.2.4: Suppression of phospholipase D (PLD) activity has no effect on HIF2α **transcription.** (a) 786-O cells were plated and shifted to media without serum for 16 h. The cells were then treated with either 1-butanol (1-BtOH) or t-BtOH (0.8%) for 2 h and HIF2α and vascular endothelial growth factor-a (VEGFa) RNA levels were determined by reverse transcription (RT)–PCR as described in 'Materials and Methods'. (b) 786-von Hippel-Lindau (VHL) cells prepared as in (a) were examined for HIF2α and VEGFa RNA in the presence and absence of CoCl2 (150 mM) as indicated as in (a). (c) 786-O cells were transfected with dominant-negative (dn) mutants of PLD1 and PLD2 as indicated. HIF2α and VEGFa RNA levels were determined 24 h later as in (a). PLD activity levels were determined by the transphosphatidylation reaction as described in 'Materials and Methods'. The PLD mutants were tagged with hemagglutinin (HA; Colley *et al* ., 1997) and the expression levels of the PLD mutants were determined by western blot using an anti-HA antibody. The levels of HIF2α protein and actin were also determined by western blot. Experiments shown are representative of experiments repeated at least two times.

We next investigated whether PLD affected the stability and half-life of HIF2α. To accomplish this, we examined whether the suppression of PLD activity could be reversed by a variety of protease inhibitors. We also investigated the effect of suppressing of PLD activity on the half-life of HIF2α. These data revealed that the dependence of HIF2α expression on PLD activity was not due to changes in the stability of HIF2α (Figures 3.2.5 and 3.2.6).

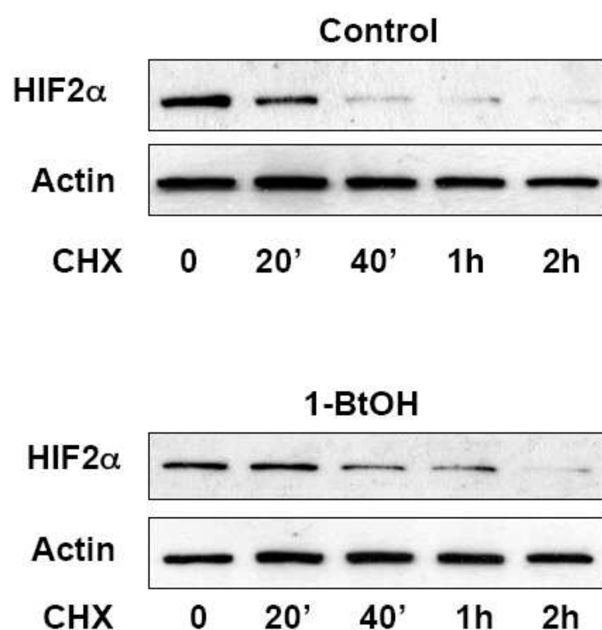

Figure 3.2.5: PLD does not affect the half-life of HIF2α. (a) 786-O cells were plated and shifted to media without serum for 16 hr. The cells were then treated with the inhibitor of translation cyclohexamide (CHX) (100 μM) for the indicated times and HIF2α levels were determined at the indicated times by Western blot analysis. (b) 786-O cells were plated and shifted to media without serum for 16 hr. The cells were then treated with 1-BtOH (0.8%) for the indicated times and HIF2α levels were determined at the indicated times by Western blot analysis. The data shown is representative of two independent experiments.

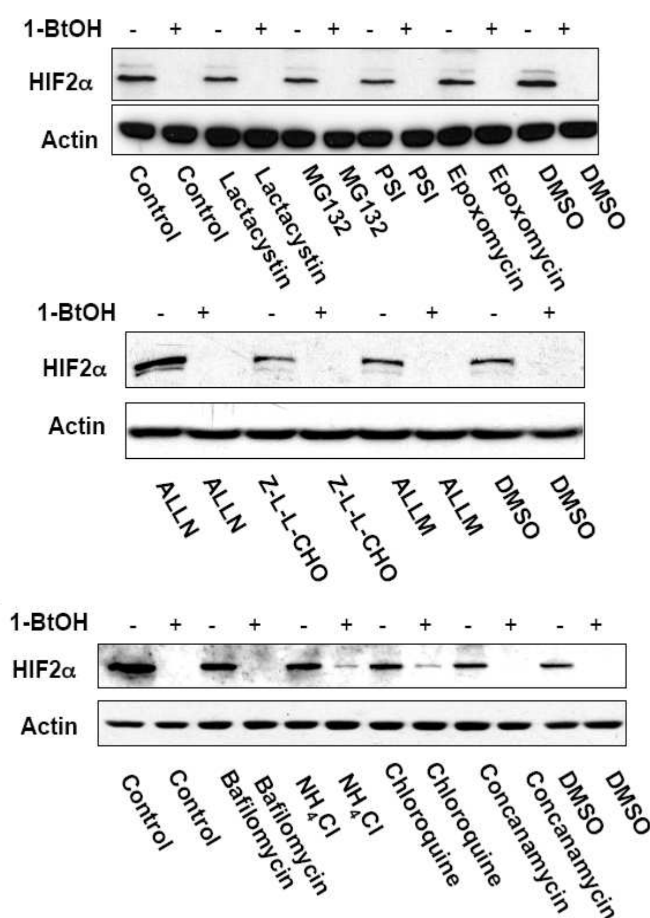

Figure 3.2.6: The effect of PLD on HIF2α expression is not due to stabilization. (a) 786-O cells were plated and shifted to media without serum for 16 hr in the presence of the proteasome inhibitors lactacystin (10 μM), MG132 (10 μM), PSI (5 μM), epoxomycin (2 μM) or DMSO vehicle control. The cells were then treated with 1-BtOH (0.8%) for 2 hr and HIF2α levels were then determined by Western blot analysis. (b) 786-O cells were plated and shifted to media without serum for 16 hr in the presence of the calpain inhibitors ALLN (50 μM), Z-L-L-CHO (40 μM), ALLM (100 nM), or DMSO. The cells were then treated with 1-BtOH (0.8%) for 2 hr and HIF2α levels were determined 2 hr later by Western blot analysis. (c) 786-O cells were plated and shifted to media without serum for 16 hr in the presence of the lysosome inhibitors bafilomycin (2 μM), NH4Cl (10 mM), chloroquine (25 μM), concanamycin (1 μM) or DMSO. The cells were then treated with 1-BtOH (0.8%) for 2 hr and HIF2α levels were determined 2 hr later by Western blot analysis. Experiments shown are representative of experiments repeated at least two times.

We next examined whether the effect of PLD on HIF2α was at the level of translation. To determine whether PLD impacted on HIF2α translation, we used the 786-O cells with restored expression of pVHL. In these cells HIF2α expression is not detected because, in the presence of Oxygen, HIF2α is ubiquitinated and degraded by the proteasome (Ohh, 2006). If these cells are treated with the proteasome inhibitor MG132, there is an increase in HIF2α that is blocked by cyclohexamide, which inhibits protein synthesis (Figure 3.2.7). Thus, the increase in HIF2α observed in the presence of the proteasome inhibitor is due to translation. We then examined the effect of 1-BtOH on the translation of HIF2α and as shown in Figure 3.2.7, 1-BtOH, like cyclohexamide, completely suppressed HIF2α expression. These data indicate that the effect of PLD in HIF2α expression is at the level of translation.

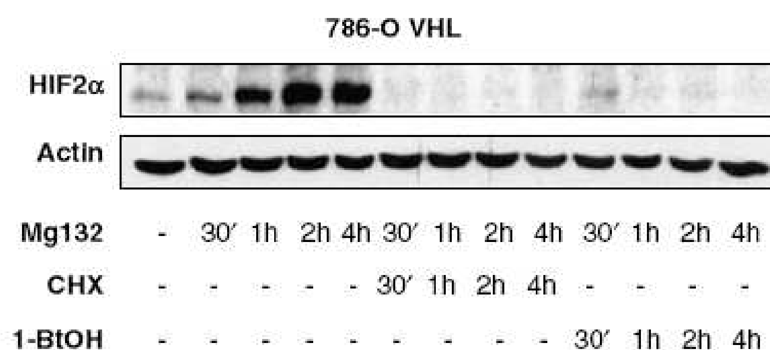

Figure 3.2.7: Inhibition of PLD prevents HIF2α **protein translation.** 786-von Hippel-Lindau (VHL) cells were plated and shifted to media without serum for 16 h. The cells were then treated for the indicated times with the proteasome inhibitor MG132 (100 mM) alone or concomitant with the translation inhibitor cyclohexamide (CHX) (100 mM) or 1-butanol (1-BtOH, 0.8%) as indicated. HIF2α levels were determined at the indicated times by western blot analysis. The data shown are representative of two independent experiments.

3.2.5 BOTH HIF1α AND HIF2α EXPRESSION IN RCC4 CELLS IS DEPENDENT ON PLD ACTIVITY

As indicated in Figure 3.2.1, RCC4 kidney cancer cells also have elevated PLD activity. Whereas the 786-O cells have elevated expression of HIF2α, the RCC4 cells have elevated expression of both HIF2α and HIF1α (Figure 3.2.8a). We reported previously that elevated expression of HIF1α in MDA-MB-231 cells was dependent on PLD activity (Zheng *et al* ., 2006). We, therefore, examined whether the expression of HIF1α and HIF2α in RCC4 cells was dependent on PLD activity. As shown in Figure 3.2.8b, 1-BtOH suppressed both HIF1α and HIF2α in the RCC4 cells. We also examined the induction of HIF1α by hypoxia-mimetic conditions generated by CoCl2 in RCC4 cells where the VHL gene had been reintroduced (Lonergan *et al* ., 1998; Ohh *et al* ., 1998). As shown in Figure 3.2.8c, restoration of pVHL expression eliminated HIF1α expression. However, when cells were treated with CoCl2, HIF2α expression was restored and importantly, the induction of HIF1α expression by hypoxia-mimetic CoCl2 was also prevented by 1-BtOH, but not by t-BtOH. These data indicate that both HIF1α and HIF2α are dependent on PLD activity. We next examined whether the impact of PLD on HIF1α, like HIF2α, is at the level of translation. RCC4 cells with reintroduced pVHL expression were treated with MG132 to increase expression of HIF1α and then treated with either cyclohexamide or 1-BtOH as in Figure 3.2.7. Both cyclohexamide and 1-BtOH prevented the increase in HIF1α expression induced by suppression of the proteasome with MG132 (Figure

3.2.8d). Thus, like HIF2α, the effect of PLD on HIF1α expression is at the level of translation.

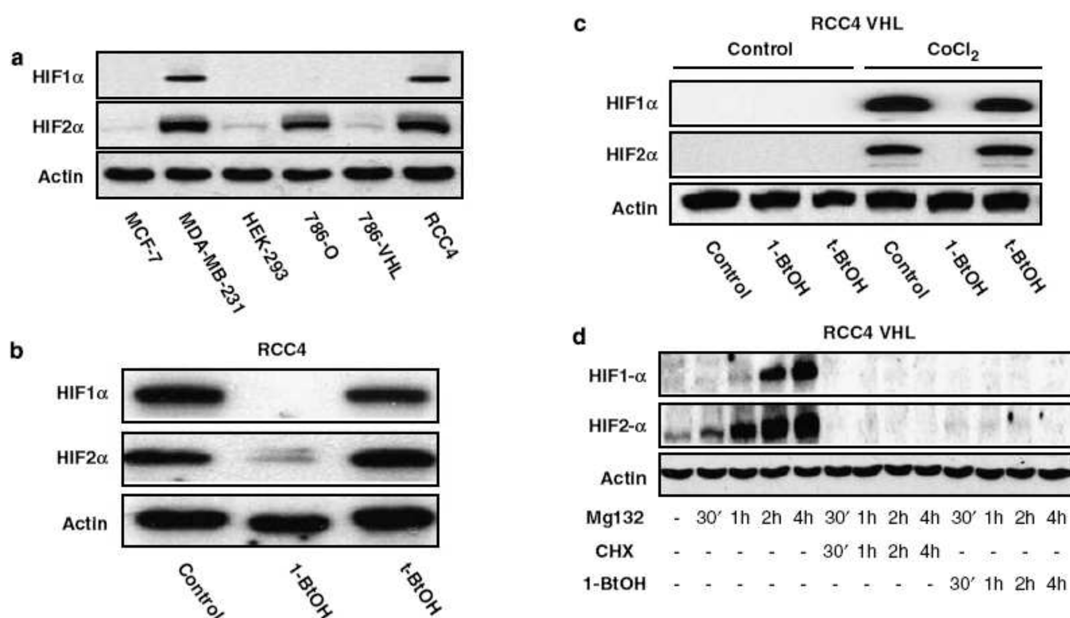

Figure 3.2.8: PLD is required for HIF1α **accumulation in RCC4 cells.** (a) The relative levels of HIF1α and HIF2α were compared with three human kidney-derived cell lines (HEK293, RCC4, 786-O and 786-VHL) and two breast cancer cell lines (MDA-MB-231 and MCF-7) by western blot. (b) RCC4 cells were plated and shifted to media without serum for 16 h. The cells were then treated with either 1-butanol (1-BtOH) where indicated and HIF1α levels were determined 2 h later by western blot analysis. (c) RCC4-
VHL cells were prepared as in (b) in the presence and absence of CoCl2 (150 mM), which was used to mimic the lack of oxygen. The cells were then treated with either 1-BtOH or t-BtOH (0.8%) as indicated and HIF1α levels were determined 2 h later by western blot analysis. (d) RCC4-VHL cells were plated and shifted to media without serum for 16 h. The cells were then treated for the indicated times with the proteasome inhibitor MG132 (100 mM) alone or concomitant with the translation inhibitor cyclohexamide (CHX, 100 mM) or 1-BtOH (0.8%). HIF1α and HIF2α levels were determined at the indicated times by western blot analysis. Experiments shown are representative of experiments repeated at least two times.

3.3 DISCUSSION

HIFα expression is elevated as a response to stressful conditions that warrant enhanced angiogenesis and blood supply (Hickey and Simon, 2006). In the presence of Oxygen, HIFα is hydroxylated and targeted by pVHL for ubiquitination and subsequent degradation by the proteasome (Kaelin, 2005; Ohh, 2006). In the absence of pVHL, HIFα is stabilized under normoxic conditions and stimulates the expression of several genes implicated in the survival of cancer cells and tumorigenesis (Maynard and Ohh, 2004; Gordan and Simon, 2007; Kaelin, 2007). We recently reported that in response to the stress of serum withdrawal in culture, there is a substantial increase in PLD activity in several cancer cell lines (Zheng *et al* ., 2006). Thus, elevated PLD activity also represents a stress response that some cancer cells have adapted to promote survival in the absence of serum growth factors. Upon serum withdrawal, the survival of the renal cancer cells used in this study was also dependent on PLD. Stable expression of HIF2α is a critical aspect of tumorigenesis in renal cell carcinoma

(Maxwell, 2005; Kaelin, 2007). The evidence provided here reveal that the expression of both HIF1α and HIF2α is dependent on the elevated levels of PLD activity in two renal cancer cells lacking pVHL. The PLD requirement was at the level of translation. Upon restoration of pVHL expression, hypoxic mimetic induction of HIFα was also dependent on PLD activity. Both PLD1 and PLD2 seem to be involved in that suppression of both PLD1 and PLD2 reduced HIF2α

expression. We have proposed previously that the activation PLD1 leads to increased PLD2 activity (Foster and Xu, 2003; Foster, 2006), and we have shown that both PLD1 and PLD2 are required for receptor endocytosis and MAP kinase activation (Shen *et al* ., 2001). This study reveals that in addition to being regulated at the level of turnover through pVHL, HIFα is also regulated at the level of translation by signals mediated by PLD. Regulation of HIFα at the level of translation was reported recently for suppression of HIFα in response to the promyelocytic leukemia tumor suppressor (Bernardi *et al* ., 2006). This report, along with data provided here, suggest multiple forms of stress impact upon HIFα expression in renal cancer cells. An emerging theme in tumorigenesis is that cancer cells need to continue proliferate under stressful conditions— such as the hypoxia that occurs early in a solid tumor prior to vascularization (Gatenby and Gillies, 2004). The expression of HIFα stimulates the expression of genes that promote angiogenesis, glycolysis and anaerobic respiration (Semenza, 2001; Hickey and Simon, 2006). Thus, elevated HIFα expression in a solid tumor would allow the cells to function better in the absence of Oxygen and enhance vascularization so that ultimately Oxygen and other nutrients can become available.

The lack of serum growth factors also represents a challenge for cells in an emerging solid tumor prior to vascularization. We have found that in several human cancer cells PLD activity is elevated as a stress response to the removal of serum (Zheng *et al* ., 2006) and that the elevated PLD activity suppresses apoptosis under these conditions (Chen *et al* ., 2005). The renal cancer cells

used in this study had elevated PLD activity in both the presence and absence of serum (data not shown). Thus, unlike some other cancer cells, the elevated PLD activity is constitutive. A possible explanation for this is that HIFα expression is needed constitutively in renal cell carcinoma and therefore a constitutive elevation of PLD activity is needed to maintain the translation of HIFα. Kaelin and colleagues have reported that suppressing HIF2α is sufficient to prevent tumorigenesis (Kondo *et al* ., 2002, 2003). It is currently not practical to suppress the expression of a protein as a therapeutic strategy in most cancers. It is also not practical to reintroduce a lost protein such as VHL back into a cancer cell. The finding here that PLD is required for the expression of both HIF1α and HIF2α suggests that targeting PLD represents a promising therapeutic strategy in cancers such as renal cancer where there is both a dependence upon HIFα for tumorigenesis and a reported high incidence of elevated PLD activity (Zhao *et al* ., 2000). While there are no drugs available that target PLD specifically, there is potential for targeting the signals that activate PLD or the downstream targets of PLD-generated PA. The data in Figure 1 indicate that the elevated PLD activity in the cancer cell lines used in this study was not due to elevated expression of either PLD1 or PLD2. Thus, there should be elevated signals that lead to increased PLD activity that could be targeted. There are also several downstream targets of PLD that might be similarly targeted. The potential for targeting PLD in cancer cells was recently reviewed (Foster, 2006). It will be critical to identify the critical upstream effector signals that activate PLD and the downstream targets of PLD that lead to the expression of HIFα. An especially

attractive target of PLD signaling is mTOR—the mammalian target of rapamycin, since rapamycin is a highly specific drug and mTOR has been implicated in the translation of proteins implicated in cell survival (Sawyers, 2003; Foster, 2004) and is activated by PA (Fang *et al* ., 2001; Chen *et al* ., 2003; Foster, 2007). Preliminary studies indicate that it may be complicated in that rapamycin suppresses HIF1α very efficiently, while having little effect on HIF2α expression (our unpublished results). Intriguingly, HIF2α seems to be dependent on the rapamycin-insensitive mTORC2. While the signals mediated by PLD required for HIFα expression need to be further elaborated, data presented here make it clear that targeting PLD-mediated signals could be a viable strategy for treating cancers that are dependent on HIFα.

CHAPTER IV

DIFFERENTIAL DEPENDENCE OF HIF1α AND HIF2α ON mTORC1 and mTORC2

4.1 INTRODUCTION

Hypoxia-inducible factor (HIF) is a critical transcriptional regulator of cellular responses to a variety of stressful conditions (Kelin, 2005; Shaw, 2006). Under non-stressful conditions HIFα is ubiquitinated by the von Hippel Lindau (VHL) gene product pVHL, a substrate conferring component of an E3 ubiquitin ligase that targets HIFα for degradation by the proteasome (Ohh, 2006). Loss of the VHL gene results in a variety of pathologies – most significantly renal cell carcinoma (RCC) (Maynard and Ohh, 2004; Maxwell, 2005; Kaelin, 2007). In the absence of pVHL, there is an up-regulation of HIFα, and elevated expression of HIFα has been strongly implicated in VHL disease and RCC (Maynard and Ohh, 2004; Maxwell, 2005; Kaelin, 2007). HIFα dimerizes with HIFβ to form a transcription factor HIF that stimulates the transcription of genes that regulate angiogenesis and other factors important for responding to hypoxic and other stressful conditions such as vascular endothelial growth factor and glycolytic enzymes (Semenza, 2001; Shaw, 2005; Hickey and Simon, 2006). There are several distinct α subunits, but it is the expression of HIF1α and HIF2α that are most frequently elevated in human cancers (Maynard and Ohh, 2004; 2007). Whereas HIF1α has both pro- and anti-proliferative properties, HIF2α lacks the anti-proliferative properties and is more strongly implicated in tumorigenesis (Gordan and Simon, 2007). The somewhat overlapping and antagonistic effects of HIF1α and HIF2α are poorly understood, but it is clear that in renal cell carcinoma, HIF2α is a critical factor – in that suppression of HIF2α blocks tumor formation by renal cancer cells (Kondo *et al* ., 2002; 2003). It is believed that the

elevated expression of HIF2α contributes to the survival signals in renal cancer cells that protects from apoptosis and facilitates angiogenesis (Gordan and Simon, 2007).

A common node for survival signals in cancer cells is mTOR, the mammalian target of rapamycin (Sawyers, 2003; Foster, 2004; Guertin and Sabatini, 2007). mTOR exists in two distinct complexes, mTORC1 and mTORC2 (Sabatini, 2006), that differ in their subunit composition and sensitivity to rapamycin. mTORC1 consists of a complex that includes mTOR and a protein known as Raptor (regulatory associated protein of mTOR), whereas mTORC2 consists of a complex that includes mTOR and a protein known as Rictor (rapamycin-insensitive companion of mTOR) (Guertin and Sabatini, 2007). mTORC1 is highly sensitive to rapamycin, whereas mTORC2 is relatively insensitive to rapamycin (Sabatini, 2006). While, there have been several reports that HIF1α is sensitive to rapamycin (Land and Tee, 2007; Bernardi *et al.*, 2006; Hudson *et al.*, 2002; Thomas *et al.*, 2006) indicating a dependence on mTORC1, HIF2α expression has been reported to be insensitive to rapamycin in VHL-deficient RCC cells (Bhatt *et al.,* 2008). We recently reported that elevated expression of both HIF1α and HIF2α in VHL-deficient RCC cell lines was dependent on Phospholipase D (PLD) (Toschi *et al* ., 2008). PLD, like HIFα, has been implicated in stress responses (Zheng *et al.*, 2006), and like mTOR, has been shown to provide a survival signal in several human cancer cell lines (Chen *et al.,* 2005; Zheng *et al.*, 2006; Shi *et al.*, 2007; Gadir *et al.*, 2007; 2008; Toschi *et al.*, 2008). Importantly, the PLD metabolite phosphatidic acid (PA) interacts

with mTOR in a manner that is competitive with rapamycin in association with the FK506 binding protein-12 (FKBP12) (Fang *et al.*, 2001; Chen *et al.*, 2003) and was recently reported that PLD1 is a downstream target of Rheb, a GTPase that activates mTORC1 (Sun *et al.,* 2008). Consistent with reports that suppression of HIF2α blocks tumor formation by renal cancer cells (Kondo *et al* ., 2002; 2003), suppression of PLD activity in RCC cell lines led to apoptosis when the cells were deprived of serum (Toschi *et al* ., 2008). Thus, PLD, like HIF2α, was able to provide a “survival signal” that suppressed apoptosis in the RCC cells.

We report here that HIF2α expression is dependent on mTORC2, whereas HIF1α is dependent upon both mTORC1 and mTORC2. Since HIF2α expression is critical for RCC tumorigenesis, this study implicates an mTORC2 signaling pathway that could be targeted in RCC and perhaps other cancers where HIF2α is critical for tumorigenesis.

4.2 <u>RESULTS</u>

4.2.1 <u>DIFFERENTIAL EFFECT OF RAPAMYCIN ON HIF1α AND HIF2α</u>

While several labs have reported that expression of HIF1α is sensitive to rapamycin (Land and Tee, 2007; Bernardi *et al* ., 2006; Hudson *et al* , 2002), the expression of HIF2α in VHL-null RCC cells was reported to insensitive to rapamycin (Bhatt *et al.,* 2008). However, both HIF1α and HIF2α are dependent upon PLD activity in VHL-null RCC cells (Toschi *et al* ., 2008). Since PLD activity has been widely implicated in the activation of mTOR (Foster, 2007), we investigated the effect of rapamycin, on the expression of HIF2α in VHL-null

786-O RCC cells, which have elevated expression of HIF2α, and in VHL-null RCC4 cells, which express both HIF1α and HIF2α. Both 786-O cells have highly elevated levels of PLD activity (Toschi *et al.,* 2008) which confers rapamycin resistance (Chen *et al.,* 2003) due to the competition of between rapamycin and phosphatidic acid for mTOR (Fang *et al.,* 2001). Consistent with these reports, 786-O cells are relatively resistant to rapamycin (Sorbassov *et al.,* 2006). We therefore used a hi dose (20μM) 24 hr treatment with rapamycin and examined HIFα expression. As shown in Figure 4.2.1 A, rapamycin partially suppressed the expression of HIF2α in the 786-O and RCC4 cells, but completely suppressed the expression of HIF1α in the RCC4 cells. Phosphorylation of the mTORC1 substrate S6 kinase was also evaluated to ensure that rapamycin was able to suppress mTORC1. We performed a shorter term kinetic analysis of the effect of rapamycin on HIF1α and HIF2α in the RCC4 cells where both HIF1α and HIF2α are expressed, and as shown in Figure 4.2.1 B, suppression of HIF1α can be detected by 2 hr, whereas there is no detectable drop in HIF2α levels by 8 hr. A rapamycin dose response for HIF2α expression in 786-O cells (Figure 4.2.1 C), and HIF1α and HIF2α expression in RCC4 cells (Figure 4.2.1 D), was also performed. The sensitivity of HIF1α corresponded to the sensitivity of S6 kinase phosphorylation with an IC50 between 20 and 200 nM, corresponding to an effect of rapamycin on mTORC1, which phosphorylates S6 kinase. These data reveal that in spite of a similar dependence of HIF1α and HIF2α on PLD activity, there was a clear difference in the sensitivity of HIF1α and HIF2α to rapamycin.

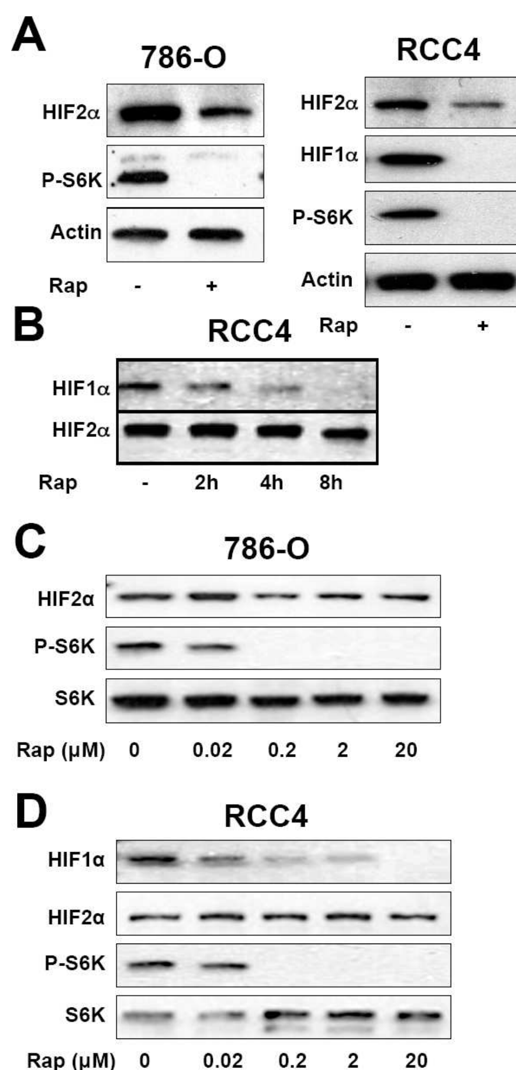

Figure 4.2.1 Differential Effect of Rapamycin on HIF1α and HIF2α. (A) 786-O and RCC4 cells were plated at 80% confluence for 24 hr in media containing 10% serum. Cells were then shifted to media without serum. Rapamycin (Rap) was added at 20 μM and the levels of HIF1α (RCC4 cells only) and HIF2α, phosphorylated S6 kinase (P-S6K) and actin were determined by Western blot analysis 18 hr later. (B) RCC4 cells were plated and then shifted to media without serum as described in (A). Rapamycin (Rap) was added at 20 μM and the levels of HIF1α and HIF2α were determined at the times indicated. (C) 786-O and RCC4 cells were plated at 80% confluence for 24 hr in media containing 10% serum. Cells were then shifted to media without serum. Rapamycin (Rap) was added at the indicated concentrations and the levels of HIF1α, HIF2α, phosphorylated S6 kinase (PS6K) and S6 kinase (S6K) were determined by Western blot analysis 18 hr later. All data shown are representative from at least three independent experiments.

4.2.2 SENSITIVITY OFf HIF1α and HIF2α TO SUPPRESSED EXPRESSION OF mTOR

The partial sensitivity of HIF2α expression with long term rapamycin treatment is consistent with recent reports from Sabatini's group indicating that mTORC2 can be suppressed by long term rapamycin treatment by preventing the formation of a complex between mTOR, Rictor and other components of mTORC2 (Sarbossov *et al.,* 2006; Zeng *et al*., 2007). To begin to determine whether the partial sensitivity of HIF2α to rapamycin was due to an mTORC2 requirement, we examined the effect of suppressing mTOR expression using siRNA for mTOR. In the 786-O cells, siRNA for mTOR strongly suppressed the expression of mTOR, and importantly, also suppressed the expression of HIF2α (Fig. 4.2.2 A). In the RCC4 cells, siRNA for mTOR suppressed the levels of both HIF1α and HIF2α (Fig. 4.2.2 B). Depleting cells of mTOR also suppressed phosphorylation of the mTORC1 substrate S6 kinase, and phosphorylation Akt at the mTORC2 site Ser473 (Sarbossov *et al* ., 2006) (Figs. 4.2.2 B and 4.2.2 C). These data indicate that while HIF1α is more sensitive to rapamycin than HIF2α, both HIF1α and HIF2α are dependent upon the expression of mTOR.

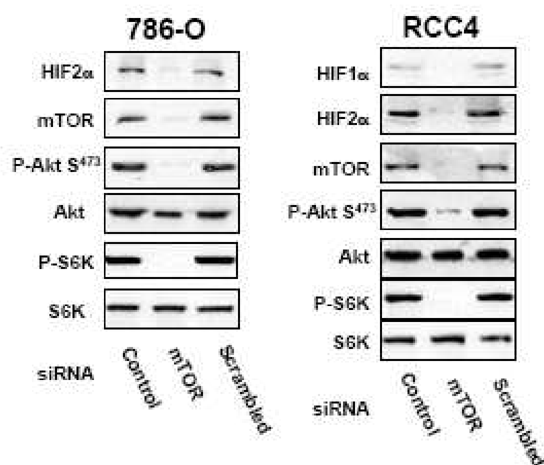

Figure 4.2.2: Both HIF1α and HIF2α are Sensitive to Suppression of mTOR Expression. (A) 786-O cells were plated at 30% confluence. 24 hr later the cells were transfected with mTOR siRNA or a scrambled siRNA as described below. 24 hr later the cells were treated with fresh media containing 10% serum for 48 additional hr. The Control cells were treated with transfection media, but without the transfection. The cells were then harvested and analyzed for levels of HIF2α, mTOR, phosphorylated Akt at Ser473 (P-Akt S473), Akt, phosphorylated S6 kinase (P-S6K), and S6 kinase (S6K) using Western blot analysis as in Fig. 4.2.1. (B) RCC4 cells were prepared and transfected with mTOR siRNA as in *A*. The cells were evaluated by Western blot analysis as in (A) except that HIF1α levels were also evaluated. All data shown are representative from at least three independent experiments.

4.2.3 DEPENDENCE OF HIF1α and HIF2α EXPRESSION ON RAPTOR AND RICTOR

The data showing a differential sensitivity of HIF1α and HIF2α to rapamycin, but equal sensitivity to mTOR depletion could be explained if HIF2α was dependent upon mTORC2, which is relatively resistant to rapamycin, and HIF1α was dependent upon mTORC1. To test this hypothesis, the 786-O and RCC4 cells were depleted of Raptor, which is a component of mTORC1, and Rictor, which is a component of mTORC2. As shown in Figure 4.2.3 A, HIF2α

expression in 786-O cells was sensitive to the depletion of Rictor, but not Raptor. These data are consistent with a dependence of HIF2α expression on mTORC2, but not mTORC1. We also examined the effect of depleting the expression Raptor and Rictor on HIF1α and HIF2α in RCC4 cells, and as shown in Figure 4.2.3 B, suppression of Rictor, but not Raptor, suppressed the expression of HIF2α - as was observed in the 786-O cells. The expression of HIF1α was dependent upon both Raptor and Rictor (Figure 4.2.3 B). Consistent with the roles of Raptor and Rictor in regulating mTORC1 and mTORC2 respectively, suppression of Raptor reduced phosphorylation of S6 kinase at mTORC1 site at Thr389 and suppression of Rictor reduced Akt phosphorylation at the mTORC2 site at Ser473. These data indicate that HIF1α is dependent upon both mTORC1 and mTORC2, whereas the expression of HIF2α is dependent only on mTORC2. Thus, the differential sensitivity of HIF1α and HIF2α to rapamycin is due to a differential dependence on mTORC1 and mTORC2.

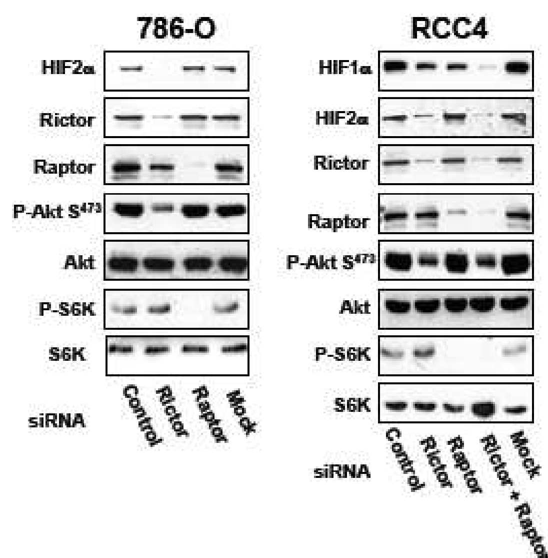

Figure 4.2.3: Dependence of HIF1α and HIF2α expression on Raptor and Rictor. (A) 786-O cells were plated as in Fig. 4.2.2. 24 hr later the cells were transfected with siRNAs for Raptor, Rictor, or a scrambled siRNA as indicated. 24 hr later the cells were treated with fresh media containing 10% serum for 48 additional hr. The cells were then harvested and analyzed for levels of HIF2α, mTOR, phosphorylated Akt at Ser473 (P-Akt S473), Akt, phosphorylated S6 kinase (P-S6K), and S6 kinase (S6K) using Western blot analysis as in Fig. 4.4.2. (B) RCC4 cells were prepared and transfected with Raptor and Rictor siRNAs as in *A*. The cells were evaluated by Western blot analysis as in (A) except that HIF1α levels were also evaluated. All data shown are representative from at least three independent experiments.

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4.2.4 DEPENDENCE OF HIF1α AND HIF2α ON AKT

The data in Fig. 4.2.3 indicate that both HIF1α and HIF2α are dependent on mTORC2. It is becoming apparent that a key target of mTORC2 is Akt, which gets phosphorylated at Ser473 (Sorbassov *et al.* 2006). Consistent with the previous reports, suppression of Rictor, but not Raptor, suppressed Akt phosphorylation at Ser473 (Fig. 4.2.3). We therefore examined whether the

expression of either HIF1α or HIF2α were dependent on Akt expression. There are three Akt isoforms - Akt1, Akt2, and Akt3. Akt1-deficient mice have developmental defects, Akt2-deficient mice have defects in glucose homeostasis, and Akt3-deficient mice have defects in brain development (Manning and Cantley, 2007). The 786-O cells were treated with siRNAs for the Akt isoforms and levels of HIF2α were evaluated by Western blot. As shown in Fig. 4.2.4 A, depleting cells of Akt2, but not Akt1 abolished HIF2α expression. There was no detectable expression of Akt 3 in the 786-O cells (Fig. 4.2.4 A lower panel). We also examined the effect of depleting cells of the Akt isoforms in the RCC4 cells, which express both HIF1α and HIF2α. In contract with the 786-O cells, the RCC4 cells expressed all three Akt isoforms. As shown in Fig. 4.2.4 B, depleting cells of Akt2, but not Akt1 or Akt3 suppressed the expression of HIF2α as was observed in the 786-O cells. Suppression of either Akt1 or Akt2 had no effect on HIF1α expression, however suppression of Akt3 completely abolished HIF1α in the RCC4 cells (Fig. 4.2.4 B lower panel). These data indicate that Akt2 is a critical downstream target of mTORC2 for HIF2α expression and that Akt3 is a critical downstream target of mTORC2 for HIF1α expression.

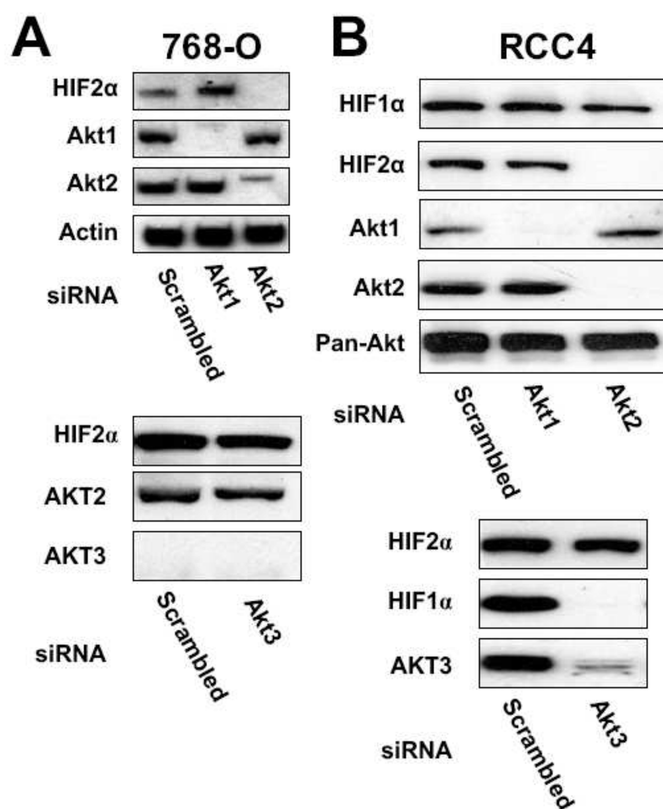

Figure 4.2.4: Dependence of HIFα expression on Akt. 786-O (*A*) and RCC4 (*B*) cells were plated as in Fig. 2. 24 hr later the cells were transfected with siRNAs for Akt1, Akt2, Akt3 or a scrambled siRNA as indicated. 24 hr later the cells were treated with fresh media containing 10% serum for 48 additional hr. The cells were then harvested and analyzed for levels of HIF1α HIF2_, Akt1, Akt2 and Akt3 using Western blot analysis as in Fig. 4.2.2. The data shown are representative of two independent experiments.

4.3 DISCUSSION

We previously reported that the expression of both HIF1α and HIF2α are dependent on PLD activity (Toschi *et al.*, 2008). The PLD metabolite PA has been implicated in the activation of mTOR (Foster, 2004; 2007). However, there is a clear differential sensitivity of HIF1α and HIF2α to rapamycin. In this report, we demonstrate that while there is a differential sensitivity of HIF1α and HIF2α to rapamycin, both HIF1α and HIF2α are dependent upon mTOR. This observation is explained by a dependence of HIF2α expression on the rapamycin resistant mTORC2, while there is a dependence of HIF1α expression on both mTORC1 and mTORC2. This study reveals an mTORC2 requirement for the expression of HIF2α.

Akt2, which is a down stream target of mTORC2, was also required for the expression of HIF2α. Of interest here is that Akt2 has been implicated in the regulation of glycolysis (Manning and Cantley, 2007) – as has HIF2α (Gordan and Simon, 2007). HIF1α, which was also dependent on mTORC2, was dependent on Akt3, which has been implicated in melanoma and ovarian cancer (Davies *et al.*, 2007; Cristiano *et al.*, 2006). At this point, it is difficult to determine the link between Akt and HIFα expression in that the regulation is very complicated (Manning and Cantley, 2007). The impact of phosphorylating Akt at Ser473 by mTORC2 is not clear. While this phosphorylation leads to increased kinase activity (Alessi *et al.*, 1996), suppressing mTORC2 activity suppressed phosphorylation of the Akt substrate FOXO, but did not prevent the phosphorylation of the tuberous sclerosis complex (Guertin *et al.*, 2006; Jacinto

et al., 2006). Thus, it has been speculated that phosphorylation of Akt by mTORC2 may influence substrate specificity. Thus, the data presented here only implicate Akt2 and Akt3 in the regulating the expression of HIF2α and HIF1α respectively. The data do not indicate how this is accomplished.

While there is a well-established connection between PLD activity and mTORC1 (Foster, 2004; 2007), there has been no connection between PLD activity and mTORC2. Since PLD activity is required for the expression of HIF2α (Toschi *et al.*, 2008), which is dependent on mTORC2, this study also suggests that PLD is required for the activation of mTORC2 as well as mTORC1. And we now have preliminary data indicating that the PLD metabolite phosphatidic acid is required for the assembly of active mTORC1 and mTORC2 complexes with Raptor and Rictor respectively (Toschi *et al.*, submitted for publication). Thus, the PLD requirement for the expression of HIF2α reported previously (Toschi *et al.*, 2008) and the dependence of HIF2α expression on mTORC2 reported here is consistent with our preliminary study indicating a role for PLD in the activation of mTORC2.

The dependence of HIF2α on mTORC2 is significant in that HIF2α expression has been shown to be critical for tumorigenesis (Kondo *et al.*, 2002; 2003). Rapamycin and rapamycin derivatives have been widely employed in clinical trials with mostly disappointing results (Sawyers, 2003). A recent clinical study (Cloughesy *et al.*, 2008) focused on glioblastoma where there are commonly defects in PTEN. Defective PTEN increases signals that lead to increased activation of mTORC1, but a role for PTEN in the regulation of

mTORC2 is not clear (Guertin And Sabatini, 2007). This clinical study indicated that there was cell cycle arrest in response to rapamycin as well as effects on S6 kinase phosphorylation – implicating mTORC1. However, mTORC1 is much more sensitive to rapamycin than mTORC2. As described here, the expression of HIF2α in renal cancer cells is dependent on mTORC2, which is much more resistant to rapamycin than mTORC1. While suppression of mTORC1 with rapamycin is achievable, it may be that to effectively target mTOR in cancer, targeting mTORC2 may be more important because HIF2α is likely more critical (Kondo *et al.*, 2002; 2003). It will be important to develop strategies that can suppress mTORC2 and HIF2α. PLD is required for the expression of both HIF1α and HIF2α (Toschi *et al.*, 2008), suggesting that PLD is required for the activation of both mTORC1 and mTORC2. Thus, targeting PLD or the signals that activate PLD may represent a viable therapeutic strategy for suppressing HIF2α in RCC and other cancers where HIF2α has been implicated. The dependence of HIF2α on mTORC2 indicates that targeting mTORC1 with rapamycin will likely have limited therapeutic effects given the apparent significance of HIF2α in RCC.

CHAPTER V

REGULATION OF mTORC1 AND mTORC2 COMPLEX ASSEMBLY BY PHOSPHATIDIC ACID – A COMPETITION WITH RAPAMYCIN

5.1 INTRODUCTION

mTOR, the mammalian target of rapamycin, is a critical node for control of cell growth and survival and has widely been implicated in cancer survival signals. mTOR exists in two complexes – mTORC1 and mTORC2. While much is known about the regulation of mTORC1, little is known about the regulation of mTORC2. Phospholipase D (PLD) and its metabolite phosphatidic acid (PA) have been implicated in the regulation mTOR, however its role has been controversial. We report here that PA, in competition with rapamycin, is required for functional mTORC1 and mTORC2 complex formation. Suppression of PLD prevented phosphorylation of the mTORC1 substrate S6 kinase at Thr389 and the mTORC2 substrate Akt at Ser473. Suppression of PLD also blocked insulin-stimulated phosphorylation of Akt and the mTORC2- and Akt-dependent phosphorylation of PRAS40. PA is required for the association of mTOR with Raptor to form mTORC1 and mTOR with Rictor to form mTORC2. The effect of PA was competitive with rapamycin with much higher concentrations of rapamycin needed to compete with the PA-mTORC2 interaction than with the PA-mTORC1 interaction. However, suppressing PA production substantially increased the sensitivity of mTORC2 to rapamycin. The data provided here reveal a PA requirement for the stabilization of both mTORC1 and mTORC2 complexes. The competition between PA and rapamycin for mTOR suggests a mechanism for the suppression of mTOR by rapamycin and explains the rapamycin resistance of mTORC2. The study also suggests that mTORC2

could be targeted therapeutically with rapamycin by suppressing signals that control PLD.

It has become apparent during the past decade that a critical aspect of tumor progression is the suppression of default apoptotic programs that constitute what is likely the most important protection against cancer. Cellular signals that suppress apoptosis have come to be known as “survival signals”. A common node for survival signals is mTOR, the mammalian target of rapamycin (Sawyers, 2003; Foster, 2004; Guertin and Sabatini, 2007). mTOR exists in two distinct complexes, mTORC1 and mTORC2 (Sabatini, 2006), that differ in their subunit composition and sensitivity to rapamycin. mTORC1 consists of a complex that includes mTOR and a protein known as Raptor (regulatory associated protein of mTOR), whereas mTORC2 consists of a complex that includes mTOR and a protein known as Rictor (rapamycin-insensitive companion of mTOR) (Guertin and Sabatini, 2007). There are also mTORC2 complexes that can be distinguished by association with different isoforms of mSin1 (Frias *et al* ., 2006). While much is known about the regulation of mTORC1 (Sabatini, 2006), very little is known about the regulation of mTORC2.

mTORC1 is highly sensitive to rapamycin, whereas mTORC2 is relatively insensitive to rapamycin (Sabatini, 2006). However, it was recently reported that long-term exposure to rapamycin prevented the formation of mTORC2 complexes and blocked the phosphorylation of the mTORC2 substrate Akt at Ser473 (Sarbassov *et al* ., 2006; Zeng *et al* ., 2007). Rapamycin, in association with the FK506 binding protein-12 (FKBP12), has been reported to interact with

mTOR in a manner that is competitive with phosphatidic acid, the metabolic product of phospholipase D (PLD) (Fang *et al.*, 2001; Chen *et al.*, 2003). PLD, like mTOR, has been implicated in survival signals in several human cancer cell lines (Chen *et al* ., 2005; Zheng *et al.*, 2006; Shi *et al.*, 2007; Gadir *et al.*, 2007; 2008; Toschi *et al.*, 2008). Since rapamycin-FKBP12, competes with PA for binding to mTOR, the studies suggesting that mTORC2 complex formation is sensitive to rapamycin suggest that PA would do the opposite - facilitate the assembly of mTORC2, and ultimately, the activation of mTORC2. We report here that the assembly of both mTORC1 and mTORC2 complexes are dependent upon PLD. The study provides mechanistic insight as to how rapamycin impacts on mTOR-mediated signals and how PLD regulates mTOR by facilitating the formation of mTOR complexes.

5.2 RESULTS

5.2.1 ACTIVATION OF mTORC1 and mTORC2 IS DEPENDENT UPON PHOSPHOLIPASE D AND PHOSPHATIDIC ACID

We previously reported that the expression of HIF2α in 786-O cells is dependent upon PLD (Toschi *et al.*, 2008) and that HIF2α is dependent on mTORC2 (Toschi *et al.*, submitted). These studies suggested a link between PLD and mTORC2. PLD, through its metabolite PA, has been implicated in the activation of the rapamycin sensitive mTORC1 in a manner whereby PA is competitive with rapamycin for binding to the FKBP12-rapamycin binding (FRB) domain of mTOR (Foster, 2007). Suppression of PLD activity reduces

phosphorylation on the mTORC1 substrate S6 kinase at Thr389 (Chen *et al.*, 2003). Sabatini and co-workers recently reported that rapamycin suppressed the assembly of mTORC2 complexes (Sarbossov *et al.*, 2006; Zeng *et al.*, 2007). This finding suggests that if rapamycin is competitive with PA for mTOR, then PA may also be required for the activation of mTORC2. mTORC2 phosphorylates Akt at Ser473 (Sarbassov *et al.*, 2005; Hresko and Mueckler, 2005) and can be used as an indicator of mTORC2 activity. To verify this in 786-O cells, we examined the dependence of Akt phosphorylation at Ser473 on mTOR and Rictor – a critical component of mTORC2. We also examined the dependence of S6 kinase phosphorylation on mTOR and Raptor – a critical component of mTORC1. As shown in Figure 5.2.1 , the phosphorylation Akt at Ser473 in 786-O cells is suppressed by siRNAs for both mTOR and Rictor, but not Raptor; and that the phosphorylation of S6 kinase at Thr389 was suppressed by siRNAs for mTOR and Raptor, but not Rictor. These data establish that the phosphorylation of Akt at Ser473 is dependent on mTORC2, which is dependent on Rictor, and that S6 kinase phosphorylation is dependent on mTORC1, which is dependent on Raptor.

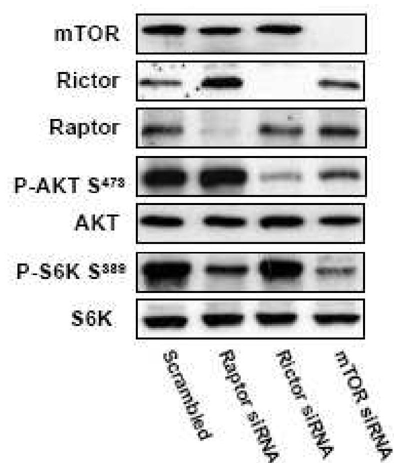

Figure 5.2.1 Phosphorylation of Akt at Ser473 in 786-O cells is dependent on mTORC2. 786-O cells were plated at 30% confluence. 24 hr later the cells were transfected with siRNAs for Raptor, Rictor, mTOR or a scrambled siRNA as described in Material and Methods. 24 hr later the cells were treated with fresh media containing 10% serum for 48 additional hr. The cells were then harvested and analyzed for levels of mTOR, Raptor, Rictor, phosphorylated Akt at Ser473 (P-Akt S^{473}), Akt, phosphorylated S6 kinase (P-S6K T^{389}), and S6 kinase (S6K) using Western blot analysis. All data shown are representative from at least three independent experiments.

To investigate whether mTORC2 is dependent on PLD activity, we examined whether the phosphorylation of Akt at the mTORC2-dependent Ser473 site was dependent upon PLD activity using the "alcohol trap" transphosphatidylation reaction whereby primary alcohols, but not tertiary alcohols, block PA production (Shen *et al.,* 2001). As shown in Figure 5.2.2 A, 1-butanol (1-BtOH) suppressed the phosphorylation of Akt at Ser473. The effect could be seen at 0.5% and complete inhibition was seen at 0.75% 1-BtOH. Significantly, S6 kinase phosphorylation was more sensitive to 1-BtOH than was Akt phosphorylation. We also examined the effect of 1-BtOH on GSK-3β

phosphorylation at Ser9/21 – sites phosphorylated by activated Akt (Shaw *et al* ., 1997). As shown in Figure 5.2.2 A, GSK-3B phosphorylation was suppressed at concentrations of 1-BtOH that were similar to those required for suppression of Akt phosphorylation at Ser473. 1-BtOH had much less of an effect on the phosphorylation of Akt at Thr308 – a site that is not phosphorylated by mTORC2 (Guertin and Sabatini, 2007). Tertiary-butanol (t-BtOH) at 1% had no effect upon the phosphorylation of the substrates examined here, indicating that the effects were due to the suppression of PA production by PLD. The effect of 1-BtOH was reversible and phosphorylation of Akt at Ser473 was restored within one hr after providing fresh media lacking 1-BtOH (Figure 5.2.2 B). If PA was added to the cell cultures treated with 1-BtOH, the suppression of Akt phosphorylation at Ser473 was reversed (Figure 5.2.2 C). This observation indicates that it is PA generated by PLD that is stimulating Akt phosphorylation. To further establish that the effect of 1-BtOH on Akt phosphorylation was due to an effect on PLD, we transiently introduced catalytically inactive mutants of PLD1 and PLD2 (Sung *et al.,* 1997; 1999), which have been shown to act effectively as dominant negative PLD mutants (Shen *et al.,* 2001; Toschi *et al*., 2008). As shown in Figure 5.2.2 D, the presence of the PLD mutants strongly suppressed the phosphorylation of Akt at Ser473, but not at Thr308. The PLD mutants also suppressed S6 kinase phosphorylation. The data in Figure 5.2.2 indicate that the mTORC2-dependent phosphorylation of Akt at Ser473, and the mTORC1-dependent phosphorylation of S6 kinase at Thr389 are dependent upon PLD and its metabolite PA.

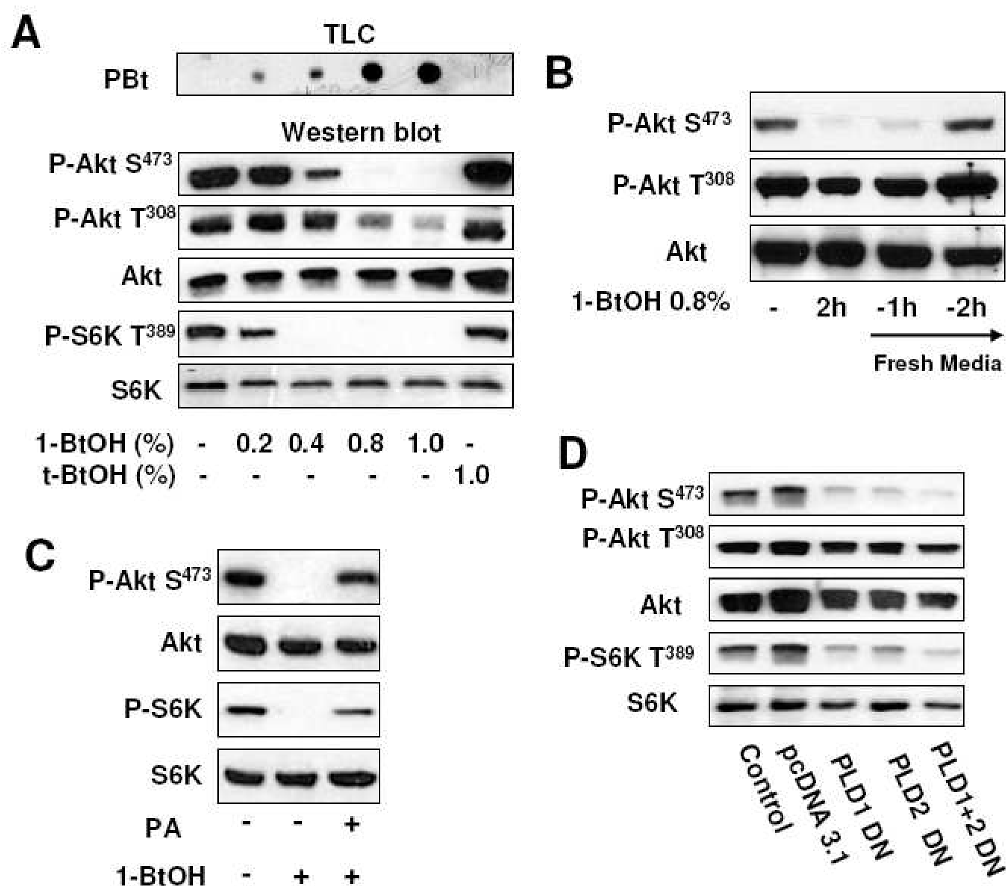

Figure 5.2.2: Activation of mTORC2 is Dependent on Phospholipase D and Phosphatidic Acid. (A) 786-O cells were plated at 5 X 10^5 cells per 60 mm plate for 24 hr at which time they were shifted to media containing 0.5% serum. 1-BtOH or t-BtOH was then added where indicated at the concentrations shown. After two hr, the cells were harvested and analyzed for levels of the transphosphatidylation product PBt using thin layer chromatography (TLC) as described in Materials and Methods. The PBt band of the TLC plate is shown in the top panel. The cells were also evaluated for levels of phosphorylated Akt at Ser473 (P-Akt S^{473}), phosphorylated Akt at Thr308 (P-Akt T^{308}), Akt, phosphorylated S6 kinase at T398 (P-S6K T^{389}), and S6 kinase (S6K) using Western blot analysis. (B) 786-O cells were plated as in (A). 1-BtOH (0.8%) was then added where indicated. After two hr, the cells were either harvested or placed in fresh media for 1 or 2 hr as indicated, at which time the cells were harvested and analyzed for levels of phosphorylated Akt at Ser473 (P-Akt S^{473}), phosphorylated Akt at Thr308 (P-Akt T^{308}), and Akt as in (A). (C) 786-O cells were prepared and treated with 0.8% 1-BtOH as in B. PA (100 μM) was prepared as described in Materials and Methods and was added with the 1-BtOH where indicated. After two hr, the cells were harvested and analyzed for levels of phosphorylated Akt at Ser473 (P-Akt S^{473}), Akt, phosphorylated S6 kinase at

Thr389 (P-S6K T^{389}) and S6 kinase (S6K) as in (A). (D) 786-O cells were plated at 5 X 10^5 cells per 60 mm plate. 24 hr later the cells were transfected with vectors expressing catalytically inactive dominant negative (DN) mutants for PLD1 or PLD2 as indicated. The parental vector pcDNA 3.1 was used as a control. 24 hr later the cells were treated with fresh media containing 10% serum for additional 24 hr. The Control cells were treated with transfection media, but without the transfection. The cells were then harvested and analyzed for levels of phosphorylated Akt at Ser473 (P-Akt S^{473}), phosphorylated Akt at Thr308 (P-Akt T^{308}), Akt, phosphorylated S6 kinase at Thr389 (P-S6K T^{389}), and S6 kinase (S6K) as in (A). All data shown are representative from at least three independent experiments.

5.2.2 PLD REQUIREMENT FOR THE FORMATION OF mTOR COMPLEXES

Sabatini and co-workers reported that prolonged rapamycin treatment prevented the formation of mTORC2 complex (Sarbassov *et al*., 2006; Zeng *et al*., 2007), suggesting that PA, which is competitive with rapamycin for binding mTOR (Fang *et al*., 2001; Chen *et al*., 2003), might be required for the assembly of the mTORC2 complex. To address this question, we examined the impact of PLD activity on the ability of mTOR to co-immunoprecipitate with Rictor and Raptor. 786-O cells were treated with increasing concentrations of 1-BtOH, and the levels of Rictor and Raptor that co-immunoprecipitated with mTOR were evaluated. As shown in Figure 5.2.3 A, treatment of the 786-O cells with increasing concentrations of 1-BtOH suppressed the level of both Rictor and Raptor that co-immunoprecipitated with mTOR. Importantly, the level of 1-BtOH required to suppress Akt phosphorylation at Ser473 correlated with level required to suppress co-immunoprecipitation of mTOR with Rictor, and the level of 1-BtOH required to suppress S6 kinase phosphorylation correlated with level required to suppress co-immunoprecipitation of mTOR with Raptor (Figure 5.2.3 A). If PA was added the effect of 1-BtOH on the association between mTOR and

both Raptor and Rictor was reversed (Figure 5.2.3 B). These data suggest that the PLD/PA requirement for mTOR activity is to facilitate or stabilize the assembly of mTOR complexes. This finding is consistent with the reported competition between PA and rapamycin-FKBP12 (Fang *et al* ., 2001; Chen *et al* ., 2003) and the observation that rapamycin suppresses the formation of mTORC2 complex (Sarbassov *et al* ., 2006; Zeng *et al* ., 2007).

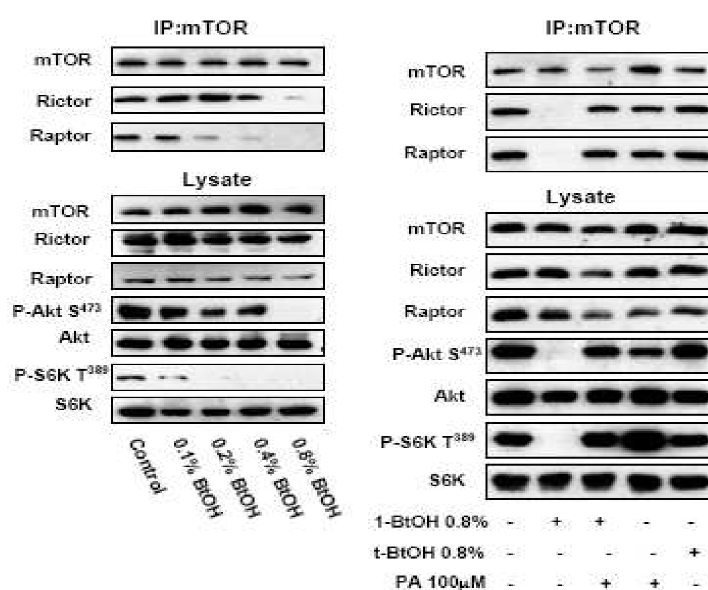

Figure 5.2.3: PLD is Required for the Formation of mTOR Complexes. (A) 786-O cells were plated at 80% confluence for 24 hr at which time they were shifted to media containing 0.5% serum. 1-BtOH was then added at the indicated concentrations. After 3 hr, lysates were prepared and subjected to immunoprecipitation with anti-mTOR antibody overnight at which time mTOR immunoprecipitates (IP: mTOR) along with the lysates, were subjected to Western blot analysis for Rictor, Raptor and mTOR. The lysates were also analyzed for the levels of phosphorylated Akt at Ser473 (P-Akt S^{473}) and for S6K and phosphorylated S6 kinase (P-S6K T^{389}). (B) 786-O cells were prepared as in (A) and then treated with 1-BtOH, t-BtOH, and PA as indicated. Lysates were prepared and subjected to immunoprecipitation with anti-mTOR antibody overnight at which time the mTOR immunoprecipitates and lysates were subjected to Western blot analysis as in (A). All data shown are representative from at least two independent experiments.

5.2.3 DIFFERENTIAL SENSITIVITY OF mTORC1 AND mTORC2 TO RAPAMYCIN.

We previously demonstrated that elevated PLD activity conferred rapamycin resistance in breast cancer cells (Chen *et al* ., 2003). If there was higher PLD activity, higher doses of rapamycin were required to suppress both cell proliferation and S6 kinase phosphorylation (Chen *et al* ., 2003). Intriguingly, higher concentrations of rapamycin were required to suppress cell proliferation than were required to suppress S6 kinase phosphorylation. These data could be explained by a differential sensitivity of mTORC1 and mTORC2 to rapamycin. To test this hypothesis, we examined the effect of increasing concentrations of rapamycin on phosphorylation of Akt at Se473 and S6 kinase at Thr389. As shown in Figure 5.2.4 A, S6 kinase phosphorylation was sensitive to concentrations of rapamycin in the low nano-molar range with an IC50 around 20 nM. In contrast, Akt phosphorylation at Ser473 was resistant to rapamycin concentrations of up to 20 μM.

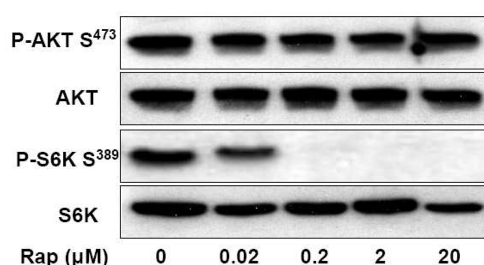

Figure 5.2.4: Differential Sensitivity of mTORC1 and mTORC2 Activity to Rapamycin and 1-BtOH. (A) 786-O cells were plated at 80% confluence for 24 hr in media containing 10% serum. Cells were then shifted to media containing 0.5% serum overnight. Rapamycin (Rap) was added at the indicated concentrations. The cells were harvested 8 hr later and the levels of phosphorylated Akt at Ser473 (P-Akt S^{473}), Akt, phosphorylated S6 kinase (P-S6K T^{389}), and S6 kinase (S6K) were determined using Western blot analysis.

The data in Fig. 5.2.2 A revealed that suppression of Akt phosphorylation at Ser473 (mTORC2) required higher concentrations of 1-BtOH than did the suppression of S6 kinase phosphorylation (mTORC1). This finding indicates that the concentration of PA must be substantially lower before mTORC2 activity is reduced – indicating that PA interacts more strongly with mTORC2 than with mTORC1 (see discussion). The observation also suggests that rapamycin-FKBP12 would disrupt the weaker interaction between PA and mTORC1 at lower concentrations than would be needed to disrupt the stronger interaction between PA and mTORC2. If true, then lowering the PA concentration should allow rapamycin to suppress mTORC2. To investigate this, we examined the effect of rapamycin on Akt phosphorylation at Ser473 in the presence of increasing concentrations of 1-BtOH, which reduces the level of PA. As shown in Figure 5.2.4 B, the presence of either 0.25% or 0.5% 1-BtOH had little or no effect on Akt phosphorylation at Ser473. However, in the presence of 1-BtOH, Akt phosphorylation was now sensitive to 200 nM rapamycin. To verify that the effect of rapamycin on Akt phosphorylation was due to an effect on mTOR, we examined the effect of rapamycin on Akt phosphorylation in the presence of FK506, which competes with rapamycin for binding to FKBP12. As shown in Figure 5.2.4 C, FK506 reversed the inhibitory effect of rapamycin on both Akt and S6 kinase. We also examined whether the effect could be reversed by PA and as shown in Fig. 5.2.4 D, the phosphorylation of both Akt at Ser473 and S6 kinase at Thr389 was restored with PA. The sensitivity of Akt phosphorylation at Ser473 to 0.5% 1-BtOH and 200 nM rapamycin suggests that the effect is due to

an effect on mTORC2. We therefore examined the sensitivity of the mTOR association with Rictor to rapamycin in the presence of 1-BtOH.

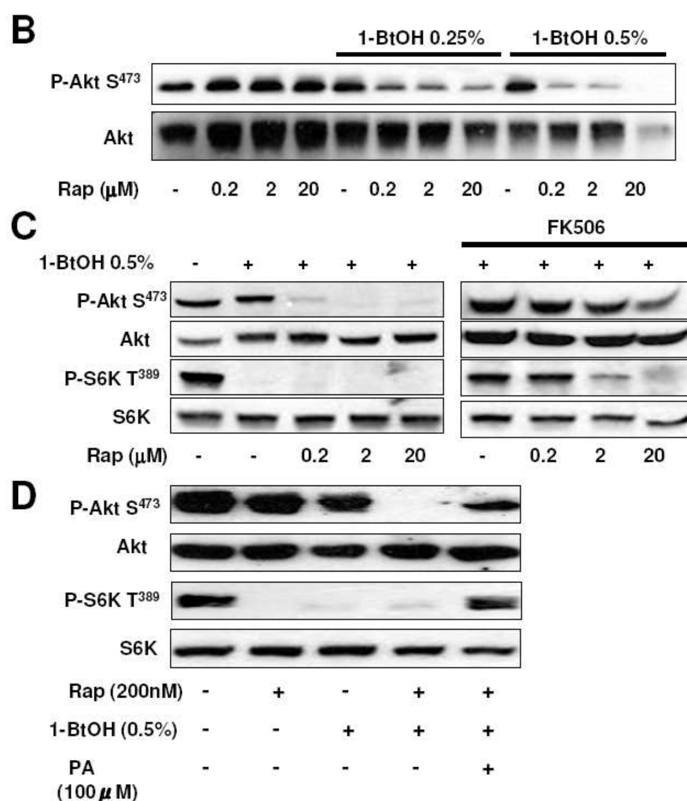

Figure 5.2.4: (Continued) Differential Sensitivity of mTORC1 and mTORC2 Activity to Rapamycin and 1-BtOH. (B) 786-O cells were plated as in (A). Rapamycin (Rap) was added at the indicated concentrations in the absence or presence of either 0.25% or 0.5% 1-BtOH as indicated. Eight hr later, the cells were harvested and the levels of phosphorylated Akt at Ser473 (P-Akt S473) and Akt were determined using Western blot analysis. (C) 786-O cells were plated as in (A). Rapamycin (Rap) was added at the indicated concentrations in the absence or presence of 0.5% 1-BtOH as indicated. FK506 (10 μM) was added where indicated along with the rapamycin and 1-BtOH. Eight hr later, the cells were harvested and the levels of phosphorylated Akt at Ser473 (P-Akt S473) and Akt were determined using Western blot analysis. (D) 786-O cells were prepared and treated with 0.5% 1-BtOH and 200 nM rapamycin as shown. PA (100 μM) was added with the 1-BtOH where indicated. After 8 hr, the cells were harvested and analyzed for levels of phosphorylated Akt at Ser473 (P-Akt S473), Akt, phosphorylated S6 kinase at Thr389 (P-S6K T389) and S6 kinase (S6K) as in (A). All data shown are representative from at least two independent experiments.

As shown in Figure 5.2.5 , the association between mTOR and Rictor was sensitive to 200 nM rapamycin in 0.5% 1-BtOH. These data support the hypothesis that the formation of mTOR complexes are mediated by PA and that rapamycin-FKBP12 interferes with the interaction between PA and mTOR.

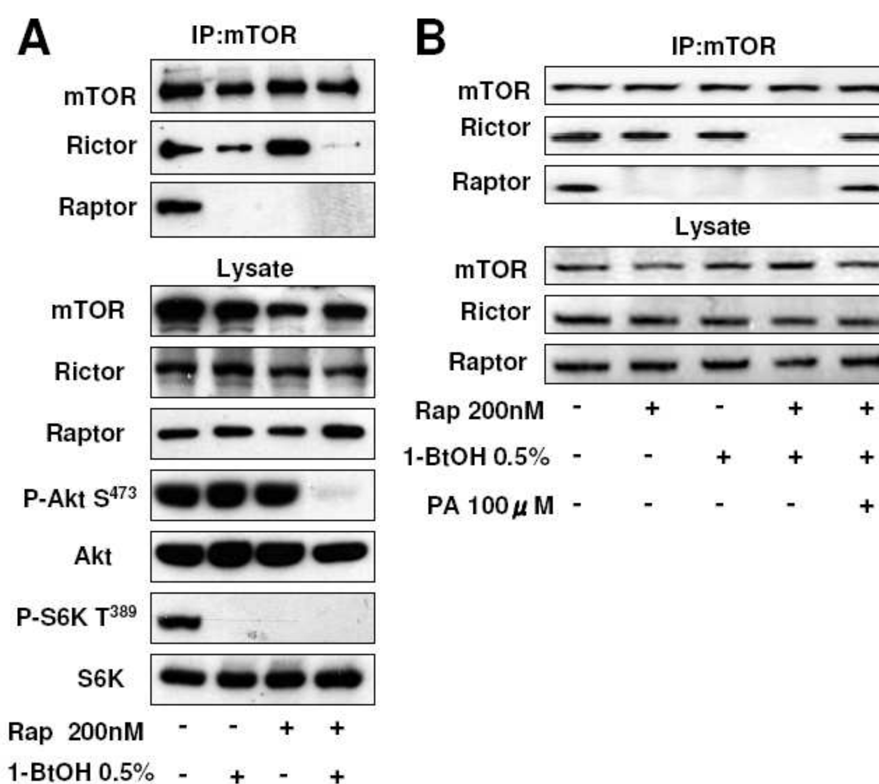

Figure 5.2.5: Differential Sensitivity of mTORC1 and mTORC2 Complex Formation to Rapamycin and 1-BtOH. 786-O cells were plated at 80% confluence for 24 hr at which time they were shifted to media containing 0.5% serum. 1-BtOH (0.25%) and the indicated concentrations of rapamycin (Rap) were added as indicated. After 6 hr, lysates were prepared and subjected to immunoprecipitation with anti-mTOR antibody overnight at which time the mTOR immunoprecipitate (mTOR-IP) was subjected along with the lysates to Western blot analysis for Rictor. The data shown are representative of three independent experiments.

5.2.4 *INSULIN-STIMULATED AKT PHOSPHORYLATION AT Ser473 IS DEPENDENT ON PLD ACTIVITY*

Akt phosphorylation at Ser473 can be stimulated by insulin (Hresko and Muekler, 2005; Frias *et al* ., 2006). We therefore wanted to examine the PLD dependence of insulin-stimulated Akt phosphorylation. The 786-O cells used above have constitutively elevated levels of Akt phosphorylation. We therefore used MDA-MB-231 cells, which have low levels of basal Akt phosphorylation (Chen *et al* ., 2005). As shown in Figure 5.2.6 A, insulin strongly induced an increase in Akt phosphorylation at Ser473. This increase was suppressed by 0.8% 1-BtOH, however if PA was added, the effect of 1-BtOH was reversed – indicating a PA requirement for the insulin-induced increase in Akt phosphorylation. We also examined the sensitivity of the insulin-induced increase in Akt phosphorylation to rapamycin. The insulin-induced increase is Akt phosphorylation at Ser473 was also suppressed in the MDA-MB-231 cells if they expressed the dominant negative PLD1 and PLD2 mutants. As shown in Figure 5.2.6 C, the insulin-induced increase in Akt phosphorylation was resistant to rapamycin concentrations of 20 μM. However, in the presence of 0.25% 1-BtOH, which had little effect on Akt phosphorylation, rapamycin was able to suppress insulin-induced Akt phosphorylation at 200 nM (Figure 5.2.6 C). These data further support the hypothesis that mTORC2 can be made rapamycin sensitive by reducing the concentration of PLD-generated PA.

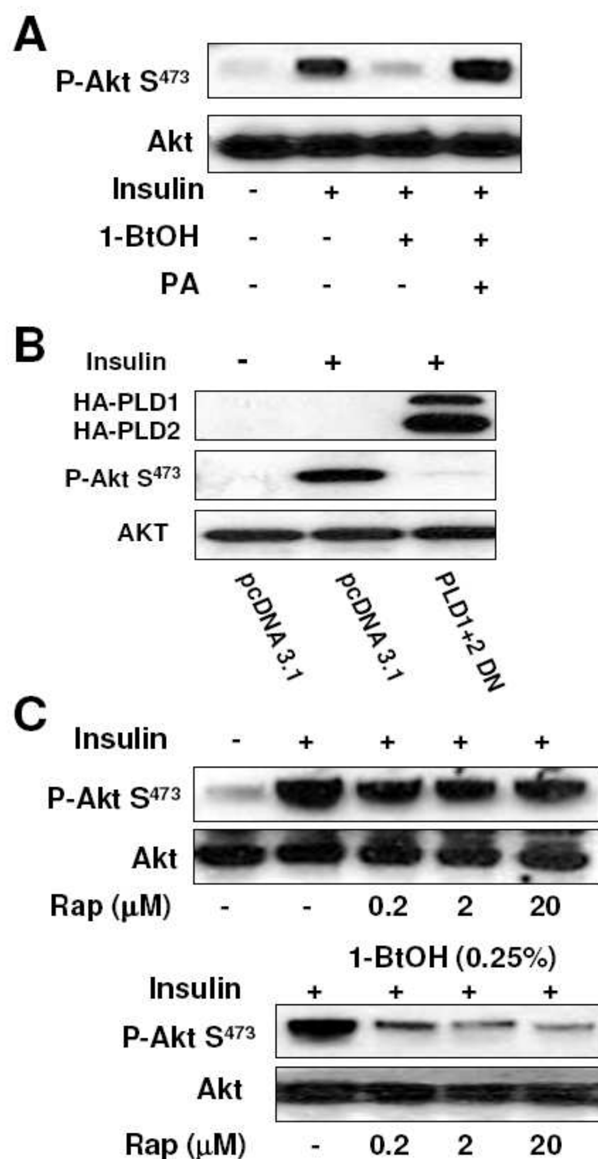

Figure 5.2.6: Insulin-stimulated Akt phosphorylation at Ser473 is dependent on PLD activity. (A) MDA-MB-231 cells were plated at 80% confluence for 24 hr in media containing 10% serum. Cells were then shifted to media containing 0.5% serum overnight. Insulin (100 nM), 1-BtOH (0.8%), and PA (100 μM) were then added as indicated. The cells were harvested 2 hr later and the levels of phosphorylated Akt at Ser473 (P-Akt S^{473}) and Akt were determined using Western blot analysis. (B) MDA-MB-231 cells were plated as in (A). Rapamycin (Rap) was added at the indicated concentrations in the absence or presence of 0.25%1-BtOH as indicated. 6 hr later, the cells were harvested and the levels of phosphorylated Akt and Akt were determined as in (A). The data shown are representative of three independent experiments.

5.2.5 *SUPPRESSION OF PLD INCREASES THE ASSOCIATION BETWEEN mTOR AND THE mTOR INHIBITORY PROTEIN PRAS40.*

A substrate of Akt is PRAS40 - Proline-Rich Akt Substrate of 40 kDa, which is an inhibitor of mTORC1 (Vander Haar *et al* ., 2007; Wang *et al* ., 2007; Sancak *et al* ., 2007). The phosphorylation of PRAS40 prevents association with mTOR. We therefore examined the effect of 1-BtOH and rapamycin on the association of PRAS40 with mTOR and the phosphorylation state of PRAS40. As shown in Figure 5.2.7 A (upper panel), neither 0.5% 1-BtOH or 200 nM rapamycin had an impact on the ability of PRAS40 to co-immunoprecipitate with mTOR. However, the combination of 0.5% 1-BtOH and 200 nM rapamycin strongly increased the association between mTOR and PRAS40. This combination of 1-BtOH and rapamycin was that needed to suppress mTORC2 and Akt phosphorylation (see Fig. 5.2.4 B). This combination of 1-BtOH and rapamycin also suppressed the phosphorylation of PRAS40 at the Akt site of Thr246 (Fig. 5.2.7 A, lower panel). The effect of the 1-BtOH on the association between mTOR and PRAS40, as well as the effect on PRAS40 phosphorylation was overcome with PA (Fig. 5.2.7 A). We also examined the effect of the dominant negative PLD mutants on the association between mTOR and PRAS40, and as shown in Fig. 5.2.7 B, the dominant negative PLD mutants suppressed PRAS40 phosphorylation and increased association with mTOR. These data further support a role for PLD in the regulation of mTORC2 and the positive feedback on mTORC1 through suppression of PRAS40 inhibition.

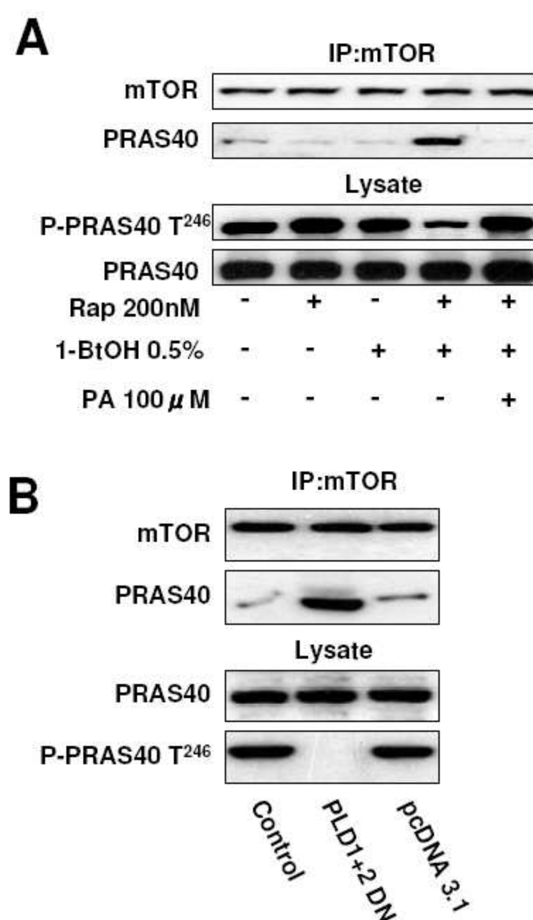

Figure 5.2.7: Suppression of PLD increases the association between mTOR and the mTOR inhibitory protein PRAS40. (A) 786-O cells were plated at 5 X 105 cells per 60 mm plate for 24 hr at which time they were shifted to media containing 0.5% serum. 1-BtOH (0.5%) and rapamycin (200nM) were added as indicated. After 6 hr, lysates were prepared and subjected to immunoprecipitation with anti-mTOR antibody overnight at which time the mTOR immunoprecipitate (mTOR-IP) was subjected along with the lysates to Western blot analysis for mTOR, PRAS40 and phosphorylated PRAS40 (P-PRAS40) at Thr246. (B) 786-O cells were plated as in (A). 24 hr later the cells were transfected with vectors expressing catalytically inactive dominant negative (DN) mutants for PLD1 or PLD2 or the parental vector pcDNA 3.1 as indicated. 24 hr later the cells were treated with fresh media containing 10% serum for 24 additional hr. The cells were then harvested and analyzed for mTOR and PRAS40 in the mTOR immunoprecipitates, and PRAS40 and phosphorylated PRAS40 (P-PRAS40) at Thr246 in the lysates as in (A). The data shown are representative of two independent experiments.

5.3 DISCUSSION

Elevated PLD activity has been observed in a large number of human cancers and in human cancer cells (Foster and Xu, 2003). PLD activity has also been implicated in survival signaling in cancer cells (Foster, 2004; 2006). The PLD metabolite PA has been implicated in the activation of mTOR (Foster, 2007), which has also been widely implicated in cancer survival signals (Guertin and Sabatini, 2007). However, a role for PLD in the survival signals mediated by mTOR has not been widely accepted. The data provided here reveal that PLD and its metabolite PA is critical for the formation of both mTORC1 and mTORC2 complexes. This study reinforces the concept that rapamycin suppresses mTOR by interfering with the interaction between mTOR and PA and that mTOR can become more sensitive to rapamycin by reducing PA levels. Data presented here demonstrate that the mTORC2-dependent phosphorylation of Akt at Ser473 requires PLD activity and PA. Akt, like mTOR, is a critical node for cancer survival signals and phosphorylation of Akt at Ser473 has been strongly correlated with elevated Akt activity (Manning and Cantley, 2007; Guertin and Sabatini, 2007). The dependence of Akt phosphorylation at Ser 473 on PLD implicates PA as a critical regulator of Akt-mediated survival signals. The PLD dependence was observed in renal cancer cells where there is elevated basal Akt phosphorylation and also on insulin-stimulated increases in Akt phosphorylation in a breast cancer cell line where there are reduced levels of Akt phosphorylation. Data were also presented that demonstrated that the suppression of mTORC1 by PRAS40 is reversed when PLD activity is

suppressed. Collectively, these data firmly establish a role for PLD-generated PA in the regulation of both mTORC1 and mTORC2 in human cancer cells and suggest that targeting PLD signaling represents a means for suppressing mTOR dependent survival signals and enhancing the efficacy of rapamycin based therapeutic strategies in cancers where PLD and mTOR are suppressing default apoptotic programs that protect against cancer.

While the specificity of rapamycin for mTOR has been known for some time, the mechanism of action has not been established. Jie Chen's group reported that PA interacted with mTOR in a manner that was competitive with rapamycin, but did not have any apparent impact on the kinase activity of mTOR (Fang *et al* ., 2001). Our group subsequently demonstrated that elevated PLD activity increased the concentration of rapamycin needed to suppress S6 kinase phosphorylation and cell proliferation (Chen *et al* ., 2003). Recent structural studies have revealed that PA interacts with the FRB domain of mTOR and causes similar structural changes observed when rapamycin-FKBP12 binds to the FRB domain (Veverka *et al* ., 2007). Sabatini's group reported recently that rapamycin could prevent the association of mTOR with other components of the mTORC2 complex. Our finding that PLD is required for the stability of complexes between mTOR and Rictor and between mTOR and Raptor is consistent with the observation that rapamycin prevents mTOR2 complex formation and that PA interacts with mTOR in a manner that is competitive with rapamycin (Fang *et al* ., 2001; Chen *et al* ., 2003; Veverka *et al* ., 2007). The implication being that PLD and PA regulate mTOR signaling by facilitating the formation of mTOR

complexes and that rapamycin inhibits mTOR by interfering with the PA-mTOR interaction.

Our previous study demonstrating that elevated PLD activity increased the dose of rapamycin needed to suppress S6 kinase phosphorylation and cell proliferation revealed something that was confusing. The amount of rapamycin needed to suppress cell proliferation was greater than that needed to suppress S6 kinase phosphorylation. Data provided in Figure 5.2.3 reveal that higher concentrations of 1-BtOH are required to suppress Akt phosphorylation at Ser 473 than are needed to suppress S6 kinase phosphorylation. This suggests that higher concentrations of 1-BtOH are required to suppress mTORC2 than are required to suppress mTORC1. The implication from this finding is that mTORC2 binds PA more strongly than mTORC1 and that lower concentrations of PA in the cell are needed when PA dissociates in order for PA to stay dissociated from mTORC2. This observation also suggests why higher concentrations of rapamycin are needed to compete for binding to mTORC2 than with mTORC1. The data provided in Figure 5.2.4 reveal a differential rapamycin dose response for HIF1α and HIF2α. The higher sensitivity of S6 kinase phosphorylation to rapamycin is consistent with a requirement for mTORC1, which has an apparent lower affinity for PA than mTORC2. The weaker association of PA with mTORC1 means that PA and mTORC1 will dissociate more often and thus, lower concentrations of rapamycin would be needed to replace PA on mTORC1 when there is a dissociation. In contrast, the higher concentration of rapamycin needed to suppress Akt phosphorylation at Ser473 reflects a requirement for only

mTORC2, which apparently binds PA more strongly and therefore, dissociations are rare – meaning that very high concentrations of rapamycin would be needed to bind the low concentrations of mTOR obtained when PA dissociates from mTORC2. Thus, the dissociation constant for PA and mTORC2 (K_{D2}) would be less than the dissociation constant for mTORC1 and PA (K_{D1}). This is depicted in a model shown in Fig. 5.3.1, where we propose that rapamycin-FKBP12 binds the FRB domain of mTOR only when PA is dissociated from the FRB domain. However, since the association of PA with mTORC2 is stronger than the association with mTORC1, the dissociation of PA from mTORC2 is less frequent and higher concentrations of rapamycin are needed interact with mTOR released from mTORC2. The rate constants described in the model are only for the interaction between mTOR and PA and therefore the model represents an oversimplification, since the involvement of Rictor and Raptor along with other components of the mTORC1 and mTORC2 complexes have been neglected. However, the model does provide a first approximation for the differential stability of mTORC1 and mTORC2 complexes that explains the observed differential sensitivities to rapamycin and PA.

We used two cell lines in this study, 786-O renal cancer cells and MDA-MB-231 breast cancer cells – both of which have high levels of PLD activity (Zheng *et al* ., 2006; Toschi *et al* ., 2008). The PLD activity MDA-MB-231 cells is highly elevated only in the absence of serum, whereas the elevated PLD activity in the 786-O cells is elevated both in the presence and absence of serum. While the significance of this difference is not clear, interestingly, Akt phosphorylation at

Ser473 is high in the 786-O cells and low in the MDA-MB-231 cells. The low level of Akt phosphorylation in the MDA-MB-231 cells in the absence of serum where PLD activity is high (Zheng *et al* ., 2006) clearly reveals that PLD activity and PA is not sufficient by itself to activate mTORC2 and cause the phosphorylation of Akt at Ser473. In contrast, the introduction of an exogenous PLD2 gene did stimulate the phosphorylation of S6 kinase (Chen *et al* ., 2005), suggesting that mTORC1 can be activated by elevated levels of PA. It will be of interest to determine what signals in addition to those that activate PLD are necessary for the activation of mTORC2. The data provided here showing that insulin can stimulate Akt phosphorylation in a PLD-dependent manner in the MDA-MB-231 cells may provide a lead as to the additional signals needed to activate mTORC2. Recently, Rosen and colleagues demonstrated that suppression of mTORC1 led to an increase in Akt phosphorylation at Ser473 that was dependent on IGF-1 (O'Reilly *et al* ., 2006). A similar observation was subsequently made by Wan *et al* . (2007), who additionally demonstrated that suppressing S6 kinase increased Akt phosphorylation. Thus, components of IGF-1 signaling may be an important component in addition to PA for activating mTORC2.

Rapamycin and rapamycin derivatives have been widely employed in clinical trials with mostly disappointing results (Sawyers, 2003). A recent clinical study focused on glioblastoma where there are commonly defects in PTEN (Cloughesy *et al* ., 2008). This study indicated that there was cell cycle arrest in response to rapamycin and effects on S6 kinase phosphorylation – implicating

mTORC1. As indicated here, mTORC1 is much more sensitive to rapamycin than mTORC2. However, Akt phosphorylation is dependent on mTORC2, indicating that mTORC2 may more critical in cancer – in that Akt phosphorylates many key substrates critical for cancer cell survival (Manning and Cantley, 2007). Thus, targeting mTOR effectively may require strategies that suppress mTORC2. As indicated in this study, suppressing PLD activity makes rapamycin effective in suppressing mTORC2 in 786-O cells, which have high levels of PLD activity (Toschi *et al* ., 2008). It is therefore possible that combining strategies that suppress PLD activity with rapamycin could improve the efficacy of rapamycin. While there are no drugs currently being used to target PLD directly, targeting the intracellular signals that increase PLD activity remains a possibility. We just recently reported that a natural product from Magnolia grandiflora known as honokiol suppresses PLD activity (Garcia *et al* ., 2008) and might therefore used in combination with rapamycin to suppress mTORC2. It will therefore be of interest to determine whether honokiol can improve the efficacy of rapamycin in cancer cells with elevated PLD activity. This study provides a rationale for targeting the signals that regulate PLD activity to increase the efficacy of rapamycin, which has had mixed results in clinical trials.

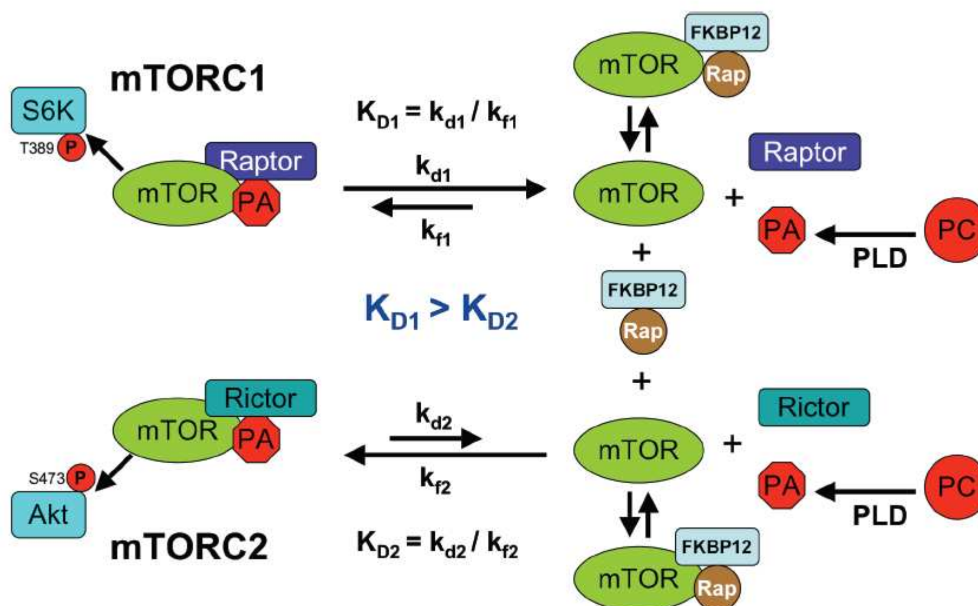

FIGURE 5.3.1: Model for the Differential Effects of Rapamycin and 1-BtOH on mTORC1 and mTORC2. The dissociation constants (K_D) for mTORC1 and mTORC2 with PA represent the ratios for the rate constants for dissociation (k_d) and formation (k_f). The data provided here are consistent with a model whereby the rate constant for the dissociation of mTORC1 to PA and mTOR (k_{d1}) is greater than rate constant for the dissociation of mTORC2 to PA and mTOR (k_{d2}). Thus, there are fewer dissociations of PA from mTORC2. The ability of rapamycin-FKBP12 to suppress mTORC1 and mTORC2 would be dependent on how frequently mTOR became available to bind rapamycin-FKBP12. There would be far more dissociated mTOR generated from mTORC1 than from mTORC2 and therefore less rapamycin would be required to compete with PA for binding to the mTOR derived from mTORC1. In contrast, the rare dissociations of mTORC2 would require much more rapamycin-FKBP12 to compete with PA to capture the rare mTOR proteins derived from mTORC2. Higher concentrations of 1-BtOH would reduce the levels of PA shifting the equilibrium in favor of dissociation of the mTOR complexes reducing the concentrations of rapamycin-FKBP12 needed to bind to and suppress mTOR.

CHAPTER VI

PHOSPHOLIPASE D-mTOR REQUIREMENT FOR THE WARBURG EFFECT IN HUMAN CANCER CELLS

6.1 INTRODUCTION

A hallmark of cancer cells is aerobic glycolysis whereby cells shut down oxidative phosphorylation in the mitochondria and rely on glycolysis for energy and the raw materials needed for cell growth (De Berardinis *et al.,* 2008). This effect is known as the Warburg effect after its discoverer (Warburg, 1956; Warburg 1956b). The effect has also been called “metabolic transformation” in that there is a profound change in cellular metabolism when cells need to double their mass prior to dividing into two daughter cells (De Berardinis *et al.,* 2008; De Berardinis *et al.,* 2008b). Glycolysis generates the precursors needed for the synthesis of lipids and nucleotides for generating membranes and nucleic acids (De Berardinis *et al.,* 2008b). A shift away from mitochondrial respiration also occurs as a response to the stress of hypoxia where oxidative phosphorylation is not an option (Hickey and Simon, 2006). Much of the response to hypoxia is due to elevated expression of hypoxia inducible factor-α (HIFα)2 – a family of transcription factors that stimulate the transcription of genes that encode glycolytic enzymes and angiogenesis factors (Hickey and Simon, 2006). HIFα expression is also elevated in a significant percentage of human cancers (Gordan and Simon, 2007).

The expression of the α subunits for both HIF1 and HIF2 is dependent upon phospholipase D (PLD) in human kidney and breast cancer cells (Toschi *et al.,* 2008; Zheng *et al.,* 2006). Elevated PLD activity in human cancer cells provides both survival and migration signals (Zheng *et al.,* 2006; Chen *et al.,* 2006). The primary metabolite of PLD is phosphatidic acid and is required for the

activation of the mammalian target of rapamycin (mTOR) (Fang *et al.,* 2001; Veverka *et al.,* Foster, 2007), which has also been implicated in survival signals and HIFα expression (Sawyers, 2003; Kim and Kaelin, 2006). mTOR has been implicated as a sensor of nutritional sufficiency and elevated mTOR promotes cell cycle progression when there is sufficient nutrition for cells to double their mass and divide (Wullschleger *et al.,* 2006). Thus, there is a connection between PLD-mTOR survival signals and the Warburg effect in cancer cells. We have investigated whether the Warburg effect is dependent on PLD-mTOR signaling in human cancer cells. We report here that the metabolic shift away from mitochondrial respiration to aerobic glycolysis in both breast and kidney cancer cells is dependent on PLD and mTOR.

6.2 RESULTS

6.2.1 GLUCOSE UPTAKE IN HUMAN CANCER CELLS CORRELATES WITH PLD ACTIVITY

Glucose uptake was examined in four human cell lines – two breast cancer cell lines (MCF-7 and MDA-MB-231), a kidney cancer cell line (786-O), and HEK293 human embryonic kidney cells. These cells have been analyzed previously for their PLD activity with MDA-MB-231 cells having high levels of PLD activity relative to the MCF-7 cells, and 786-O cells having high levels of PLD activity relative to the HEK293 cells (Toschi *et al.,* 2008; Chen *et al.,* 2003; Zhong

et al., 2002). This is shown graphically in Figure 6.2.1 A. The level of glucose uptake in these cells was investigated by examining the uptake of a fluorescent-tagged glucose 2-NBDG. As shown in Figure 6.2.1 B, the level of glucose uptake was strongly correlated to the level of PLD activity in these cell lines. The uptake of glucose is represented graphically in Figure 6.2.1 C.

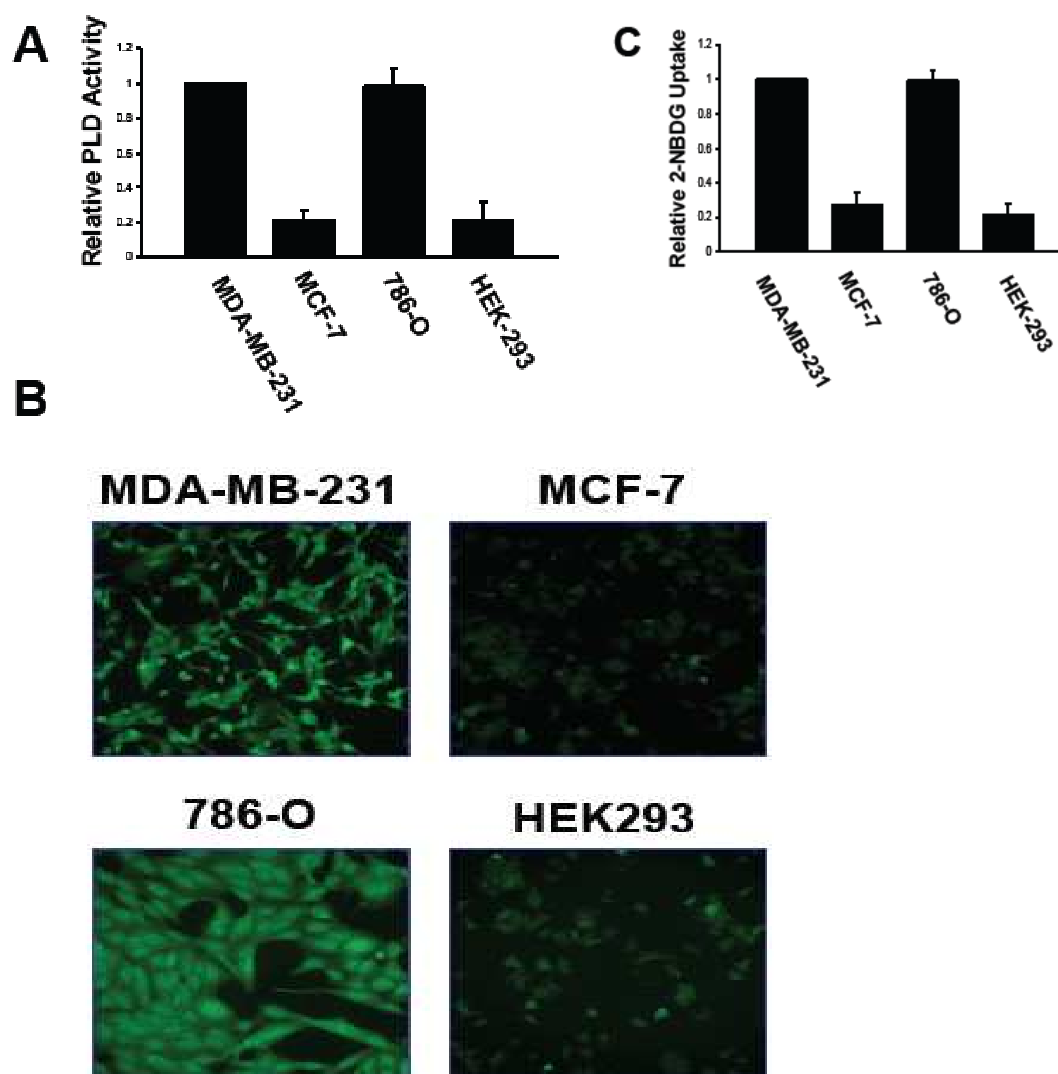

FIGURE 6.2.1: Glucose uptake in human cancer cells correlates with PLD activity. A) MDA-MB-231, MCF-7, 786-O, and HEK293 cells were plated at 80% confluence. 24 hr later, the cells were shifted to media containing 0.5% serum overnight prior to performing the assay. PLD activity was determined using the transphosphatidylation reaction in the presence of 0.8% 1-BtOH and the relative values of phosphatidyl-BtOH were determined using thin layer chromatography as described in Experimental Procedures. The PLD activity was normalized to that observed in the MDA-MB-231 cells which was given a value of one. The error bars represent the standard error for three independent experiments. The relative levels of PLD activity were obtained by normalizing to the level of PLD activity in the MDA-MB-231 cells, which was given a value of 100%. B) MDA-MB-231, MCF-7, 786-O, and HEK293 cells were prepared as in A. The cells were then treated with 2-NDBG (200μM) and incubated for 2 hr. The fluorescence

in the cells was then visualized by inverted fluorescent microscopy. C) Triplicate samples were prepared as in B the fluorescence was quantified by spectrofluorimetry as described in Experiments Procedures. The fluorescence was normalized to that observed in the MDA-MB-231 cells which was given a value of one. Error bars represent the standard error for a representative experiment. All experiments shown are representative of at least three independent experiments.

6.2.2 GLUCOSE UPTAKE IS BLOCKED BY DOMINAND NEGATIVE PLD MUTANTS

We next examined whether the elevated glucose uptake observed in the MDA-MB-231 and 786-O cells was dependent on the elevated PLD activity in these cells. The MDAMB-231 and 786-O cells were transiently transfected with catalytically inactive PLD1 and PLD2 mutants, which function asdominant negative PLD mutants (Toschi *et al.,* 2008; Shen *et al.,* 2001). As shown in Figure 6.2.2 A, the dominant negative mutants for both PLD1 and PLD2 suppressed the uptake of glucose into the cytoplasm of both MDA-MB-231 and 786-O cells. The expression of both PLD1
and PLD2 mutants together was even more effective in suppressing glucose uptake – especially in the 786-O cells (Fig.2A).

The 786-O cells have elevated HIF2α expression by virtue of the lack of the von Hippel-Lindau (VHL) gene product pVHL, a substrate-conferring component of an E3 ubiquitin ligase that targets HIFα for degradation by the proteasome (Ohh, 2006). The VHL gene has been re-introduced into the 786-O cells (786-VHL), which suppresses HIF2α expression. HIF2α expression can be induced with hypoxia or hypoxia-mimetic conditions in these cells (Lonergan *et al.,* 1998; Ohh *et al.,* 1998). Since HIF2α has been implicated in the expression

of glycolytic genes, we investigated the effect of the PLD mutants on glucose uptake stimulated by hypoxia-mimetic conditions in the 786-VHL cells. As expected, glucose uptake in the 786-VHL cells was reduced relative to the parental 786-O cells (Figure 6.2.2 A and B). However, CoCl2, which acts as a hypoxia-mimetic agent by suppressing the prolyl-hydroxylase activity that targets HIFα for degradation in presence of O2 (Epstein *et al.,* 2001), restored the elevated glucose uptake (Figure 6.2.2 B). We then investigated the effect of dominant-negative PLD1 and PLD2 on the CoCl2- induced increase in glucose uptake, and as shown in Figure 6.2.2 B, glucose uptake was suppressed in the presence of the PLD mutants. We also stimulated the MCF-7 cells with CoCl2 and found a similar increase in glucose uptake. And as shown in Figure 6.2.2 B, the CoCl2-induced increase in glucose uptake in these cells was similarly blocked by the dominant-negative PLD mutants. The lack of glucose uptake in the 786-VHL cells suggests that the increased glucose uptake is dependent on HIF2α, which is consistent with our previous report that HIF2α expression is dependent on PLD activity (Toschi *et al.,* 2008). Collectively, the data in Figure 6.2.2 demonstrate that glucose uptake is dependent on PLD activity in both kidney and breast cancer cells.

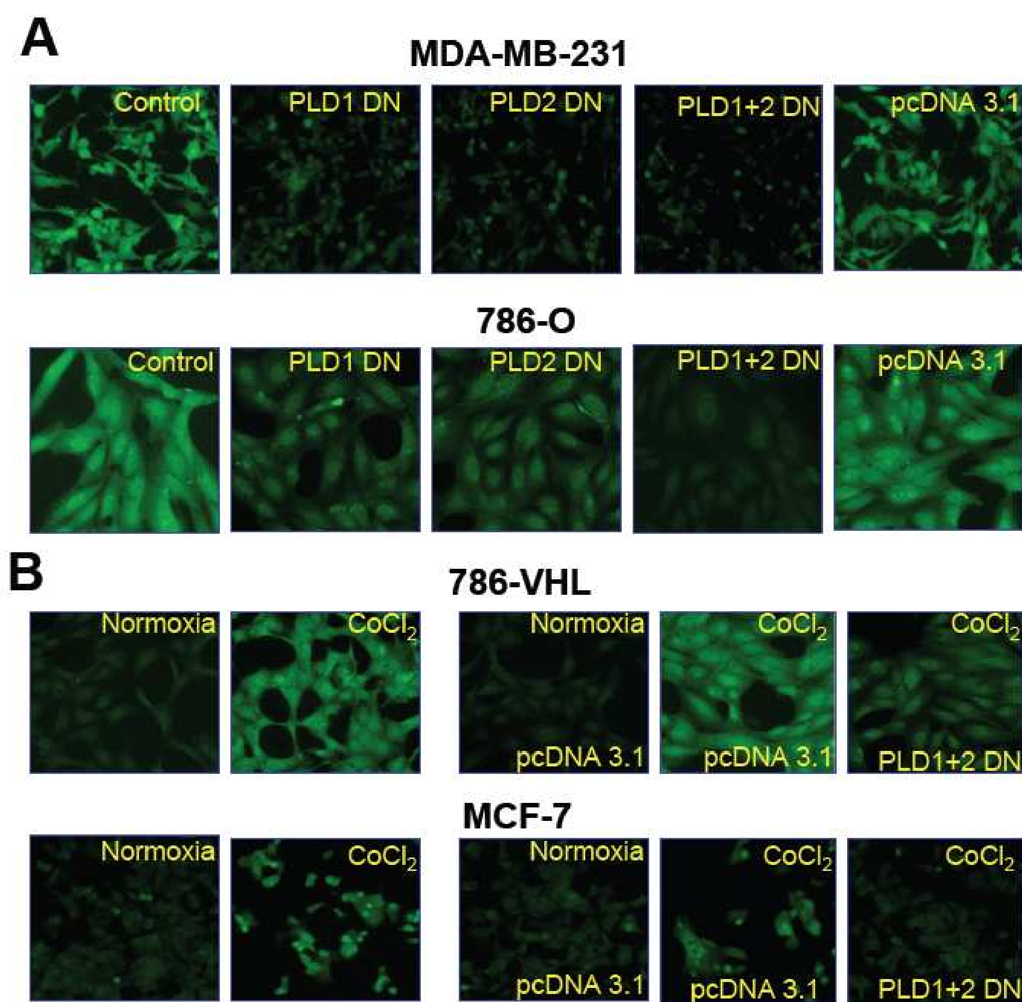

FIGURE 6.2.2: Increased glucose uptake in MDA-MB-231 and 786-O cells is blocked by dominant negative PLD mutants. A) MDA-MB-231 and 786-O cells were plated at 80% confluence. 24 hr later, the cells were transfected with plasmid vectors expressing either PLD1 or PLD2 as indicated. The parental vector was pcDNA 3.1, which was used as an empty vector control. The control cells shown were treated with transfection reagent, but without DNA. 24 hr later, the cells were given fresh media with 0.5% serum and incubated for an additional 24 hr. The following day 2-NDBG uptake was evaluated as in Figure 6.2.1 B. B) Two left panels: 786-VHL and MCF-7 cells were prepared as in Figure 6.2.1 and placed in media containing 0.5% serum in the presence or absence of CoCl2 (150 µM) overnight. The following day 2-NDBG was added and fluorescence was determined as in Figure 6.2.1 . In the three right panels, cells were prepared as in A and transfected with the indicated plasmids. 24 hr later the cells were placed in media containing 0.5% serum overnight in the presence or absence of CoCl2 (150 µM). The following day 2-NDBG was added and fluorescence was determined as in Figure 6.2.1.

6.2.3 GLUCOSE TRANSPORTER EXPRESSION IS DEPENDENT ON PLD

A key aspect of increased uptake of glucose is the expression level of glucose transporters (GLUT) – the membrane proteins that facilitate passage of glucose through the plasma membrane. We therefore examined the effect of the dominantnegative PLD mutants on the expression levels of GLUT1, GLUT3 and GLUT4 in 786-O and in 786-VHL cells. As shown in Figure 6.2.3 A, the levels of all three glucose transporters was substantially reduced in the 786-O cells in the presence of the dominant negative PLD1 and PLD2 mutants. GLUT expression was also suppressed by the PLD mutants in the 786-VHL cells treated with CoCl2 (Figure 6.2.3 A). Of interest was that GLUT1 expression was increased the most by CoCl2 treatment in the 786-VHL cells and correlated most strongly with HIF2α expression (Figure 6.2.3 A). We also examined the effect of the dominant-negative PLD mutants on GLUT expression in MDAMB-231 cells, and as shown in Figure 6.2.3 B, the expression of GLUT1, GLUT3 and GLUT4 were all suppressed by the PLD mutants. These data indicate that PLD activity is required for the expression of GLUTs and is consistent with the PLD requirement for increased glucose uptake in cancer cells.

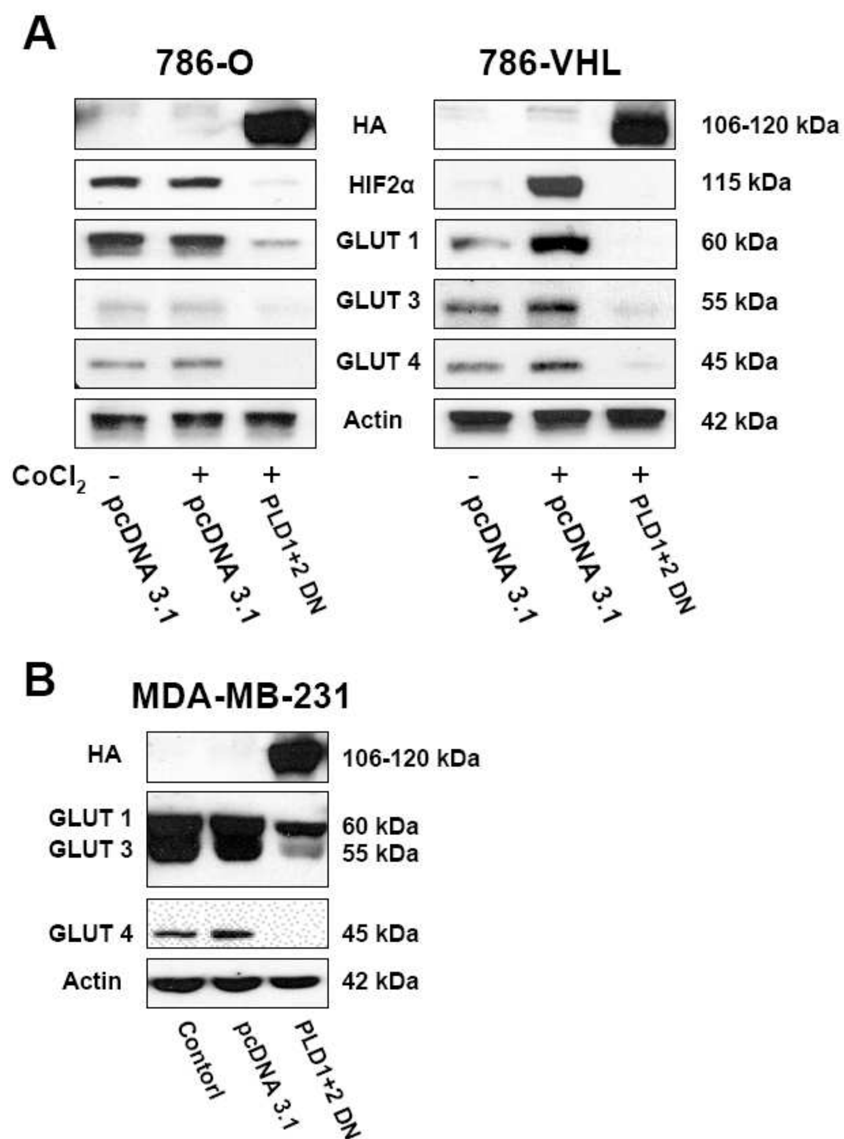

FIGURE 6.2.3: Glucose transporter expression is dependent on PLD activity. 786-O and 786-VHL (*A*) and MDA-MB-231 (*B*) cells were plated and 24 hr later were transfected with the indicated plasmid vectors as in Figure 6.2.2 . 24 hr later the cells were shifted to DMEM containing 0.5% serum and CoCl2 (150 μM) overnight where indicated. The following day the cells were evaluated for the expression of GLUT1, GLUT3, and GLUT4 by Western blot analysis. We also evaluated the expression of HIF2α in the 786-0 and 786-VHL cells. Expression of the PLD mutants was evaluated by probing the blots for the HA tags on the PLD mutants. Blots were also probed for actin as loading controls. The experiments shown are representative of at least two independent experiments.

6.2.4 LACTATE PRODUCTION IS DEPENDENT ON PLD ACTIVITY

A hallmark of the Warburg effect is the reduction of pyruvate, the end product of glycolysis, to lactic acid, which is accompanied by the oxidation of NADH to NAD+. This reaction takes place instead of the conversion of pyruvate to acetyl-CoA, which can enter the tri-carboxilic acid (TCA) cycle (Figure 6.2.4 A).

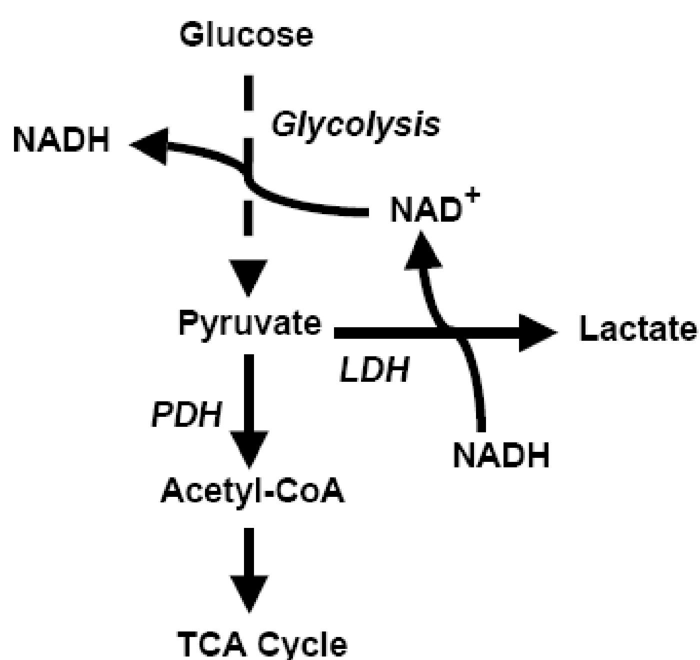

FIGURE 6.2.4: Lactate production is dependent on PLD activity. A) A model for the different fates of pyruvate generated by glycolysis is shown where pyruvate is converted either to lactic acid by lactate dehydrogenase (LDH) or to acetyl-CoA by pyruvate dehydrogenase (PDH).

We therefore examined the levels of lactic acid in the media of 786-O and MDA-MB-231 cells with and without the dominant negative PLD mutants. As shown in Figure 6.2.4 B, the levels of secreted lactic acid were substantially reduced in the cells that were transfected with the PLD mutants. The production of lactic acid should lower the pH of the media and as expected, the pH was higher in the cells with the PLD mutants (Figure 6.2.4 C). These data reveal that PLD activity is required for the shift to lactate production that is a characteristic of the Warburg effect.

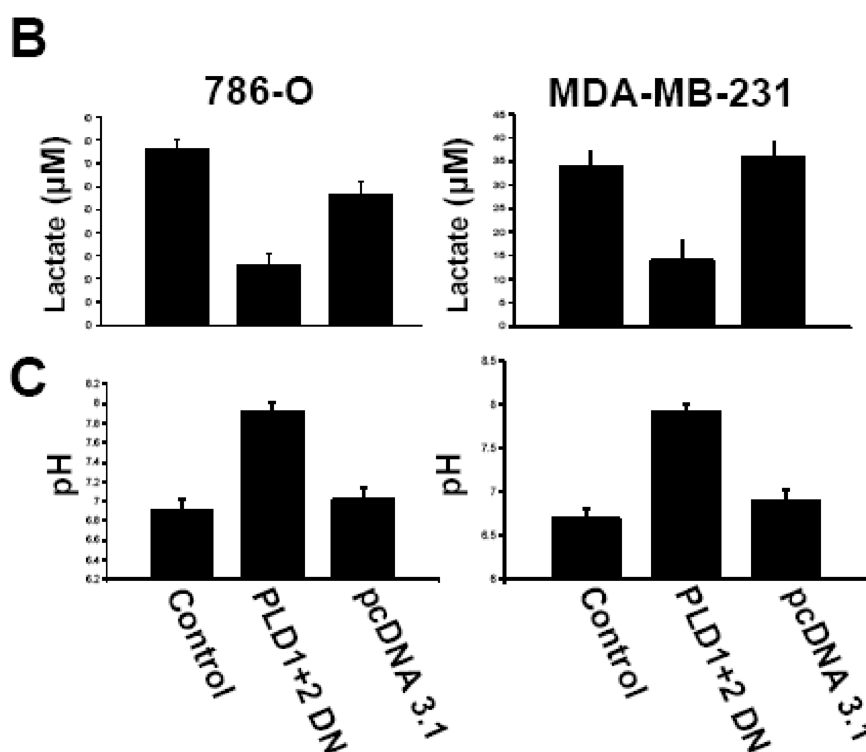

FIGURE 6.2.4: (CONTINUED). MDA-MB-231 and 786-O cells were prepared and transfected with the indicated vectors as in Figure 6.2.2 . 24 hr after transfection, the cells were counted and replated in 60mm plates at 8.0 x 105 cells/plate, in media containing 0.5% serum. The levels of lactic acid (*B*) and pH of the media (*C*) were determined 24 hr later as described in Materials and Methods. The experiments shown are representative of at least two independent experiments.

6.2.5 PLD IS REQUIRED FOR PYRUVATE DEHYDROGENASE KINASE 1 EXPRESSION

Another characteristic of the Warburg effect is the suppression of the TCA cycle, which is the step after pyruvate generation in the oxidation of glucose to CO2 and H2O. It is during the TCA cycle that most of the NADH is generated to be oxidized during subsequent mitochondrial respiration. A key enzyme for controlling the TCA cycle is pyruvate dehydrogenase, which converts pyruvate to acetyl-CoA, which in turn reacts with oxaloacetate to form TCA and begin the cycle. Pyruvate dehydrogenase is regulated by pyruvate dehydrogenase kinase 1 (PDK1). PKD1 phosphorylates pyruvate dehydrogenase and suppresses its activity. PDK1 expression is regulated by HIF1α in mouse embryo fibroblasts (Kim *et al.,* 2006). Elevated HIF1α expression in MDA-MB-231 cells is dependent on PLD (Zheng *et al.,* 2006). The 786-O cells do not express HIF1α, but do express HIF2α (Toschi *et al.,* 2008), which also stimulates the expression of glycolytic enzymes. The expression of HIF2α in these cells is also dependent on PLD activity (Toschi *et al.,* 2008). We therefore examined the effect of the dominant negative PLD mutants on the level of PDK1. As shown in Figure 6.2.5 A, introduction of the dominant negative PLD mutants suppressed the expression of PDK1 in both the MDA-MB-231 and 786-O cells.

We also investigate the effect primary alcohol treatment, which prevents the conversion of phosphatidylcholine to phosphatidic acid by PLD (20). 1-BtOH serves as a better substrate that H2O and phosphatidyl-BtOH is generated instead of phosphatidic acid (Shen *et al.,* 2001). As shown in Figure 6.2.5 B, a

two hour treatment with 1-BtOH reduced the expression of PDK1. This was not observed if t-BtOH, which is not utilized by PLD, was used. These observations further support a PLD requirement for the Warburg effect in these cancer cells.

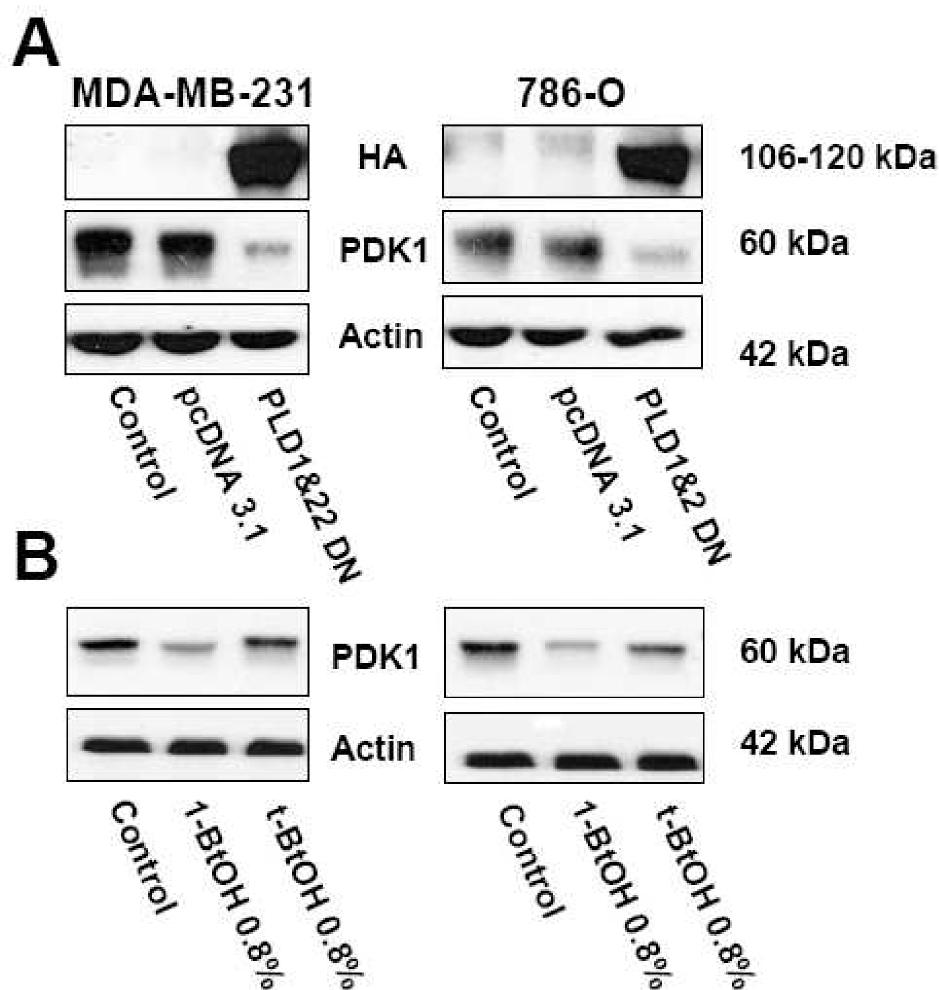

Figure 6.2.5: PLD is required for PDK1 expression. A)MDAMB-231 and 786-O cells were transfected with the indicated plasmid vectors as in Fig. 3. The levels PDK1, HA, and actin were determined by Western blot analysis 18 hr later. B) MDAMB-231 and 786-O cells were plated and 24hr later were placed in media containing 0.5% serum overnight. The cells were then treated with either 0.8% 1-BtOH or 0.8% t-BtOH for 2 hr. The cells were then harvested and the expression of PDK1 and actin was evaluated by Western blot analysis. The experiments shown are representative of at least two independent experiments.

6.2.6 SUPPRESSION OF PLD RESTORES OXYDATIVE PHOSPHORYLATION

Another hallmark of the Warburg effect is the shut down of oxidative phosphorylation in the mitochondria. Oxidative reactions in the mitochondria result in the production of reactive oxygen species (ROS), which are converted to H2O2 by superoxide dismutase. We therefore examined the effect of suppressing PLD activity on the production of ROS and H2O2 in both MDA-MB-231 and 786-O cells. We examined the effect of the dominant negative PLD mutants on the level intracellular oxidants by staining cells with H2DCFDA, which is oxidized by ROS to the highly fluorescent DCF. As shown in Figure 6.2.6 A, introduction of the dominant negative PLD1 and PLD2 mutants significantly increased the level of DCF. The effect was reversed by the mitochondrial complex 1 inhibitor rotenone, indicating that increased level of ROS was due to mitochondrial activity. We also examined the level of H2O2 in the MDA-MB-231 and 786-O cells. As indicated in Figure 6.2.6 B, the level of H2O2 in both cell lines was dramatically increased when the dominant negative PLD mutants were introduced. These data further support the hypothesis that PLD activity is required for suppression of mitochondrial oxidative phosphorylation.

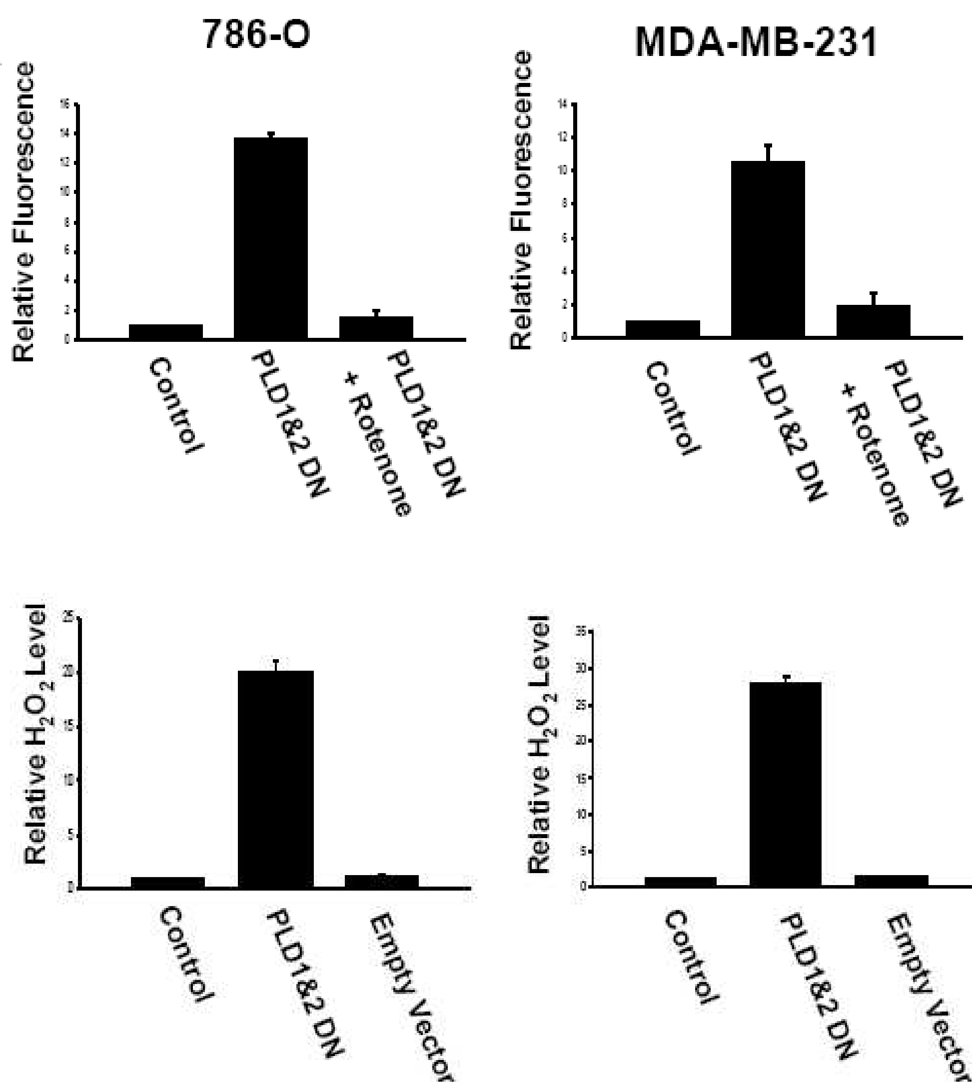

FIGURE 6.2.6: Suppression of PLD restores oxidative phosphorylation. A) MDA-MB-231 and 786-O cells were prepared and transfected with the indicated vectors as in Figure 6.2.2 . 24 hr later, the media was replaced with fresh media containing 0.5% serum and the cells were incubated for an additional 24 hr in the absence or presence of rotenone (0.1 μM). H2DCFDA (5 μM) was then added for 1 hr, after which the cells were washed and incubated in fresh media without H2DCFDA for an additional 30 min. The fluorescent oxidized DCF was then evaluated as described in Experimental Procedures. B) MDA-MB-231 and 786-O cells were prepared and transfected with the indicated vectors as in Figure 6.2.2 and the levels of H2O2 were determined as described in Experimental Procedures. The experiments shown are representative of at least two independent experiments.

Although mitochondrial respiration generates ATP more efficiently, ATP is generated more rapidly with elevated glucose uptake and glycolysis (Bui and Thompson, 2006). Shutting down the TCA cycle with forced expression of PDK1 was shown to increase cellular ATP levels (Kim *et al.,* 2006). We therefore examined the impact of the PLD dominant negative mutants on cellular ATP levels in the MDAMB-231 and 786-O cells, and as shown in Figure 6.2.6 C, the PLD mutants reduced cellular ATP levels. Collectively, the data in Figure 6.2.6 reveal that the elevated PLD activity in both MDA-MB-231 and 786-O cancer cell lines is required for suppression of mitochondrial respiration – a hallmark of the Warburg effect in cancer cells.

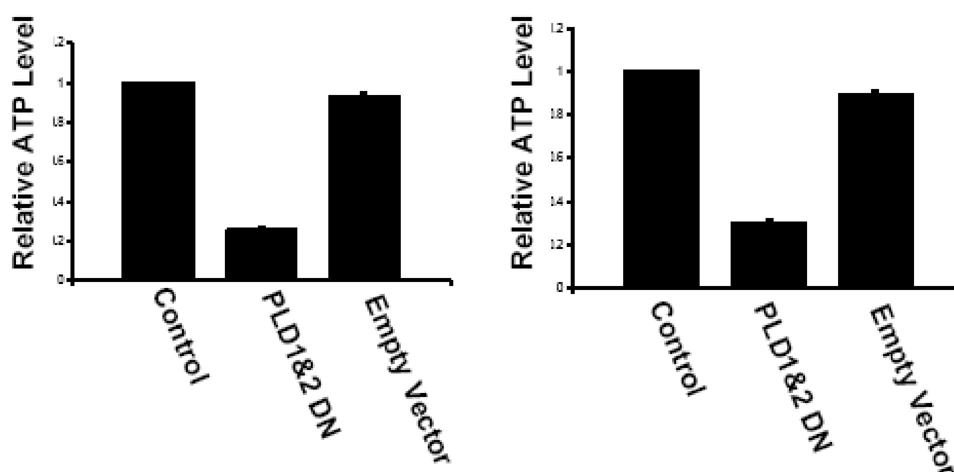

FIGURE 6.2.6: (Continued). MDA-MB-231 and 786-O cells were prepared and transfected with the indicated vectors as in Figure 6.2.2 and the levels of ATP were etermined as described in Experimental Procedures. The experiments shown are representative of at least two independent experiments.

6.2.7 THE EFFECT OF PLD ON GLUCOSE UPTAKE IS MEDIATED BY mTOR

A critical downstream target of PLD is mTOR – the mammalian target of rapamycin (Foster, 2007; Foster, 2004). We therefore examined whether the dependence of glucose uptake was also dependent on mTOR. mTOR exists as two complexes – mTORC1 and mTORC2, which have differential sensitivities to rapamycin. The mTOR complexes have different components that are required for their activity – mTORC1 is dependent upon a protein known as Raptor and mTORC2 is dependent on Rictor (Guertin and Sabatini, 2007). We examined the effect of depleting cells of mTOR, Raptor, and Rictor on glucose uptake in the MDA-MB-231 and 786-O cells. In the 786-O cells, we found that siRNA for mTOR strongly suppressed glucose uptake (Figure 6.2.7 A). Rictor siRNA also strongly suppressed glucose uptake, whereas the siRNA for Raptor did not strongly suppress glucose uptake. These data indicate that the elevated glucose uptake in the renal cancer cell line 786-O is dependent on mTORC2. We also examined the effect of suppressing mTOR, Raptor, and Rictor on glucose uptake in the MDA-MB-231 cells. And as shown in Figure 6.2.7 B, glucose uptake was strongly suppressed by mTOR siRNA. However, we saw the reverse effect with Raptor and Rictor siRNA in the MDA-MB-231 cells whereby the Raptor siRNA suppressed glucose more strongly than the Rictor siRNA. Thus, in the MDA-MB-231 cells, the elevated glucose uptake is apparently dependent upon mTORC1.

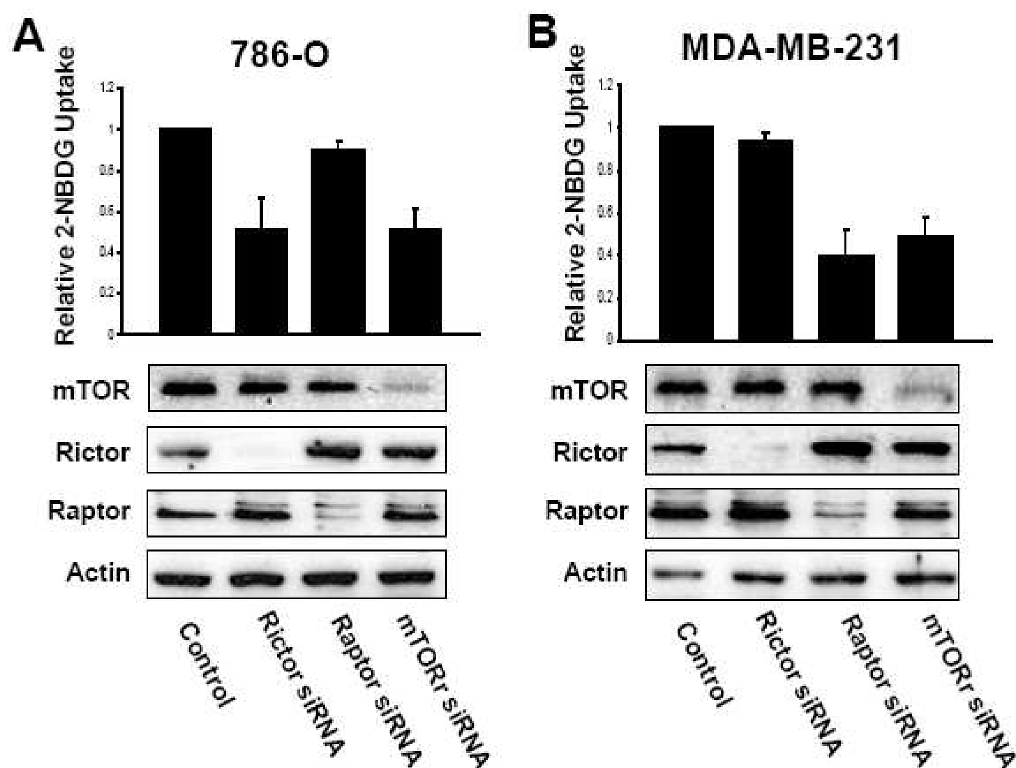

FIGURE 6.2.7: The effect of PLD on glucose uptake is mediated by mTOR. A) 786-O cells were plated at 30% confluence. 24 hr later the cells were transfected with siRNAs for mTOR, Raptor and Rictor as described in the Experimental Procedures. 24 hr later, the cells were shifted to media containing 0.5% serum and 2-NDBG uptake was evaluated 24 hr later as in Fig. 1. The expression of mTOR, Raptor, and Rictor was evaluated by Western blot. B) MDA-MB-231 were treated as in A and evaluated for 2-NDBG uptake and the expression of mTOR, Raptor and Rictor. The experiments shown are representative of at least two independent experiments.

6.2.8 ELEVATED GLUCOSE UPTAKE IN 786-O CELLS IS DEPENDENT ON AKT2

A target of mTORC2 is Akt, which gets phosphorylated by mTORC2 at Ser473 (Sorbasso *et al.,* 2005). Akt has also been shown to stimulate aerobic glycolysis (Elstrom *et al.,* 2004) There are three Akt isoforms, of which Akt1 and Akt2 are expressed ubiquitously (Manning and Cantley, 2007). Akt1-deficient mice have developmental defects and Akt2-deficient mice have defects in glucose homeostasis. The MDA-MB-231 and 786-O cells were treated with siRNAs for Akt1 and Akt2 and the levels of glucose uptake was evaluated. As shown in Figure 6.2.8 , depleting cells of Akt2, but not Akt1 abolished glucose uptake in the 786-O, but not in the MDA-MB-231 cells. Depleting cells of Akt1 did not have any significant impact on glucose uptake in either the MDAMB-231 or 786-O cells. These data indicate that Akt2, a critical downstream target of mTORC2, is critical for the elevated glucose uptake in 786-O cells. These data are consistent with the dependence of glucose uptake in these cells on mTORC2 observed in Figure 6.2.7 . They are also consistent with the dependence of glucose uptake on mTORC1 in MDA-MB-231 cells – in that Akt is not a target of mTORC1 (Guertin and Sabatin, 2007). This finding is consistent with the observation that mice with defective Akt have defects in glucose homeostasis (Dummler and Hemmings, 2007).

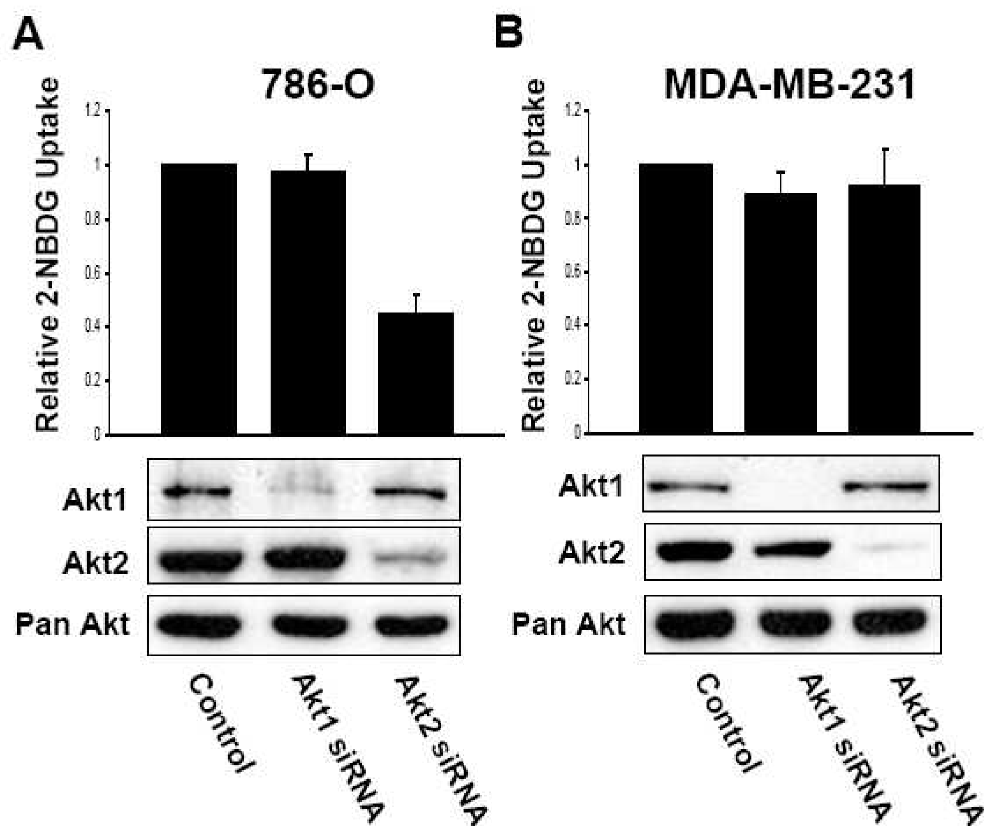

FIGURE 6.2.8: Elevated glucose uptake in 786-O cells is dependent on Akt2. 786-O (*A*) and MDA-MB-231 (*B*) cells were transfected with siRNAs for Akt1 and Akt2 as described in Figure 6.2.7 . 24 hr later, the cells were shifted to media containing 0.5% serum and 2-NDBG uptake was evaluated 24 hr later. A Western blot is shown that reveals that the siRNAs successfully suppressed the expression of Akt1 and Akt. The experiments shown are representative of at least two independent experiments.

6.3 DISCUSSION

The metabolic transformation that takes place in most cancer cells, first observed by Otto Warburg in the 1920s (Warburg, 1956; Warburg, 1956b),has attracted renewed attention of late as it has become apparent that the altered metabolism is closely integrated into the oncogenic transformation of cancer cells. Increasingly, it is being discovered that the intra-cellular signals altered in cancer cells result in metabolic as well as oncogenic transformation. One of the key regulators of the metabolic shift is HIFα, which is stabilized in response to hypoxic and other stressful conditions. In a majority of renal cancers, there is a defect in the VHL gene, which encodes a component of the E3 ubiquitin ligase complex that targets HIFα for degradation (Kaelin, 2005; Kaelin, 2007). The metabolic shift that occurs in cancer cells confers several advantages that allow cells to survive in an emerging tumor mass where there is inconsistent vascularization. The metabolic shift also allows for the products of glycolysis to be shunted off for the synthesis of nucleotides and fatty acids needed for cell growth (Kroemer and Pouyssegur, 2008). By dramatically increasing the levels of glucose uptake, the cancer cells actually produce more ATP than by the complete oxidation to CO2 and H2O via the respiratory chain in the mitochondria, which produces dramatically more ATP per molecule glucose (Bui and Thompson, 2006). We have previously reported that elevated PLD activity in human cancer cells provides a survival signal that suppresses apoptosis under the stress of serum withdrawal (Toschi *et al.,* 2008; Zheng *et al.,* 2006; Chen *et al.,* 2005; Zhong *et al.,* 2002; Gadir *et al.,* 2008). We also reported that elevated

PLD activity in VHL-null renal cancer cells is required for the expression of both HIF1α and HIF2α (Toschi *et al.,* 2008). In this report, we established that the elevated PLD activity in both breast and renal cancer cells is required for the metabolic shift that results in increased glucose uptake, suppression of the TCA cycle, and the shutdown of oxidative phosphorylation.

A critical target of the phosphatidic acid generated by PLD is mTOR (Foster, 2007). Consistent with a role for mTOR in the PLDdependent increase in glucose uptake, suppression of mTOR expression reduced glucose uptake in both the 786-O and MDAMB- 231 cells. Interestingly, glucose uptake in the MDA-MB-231 cells was dependent mTORC1, whereas in the 786-O cells, glucose uptake was dependent on mTORC2. There is substantial data supporting a role for PA in the regulation of mTORC1 (Foster, 2007) including the very recent finding that Rheb, a GTPase that activates mTORC1, activates PLD1 directly (Sun *et al.,* 2008). However, the mTORC2 requirement for glucose uptake demonstrated here suggests that PA may also regulate mTORC2. Consistent with this hypothesis, we recently found that there is a phosphatidic acid requirement for the assembly of both mTORC1 and mTORC2 complexes (Toschi *et al.,* Provisionally Accepted). The lack of an mTORC2 requirement in the MDA-MB-231 cells is consistent with the observation that there are very low levels of Akt phosphorylation at the mTORC2 site at Ser473 (Chen *et al.,* 2005). The observation here that Akt2 is required for the effect in 786-O, but not in the MDA-MB-231 cells, is also consistent the differential dependence on mTORC1 and

mTORC2. The data are consistent with a model whereby both mTORC1 and mTORC2 can contribute to the metabolic transformation in human cancer cells.

We demonstrated previously that HIF1α levels are elevated in response to PLD activity in the MDA-MB-231 cells (Zheng *et al.,* 2006). The 786-O cells used here express only HIF2α, which is also dependent on PLD activity (Toschi *et al.,* 2008). Since both HIF1α and HIF2α have been implicated in the Warburg effect and the metabolic shift to aerobic glycolysis in cancer cells, the data provided here suggest that there are two independent routes to metabolic transformation – one that goes through mTORC1 and HIF1α and another that goes through mTORC2, Akt2 and HIF2α. PLD would seem to be a common denominator for both pathways. There is clearly more to be learned about the signals that regulate the metabolic shift that occurs in cancer cells and there is already evidence that targeting of these signals and the altered metabolism in cancer cells has therapeutic potential.

The tumorigenicity of cancer cells was severely diminished by knockdown of the lactate dehydrogenase gene (Fantin *et al.,* 2006). By suppressing the PLD activity necessary for the increased glucose uptake in both the MDA-MB-231 and 786-O cells used in this study, we induce apoptosis in the absence of serum (Toschi *et al.,* 2008; Chen *et al.*, 2005). Suppression of HIF2α expression in renal cancer cells suppressed tumorigenesis (Kondo *et al.,* 2002; Kondo *et al.,* 2003). Thus, targeting both the metabolic changes and the signals that bring about these metabolic changes has already been shown to have therapeutic

potential. In fact, it was recently proposed that tumor cell metabolism might be the Achilles' heel of cancer cells (Gadir *et al.,* 2008). The appetite of cancer cells for glucose could also be used to improve drug delivery specifically to cancer cells. Consistent with this theory, it was reported that a saccharide conjugated to an agent used in photodynamic therapy increased the specificity of uptake of the reagent for cancer cells (Chen *et al.,* 2004). Thus, the increased glucose uptake by cancer cells that occurs with metabolic transformation may represent a means for improving drug delivery specifically to cancer cells. Learning more about the metabolic shift that occurs in cancer cells will likely reveal many new possibilities for therapeutic intervention in cancer.

CHAPTER VII

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