THE ROLE OF PHOSPHOLIPASE D IN HUMAN CANCER CELLS WITH ACTIVATED RAS

by

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A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2008

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This manuscript has been read and accepted for the
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ABSTRACT

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Ming Shi

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Phospholipase D (PLD) is a ubiquitous enzyme which hydrolyzes phosphatidylcholine to phosphatidic acid and choline. PLD can be activated by various factors and plays a vital role in cell transformation, survival and other cellular processes. Activating Ras mutations are common in many human cancers and have been found to play important roles in cell proliferation and survival. In this dissertation we establish a role for PLD in the survival of human cancer cells with activating mutations in both H-Ras and K-Ras.

T24 bladder carcinoma cells express an activated H-Ras gene and Calu-1 lung carcinoma cells express an activated K-Ras gene. We found that both of these cancer cell lines have highly elevated levels of PLD activity and that the PLD activity is dependent on Ras. The PLD activity was also dependent on two downstream targets of Ras signaling RalA and phosphatidylinositol-3-kinase. Suppression of Ras, RalA and phosphatidylinositol-3-kinase resulted in apoptosis in the absence of serum. Suppression of PLD activity in the T24 and Calu-1 cells resulted in apoptotic cell death in the absence of serum, indicating that the elevated PLD activity provides a survival signal in these cancer cell lines. These data indicate that a critical component

of Ras signaling in human cancer cells is the activation of PLD.

We also examined the effects of H-Ras and K-Ras in 3Y1 rat fibroblasts. We found that H-Ras reduced cell viability whereas K-Ras did not. These data suggest a rationale for the high frequency of K-Ras mutation in human cancers relative to H-Ras because human cancers with activated H-Ras are more likely to undergo apoptosis unless a survival signal is provided to prevent this from taking place.

Collectively, the studies described in this dissertation suggest that targeting PLD survival signals in cancer cells could be an effective strategy to induce apoptosis in human cancer cells with activated Ras.

Acknowledgements

I would like to thank my mentor Dr. David A. Foster for his guidance and support during my Ph.D. study. Dr. Foster is passionate for science research and is a great mentor, he has set an excellent role model for me.

I also like to thank my thesis committee members Dr. Derrick Brazill, Dr. Patricia Rockwell, Dr. Lu-Hai Wang, and Dr. Susan Rotenberg for their constructive and helpful comments and suggestions.

I am also grateful to my former and current colleagues Dr. Lizhong Xu, Dr. Yang Zheng, Donggon Lyo, Noga Gadir, Alfredo Toschi, and Avalon Garcia for their help and support.

I also want to thank Department of Biological Sciences of Hunter College and Biochemistry Ph.D. Program of CUNY Graduate Center for their financial support.

Finally I want to dedicate this dissertation to my parents and thank them for bringing me into this world and teaching me great values.

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List of Abbreviations

ARF, adenosine-diphosphate (ADP)-ribosylation factor
b-FGF, basic-fibroblast growth factor
CEMM, caveolin-enriched (light) membrane microdomain
EGF, epidermal growth factor
EGFR, epidermal growth factor receptor
GAP, GTPase-activating proteins
GDS, GDP dissociation stimulator
GEFs, guanine nucleotide exchange factors
GPCR, G protein-coupled receptors
HKD domains, H(x)K(xxxx)D motif with histidine (H), lysine (K) and aspartate (D)
IP_3, inositol triphosphate
LysoPA, lysophosphatidic acid
MAP kinase, mitogen-activated protein kinase
PA, phosphatidic acid
PAP, phosphatidic acid phosphohydrolase
PC, phosphatidylcholine
PE, phosphatidylethanolamine
PDGF, platelet-derived growth factor
PDGFR, PDGF receptor
PI, phosphoinositide
PI3K, PI3-kinase, phosphatidylinositol-3 kinase
PI-PLC, phosphoinositide-specific PLC
PIP_2, $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate
PKC, protein kinase C
PLA, phospholipase A
PLC, phospholipase C
PLD, phospholipase D
PS, phosphatidylserine
PTK, protein tyrosine kinase
1-BtOH, primary butanol

Chapter I. Introduction

1.1. Phospholipase D and Its regulation

1.1.1. General properties of PLD

Phospholipase D (PLD) is a ubiquitous enzyme found in bacteria (Hodgson et al., 1990), fungi, yeasts (Rose et al., 1995), plants (Wang et al., 1994) and animals (Hammond et al., 1995; Lopez et al., 1998; Steed et al., 1998, Colley et al., 1997a; 1997b, Katayama et al., 1998; Kodaki and Yamashita, 1997; Park et al., 1997). PLD belongs to a class of lipolytic enzymes called phospholipases which also include PLA1, PLA2, and PLC. PLD has been implicated in many important cellular signaling pathways and can be activated by many hormones, neurotransmitters, growth factors and cytokines. PLD has also been implicated in several human cancers (Foster and Xu, 2003).

PLD hydrolyzes the substrate phosphatidylcholine (PC) to generate phosphatidic acid (PA) and choline (Fig.1.1.). PA can be further converted into diacylglycerol (DAG) or lysoPA (LPA) (Gomez-Cambronero and Keire, 1998; Hodgkin et al., 1998). PA is widely believed to act as an important lipid 2nd messenger in cellular signaling pathways.

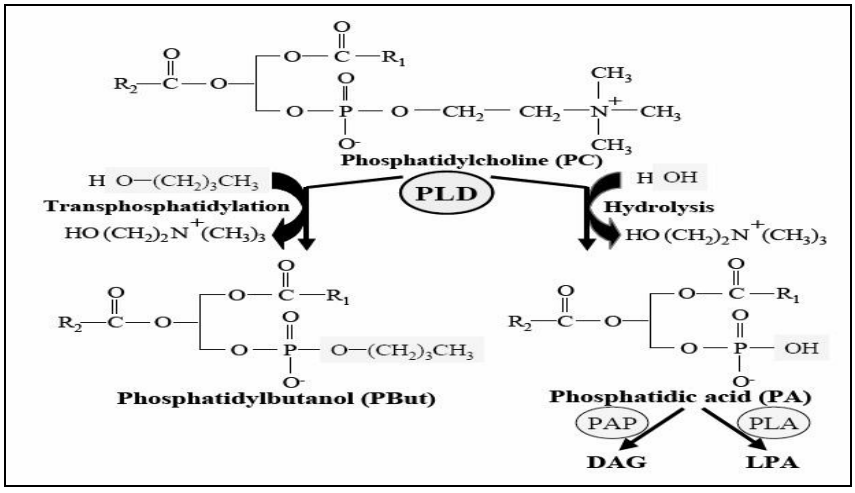

FIG. 1.1. PLD-catalyzed hydrolysis and transphosphatidylation reactions. PLD hydrolyzes the distal phosphodiester bond in phospholipids such as PC, using H_2O as an electron donor to generate PA and choline. PA can be subsequently converted into DAG or lysoPA by PA phosphohydrolase (PAP) or PLA_2, respectively. In the presence of short-chain primary alcohols such as 1-BtOH, PLD can catalyze the transphosphatidylation reaction to generate phosphatidylalcohol such as phosphatidylbutanol instead of PA.

The preferred substrate of phosphoinositide-specific PLC (PI-PLC) are inositol head group-containing phospholipids, which make up 5-10% of the total phospholipid content of most mammalian cells, and they are mainly polyunsaturated (Hodgkin et al., 1998). PIP2, a main substrate of PLC, is present at a very low concentration (<0.1% of total phospholipids) (Exton, 1997). The preferred substrate of PLD is PC, which is much more abundant, making up approximately 30-50% of the total mammalian cell phospholipids. In many cells, PC predominantly contains saturated and mono-unsaturated fatty acids (Hodgkin et al., 1998). It was proposed that only polyunsaturated fatty acids-containing DAGs generated by PLC and saturated/mono-unsaturated fatty acid-containing PAs generated by PLD are intracellular signaling molecules (Hodgkin et al., 1998).

PLD is a member of a superfamily including phosphatidylserine synthase, cardiolipin synthase, tyrosyl-DNA phosphodiesterase, two bacterial endonucleases, a murine toxin of Yersinia pestis and two poxvirus envelope proteins (Exton, 2002). There are two mammalian PLD isoforms: PLD1 and PLD2. PLD1 and PLD2 are

~50% identical in structure. They both have four conserved regions (CRI~CRIV), a pleckstrin homology (PH)-like domain, a phox homology (PX) domain at their N-termini, as well as a conserved C-terminal motif. In addition, PLD1 contains a "loop" sequence between CRII and III that is not found in PLD2 (Fig. 1.2.). The N-terminal regions and the "loop" sequence are believed to be negative regulatory elements. The conserved carboxyl termini of both PLD1 and PLD2 (Sung et al., 1999a; 1999b), especially the last four amino acids (Liu et al., 2001b; Xie et al., 2000), are critical for PLD activity, but not for membrane association (Sung et al., 1999b). Any change in these residues including deletion, mutation, or addition can cause loss of PLD activity. CRII and CRIV are catalytic domains, or transphosphatidylation domains. They are also called HKD domains, due to the presence of a H(x)K(xxxx)D motif. In this motif, the conserved histidine (H), lysine (K), and aspartate (D) residues are believed to coordinate the transphosphatidylation reaction. The HKD motif presents itself twice without exception in all known PLD enzymes of species ranging from bacteria to humans (reviewed by Exton, 2002a; Frohman et al., 1999). Structural analysis studies and the use of mutant proteins have suggested a mechanism that involves two HKD domains acting in concert for catalytic activity (reviewed in Exton, 2002b; Waite, 1999).

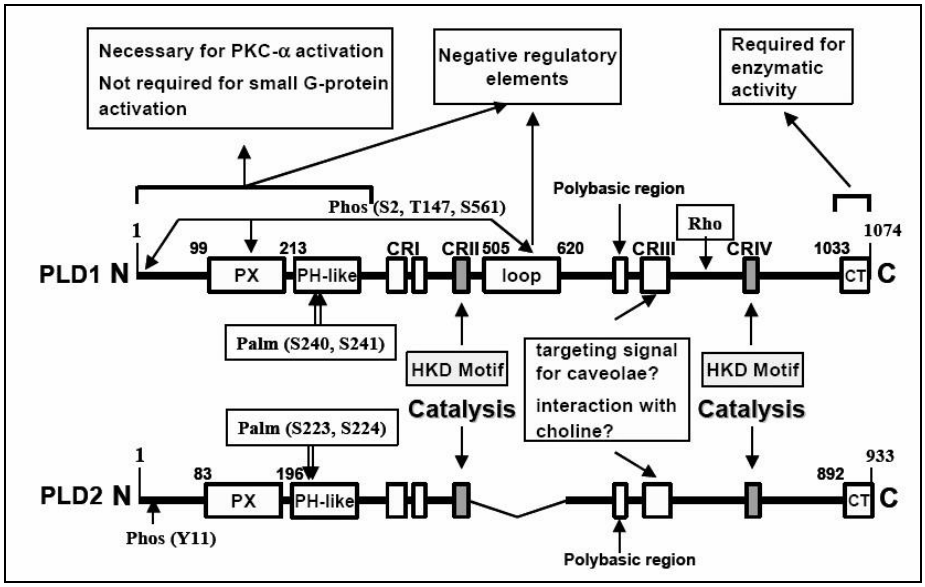

FIG. 1.2. Conserved and unique features for mammalian PLD1 and PLD2. The PLD amino acid sequences encode regions that are either unique to PLD1 (loop region) or that are conserved among mammalian PLDs and some or all PLDs from non-mammalian species (other boxed regions). Possible functions that have been proposed for these regions are listed. The site of interaction of Rho, a second PIP_2 binding site besides PH domain (polybasic region between CRII~CRIII), three serine/threonine phosphorylation (Phos) sites of PLD1, one tyrosine phosphorylation site (Y11) of mouse and rat PLD2 that is not conserved in human, and two palmitoylation sites (Palm) for both PLD1 and PLD2, are indicated. See text for details. PX, phox; CR, conserved region; CT, carboxyl terminus.

PLD1 is primarily localized in the Golgi and perinuclear area, whereas PLD2 is often found associated with plasma membrane, especially in the caveolin-enriched membrane microdomains known as lipid rafts. It appears that PLD activity is differentially regulated by its localization. PLD1 is activated in response to mitogenic signals via ADP ribosylation factor (ARF) and RalA (Xu et al., 2003). Protein kinase C-α (PKC-α) is also implicated in mitogenic signaling through growth factors (Hornia et al., 1999), suggesting that PLD1 is involved. However, mitogenic PLD activity has been found primarily in caveolin-enriched plasma membrane microdomains (Xu et al., 2000) where PLD2 is the predominant PLD isoform. PLD1 is also found in these light membrane fractions (Xu et al., 2000), but a substantial majority of PLD1 is localized in perinuclear heavy membrane. Thus genetic evidence links mitogenic signaling with

PLD1, but circumstantial evidence implicates PLD2. As shown in Fig.1.3, a model has been proposed to resolve this apparent paradox (Foster and Xu, 2003). In this model, in response to growth factor stimulation, PLD1 is activated by ARF6 and RalA and moves to plasma membrane upon phosphorylation by PKC-α. Activated PLD1 then hydrolyzes PC to generate PA which can activate phosphatidylinositol 4-P-5 kinase (PIP5K). PIP5K generates phosphatidylinositol 4, 5-biphosphate (PIP2) from phosphatidylinositol 4-P (PIP). PIP2 then activates PLD2. Therefore, both PLD1 and PLD2 are thought to be involved in mitogenic signaling.

Both PLD1 and PLD2 are palmitoylated and phosphorylated on Ser/Thr residues under basal conditions, but these modifications are not required for catalytic activity, they are believed to contribute to membrane association (Exton, 2002).

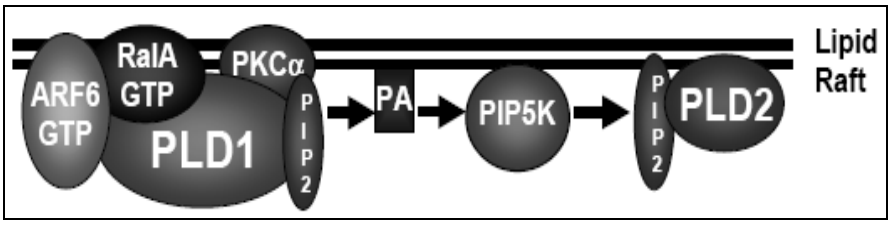

FIG. 1.3. Mitogenic signaling through PLD1-dependent activation of PLD2. In this model, it is proposed that PLD1 is activated through the synergistic actions of ARF6 and RalA in lipid rafts as described in Fig. 1. PIP2 is required for both PLD1 and PLD2 activity. Upon activation of PLD1 all available PIP2 is utilized 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.

1.1.2. Regulation of PLD

PLD can be regulated at activity and expression levels. However, the regulation of PLD activity is believed to play a more important role in cellular signaling. Therefore, the regulation of PLD activity will be discussed in detail.

1.1.2.1. Regulation by PKC

PKCs are involved in a multitude of physiological functions in the cell and play a fundamental role in signaling mechanisms leading to mitogenesis and

proliferation of cells, apoptosis, platelet activation, remodeling of the actin cytoskeleton, modulation of ion channels, and secretion (reviewed in Toker, 1998). There are three subfamilies of PKCs: conventional PKCs including α, βI, and the splice variant βII, and γ, which are activated by PS, DAG, and Ca^{2+}; novel PKCs including δ, ε, η, and θ, which are activated by PS and DAG, but have lost the requirement for calcium; and atypical PKCs including ζ and λ (also known as ι), which require PS as a cofactor but do not respond to either DAG or calcium. In addition, PKCμ □ may be considered to constitute a fourthsubfamily member of PKCs or a distinct family called protein kinase D (PKD), which does not respond to either DAG or calcium but requires PS as a cofactor (reviewed by Toker, 1998).

PKC has been implicated in the activation of PLD by phorbol esters in various cell types. Although it has been shown that isoforms from all PKC subfamilies are involved in regulation of PLD activity, only PKCα and PKCβ in the conventional subfamily have been clearly shown to bind directly to and activate PLD1 (Exton, 2002). PKCδ and PKCε have been shown to play an inhibitory role in the regulation of PLD activity (Hornia et al., 1999, Kiss et al., 1999). There are some controversies about PKCδ in the regulation of PLD. For instance, PKCδ is required for the activation of PLD by sphingosine 1-phosphate (Ghelli et al., 2002), bradykinin, and TPA (Han et al., 2002a). However, in membranes, inhibition of PKCδ by rottlerin blocks the PLD activity mediated by sphingosine 1-phosphate, but enhances the PLD activity by bradykinin (Meacci et al., 2003). It has also been reported that the dominant-negative PKCδ potentiates both basal and EGF-induced PLD activity (Han et al., 2002b).

PKCα has been shown to mediate the activation of PLD by EGF (Hornia et al., 1999), b-FGF (Yeo and Exton, 1995), PDGF (del Peso et al., 1997), insulin

(Slaaby et al., 2000), the antigen that cross-links IgE receptors (Powner et al., 2002) or IgG receptors (FcεRI) (Melendez et al., 2001); bombesin (Yeo and Exton, 1995), thrombin (Pachter et al.,1992), and carbachol (Zhang et al., 1999); and phorbol ester (Kotter et al., 2000) and H_2O_2 (Min et al., 2001). PKCα was reported to constitutively associate with PLD2 (Slaaby et al., 2000), however, PKCα interacts with and activates PLD1 in a ligand dependent manner (Min and Exton, 1998; Oka et al., 2002). In the early studies it was not clear if PKC activates PLD through kinase activity. Some reports indicated that PKCα activates PLD1 *in vitro* in a kinase-independent manner (Melendez et al., 2001); whereas other reports indicate that phosphorylation of PLD1 is needed for its activation (Kim et al., 2000). For example, it was found that PLD1 was activated by PKCα and phosphorylated exclusively in caveolin-enriched microdomains at serine 2, threonine 147 and serine 561 residues (Han et al., 2002b; Kim et al., 2000). A triple mutation of these phosphorylation sites reduces but does not block PMA-induced PLD1 activity (Kim et al., 2000), but it was unknown if the mutant PLD1 localizes properly to the membrane or is still responsive to other factors. Cholesterol depletion by methyl-b-cyclodextrin dramatically reduces the phosphorylation of PLD in the light membranes, but does not affect the PLD1 activity (Kim et al., 2000).

PKC appears to regulate PLD1 through interaction and phosphorylation (Oka et al., 2002). It has been reported that both the regulatory and catalytic domains of PKCα are required for the binding activation of PLD1 (Hu and Exton, 2003), but phosphorylation is associated with the decrease of PLD activity during the time course of PLD1 activation by PMA. The PKC inhibitor staurosporine and a kinase deficient PKCα can both eliminate PMA-mediated PLD1 phosphorylation and block the later decline in PLD activity (Hu and Exton, 2003). These results suggest that

PKCα-PLD interaction has a role for activation, but PLD1 phosphorylation by PKCα was associated with inactivation. However, it was also reported that the phosphorylation of PLD1 by PKCα is required for its translocation from perinuclear region to plasma membrane where it is activated by PIP2 (Kim et al., 1999). It is clear that PKCα regulation of mitogenic PLD activity is very complex and that there is still much to be researched as to how PKCα is involved.

1.1.2.2. Regulation by tyrosine phosphorylation

Tyrosine phosphorylation of PLD1 can be induced by agents that increase reactive oxygen species, such as H_2O_2 in the presence of vanadate (Marcil et al., 1997; Min et al., 2001; 1998). In contrast, it has also been shown that H_2O_2 (Min et al., 2001), EGF (Slaaby et al., 1998), and agents acting on G protein-linked receptors (Parmentier et al., 2001) can induce tyrosine phosphorylation of PLD2. PLD2 complexes with EGF receptor in a ligand-independent manner, but becomes tyrosine phosphorylated upon the receptor activation (Min et al., 2001). Interestingly, both H_2O_2 and EGF can induce tyrosine phosphorylation of EGF receptor, but only H_2O_2 can induce both PLD1 and PLD2 phosphorylation whereas EGF can only cause the tyrosine phosphorylation of PLD2 (Min et al., 2001). PKC activation has been reported to be involved in the activation of PLD by H_2O_2 and EGF (Min et al., 2001; 1998). It was also reported that tyrosine phosphorylation of PLD2 does not directly mediate PLD activity by peroxyvanadate (Mehta et al., 2003). Mutation on the tyrosine phosphorylation site (Tyr-11) of mouse PLD2 enhanced PLD activation, but it did not alter the magnitude of the increase of PLD2 activity by EGF (Slaaby et al., 1998). So far, the exact role of this tyrosine phosphorylation for PLD activation is not clear yet, since this Tyr residue is not conserved in humans. It has been recently reported that c-Src can tyrosine phosphorylate PLD2 and to a lesser extent the PLD1

isoform without affecting the PLD activity; however, coexpession of PLD1 or PLD2 with c-Src synergistically enhances cellular proliferation compared to the expression of either molecule (Ahn et al., 2003).

1.1.2.3. Regulation by small GTPases

Several members of the small GTPase family have been implicated in the regulation of PLD, including members of the Rho-, ARF-, and Ral-family GTPases.

Regulation by Rho family

There is evidence demonstrating that Rho family small G proteins (Rho/Rac/Cdc42) activate PLD. Rho family small G proteins have been shown to mediate the activation of PLD by insulin (Karnam et al., 1997), PDGF (Malcolm et al., 1996), the antigen that cross-links IgE receptors (FcεRI) (Ojio et al., 1996; Powner et al., 2002), lysoPA (Malcolm et al., 1996), sphingosine 1-phosphate (Meacci et al., 2002), bradykinin (Meacci et al., 1999), endothelin I (Malcolm et al., 1996), carbachol (Zhang et al., 1999), fMLP (Fensome et al., 1998), glutarmate receptor agonists (Kanumilli et al., 2002), phorbol ester (Kotter et al., 2000), and the Ca^{2+}-mobilizing agent thapsigargin (Cissel et al., 1998).

Rho is also required for PLD activity in v-Raf transformed cells (Frankel et al., 1999). The C-terminus of PLD1 is thought to provide the interaction site with RhoA (Exton, 2002). RhoA can activate PLD directly by binding with PLD1 or indirectly through the activation of PIP5K to generate PIP_2, a cofactor for both PLD1 and PLD2 activity, or through other undefined pathways (Exton, 2002).

There has been reports implicating Rho family GTPases in mitogenic signaling through PLD (Frankel et al., 1999; Hess et al., 1997), however, Rho family GTPases have been more convincingly demonstrated to mediate PLD responses through agonists that stimulate secretion (Powner et al., 2002) or activate heptahelical

receptors and heterotrimeric GTPases not usually implicated in mitogenic signaling (Exton, 1998). It is likely that the major role for the PLD activity stimulated by Rho family GTPases is the formation of actin stress fibers and regulation of cell motility since PLD activity has been implicated in the formation of stress fibers (Cross et al., 1996; Kam and Exton, 2001). This would be consistent with many studies on Rho family GTPases where it has been clearly established that the Rho family GTPases regulate membrane traffic and actin dynamics (Schmitz et al., 2000). Thus, while available evidence does not indicate a major role for Rho family GTPases in mitogenic signal transduction, the Rho-family GTPases may play a critical role in the regulation of PLD activity in cell migration which is critical for progression to a malignant metastatic cancer.

Regulation by ARF

ARF was first identified as a direct regulator of PLD1 (Brown et al., 1993). All mammalian ARF isoforms can activate PLD in vitro. There are three classes of ARF family G proteins: class I (ARF1-3), class II (ARF4 and 5), and class III (ARF6) (Moss and Vaughan, 1998).

ARF has been reported to be required for the activation of PLD by insulin (Karnam et al., 1997), EGF (Kim et al., 2003), PDGF (Shome et al., 1998); agents that cross-link IgE receptors (FcεRI) (Powner et al., 2002) or IgG receptors (FcεRI) (Melendez et al., 2001); angiotensin II (Shome et al., 2000), endothelin-1 (Shome et al., 2000), fMLP (Kaldi et al., 2002), ligands binding VPAC receptors (McCulloch et al., 2001), agents stimulating M3 muscarinic receptors (Rumenapp et al., 1995), phorbol ester (Kotter et al., 2000; Shome et al., 1998), and NaF (Bourgoin et al., 1996). ARF is also required for the activation of PLD1 by H-Ras (Xu et al., 2003).

PLD can be activated by ARF 1, 3, 5, and 6 in vitro. The myristoylated ARFs are more effective than the non-myristoylated forms (Brown et al., 1995). ARF4 has been reported to mediate EGFR-dependent PLD2 activation (Kim et al., 2003), but ARF does not activate PLD2 *in vitro*. PLD1 can be activated by ARF1 *in vitro*, but the role of ARF1 in activation of PLD1 *in vivo* is not clear. ARF6 is required to activate PLD1 by RalA in H-Ras-transfected NIH3T3 cells (Xu et al, 2003). PLD1 has also been reported to co-localize with ARF6 in endosomes (Toda et al., 1999) and ARF6 has been implicated in the activation of PLD1 by the high affinity immunoglobulin G receptor FcεRI (Melendez et al., 2001). Both ARF6 and PLD have been reported to activate phosphatidylinositol 4-phosphate 5-kinase [PI(4)P5-kinase, PI(4)P5K] (Honda et al., 1999), which may further activate PLD by generating PIP2.

Regulation by Ral

Another GTPase implicated in mitogenic PLD signaling is RalA (Feig et al., 1996), a Ras family GTPase that interacts directly with PLD1, but does not activate it (Jiang et al., 1995; Luo et al., 1997). While RalA is not sufficient to activate PLD1 either *in vitro* or *in vivo* (Jiang et al., 1995), RalA is required for the activation of PLD activity by EGF (Lu et al., 2000), PDGF (Voss et al., 1999), insulin (Voss et al., 1999), Src (Jiang et al., 1995), Ras (Jiang et al., 1995; Urano et al., 1996), Raf (Frankel et al., 1999), and phorbol esters (Voss et al., 1999). These findings suggest that RalA plays a critical role in the activation of PLD activity in response to mitogenic and oncogenic signals. Ras activates RalA through recruitment of GTP/GDP exchange factor for RalA, Ral-GDS (Feig et al., 1996; Wolthuis et al., 1998), and importantly, the Ral pathway emanating from H-Ras was recently reported to be the most critical for the transformation of human cells (Hamad et al., 2002). Although activated RalA is not sufficient to activate PLD1, RalA can be co-immunoprecipitated with ARF proteins

(Luo et al., 1998). Moreover, it was demonstrated that RalA is able to work synergistically with ARF1 to elevate PLD1 activity *in vitro* (Kim et al., 1998). It was also shown that a combination of activated RalA and activated ARF6 was sufficient to elevate PLD activity *in vivo* (Xu et al., 2003). Activated ARF1 could not work synergistically with activated RalA in this *in vivo* study – most likely because ARF1 and RalA do not co-localize in the cell, whereas both ARF6 and RalA do co-localize to lipid rafts where mitogenic PLD activity is localized (Xu et al., 2003). A model for PLD activation by Ras was proposed whereby mitogenic signals mediated by Ras activate two parallel pathways leading to the activation of guanine nucleotide dissociation stimulator (GDS) proteins for both RalA and ARF6. This results in the activation RalA, which is already in a complex with PLD1. The activated form of RalA is proposed to recruit activated ARF6 into the RalA-PLD1 complex and the activated ARF6, now in a RalA/PLD1/ARF6 complex, then activates PLD1 through a direct interaction. This model for the synergistic activation of PLD by RalA and ARF6 is shown in Fig. 1.4. (Foster and Xu, 2003).

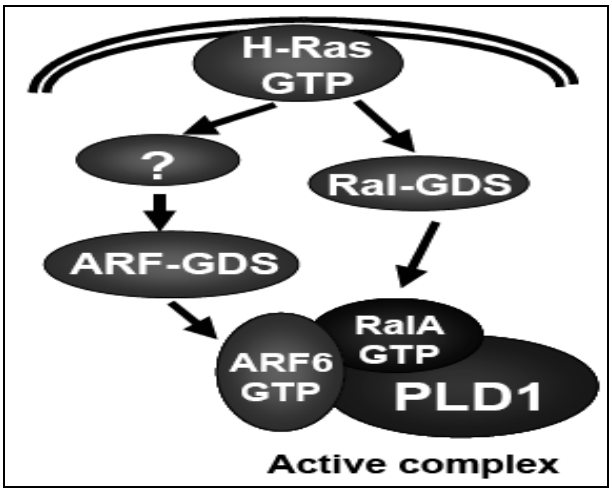

FIG. 1.4. Activation of PLD1 by oncogene signaling.
Oncogenic activation of PLD1 through the synergistic action of RalA and ARF6. It is proposed that parallel pathways are activated, in this case by H-Ras, leading to the activation of Ral-GDS and an as yet unspecified ARF-GDS. Activation of Ral-GDS activates RalA, which is already in a complex with PLD1. The activation of RalA then recruits activated ARF6 into the RalA-PLD1 complex. The activated ARF6 then activates PLD1.

Regulation by Ras

Ras does not directly activate PLD. However it can activate PLD through other factors. It was reported that H-Ras elevated PLD activity through RalA and ARF6 (Xu et al., 2003). Ras can activate many downstream effectors including GEF for Ral, Raf, JNK and PI3K. These effectors may mediate the activation of PLD by Ras. It has been reported that Ras can mediate the activation of PLD by v-Src (Jiang et al., 1995a). The data from Chapter III implicate the activation of PLD by Ras through RalA and PI3K in human cancer cells.

1.1.2.4. Regulation by PIP2

Both PLD1 and PLD2 have a stringent requirement for PIP2, indicating a requirement for the PI kinases that generate PIP2. In this regard, it is of interest that PIP5K, which generates PIP2 from PIP, is a downstream target of PLD signaling (Moritz et al., 1992; Jenkins et al., 1994) and may therefore represent a positive feedback loop (see Fig.3.11. for a model). Whether the formation of PIP2 regulates PLD activity in response to mitogenic stimuli remains to be determined, but it does provide an attractive hypothesis for regulating PLD2, which is constitutively active *in vitro*. Disruption of the PH domain of PLD2 results in relocalization of the protein from the plasma membrane to endosomes and renders PLD2 unresponsive to activation *in vivo* (Sciorra et al., 2002). Moreover, elevating PIP2 levels with PI-4-P 5-kinase elevated PLD2 activity *in vivo* (Divecha et al., 2000). This suggests that the generation of PIP2 could be critical for regulating PLD2 *in vivo*.

1.2. Ras

1.2.1. General properties of Ras

Ras proteins are small GTPases that regulate cell growth, proliferation and differentiation. It binds and activates many downstream effectors. There are three Ras

isoforms: H-Ras, K-Ras (K-Ras4A and K-Ras4B) and N-Ras. The amino-terminal catalytic domains (amino acids 1-165) of H-Ras, K-Ras and N-Ras are highly conserved (90-100% identical). The carboxyl-terminal sequences vary significantly and are referred to as the hypervariable region (HVR). The HVR consists of the well-characterized anchor region which is necessary for Ras to associate with the inner plasma membrane. The anchor region contains a motif called CAAX that acts as a Ras trafficking signal. Mutation of the CAAX motif disrupts plasma-membrane localization and signaling of Ras.

Ras proteins are synthesized in the cytosol as precursors that undergo post-translational processing that enables their association with the plasma membrane. The initial triplet of modifications is directed by the carboxyl-terminal CAAX motif and is common to all Ras proteins (Hancock et al., 1989; Casey et al., 1989). First, protein farnesyl transferase attaches a farnesyl group to the cysteine residue of the CAAX motif. Second, the farnesylated CAAX sequence targets Ras to the cytosolic surface of the ER where AAX tripeptide is removed by Ras-converting enzyme (Rce1) (Boyartchuk et al., 1997). Third, the α-carboxyl group on the now carboxyl-terminal farnesyl-cysteine is methylated by isoprenylcysteine carboxyl methyltransferase. K-Ras is more efficiently methylated than H-Ras and N-Ras (Hancock, 2003). Finally, after methylation, Ras proteins move to the cell surface. This process is mediated by a second targeting signal located beside farnesyl cysteine at amino-terminal. H-Ras and N-Ras undergo palmitoylation on cysteine residues in their HVRs and enter the exocytic pathway, going through the Golgi to the plasma membrane. K-Ras, however, does not accumulate on Golgi, it goes to the plasma membrane directly because of its polylysine sequence which assists its association with anionic membrane lipids, possibly through electrostatic interaction. This process is thought to be regulated by

phosphorylation and surface charge of K-Ras.

H-Ras and K-Ras represent the two most cell surface-localized isoforms, whereas in many cell types, N-Ras is predominantly localized in endomembranes. Genetic and electron microscopy data have shown that H-Ras associates with lipid rafts, whereas K-Ras operates from a distinct non-lipid raft microdomain (Prior et al., 2001). H-Ras exists in dynamic equilibrium between raft and non-raft microdomains; exit from lipid rafts is needed for efficient H-Ras signaling (Omerovic et al., 2007). The differential Ras isoform distribution reveals how microdomain occupancy has a profound effect on the efficiency of signaling. A prevailing model within the Ras field is that differential localization enables Ras isoform-specific signaling by bringing isoforms into contact with distinct pools of effectors and activators. This further implies that not all microdomains have the same capacity to sustain signaling and that correct and dynamic microlocalization significantly enhances signal propagation (Omerovic et al., 2007).

1.2.2. Ras Signaling Pathways and Transformation

Ras participates in the regulation of cell proliferation, differentiation, and morphology. Activated Ras oncogenes have been identified in various forms of human cancer including epithelial carcinomas of the lung, colon, and pancreas. The cells of these cancers and those that have been experimentally transformed by the activated Ras gene exhibit abnormal growth, morphological changes and alterations of cell adhesions. Although the main effector protein has been thought to be Raf serine/threonine kinase, research has revealed that the Ras-induced signaling pathway is mediated by multiple effector proteins and has the crosstalk with various factors containing other small GTPases (Yamamoto et al., 1999).

Three primary downstream effectors of Ras signaling implicated in

transformation are phosphatidylinositol 3-kinase (PI3K), Raf kinase, and the guanine nucleotide exchange factor (GEF) for RalA GTPase (Shields et al., 2000). PI3K can convert PIP2 into phosphatidylinositol 3,4,5-triphosphate (PIP3) which activates Akt. Akt can activate mammalian target of rapamycin (mTOR) to provide survival signals. Raf promotes cell proliferation and differentiation through the mitogen activated protein kinase (MAPK) pathway. Raf-1 has been reported to promote cell survival by antagonizing apoptosis (Chen et al., 2001). RalA has been shown to be required for the activation of PLD1 in transformed 3Y1 cells induced by EGF (Shen et al., 2000). RalA is also required for the activation of PLD1 in H-Ras-transformed NIH3T3 cells (Xu et al., 2003). These effectors have been implicated in Ras-induced transformation (Yamamoto et al., 1999).

1.3. Ras and Cancer

Ras mutations are very common in human cancers. Ras mutations are found in 30% of human cancers. Activating mutations of Ras are found in humans in nearly all pancreatic cancers (90%), one-half of colon and thyroid tumors, and one-third of lung tumor. H-Ras mutation is often found in bladder, and kidney cancers, while K-Ras mutation is often found in colon, lung (10-30%), and pancreas cancers.

One of the most common gain-of-function mutations found in human cancers are activating mutations to genes encoding Ras family GTPases – most commonly to the K-Ras gene (Bos, 1989). There are several downstream targets of Ras signaling that includes PI3K, Raf kinase, and RalA-GEF (Shields et al., 2000). Interestingly, whereas Raf was the most critical downstream target of Ras for the transformation of murine cells, RalA-GEF was the most critical Ras target for the transformation of human cells (Hamad et al., 2002). RalA-GEF leads to the activation of RalA, which is associated with PLD1 (Luo et al., 1997) and is required for the activation of PLD

activity by epidermal growth factor and Ras (Shen et al., 2001; Jiang et al., 1995b). RalA has been implicated in cell transformation (Urano et al., 1996; Lim et al., 2006) and in transformation of human cells by Ras (Lim et al., 2005). The transformation of rat fibroblasts by H-Ras was reported to be dependent upon PLD1 (Buchanan et al., 2005), suggesting that a key aspect of RalA in cell transformation by Ras is the activation of PLD.

An important aspect of tumorigenesis is the activation of cellular signals that suppress default apoptotic programs that protect against cancer. Signals that suppress apoptosis have been referred to as survival signals because they allow the survival of cells under conditions where the cells would ordinarily undergo programmed cell death. A common target of survival signals is mTOR. mTOR is activated in response to signals mediated by PI3K and PLD (Foster, 2004; Foster, 2007; Fang et al., 2001). Both PI3K and PLD activity have been reported to be elevated in a large number of human cancers (Luo et al., 2003; Foster, 2006). Moreover, PI3K and PLD have been shown to suppress apoptosis in many cancer cells (Luo et al., 2003; Foster and Xu, 2003; Foster, 2006).

While activated H-Ras has been reported to stimulate PLD activity in a variety of rodent cell culture systems (Carnero et al., 1994; Jiang et al., 1995a), activated K-Ras was unable to stimulate PLD activity in mouse fibroblasts (Xu et al., 2003). This is of significance since activating K-Ras mutations are common in human cancers, whereas activating mutations to H-Ras are rare (Bos, 1989). In this dissertation we have examined the PLD activity in two human cancer cell lines that have activating mutations to H-Ras and K-Ras. We believe that investigation of PLD's role of providing a survival signal would be significant to the current study of human cancer and its therapeutic intervention through a new strategy.

Chapter II. Materials and Methods

In this chapter, the materials and methods that we used in the dissertation are described.

2.1. Cells and Cell Culture Conditions

Parental and v-H-Ras, v-K-Ras-transformed 3Y1 rat fibroblast cells were characterized previously (Frankel et al., 1999; Hornia et al., 1999; Jiang et al., 1995a; 1995b) and were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine calf serum (HyClone). To reduce background PLD activity, subconfluent cell cultures were placed in fresh media containing 0.5% bovine serum for one day. For transfection, cells were plated at a density of 10^5 cells/100 mm dish 18 h prior to transfection. Transfections were performed by using LipofectAMINE PLUS reagent (GIBCO) according to the vendor's instructions, in which PLUS reagent was included to enhance the transfection efficiency of LipofectAMINE. For transient transfection, control for efficiency was determined by transfection of pEGFP-C1 (Clonetech), which expresses green fluorescent protein. The percentage of green cells was determined microscopically and was always in excess of 80%. For stable transfections, cells were selected in the presence of G418 for 2 weeks as described previously (Lu et al., 2000) and pooled clones were used for experiments.

2.2. Materials

Dipalmitoyl-PC, phosphatidylethanolamine, and hosphatidylinostol-4, 5-bisphosphate were purchased from Sigma; [^{3}H]-myristate, [^{3}H]-arachidonate, [^{3}H]-stearate and {choline-methyl-[^{3}H]-dipalmitoyl}-PC were obtained from New England Nuclear. Precoated silica 60A thin layer chromatography plates were from

Whatman; protease inhibitor cocktail (Set I) was from Calbiochem. Monoclonal antibody 1D9, which recognizes most ARF isoforms, was obtained from Dr. Richard Kahn (Emory University, Atlanta). Antibodies against ARF1 and ARF6 were provided by Dr. Sylvain Bourgoin (Universite Laval, Quebec). Antibodies against Caveolin 1, the endoplasmic reticulum binding protein BiP, Na^+,K^+-ATPase II, the trans-Golgi protein TGN38, RalA, and Raf were from Transduction Laboratories. The anti-Ras antibodies were obtained from Santa Cruz Biotechnology. Antibodies against PLD1 and PLD2 were purchased from Quality Control Biochemicals (QCB). The v-Src antibody was from Calbiochem. A monoclonal antibody to the EGF receptor (LA22) was obtained from Upstate Biotechnology. For non-immune controls we used ChromPure Rabbit or Mouse IgG from Jackson ImmunoResearch.

2.3. Plasmid Expression Vectors

The mammalian expression plasmids, pcDNA3.1-ARF6Q67L, pcDNA3.1-ARF6T27N, pcDNA3.1-ARF1Q67L, and pcDNA3.1-ARF1T31N, have been previously described (Boshans et al., 2000; D'Souza-Schorey et al., 1995). They were constructed by PCR amplification of the corresponding cDNAs and cloned into the EcoRI site of pcDNA3.1 (-) (Invitrogen). The mammalian expression plasmids for RalA Q72L, Ki-Ras4B (G12V), and Ha-Ras (G12V) were expressed in the pZIP-NeoSV(X)1 vector and have been described previously (Jiang et al., 1995b).

2.4. Isolation of Membranes

The strategy for separation of light and heavy membrane fractions was based on one developed by Lisanti and colleagues (Song et al., 1996a; 1996b) with modifications that excluded the use of sodium carbonate and high pH. Quiescent confluent cells grown in 150 mm dishes were washed twice with ice-cold phosphate-buffered saline, scraped into 2 ml of buffer M (25mM MES, pH 6.5; 250

mM sucrose; 1 mM EDTA) or MBS (25mM MES, pH 6.5; 150 mM NaCl; 1 mM EDTA) with 1X protease inhibitor cocktail. Homogenization was carried out on ice using a Wheaton Dounce homogenizer (20~25 strokes), a Polytron homogenizer (22-25k rpm for 45 sec; PT3000, Brinkman), and then by sonication (three 20 s bursts; VC 300, Sonics & Materials Inc., Danbury, CT). Protein concentration was determined using the Bradford method (BioRad). 5 mg of homogenate protein was diluted to 2 ml in Buffer M or MBS, adjusted to 45% sucrose (w/v) by adding 90% sucrose (w/v) prepared in 25mM Mes, pH 6.5. This solution (4 ml) was then overlaid with 4 ml of 35% and 4 ml of 5% sucrose (w/v) in 25 mM MES, pH 6.5 to form a discontinuous gradient in an ultracentrifuge tube. The gradient was centrifuged at 39,000 rpm for 16~18 h in an SW41 rotor (Beckman). 1 ml fractions were collected from top to bottom and analyzed for PLD activity and proteins as described in the text. The pellet was sonicated in 1ml of buffer M and analyzed along with the collected fractions.

2.5. Immunoprecipitation

Quiescent confluent cells were washed twice with ice cold phosphate-buffered saline, scraped into the modified RIPA buffer [Tris-HCl, 50mM, pH 7.6; IGEPAL CA-630, 1%; sodium deoxycholate, 0.25%; NaCl, 150 mM; $MgCl_2$, 10 mM; EDTA, 1 mM;Na_3VO_4, 1mM; NaF, 1 mM; 1X protease inhibitor cocktail (0.5mM AEBSF, 1 mM leupeptin, 0.15 mM aprotinin, 1 mM protease inhibitor E-64)], incubated at 4 ^{0}C for 15 min by gently rocking, sonicated for 20 seconds on ice, and centrifuged at 12,000Xg at 4 ^{0}C for 10 min. The supernatant was precleared with Protein G Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech), and 500 mg of the precleared proteins was adjusted to 500 ml in the modified RIPA buffer, and then incubated with the antibody for 1h as described in the text. The immunocomplex

was captured by incubating with 50 ml of Protein G Sepharose 4 Fast Flow bead slurry, collected by centrifugation at 12,000 X g for 20 sec at 4^0C. The beads were washed 3 times with the modified RIPA buffer and once with wash buffer (50mM Tris, pH7.6), and subjected to Western blot analysis.

2.6. Western Blot Analysis

Samples were adjusted into gel-loading buffer (50mM Tris-HCl, pH 6.8; 100 mM dithiothreitol; 2% SDS; 0.1% bromophenol blue; 10% glycerol), and then heated for 3 minutes at 100$_0$C prior to separation by SDS-polyacrylamide gel electrophoresis. After transfer to polyvinylidene difluoride (for caveolin) or nitrocellulose membrane (for other proteins), the membrane filters were blocked with 5% non-fat dry milk in phosphate buffered saline with 0.05% Tween-20 (PBS-T) and then incubated with the appropriate antibody diluted in 5% milk in PBS-T. 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 using the enhanced chemiluminescent detection system (Pierce).

2.7. Assay of PLD Activity

PLD activity was determined by the transphosphatidylation reaction in the presence of 0.8% butanol as described previously (Song and Foster, 1993). Cells in 100-mm culture dishes were prelabeled with [^{3}H]-myristate for 4-5 h in DMEM containing 0.5% bovine serum. Lipids were extracted and characterized by thin layer chromatography as described in the next section. Relative levels of PLD activity were then determined by measuring the intensity of the corresponding phosphatidylbutanol band in the autoradiograph using a Molecular Dynamics scanning densitometer and Image-Quant software**.**

Phospholipid metabolites were characterized by TLC (silica gel 60A plates) using procedures described previously (Song and Foster, 1993; Song et al., 1991). Lipid standards were visualized by treating TLC plates with iodine vapor. The following solvent systems were used: For PC, $CHCl_3$: methanol:glacial acetic acid:H_2O (50:25:8:4; v/v); for phosphatidylbutanol, the upper phase of ethylacetate: trimethylpentane:acetic acid:H_2O (90:50:20:100; v/v).

2.8. RalA Activation Assay

The detection of activated RalA was performed essentially as described previously (Lu et al., 2000). Cells were lysed with 15% glycerol, 50mM Tris-HCl, pH 7.4, 1% IGEPAL CA-630, 200 nM NaCl, 10mM $MgCl_2$, 1X protease inhibitor cocktail, and precleared with glutathione-agarose beads. Lysates were then treated with glutathione-S-transferase (GST)-Ral-BD fusion protein immobilized with glutathioneagarose beads (Upstate Biotechnology). Ral-BD is the Ral binding domain of Ral-BP1 that binds activated GTP-bound Ral proteins (Wolthuis et al., 1998a; 1998b). Activated Ral proteins were recovered by centrifugation at 14,000xg at 4^0C for 5 sec and washed 3 times with lysis buffer and subjected to Western blot analysis using an antibody raised against RalA (Transduction Laboratories).

2.9. Cell Transfection

Cells were trypsinized, counted and plated so that they are 80-90% confluent the day of transfection. Antibiotics were not used at the time of plating and during transfection. DNA/siRNA was diluted into DMEM medium without serum and mixed and incubated at room temperature for 15 mins. Lipofectamine reagent was diluted into DMEM medium without serum in a second tube and mixed. Pre-complexed DNA/siRNA and diluted lipofectamine reagent were combined, mixed and incubated for 15 mins at room temperature. The complexes were added to each plate directly

into the growth medium while gently rocking the plate. Transfected cells were incubated at 37 ^{0}C at 5% CO_2. Cell extracts were assessed after 24-48 hr after the start of transfection.

2.10. Colony Selection

Cells were treated with G418 400 μg/ml every two days for two weeks after transfection. Then cells were incubated with crystal violet for 5 minutes and the plates were rinsed with water, blue colonies were counted after dry-out of the plates.

2.11. Cell Viability Assay

Cell viability was determined by trypan blue exclusion. Cells were treated according to the experimental protocol. Then cells were harvested, washed, and treated with trypan blue at a concentration of 0.4% v/v. After 5 mins, trypan blue uptake (dead cells) was counted using a hemocytometer.

Chapter III. PLD Provides Survival Signal in T24 and Calu-1 with Activated H- and K-Ras

3.1. Introduction

An important aspect of tumorigenesis is the activation of cellular signals that suppress default apoptotic programs that protect against cancer. Signals that suppress apoptosis have been referred to as survival signals because they allow the survival of cells under conditions where the cells would ordinarily undergo a programmed cell death. A common target of survival signals is mTOR. mTOR is activated in response to signals mediated by PI3K and PLD(Foster, 2004; Foster, 2007). Both PI3K and PLD activity have been reported to be elevated in a large number of human cancers (Luo et al., 2003; Foster, 2006). Moreover, PI3K and PLD have been shown to suppress apoptosis in many cancer cells (Luo et al., 2003; Foster and Xu, 2003; Foster, 2006).

One of the most common gain-of-function mutations found in human cancers are activating mutations to genes encoding Ras family GTPases – most commonly to the K-Ras gene (Bos, 1989). There are several downstream targets of Ras signaling that includes PI3K, Raf kinase, and RalA-GEF (Shields et al., 2000). Interestingly, whereas Raf was the most critical downstream target of Ras for the transformation of murine cells, RalA-GEF was the most critical Ras target for the transformation of human cells (Hamad et al., 2002). RalA-GEF leads to the activation of RalA, which is associated with PLD1 (Luo et al., 1997) and is required for the activation of PLD activity by epidermal growth factor and Ras (Shen et al., 2001; Jiang et al., 1995b). RalA has been implicated in cell transformation (Urano et al., 1996; Lim et al., 2006), and in transformation of human cells by Ras (Lim et al., 2005). The transformation of

rat fibroblasts by H-Ras was reported to be dependent upon PLD1 (Buchanan et al., 2005), suggesting that a key aspect of RalA in cell transformation by Ras is the activation of PLD.

While activated H-Ras has been reported to stimulate PLD activity in a variety of rodent cell culture systems (Carnero et al., 1994; Jiang et al., 1995a), activated K-Ras was unable to stimulate PLD activity in mouse fibroblasts NIH3T3 (Xu et al., 2003). This is of significance since activating K-Ras mutations are common in human cancers, whereas activating H-Ras mutations are rare (Bos, 1989). We have examined the PLD activity in two human cancer cell lines T24 and Calu-1 that have activating H-or K-Ras mutations. We found that there are very high levels of PLD activity in both T24 bladder and Calu-1 lung cancer cells that harbor mutations in H-Ras and K-Ras, respectively (Santos et al., 1982; Taparowsky et al., 1982; Shimizu et al., 1983). The PLD activity stimulated by Ras in these cells provides a survival signal that prevents apoptosis in the absence of serum.

3.2. Results

3.2.1. PLD activity is elevated in T24 bladder and Calu-1 lung cancer cells

PLD activity is elevated in rodent fibroblasts expressing activated H-Ras (Carnero et al., 1994; Jiang et al., 1995a; Xu et al., 2003) and PLD activity is required for the transformation of rodent cells by H-Ras (Buchanan et al., 2005). Since activating Ras mutations are present in a large number of human cancers, we investigated whether there is elevated PLD activity in human cancer cells with activated H-Ras and K-Ras genes. The T-24 bladder carcinoma cells express an activated H-Ras gene (Santos et al., 1982; Taparowsky et al., 1982) and Calu-1 lung carcinoma cells express an activated K-Ras gene (Shimizu et al., 1983). The PLD activity in these two cell lines was evaluated and compared with the PLD activity in

two breast cancer cell lines (MDA-MB-231 and MCF7), which express relatively high and low PLD activity respectively (Zhong et al., 2003; Zheng et al., 2006). As shown in Fig. 3.1., both the T24 and Calu-1 cells express levels of PLD activity that were substantially higher than that observed in the MBA-MB-231 cells. These data reveal that two human cancer cell lines with activating mutations in either H-Ras or K-Ras have substantially elevated levels of PLD activity.

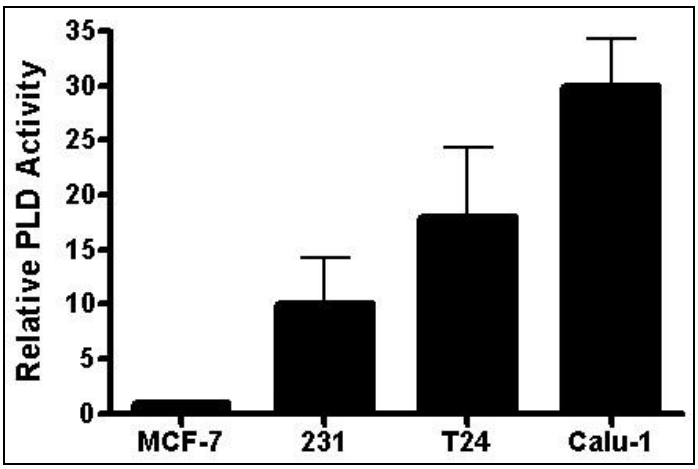

FIG. 3.1. PLD activity is elevated in T24 bladder and Calu-1 lung cancer cells. MCF-7, MDA-MB-231, T24 and Calu-1 cells were plated in media containing 10% serum for 24 hr. At that point cells were shifted to media containing 0.5% serum overnight. BtOH was added 20 min prior to harvesting of cells, and the transphosphatidylation product phosphatidylbutanol (PtBt) was determined by thin layer chromatography as described in Materials and Methods. The PLD activity in the MDA-MB-231, T24, and Calu-1 cells was normalized to that observed in the MCF-7 cells which was given a value of 1. Error bars represent the standard deviation for two independent experiments.

3.2.2. Elevated PLD activity in T24 and Calu-1 cells is dependent upon Ras

We next examined whether the elevated PLD activity in the T24 and Calu-1 cells was dependent on the activated Ras genes in these cells. To accomplish this, the T24 and Calu-1 cells were transfected with H-Ras and K-Ras siRNAs or a scrambled siRNA control. The PLD activity and Ras protein levels were then determined. As shown in Fig. 3.2, siRNA for H-Ras and K-Ras suppressed both Ras expression and PLD activity in the T24 and Calu-1 cells. These data indicate that elevated PLD activity in T24 and Calu-1 cells is dependent on Ras.

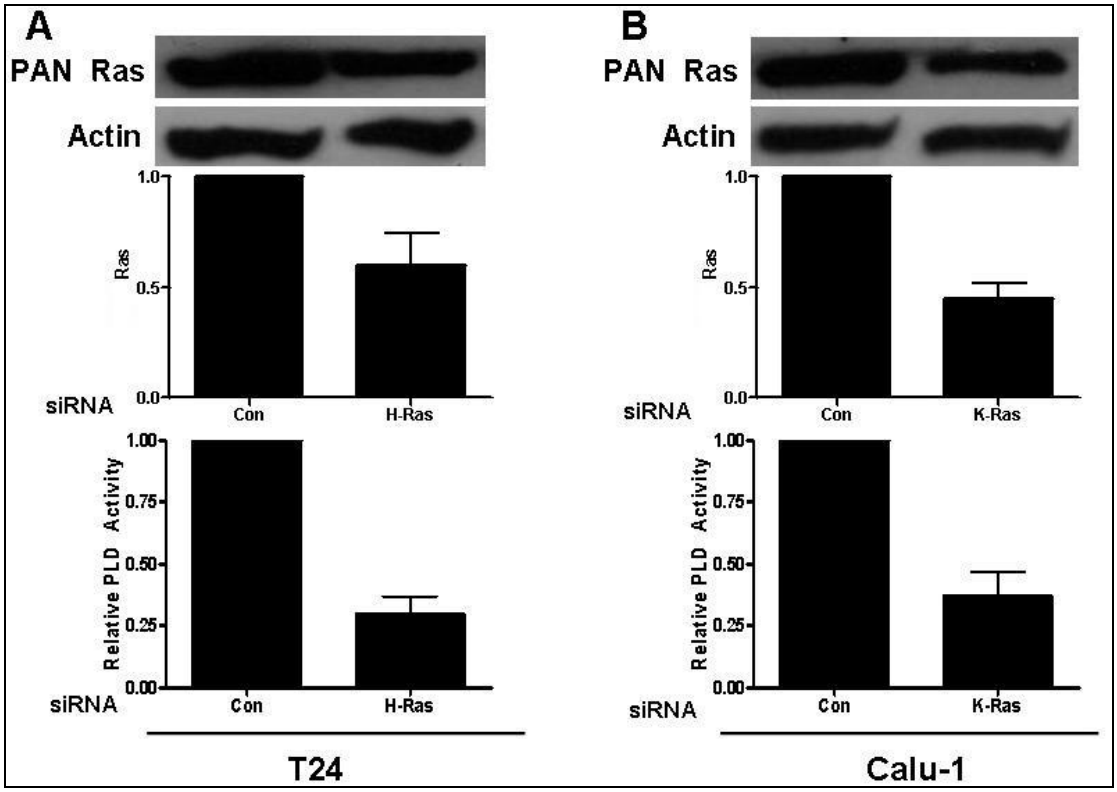

FIG. 3.2. Elevated PLD activity in T24 and Calu-1 cells is dependent upon Ras. T24 (A) and Calu-1 (B) cells plated at a density of 10^5 cells per 60 mm plate. 24 hr later the cells were transfected with either control (Con) scrambled siRNA or siRNA specific for H-Ras (A) or K-Ras (B) as indicated. The levels of Ras and actin were then determined by Western blot and the PLD activity was determined twenty four hr later as in Figure 3.1. The PLD activity in the Ras siRNA samples was normalized to the PLD activity in the controls, which was given a value of 1.00. Error bars represent the standard deviation for two independent experiments. The data showing the levels of Ras and actin were representative of an experiment that was performed at least two times.

3.2.3. Elevated PLD activity in T24 and Calu-1 cells is dependent upon RalA

We demonstrated previously that the activation of PLD by Ras was dependent upon RalA in mouse cells (Jiang et al., 1995b; Luo et al., 1998). We therefore examined whether the elevated PLD activity in the T24 and Calu-1 cells was dependent upon RalA. Cells were transfected with RalA siRNA and the levels of RalA and PLD activity were examined. As shown in Fig. 3.3., the suppression of RalA siRNA reduced the PLD activity in both the T24 and Calu-1 cells. The suppression of RalA also led to cell apoptosis indicated by increased PARP cleavage. These data indicate that the elevated PLD activity in T24 and Calu-1 cells is dependent on RalA.

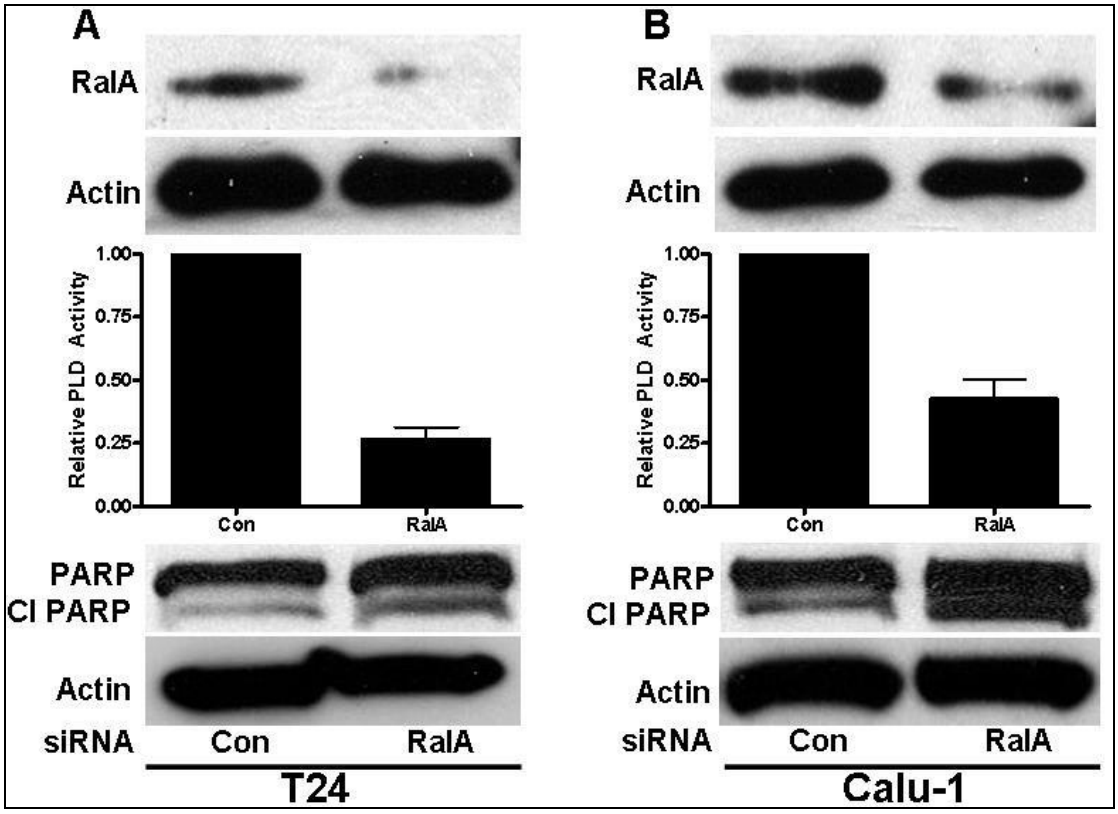

FIG. 3.3. Elevated PLD activity in T24 and Calu-1 cells is dependent upon RalA. T24 (A) and Calu-1 (B) cells were transfected with either control (Con) scrambled siRNA or siRNA specific for RalA as indicated. The levels of RalA and actin were then determined by Western blot and the PLD activity was determined as in Figure 3.1. The PLD activity in the RalA siRNA samples was normalized to the PLD activity in the controls, which was given a value of 1.00. Error bars represent the standard deviation for two independent experiments. The data showing the levels of RalA and actin were representative of an experiment that was performed at least two times.

3.2.4. Elevated PLD activity in T24 and Calu-1 cells is dependent upon PI3K

Since PI3K is one of the main downstream effectors of Ras, we decided to investigate if PI3K pathway is involved in the elevation of PLD activity. T24 and Calu-1 cells were treated with PI3K inhibitor LY294002 for overnight at 0.5% serum condition, then PLD activity assay and western blot of cleaved PARP were conducted to examine the effect of LY294002. As shown in Fig. 3.4, LY294002 treatment led to reduction of PLD activity in T24 and Calu-1 cells. It also resulted in cell apoptosis indicated by increased PARP cleavage. These data reveal that activating Ras in T24 and Calu-1 cells activates PLD through the intermediation of PI3K and contributes to the cell survival in these cells. This result is unusual because elevated PLD activity in

the MDA-MB-231 human breast cancer cells generate an mTOR-dependent survival signal that is independent of PI3K, whereas MDA-MB-435S breast cancer cells, which have very low levels of PLD activity, are dependent on PI3K for survival signals (Chen et al., 2005). Given the fact that PIP2, a co-factor required for the activation of PLD, is also required for the generation of PIP3 by PI3K, it appears that cancer cells use either mTOR or PI3K to generate survival signal. However highly elevated PLD activity in T24 and Calu-1 cells seems to indicate the existence of mTOR survival pathway, while the data from Fig.3.4 also indicate the existence of PI3K survival pathway.

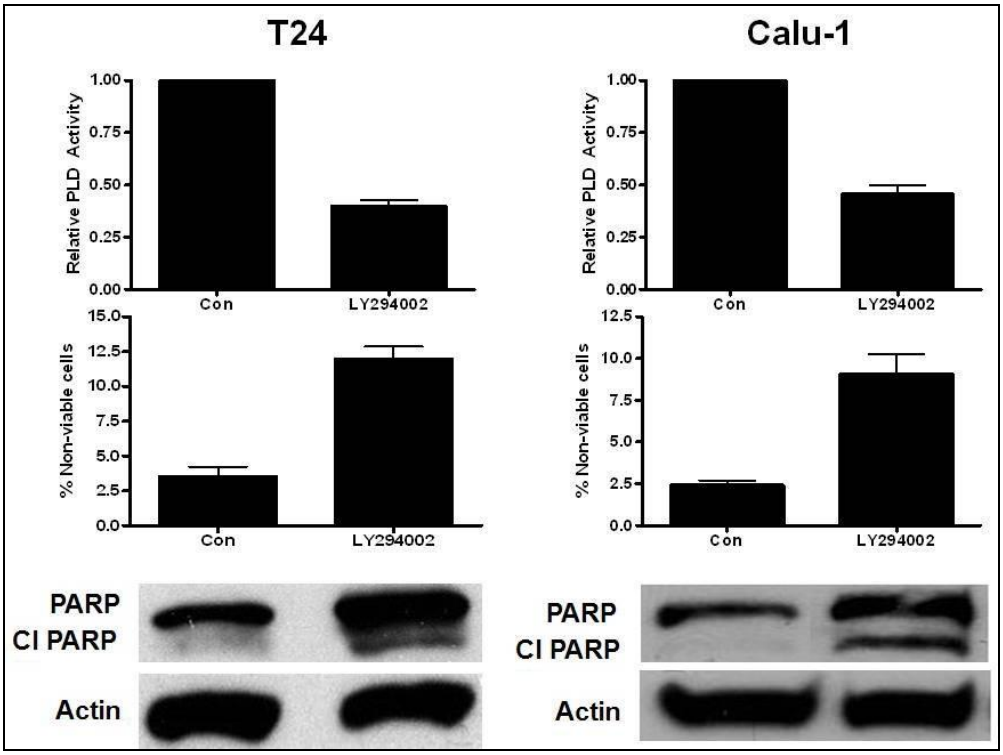

FIG. 3.4. Elevated PLD activity in T24 and Calu-1 cells is dependent upon PI3K LY294002 was added to T24 and Calu-1 cells and was incubated for 4 hours before the cells were subject to PLD activity assay. The PLD activity in the LY294002-added samples was normalized to the PLD activity in the controls, which was given a value of 1.00. PARP cleavage was detected with anti-PARP antibody. Error bars represent the standard deviation for two independent experiments. The data showing the levels of Ras and actin was representative of an experiment that was repeated at least two times.

3.2.5. Elevated PLD activity in T24 and Calu-1 cells is not dependent upon JNK and MAPK

Since Jun N-terminal kinase (JNK) and MAPK are also implicated in transformation mediated by Ras (Yamamoto et al., 1999), we wanted to investigate whether elevated PLD activity is dependent upon these two pathways. 10 uM U0126 which blocks phosphorylation of MAPK by MEK and 10uM SP600125 which blocks JNK were incubated T24 and Calu-1 cells for 4 hours before the samples were subject to PLD activity assay. As shown in Fig. 3.5, the inhibitors have little impact on the PLD activity, suggesting that the elevated PLD activity in T24 and Calu-1 cells is not dependent upon JNK and MAPK pathways.

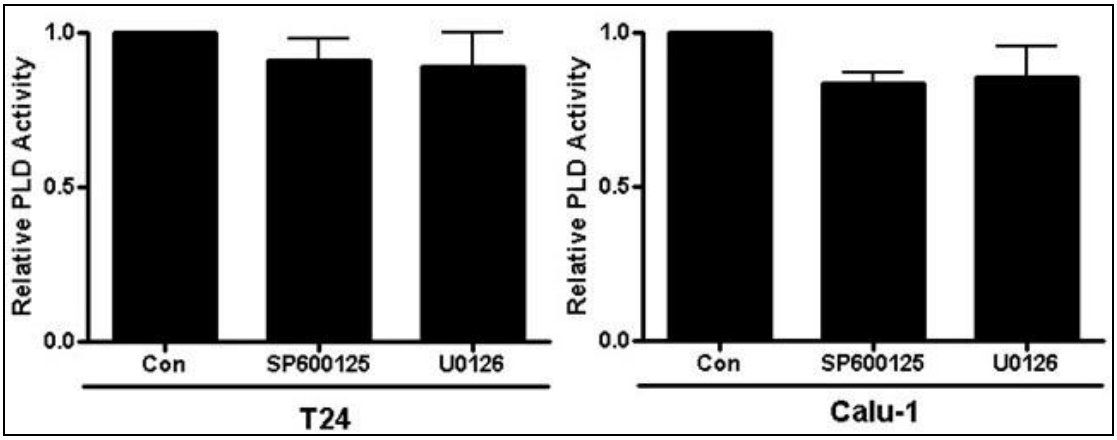

FIG. 3.5. Elevated PLD activity in T24 and Calu-1 cells is not dependent upon JNK and MAPK

10 uM JNK and MEK kinase inhibitors SP600125 and U0126 were added to T24 and Calu-1 cells and were incubated for 4 hours before the cells were subject to PLD activity assay. The PLD activity in the inhibitors-added samples was normalized to the PLD activity in the controls, which was given a value of 1.00.

3.2.6. Suppression of Ras expression in T24 and Calu-1 cells results in apoptosis upon serum withdrawal

We reported previously that elevated PLD activity in MDA-MB-231 breast cancer cells provided a survival signal for these cells when deprived of serum (Zhong et al., 2003; Chen et al., 2005). When either the T24 or Calu-1 cells were placed in low serum there was very little apoptosis observed as determined by cell viability and cleavage of the caspase-3 substrate PARP. However, as shown in Fig. 3.6, if these cells were transfected with siRNA for H-Ras (T24) or K-Ras (Calu-1) there was a

substantial decrease in cell viability and a corresponding increase in PARP cleavage indicating apoptosis. These data indicate that activated Ras is generating a survival signal that suppresses apoptosis stimulated by serum withdrawal.

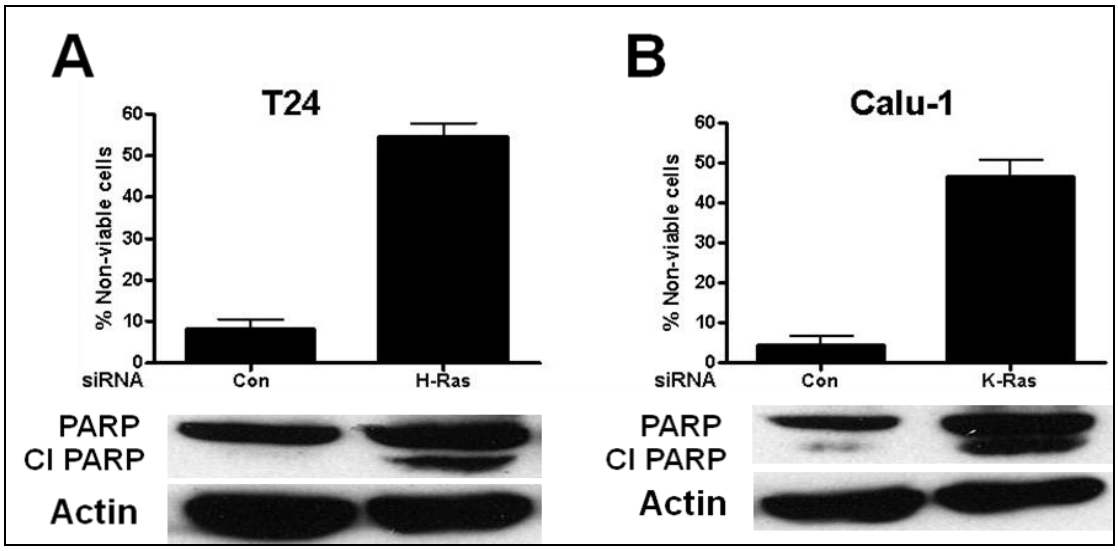

FIG. 3.6. Suppression of Ras expression in T24 and Calu-1 cells results in apoptosis upon serum withdrawal.
T24 and Calu-1 cells were plated at a density of 10^5 cells/60 mm plate. 24 hr later the cells were transfected with either control (Con) scrambled siRNA or siRNA specific for H-Ras (A) or K-Ras (B) as in Figure 2. Twenty four hr later the cells were shifted to media containing 0.5% serum. 18 hr later cell viability (upper graph) and PARP cleavage (lower blot, Cl PARP) were determined as described in Materials and Methods. The Western blot for PARP was examined for loading by reprobing with an antibody raised against actin. Error bars represent the standard deviation for duplicate samples from a representative experiment repeated at least two times.

3.2.7. Suppression of PLD in T24 and Calu-1 cells results in apoptosis upon serum withdrawal

To determine whether the PLD activity stimulated by the Ras proteins in the T24 and Calu-1 cells was critical for that survival signal generated by Ras, we employed the "alcohol trap" assay (Shen et al., 2001) whereby primary, but not tertiary, alcohols are preferentially utilized over water in the hydrolysis of phosphatidylcholine to a corresponding inert phosphatidylalcohol rather than phosphatidic acid. As shown in Fig. 3.7, primary butanol (1-BtOH), but not tertiary butanol (t-BtOH) led to apoptosis in serum deprived T24 and Calu-1 cells as indicated

by decreased cell viability and increased PARP cleavage.

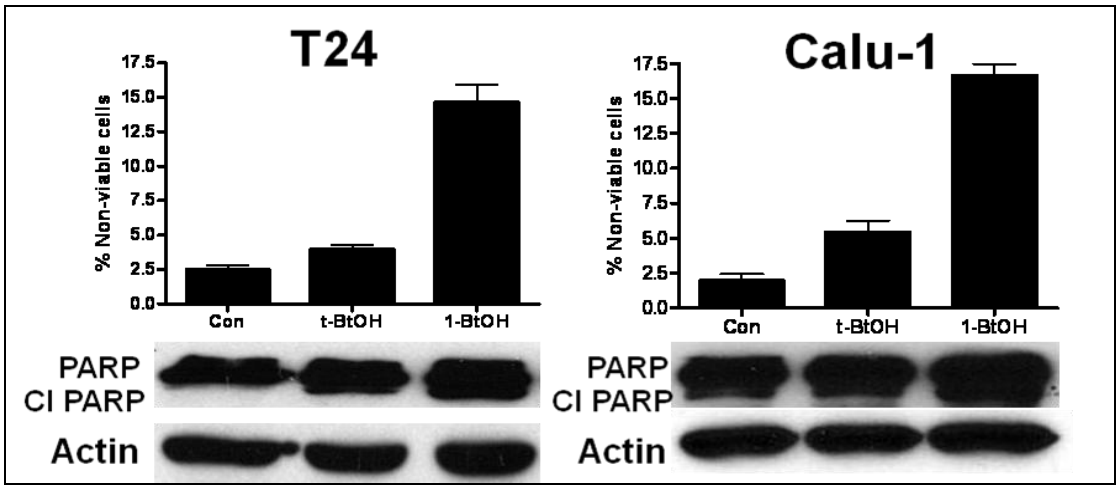

FIG. 3.7. Suppression of PLD activity by primary butanol in T24 and Calu-1 cells results in apoptosis upon serum withdrawal.
T24 and Calu-1 cells were plated at a density of 10^5 cells/60 mm plate. Twenty four hr later the cells were shifted to media containing 0.5% serum. 18 hr later the cells were either untreated (Con), or treated with either t-BtOH or 1-BtOH and cell viability (upper graph) and PARP cleavage (lower blot, Cl PARP) was determined 6 hr later. Error bars represent the standard deviation for duplicate samples from a representative experiment repeated at least two times.

To further establish that PLD activity was providing a survival signal, we transfected the T24 and Calu-1 cells with PLD1 siRNA. The T24 and Calu-1 cells were then subjected to low serum and cell viability and PARP cleavage was evaluated. As shown in Fig. 3.8., suppression of PLD1 expression made both the T24 and Calu-1 cells sensitive to serum withdrawal as indicated by decreased cell viability and increased PARP cleavage. These data reveal that the PLD activity elevated by H-Ras and K-Ras in the T24 and Calu-1 cells is critical for the Ras-generated survival signals in these cells that suppress apoptosis under the stress of serum withdrawal.

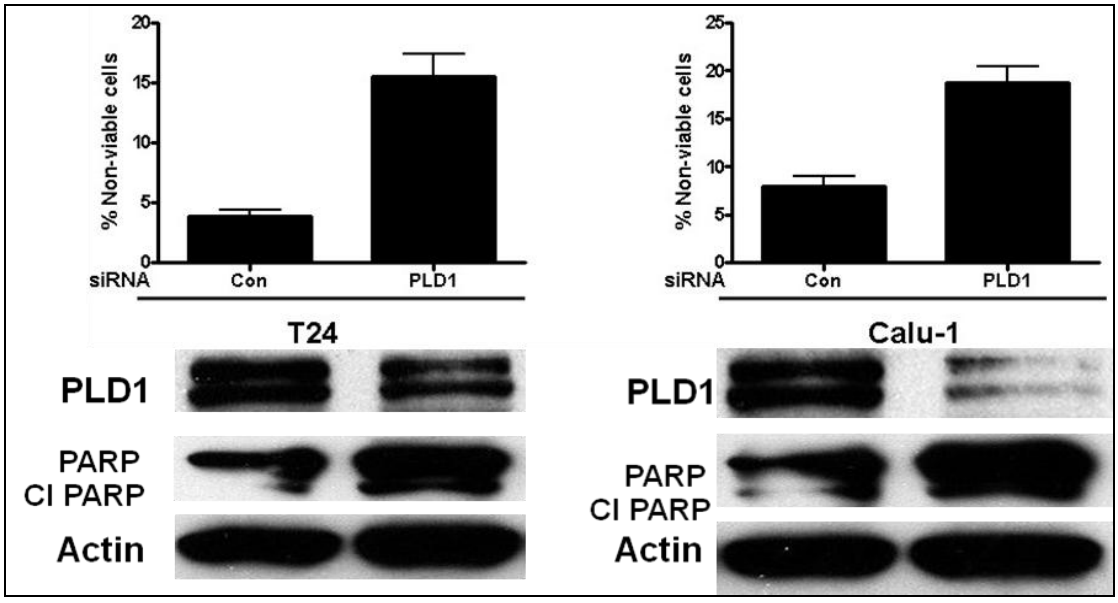

FIG. 3.8. Suppression of PLD activity by PLD1 siRNA in T24 and Calu-1 cells results in apoptosis upon serum withdrawal.
T24 and Calu-1 cells were plated at a density of 10^5 cells/60 mm plate. 24 hr later the cells were transfected with either control (Con) scrambled siRNA or siRNA specific for PLD1 as in Figure 2. Twenty four hr later the cells were shifted to media containing 0.5% serum. Eighteen hr later cell viability (upper graph) was determined as in Figure 4. In the lower panel, PLD1 levels and PARP cleavage (Cl PARP) were determined using Western blot analysis. Error bars represent the standard deviation for duplicate samples from a representative experiment repeated at least two times.

3.2.8. Rapamycin suppresses PLD activity in T24 and Calu-1 cells

We examined the effect of rapamycin which blocks mTOR on the PLD activity in T24 and Calu-1 cells. 10uM rapamycin was incubated with T24 and Calu-1 cells and DMSO was added to Control for 4 hours before the samples were subject to PLD activity assay. As shown in Fig. 3.9., rapamycin suppressed elevated PLD activity in T24 and Calu-1 cells. This surprising result indicates that there is a positive feedback between mTOR and PLD whereby PLD stimulates mTOR and mTOR somehow feeds back to keep PLD activated. We currently do not understand how this happens mechanistically.

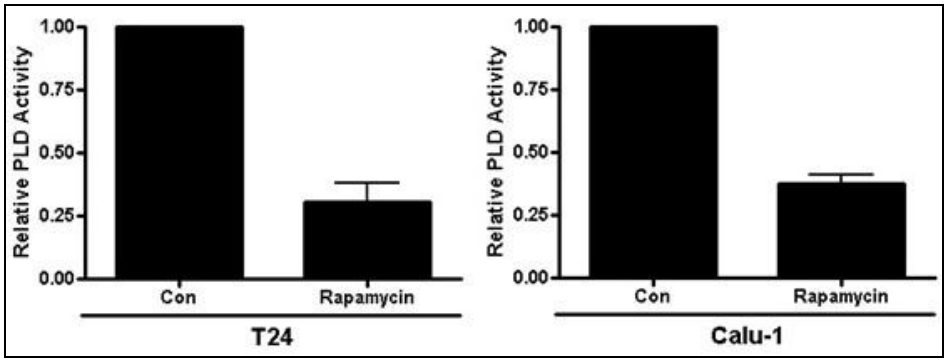

FIG. 3.9. Rapamycin suppresses PLD activity in T24 and Calu-1 cells
10uM of Rapamycin was added to T24 and Calu-1 cells and DMSO was added to Control for 4 hours before the cells were subject to PLD activity assay. The PLD activity in the rapamycin-treated samples was normalized to the PLD activity in the controls, which was given a value of 1.00. DMSO was used in the Con sample.

3.2.9. T24 cells are resistant to rapamycin

It has been shown that elevated PLD activity in MDA-MB-231 human breast cancer cells confers rapamycin resistance due to competition of PLD-generated PA and rapamycin to bind mTOR (Chen et al., 2003). Since T24 cells have higher PLD activity than 231 cells, we wanted to investigate whether elevated PLD activity in T24 cells confers stronger rapamycin resistance. We treated 231 and T24 cells with 20 uM of rapamycin for 4 hours before we measured the non-viable cells using cell viability assay. As shown in Fig. 3.10, rapamycin induced about 60% of non-viable cells in 231 cells, whereas it only induced about 7% of non-viable cells in T24. This result indicates that T24 cells do confer stronger rapamycin resistance than 231 cells.

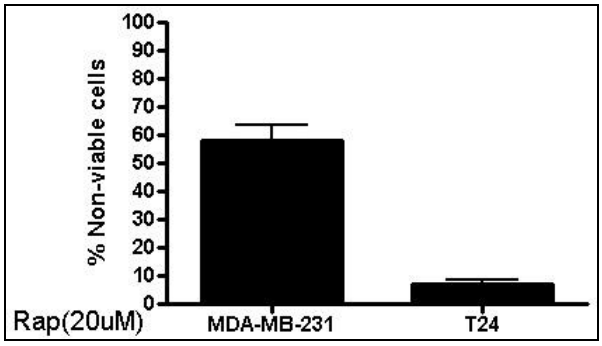

FIG. 3.10. T24 cells are resistant to rapamycin
MDA-MB 231 cells and T24 cells were treated with 20 uM of rapamycin for 4 hours before the cells were subject to cell viability assay.

3.3. Discussion

During progression to a malignant tumor, several genetic changes must occur that will allow the proliferation cells under conditions where normal cells will either stop proliferating or undergo apoptosis. During the formation of a solid tumor prior to vascularization, a cell must continue to divide and survive in the absence of growth factors. We have provided evidence here that human cancer cells with either an activated H-Ras or K-Ras depend upon highly elevated levels of PLD activity for survival in the absence of serum that contains growth factors. Suppression of PLD activity under these conditions results in apoptosis.

Although the study here examined only two cell lines, it is quite apparent that both H-Ras and K-Ras are capable of stimulating the activation of PLD. The dependence on RalA suggests that it is PLD1 that is being activated in response to both H-Ras and K-Ras, since RalA associates with PLD1 (Jiang et al., 1995b; Luo et al., 1997). Consistent with this hypothesis, PLD1 siRNA suppressed the survival signals in the T24 and Calu-1 cells. We have demonstrated previously that both PLD1 and PLD2 can work together in signals generated by epidermal growth factor (Shen et al., 2001) and that both PLD1 and PLD2 can contribute to the transformation of rat fibroblasts (Lu et al., 2000; Joseph et al., 2001). Thus, it is possible that PLD2 could also be involved. We have speculated that activation of PLD1 leads to the activation of PLD2 through stimulation of the production of the PLD co-factor PIP2 (Foster and Xu, 2003). The model in Fig.1.3 illustrates a possible mechanism.

The finding that both H-Ras and K-Ras strongly activate PLD activity in the human cancer cells was somewhat surprising in that we previously reported that activated H-Ras, but not activated K-Ras, induced PLD activity in NIH-3T3 mouse fibroblasts (Xu et al., 2003). The Calu-1 lung cancer cells, which have activating

mutation in K-Ras, had higher levels of PLD activity than the T24 bladder cancer cells, which have an activating mutation in H-Ras. Significantly, the PLD activity in the Calu-1 cells was dependent upon RalA. Several reports have linked RalA to H-Ras via the RalA-GEF, which is a direct downstream target of H-Ras (Feig, 1996; Chien and White, 2003). The data reported here implicate RalA in signals mediated by H-Ras as well as K-Ras. Importantly, K-Ras is mutated in many cancers, whereas H-Ras is activated in very few human cancers (Bos, 1989). Thus, the ability of K-Ras to activate RalA and PLD activity reveals that K-Ras, like H-Ras, takes advantage of PLD survival signals in the many cancers that harbor activating K-Ras mutations.

The signals generated by PLD have been shown to impact upon several proteins implicated in tumorigenesis. Elevated PLD activity has been shown to suppress the expression of tumor suppressor p53 (Hui et al., 2004). PLD has also been reported to inhibit the activity of another tumor suppressor – protein phosphatase 2A (PP2A) (Hui et al., 2005). PLD increases the stability, and consequently the expression of Myc (Rodrik et al., 2005; 2006). But perhaps the most important target of PLD-generated PA is mTOR. Phosphatidic acid has been reported to interact directly with mTOR in a manner that is competitive with rapamycin (Fang et al., 2001; Foster, 2007) and consistent with this report, elevated levels of PLD activity lead to rapamycin resistance (Chen et al. 2003). mTOR is apparently an important component in tumorigenesis in that survival signals generated by PI3K also target mTOR. These previous studies linking PLD activity with the suppression of tumor suppressors and the activation of Myc and mTOR indicate that activating PLD activity has many outputs that have been shown to promote tumorigenesis. The ability of both activated H-Ras and K-Ras to stimulate PLD activity in human cancer cells may be important for Ras genes to cause cancer. While suppression of Ras expression has been shown

to reverse tumorigenesis in mouse models where the tumors were caused by activated Ras genes (Pau et al., 2003), attempts to target Ras pharmaceutically have been largely disappointing (Prendergast and Rane, 2001). If PLD is a critical target of Ras as indicated here, then targeting PLD or PLD targets such as mTOR might prove valuable in the large number of human cancers with mutations in Ras genes.

Our study of MDA-MB-231 cells has established a model in which PLD-generated PA competes with rapamycin for binding with mTOR (Fang et al., 2001; Chen et al., 2003). As shown in Fig. 3.11., a feedback loop appears to exist involving PIP2, PLD, PA and PIP5K. In this loop, PA can activate PIP5K which converts PI4P into PIP2. PIP2 is required for PLD activation. The elevated PLD activity can then further generate more PA to feed into this loop to reinforce the effect. We have shown that ramamycin, surprisingly, suppresses elevated PLD activity in T24 cells, this appears to contradict with the above model in which PLD lies upstream of mTOR. As shown in Fig.3.11, a PLD-mTOR positive feedback loop model is proposed here to offer a possible explanation for this result. In this model PA activates mTOR which in turn further activates PLD to enhance the survival signal in the system. It is not clear how mTOR could stimulate PLD activity.

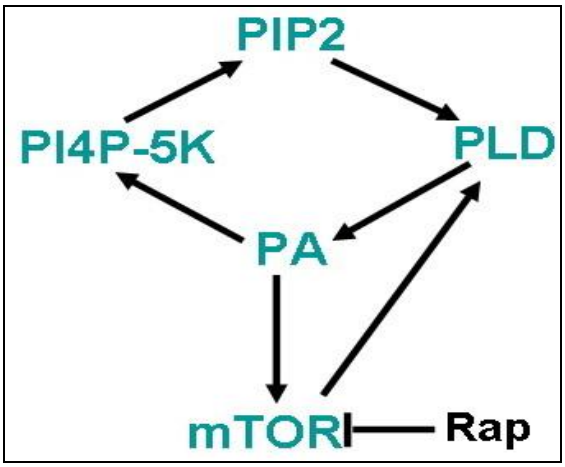

FIG.3.11. PLD-mTOR Positive Feedback Loop Model
In this model PLD-generated PA activates PI4P-5K which generates PIP2 from PI4P. PIP2 further activates PLD to generate more PA. PA also activates mTOR which in turn further activates PLD to enhance the survival signal in the system.

As shown in Fig. 3.10, T24 cells are more resistant to rapamycin than MDA-MB-231 human breast cancer cells. 20 uM of rapamycin results in almost 60% non-viable cells in MDA-MB-231 cells (Chen et al., 2003), whereas only 7% non-viable cells are found in T24 cells. This is in line with our expectation given the much higher PLD activity in T24 cells. The model we have established through the study of MDA-MB-231 cells shows that PLD-generated PA competes with rapamycin to bind with mTOR (Chen et al., 2005; see Fig. 5.2. for the model). Thus cells with higher level of PLD should have higher level of PA which makes it more difficult for rapymycin to bind with mTOR, so higher concentration of rapamycin will be needed to achieve the same result and the cells therefore become resistant to rapamycin. This may explain why early clinical trials with rapamycin and rapamycin analogues have been largely disappointing probably because of high level of PLD activity. This poses a real challenge for the effective treatment of human cancers with rapamycin. However, an alternative way involving a combination of rapamycin and PLD/PA inhibitor could theoretically enhance the therapeutic effect of rapamycin treatment in human cancers with high level of PLD activity.

Chapter IV. Different Effects of H-Ras and K-Ras in 3Y1 Rat Fibroblasts

4.1. Introduction

As indicated in Chapter III, there is elevated PLD activity in both human cancer cells with activated H- and K-Ras and the elevated PLD activity induced by both H-Ras and K-Ras in T24 and Calu-1 cells provides a survival signal to prevent cell apoptosis. Activating mutations to both H-Ras and K-Ras involve single base pair changes and should therefore be frequent. However, whereas H-Ras mutations can be found in human cancer, they are relatively rare (Bos, 1989). In contrast, K-Ras mutations are far more common (Bos, 1989). We reported previously that in NIH3T3 cells, H-Ras, but not K-Ras induced PLD activity (Xu et al., 2003). However, as described in Chapter III, both H- and K-Ras can activate PLD activity in human cancer cells. To further investigate the effect of activated Ras in non-transformed cells, we examined 3Y1 rat fibroblasts which resemble more primary cells than NIH3T3 mouse fibroblasts.

4.2. Results

4.2.1. H-Ras reduces cell viability in 3Y1 rat fibroblasts

We transfected rat fibroblasts 3Y1 with plasmids containing constitutively active H-Ras (G12V) and K-Ras (G12V) genes and G418 resistance gene and subjected them to G418 (also known as Geneticin, an antibiotic) selection for two weeks. Those cells which successfully express G418-resistant genes are expected to survive and form colonies. As shown in Fig. 4.1, less G418-resistant colonies were generated in the H-Ras and K-Ras-transfected 3Y1 cells compared with Control which contains only empty vector. However, there were substantially fewer

G418-resistant colonies generated in the H-Ras-transfected 3Y1 cells than in the K-Ras-transfected cells. These data indicate that H-Ras reduces cell viability relative to K-Ras and could explain in part why K-Ras mutations are observed with much higher frequency in human cancers.

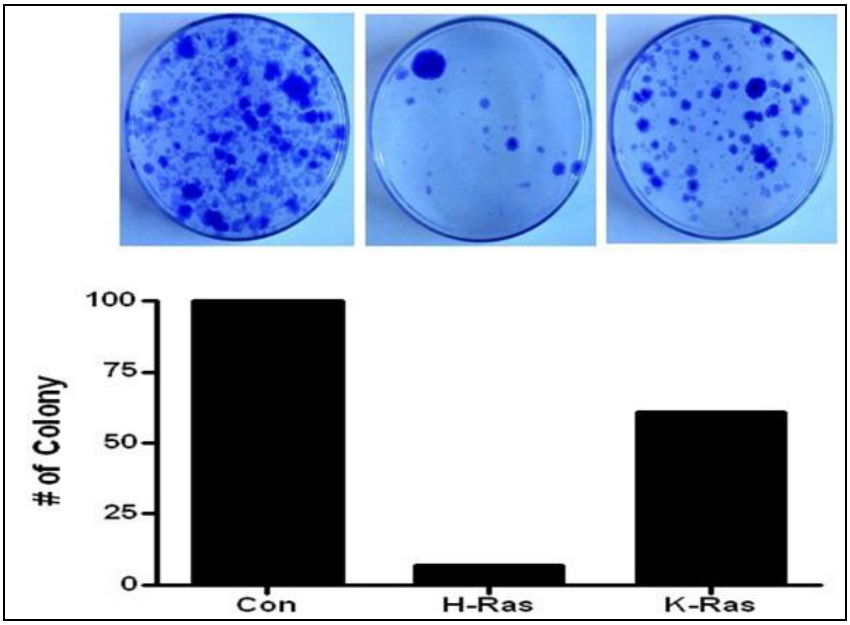

FIG. 4.1. H-Ras reduces cell viability in 3Y1 rat fibroblasts
3Y1 cells were transfected with empty plasmid vector (Con) and vector containing active H- and K-Ras genes. After transfection cells were grown under normal cell culture condition. 400ug/ml G418 was added to the medium every two days for two weeks. Then cells were incubated with crystal violet for 5 mins and rinsed. Blue colonies were counted.

4.2.2. H-Ras induces cell apoptosis in 3Y1 rat fibroblasts

To further investigate what caused substantially fewer G418-resistant colonies in the H-Ras-transfected 3Y1 cells, we transiently transfected 3Y1 cells with H- and K-Ras plasmids and investigated the effect of H- and K-Ras overexpression. As shown in Fig. 4.2, H-Ras induced more cell apoptosis than K-Ras as indicated by PARP cleavage, suggesting an apoptotic effect exerted by H-Ras in 3Y1 cells. In contrast, K-Ras seemed to suppress apoptosis, indicating that K-Ras generates a survival signal to prevent the cell apoptosis.

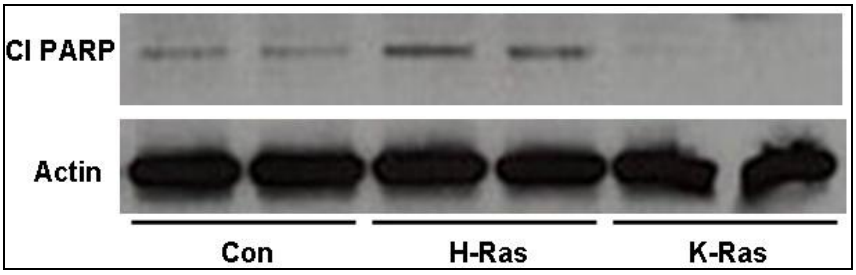

FIG.4.2. H-Ras induces cell apoptosis in 3Y1 rat fibroblasts
3Y1 cells were plated at a density of 10^5 cells/60 mm plate. Twenty four hr later the cells were transfected with empty plasmid vector (Con) or vector containing active H- and K-Ras genes. Twenty four hr later the cells were shifted to media containing 0.5% serum. 18 hr later cell apoptosis was determined using anti-PARP cleavage antibody during western blot.

4.2.3. K-Ras induces elevated PLD activity

Next we wanted to investigate what caused the reversal of cell apoptosis in the K-Ras-transfected 3Y1 cells. H-Ras can induce PLD activity through a synergistic interaction of RalA and Arf6 in NIH3T3 mouse fibroblasts and that this elevated PLD activity contributes to cell survival (Xu et al., 2003). Therefore we decided to assay PLD activity in H- and K-Ras-transfected 3Y1 cells. We transiently transfected 3Y1 cells with H-Ras and K-Ras and subjected them to PLD activity assay, as shown in Fig. 4.3, K-Ras induced elevated PLD activity compared to control cells (empty transfectants). Western blots showed that H- and K-Ras proteins had been successfully overexpressed in 3Y1 cells. These data indicate that K-Ras induces elevated PLD activity relative to H-Ras in these cells.

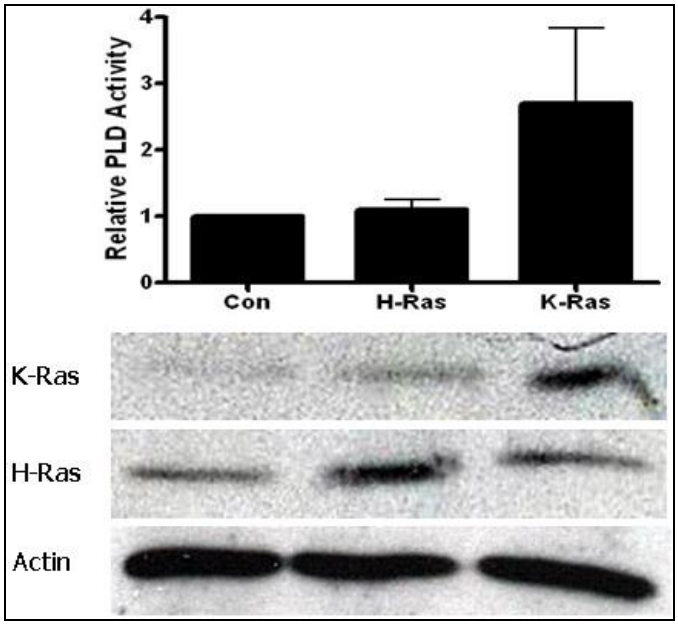

FIG. 4.3. K-Ras induces elevated PLD activity

3Y1 cells were plated in media containing 10% serum for 24 hr. Then cells were transfected with empty plasmid vector (Con) or a vector containing active H- and K-Ras genes. After transfection, cells were shifted to media containing 0.5% serum overnight. 1-BtOH was added 20 min prior to harvesting of cells, and the transphosphatidylation product phosphatidylbutanol (PtBt) was determined by thin layer chromatography as described in Materials and Methods. The PLD activity in these cells was normalized to that observed in the control 3Y1 cells which were given a value of 1. Error bars represent the standard deviation for two independent experiments. 3Y1 cells transfected with H- and K-Ras were collected and subject to Western blotting to detect specific H- and K-Ras overexpression.

4.2.4. Suppression of PLD activity results in apoptosis in K-Ras-transfected 3Y1 cells

We have established that elevated PLD activity can provide a survival signal in human cancer (Zhong et al., 2003, Chen et al., 2005). We suspected that elevated PLD activity induced by K-Ras in 3Y1 cells is responsible for the cell survival during G418 colony selection. 1-BtOH has been used to create an “alcohol trap” to suppress PLD activity (Shen et al., 2001). Therefore we used 1-BtOH to suppress K-Ras-induced PLD activity to study whether K-Ras-induced elevated PLD activity provides a survival signal. As shown in Fig. 4.4, 1-BtOH induced apoptosis in the K-Ras-transfected 3Y1 cells subjected to low serum, suggesting that the elevated PLD activity provides a survival signal.

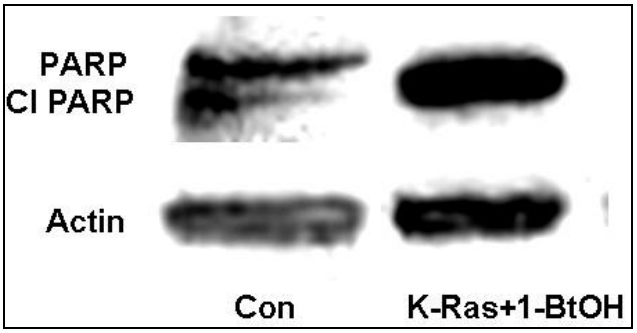

FIG. 4.4. Suppression of PLD activity by primary butanol results in apoptosis 3Y1 cells were plated at a density of 10^5 cells/60 mm plate. Twenty four hr later the cells were transfected with empty plasmid vector (Con) or vector containing active K-Ras genes. Twenty four hr later the cells were shifted to media containing 0.5% serum. 18 hr later 0.8% of 1-BtOH was also added for 4 hours before cell apoptosis was determined using anti-PARP cleavage antibody for western blot.

To further pinpoint whether it is the elevated PLD activity that provides a survival signal in K-Ras-transfected 3Y1 cells, we transfected/co-transfected 3Y1 cells with K-Ras, catalytically inactive mutants PLD1DN and PLD2DN (Shen et al., 2001), then we assayed the transfectants for cell viability in low serum using the trypan blue exclusion assay. As shown in Fig. 4.5, co-transfection of K-Ras and PLD1DN and PLD2DN substantially increased cell apoptosis compared to K-Ras transfection alone. These data indicate that suppression of PLD activity by catalytically inactive PLDs results in apoptosis in K-Ras-transfected 3Y1 cells, indicating that elevated PLD activity provides a survival signal.

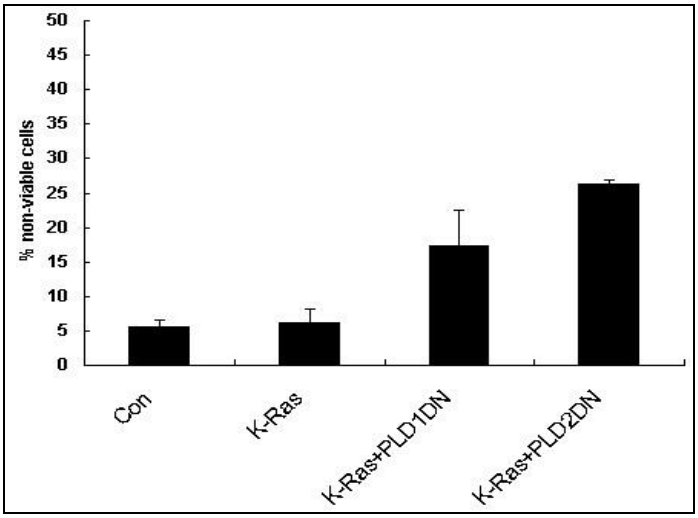

FIG. 4.5. Suppression of PLD activity by catalytically inactive PLDs results in apoptosis in K-Ras-transfected 3Y1 cells

3Y1 cells were plated at a density of 10^5 cells/60 mm plate. Twenty four hr later the cells were transfected with empty plasmid vector (Con) or vector containing active K-Ras genes. Cells were also co-transfected with active K-Ras, catalytically inactive PLD1DN, and PLD2DN. Twenty four hr later the cells were shifted to media containing 0.5% serum. 18 hr later cell viability was determined as described in Materials and Methods. Error bars represent the standard deviation for duplicate samples from a representative experiment performed at least two times.

4.3. Discussion

Activating Ras mutations involve a single base pair change and should therefore be very common. Ras mutations are commonly found in human cancer. However, H-Ras mutation is rare, whereas K-Ras mutation is far more common (Bos, 1989). Our data show that H-Ras induces apoptosis in H-Ras-transfected 3Y1 rat fibroblasts, whereas K-Ras is tolerated. K-Ras induced PLD activity suppressed apoptosis in these cells and the PLD activity was essential for their survival. While the research we conducted in 3Y1 cells is preliminary, but the data may help explain why H-Ras mutations are rare, whereas K-Ras mutations are more common in human cancer.

Chapter V. PLD in Human Cancers

PLD has emerged as an attractive player in cell survival signaling in cancer cells. The ability of PLD to suppress apoptosis makes PLD signaling an ideal target for therapeutic treatment in many human cancers with elevated PLD activity. Targeting PLD signals or the signals that lead to elevated PLD activity in cancer cells holds great promise for treating many cancers with elevated PLD activity. More research on PLD will possibly change the way of diagnosing and treating human cancers with elevated PLD activity. In this chapter, PLD-mediated survival signaling and targeting PLD signals in human cancers will be discussed.

5.1. Survival Signals and Tumorigenesis

Apoptosis is a self-protection mechanism to eliminate unwanted cells when something detrimental happens during cell cycle or development. It has evolved to become an important protection against the disease, especially human cancer. When cells detect incomplete or inappropriate growth signals such as a Ras mutation, cells activate default apoptotic programs. The default apoptotic programs must be overcome for a cell to become cancerous. During this process, survival signals are activated to suppress these default apoptotic programs. Fig. 5.1 shows a model for tumorigenesis involving the early activation of survival signals (Foster, 2006). In this model, an activated oncogene such as Ras sensitizes cells to default apoptotic programs, and therefore, mutations such as those that activate Ras will result in apoptosis. However, if a survival signal is activated or a tumor promoting agent is present to suppress apoptosis, Ras mutations will not induce apoptosis and the cells can proliferate and form a tumor mass. Suppression of the survival signal, in theory, resurrects the cellular apoptotic programs resulting in the death of the cancer cells.

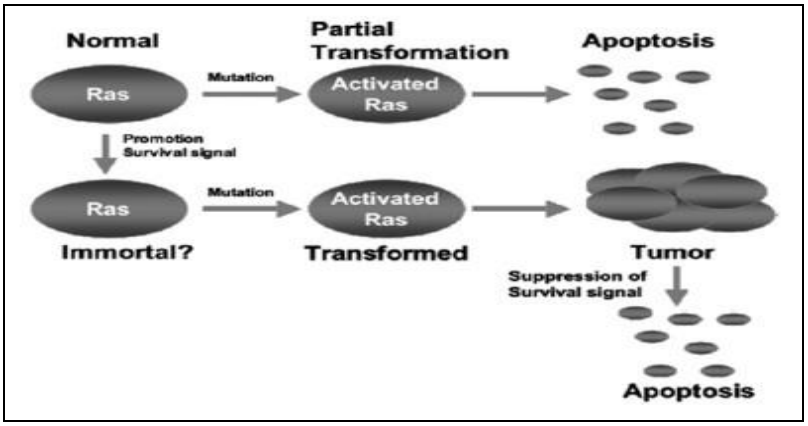

FIG. 5.1. Survival signals as targets in cancer.
An activated oncogene such as Ras sensitizes cells to default apoptotic programs, and therefore, mutations such as those that activate Ras will result in apoptosis. However, if a survival signal is activated or a tumor promoting agent is present to suppress apoptosis, Ras mutations will not induce apoptosis and the cells can proliferate and form a tumor mass. Suppression of the survival signal, in theory, resurrects the cellular apoptotic programs resulting in the death of the cancer cells.

5.2. PLD and Survival Signals

mTOR has been identified as an important player in cell survival signaling. mTOR is stimulated by amino acids, hormones, and mitogens and is critical for progression through the cell cycle. It has been reported that PLD can activate mTOR through PA in malignant human breast cancer cell line MDA-MB-231 (Chen et al., 2005). PLD-generated PA competes with rapamycin to interact with mTOR (Fang et al., 2001; Chen et al., 2003). PLD-generated survival signals in breast cancer cells are dependent on mTOR (Chen et al., 2004). The PI3K/Akt pathway is often activated to provide survival signals in many human cancers (Vivanco and Sawyers, 2002). Therefore, elevated PLD activity in human cancer cells represents an alternative mechanism for activating mTOR and preventing the stress response that occurs when mTOR is suppressed. Fig. 5.2 shows a model of the PLD and PI3K survival pathways targeting mTOR. Intriguingly, the two survival pathways, PLD/mTOR and PI3K/Akt, are connected by their dependence on PIP2 and PIP3, The levels of PIP2 and PIP3 are controlled in part by PI3K and PTEN (Vivanco and Sawyers, 2002). Thus, the control of these two survival pathways may be coordinately regulated through the level of

phosphorylated phosphoinositides as indicated in Fig. 5.2.

In the model shown in Fig. 5.2, alternative survival signals are generated by activation of either PLD or PI3K. 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 dephosphorylates PIP3 to PIP2. PDK1 phosphorylates and activated Akt, which then phosphorylates and inactivates several substrate proteins that negatively regulate cell proliferation or stimulate apoptosis. These include GSK3β, BAD, forkhead family transcription factors (FKHR), 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. It is therfore possible that phospholipase C could be a critical negative regulator of both survival pathways by removing the PIP2 required for both pathways.

Many studies have shown that PLD is implicated in cell proliferation and transformation. PLD elevation responds to some growth factors such as PDGF (Plevin et al., 1991), FGF (Motoike et al., 1993), and EGF (Song et al., 1994). PLD activity is elevated in cells transformed by oncogenes v-Src (Song et al., 1991), v-Ras (Canero et al., 1994), v-Fps (Jiang et al., 1994), and v-Raf (Frankel et al., 1999). It has been shown that elevated PLD activity contributes to cell transformation in Ras-transformed cells (Buchanan et al., 2005). Elevated PLD activity has also been shown to suppress senescence and apoptosis induced by high intensity Raf signaling (Joseph et al., 2002), suggesting that the role of PLD in transformation is to prevent cell cycle arrest and apoptosis.

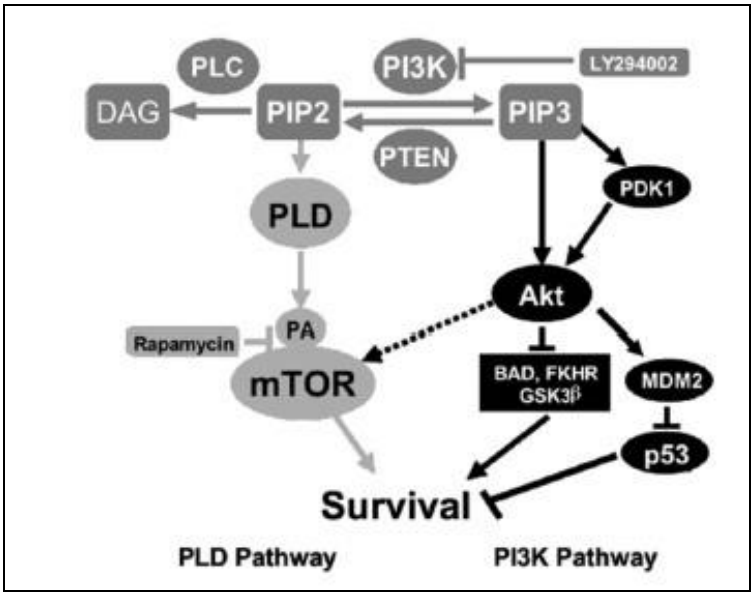

FIG. 5.2. Alternative PLD and PI3K survival signals.
Alternative survival signals are generated by activation of either PLD or PI3K. PI3K generates PIP3 from PIP2. The presence of PIP3 leads to the recruitment of PDK1 and Akt. PIP3 levels are also regulated by PTEN, a phosphatase that dephosphorylates PIP3 to PIP2. PDK1 phosphorylates and activated Akt, which then phosphorylates and inactivates several substrate proteins that negatively regulate cell proliferation or stimulate apoptosis. These include GSK3β, BAD, forkhead family transcription factors (FKHR), 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. It is therfore possible that phospholipase C could be a critical negative regulator of both survival pathways by removing the PIP2 required for both pathways.

5.3. PLD - A Gatekeeper Override in Cancer Cells

Elevated PLD activity has been shown to suppress p53 expression and apoptosis induced by DNA damage (Hui et al., 2004). The ability of PLD to suppress p53 expression allows the cell to progress through cell cycle checkpoints regulated by p53 and also restricts the cellular response to DNA damage, which increases genomic instability (Foster, 2006). Moreover, elevated PLD activity also suppresses PP2A (Hui et al., 2005). Thus, elevated PLD activity is able to accomplish much of what the SV40 early region genes accomplish – that being the suppression of the tumor suppressor genes p53 and PP2A (Foster, 2006). Elevated PLD activity has also been shown to be able to induce the phosphorylation and inactivation of the retiblastoma tumor suppressor gene Rb (Gadir et al., 2007). These findings indicate that PLD can

actually accomplish all of what the SV40 early region genes accomplish to transform human cells because it has been reported that SV40 early region genes, large T and small T antigens inactivate p53, pRb and PP2A (Land et al., 1983; Dotto et al., 1985; Hahn et al., 1999; Elenbaas et al., 2001).

5.4. Targeting PLD Signaling in Cancer Cells

The important role of PLD signaling in cancer cell proliferation and survival makes it an ideal target in the therapeutic intervention in human cancers with elevated PLD activity.

Upstream Targets

In our research, we have shown that human cancers with activated H- and K-Ras genes have elevated PLD activity, which is induced by activated H- and K-Ras. Thus, targeting activated H- and K-Ras becomes a better way to shut down PLD signaling and eventually induce cell apoptosis. There have been some Ras inhibitors on the market which have shown some effects in inhibiting Ras expression (Caraglia et al., 2007; Wesierska-Gadek et al., 2007). As shown in Fig. 5.3, Ras can activate PLD via its downstream effectors RalA and PI3K, therefore RalA and PI3K can also be targets of PLD signaling in anticancer therapies. Inhibitors of Ras, RalA and PI3K should theoretically block the activation of PLD and therefore block the survival signal.

PIP2 is a co-factor required for the activation of PLD. As shown in Fig. 5.3, PI4P5K converts PIP into PIP2, therefore PI4P5K can be a good target in anticancer therapies. Inhibition of this enzyme should reduce the amount of PIP2 and thereby result in less activation of PLD.

PLD

Since PLD is the center of PLD signaling in cancer cells, PLD itself can be a good target in anticancer therapies. However, at present, there are no good inhibitors of PLD other than alcohol, which prevents the production of PA by competing with water to form an inert phosphatidyl-alcohol (Exton, 2002a). Our research has shown that honokiol, a natural plant product, can effectively inhibit PLD activity *in vitro* through an unidentified mechanism. It is also reported that honokiol inhibits *in vitro* and *in vivo* growth of breast cancer through induction of apoptosis and cell cycle arrest (Wolf et al., 2007), possibly through the inhibition of PLD since elevated PLD activity has been found in human breast cancer cells. These findings indicate that honokiol may represent an interesting PLD inhibitor.

Downstream Targets

PLD may have multiple downstream targets. Among these targets, mTOR has emerged as a very attractive one. mTOR has been widely implicated as a critical target of survival signals generated by PLD and PI3K (Foster, 2004). Rapamycin is an effective mTOR inhibitor and can thus be used to shut down mTOR-mediated PLD survival signaling in cancer cells. However, the apparent competition between PA and rapamycin leads to rapamycin resistance because higher concentrations of rapamycin are required to out-compete the PA. Reducing PLD activity, and consequently PA levels, makes rapamycin effective at lower concentrations. Therefore a combination of PLD inhibitor and rapamycin should generate better therapeutic results in treating human cancers with elevated PLD activity

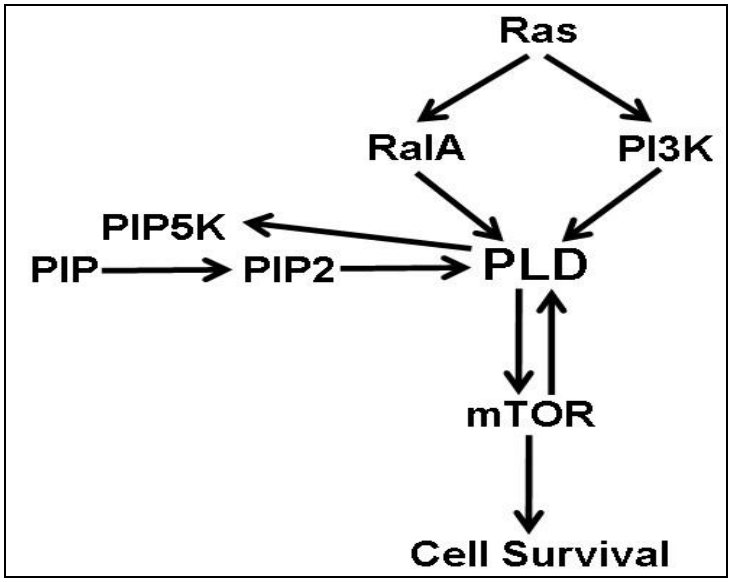

FIG. 5.3. Targeting PLD Signaling in anticancer therapies.
Upstream, downstream and PLD itself can be good targets of PLD signaling in anticancer therapies.

5.5. Summary and Future Directions

Apoptosis or programmed cell death evolved as a mechanism for getting rid of unwanted cells during development. It has become apparent that the ability of a cell to die when appropriate has been adapted as means of preventing cancer and is likely our most important protection against the disease (Foster, 2006). In principle, drugs that inactivate survival signals are ideal targets for anticancer therapies because inactivation of the survival signal can, in principle, lead to the apoptotic death of the tumor cells. PLD has been demonstrated to provide survival signals in breast cancer cells where there is elevated PLD activity (Chen et al., 2004). PLD suppresses the tumor suppressing effects of p53 (Hui et al., 2004), PP2A (Hui et al., 2005), and Rb (Gadir et al., 2007), indicating that PLD can suppress important gatekeeper functions that protect from cancer. PLD also targets mTOR (Fang et al., 2001; Chen et al., 2003), which has been widely implicated in survival signaling (Foster, 2004). Thus, targeting PLD signals or the signals that lead to elevated PLD activity in cancer cells

holds great promise for treating an apparent large number of cancers that have elevated PLD activity (Foster, 2006).

A critical aspect of targeting survivals signals is to determine the survival signals that individual cancers are dependent upon. Obtaining useful and relevant information on individual tumors will likely require a more proteomic-based approach. Currently, antibodies are available that are specific for activated signaling proteins such as phosphorylated Akt, which is indicative of an active PI3K pathway. More generally, antibodies against phosphotyrosine could indicate whether drugs targeted against a tyrosine kinase would be effective. Enzymatic assays could also be employed. Tyrosine kinase and GTPase assays could be particularly useful. And in this regard, PLD assays could also be of use to establish whether this survival pathway is activated and also as an indicator as to whether rapamycin is likely to be effective. The critical need now is to develop systematic molecular approaches to the characterization of molecular defects in individual tumors. Many tools for such an approach are currently available and the information that could be generated would allow for the rationale targeting of survival signals that keep cancer cells alive.

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