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A Role for Phospholipase D in Cell Cycle Control and Transformation.

by

TROY W. JOSEPH

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

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ABSTRACT

A Role for Phospholipase D in Cell Cycle Control and Transformation

by

TROY W. JOSEPH

Advisor: David A. Foster, Ph.D

Phospholipase D (PLD) activity is elevated in response to most mitogenic signals. Two mammalian PLD genes (PLD1 and PLD2) have been cloned and their gene products characterized. PLD1 is a downstream target of the Ras/RalA GTPase cascade implicated in mitogenic and oncogenic signaling. Consistent with a role in mitogenic signaling, elevated expression either of PLD1 or PLD2 results in the transformation of cells overexpressing either c-Src or the epidermal growth factor (EGF) receptor (EGFR). Both PLD1 and PLD2 were also able to overcome cell cycle arrest induced by high intensity Raf signaling.

These data suggest that elevated PLD activity may be an important factor in progression to a malignant phenotype in cells with elevated tyrosine kinase activity. The overexpression of a tyrosine kinase is a common genetic alteration in several human cancers. Also, PLD activity provides a novel survival signal(s) that overcomes the inhibitory effects of high intensity Raf signaling on cell cycle progression.

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To my parents for your love, encouragement and support.

Finally, I dedicate this dissertation to my loving and caring wife Evelin Joseph. Without your love and support I could never have achieved all that I have. You are the best. Thank you.

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LIST OF ABBREVIATIONS

DAG	Diacylglycerol
DMEM	Dulbecco Eagle modified Medium
Ecd	Ecdysone
EDTA	Ethylenediaminotetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
KDa	KiloDaltons
MAPK	mitogen- activated protein kinase
MEK	(MAPK/ERK kinase)
MKP	MAPK phosphatase
PA	Phosphatidic acid
PBS	Phosphate buffered saline
PKC	Protein kinase C
PDGF	Platelet-derived growth factor
PH	Pleckstrin homology
PI-3 K	Phosphatydilinositol 3-Phosphate kinase
PKC	Protein kinase Ca++-dependent
PLC	Phospholipase C
PLD	Phospholipase D
Pon A	Ponasterone A
PTK	Protein tyrosine kinase
PTP	protein tyrosine phosphatase
SDS	Sodium dodecyl sulfate
SH	Src Homology

CHAPTER 1

INTRODUCTION

PHOSPHOLIPASE D

Introduction

Phospholipase D (PLD) was first discovered in plants as a distinct phospholipid specific phosphodiesterase activity that hydrolyses phosphatidylcholine (PC) to phosphatidic acid (PA) and choline.(Hanahan *et al.*, 1947). However, widespread interest in this enzyme began once experiments in cultured animal cells revealed its rapid and dramatic activation to extrcellular stimuli. (Cockcroft *et al.*, 1984, Exton *et al.*, 1990).

Phospholipase D (PLD) is a widely distributed enzyme that is under the elaborate control of hormones, neurotransmitters, growth factors and cytokines in mammalian cells. Phospholipase D belongs to a class of enzymes known as Phospholipase, they includePLA_1, PLA_2, PLC and PLD. They function to hydrolyze membrane phospholipids and generate different biological molecules as shown in Figure 1. The activation of PLD is believed to play an important role in the regulation of cell function and cell fate. Multiple PLD activities were characterized in eukaryotic cells, and more recently, several PLD genes have been cloned. A PLD gene superfamily, defined by a number of structural domains and sequence motifs, also includes phosphatidyltransferases, and certain phosphodiesterases.

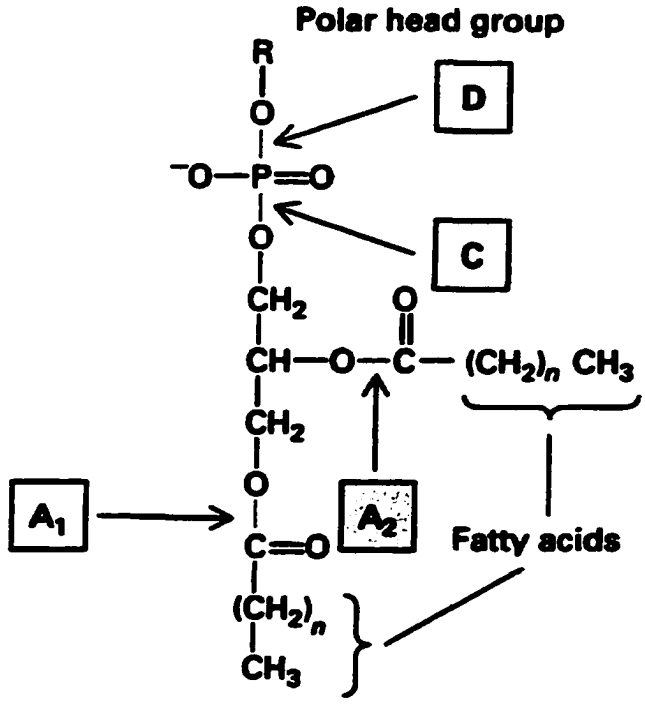

FIGURE 1. Phospholipase cleavage sites on phospholipids.

Phospholipase D major substrate is phosphatidylcholine (PC), when it is hydrolyzed by PLD; the products are phosphatidic acid and choline. PLD catalyses a phosphatidyl transfer reaction in which a primary alcohol, such as n-ethanol acts as a nucleophilic acceptor in place of water. The resulting production of phosphatidyl ethanol represents a specific assay for PLD (Figure 2).

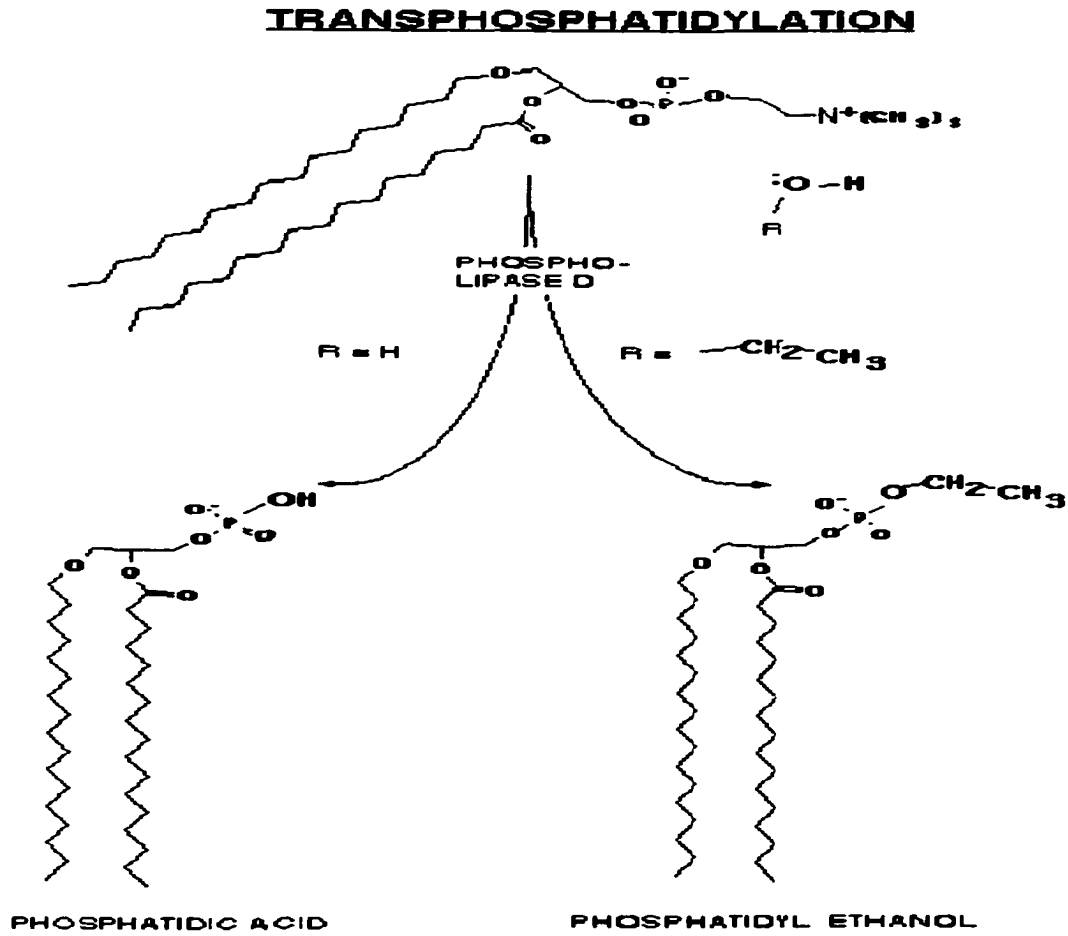

Figure 2. PLD specific transphosphatidyl reaction.

PLD STRUCTURE AND LOCALIZATION

PLD has recently been cloned from yeast, bacteria, plant, and mammalian sources (Morris *et al.*, 1996). Two separate mammalian PLD genes approximately 50% identical have been reported (hPLD1 and hPLD2;), (Colley *et al.*, 1997), with hPLD1 having two splice variants hPLD1a and hPLD1b. hPLD1a has 1072 amino acids and a molecular mass of 124 kDa (Hammond *et al.*, 1995). It is specific for PC and was obtained by using the yeast PLD gene (SPO14) (Rose *et al.*, 1995) to identify a human expressed sequence tag for screening a HeLa cDNA library. A shorter splice variant of hPLD1a with 1034 amino acids (hPLD1b) (Figure 3), which has similar regulatory properties, has been identified (Hammond *et al.*, 1997), and another PLD (PLD2) (Figure 3), which has 932 amino acids and 51% amino acid sequence identity to hPLD1a, has been cloned from a mouse embryonic library (Colley *et al.*, 1997). The amino acid sequence of PLD1 contains regions that are conserved with PLD2 as well as other nonmammalian species. In addition it contains a "loop region" that is unique to PLD1. Possible functions that have been proposed or demonstrated for these regions are shown in (Figure 4A,B).

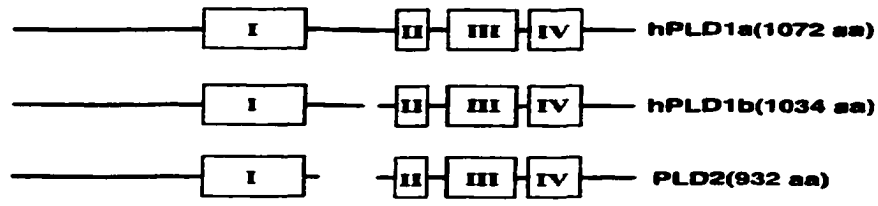

Figure 3. Alignment of conserved regions common to hPLD1a, hPLD1b, and PLD2.

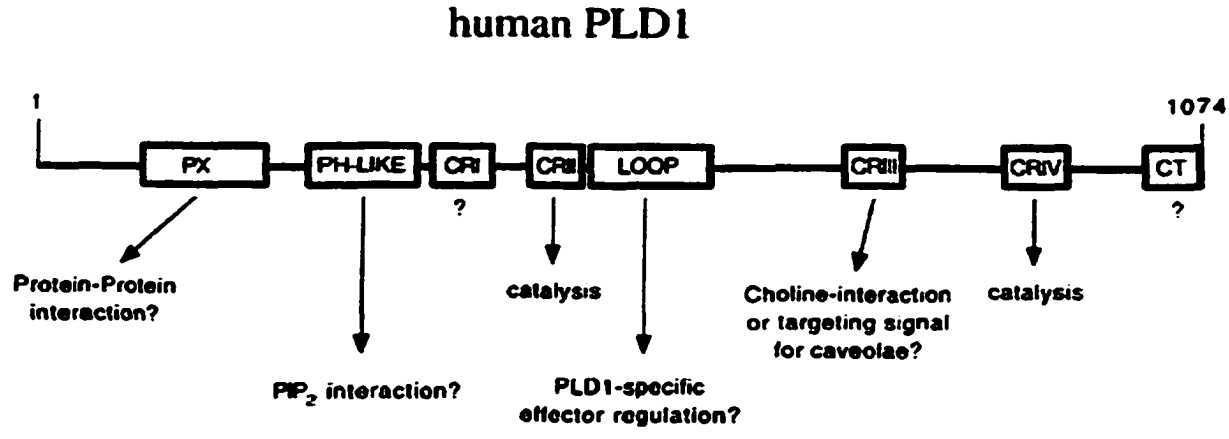

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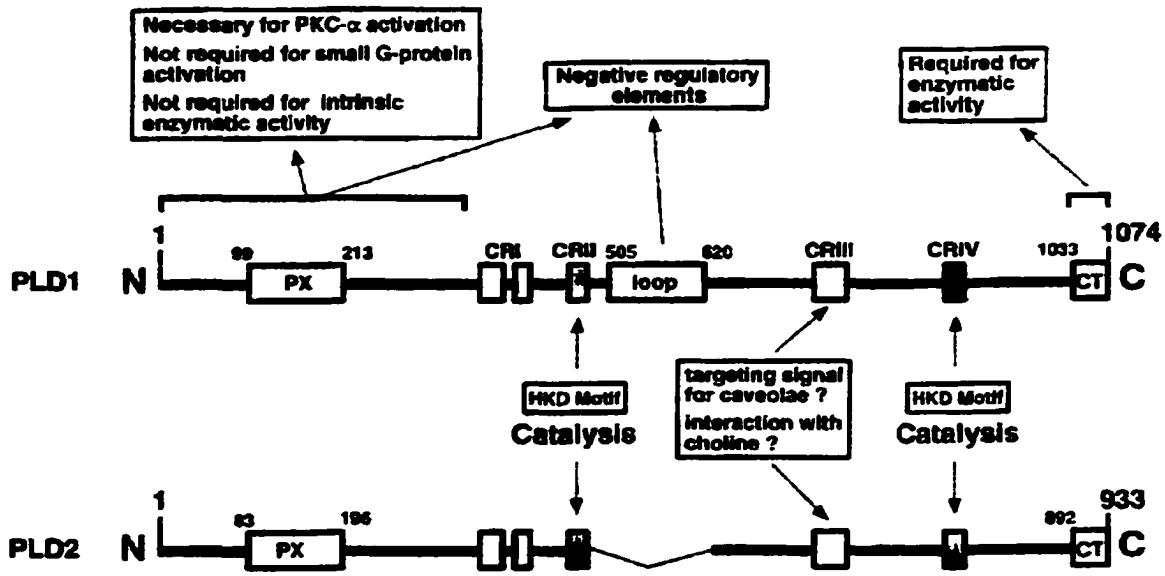
Figure 4A. Conserved and unique features for human PLD1.



Borrowed from Sung et.al., *J Biol Chem.* **274(1):** 494-502

Figure 4B. Conserved and unique features for human PLD1 and PLD2

The PLD1 amino acid sequence encodes regions of sequence that either is unique to PLD1 (loop region) or is conserved with mammalian PLD2 and some or all PLD's from nonmammalian species (other boxed regions). Possible functions that have been proposed or demonstrated for these regions are listed underneath each box. CT, carboxyl terminus; LOOP, loop region. (Borrowed from Sung *et al.*, 1999).

The subcellular localization of PLD is still unclear at this time. Some groups have reported that in fibroblasts PLD2 localizes predominantly in the caveolin-rich membrane domains of the plasma membrane, whereas PLD1 is perinuclear, i.e. in endoplasmic reticulum, Golgi, and late endosomes (Colley *et al.*, 1997; Czarny *et al.*2000). While other groups, including our own, have found PLD1 and PLD2 to be localized in the cavolin enriched membrane fraction (Kim *et al.*, 1999; Xu *et al.*, 2000) (Figure 5).

Yes	No	PKC/ARF/Rho Responsive
Yes	Yes	PIP2 Dependence
~120	~106	Molecular Weight kDa
Low	High	Basal activity
PC	PC	Substrate Specificity
Yes	Yes	Transphosphatidylation
PM,CEM,ES	PM,CEM	Subcellular localization

Figure. 5 Biochemical properties of Phospholipase D 1 and 2

Presented are various characteristics of PLD 1 and 2, corresponding references are presented in the text. PM-plasma membrane; CEM-caveolae enriched membrane; ES-endosomes; PIP2-phosphatidylinositol-4, 5-bisphosphate.

Regulation of PLD

PLD has been shown to be responsive to several signaling proteins including, Protein Kinase C alpha (PKCα), and the small GTPases RhoA, and ADP ribosylation Factor (ARF). In addition, we have recently shown that a PLD activity was found to be associated with the Ras-family GTPase RalA, which is required for the activation of PLD by both v-Src and v-Ras (Jiang *et al.*, 1995b) and the active complex includes PLD1, RalA and Arf (Luo *et al.*, 1997; 1998).

The ADP ribosylation factor (ARF) was first discovered as a factor that stimulated cholera toxin-induced ADP-ribosylation of Gs (Lopez *et al.*, 1995). It is now recognized to play a role in vesicle trafficking in Golgi and has been implicated in the fusion of microsomal vesicles and endosomes, the assembly of nuclear membranes, and the formation of clathrin-coated vesicles (Moss *et al.*, 1995; Springer *et al.*, 1999; Roth MG. 1999). The activation of PLD by ARF was first recognized by the groups of Sternweis and Cockcroft (Brown *et al.*, 1993; Cockcroft *et al.*, 1994) and has now been shown using PLD from many sources (Exton JH. 1997). Studies with cloned PLD purified from Sf9 cells indicate that ARF interacts directly with the enzyme (Hammond *et al.*, 1997). Some reports have indicated that cytosolic factors greatly enhance the effect of ARF on PLD (Singer *et al.*, 1996; Lambeth *et al.*, 1995; Bourgoin *et al.*, 1995; Takahashi *et al.*, 1996). Two of these factors are PKC (Singer *et al.*, 1996) and calmodulin (Takahashi *et al.*, 1996). It is interesting to note that RalA has been shown to contain a calmodulin binding site (Wang *et al.*, 1997) and in this way may act to stabilize a RalA/PLD1/Arf complex.

The activation of PLD by the oncogenic tyrosine kinase v-Src is mediated by a GTPase cascade of Ras and RalA (Jiang *et al.*, 1995a, 1995b). An active PLD can be precipitated from cell lysates with immobilized GST-RalA fusion protein (Jiang *et al* 1995b). Subsequent studies demonstrated that the PLD associated with RalA is PLD1 and that this interaction is direct (Luo *et al.*, 1997). Further investigation of the mechanism of PLD activation in RalA-PLD1 complexes revealed that Arf is associated with an active RalA-PLD1 complex

(Luo *et al.*, 1998). The level of Arf protein associated with RalA correlated well with the level of PLD activity in the RalA-PLD1 complexes, and the levels of Arf associated with RalA were substantially elevated in the presence of a non-hydrolyzeable analogue of GTP (GTPγS) that stimulated Arf membrane association. In addition, Brefeldin A (BFA), which inhibits GDP to GTP exchange on Arf (Chardin *et al.*, 1999), blocked the v-Src and v-Ras induced PLD activity..

The interaction between RalA and Arf is likely an indirect one. Immobilized RalA was unable to precipitate significant levels of Arf from a partially purified preparation of Arf, suggesting that the association between RalA and Arf is facilitated by another factor. Thus, there is a GTP-dependent association between Arf and a RalA-PLD1 complex that is involved in the activation PLD1 in response to the oncogenic signals generated by v-Src and v-Ras. In addition, one group has found that RalA interacts directly with PLD1 but unlike our results they find that RalA synergistically enhances Arf dependent PLD1 activity (Kim *et al.*, 1998). While another group has found that RalA is able to restore PMA induced PLD activity blocked by treatment with bacterial toxins, TcsL and TcdB-1470, which glucosylates and inactivates Rac, Rap, and Ral GTPases. Indicating a role for Ral in PKC dependent PLD activation (Schmidt *et al.*, 1998).

Rho family proteins regulate many cellular activities including those involving the actin cytoskeleton. The proteins include Rho, which controls the

formation of focal adhesions and actin stress fibers, Rac, which regulates lamellipodia formation and membrane ruffling, and Cdc42, which controls the formation of filopodia (Machesky *et al.*, 1996; Ridley *et al.*, 1996). The first evidence that PLD could be regulated by Rho proteins came from a study by (Bowman *et al.*, 1993). They showed that the stimulatory effect of GTPγS on PLD in neutrophil plasma membranes was inhibited by RhoGDI, a protein that inhibits GDP dissociation from Rho proteins and thereby blocks their activation. In subsequent studies using plasma membranes from rat liver, HL-60 cells, and neutrophils, it was found that RhoA was the most effective Rho protein to activate PLD, but Rac1 or Cdc42Hs showed some activity (Malcolm *et al.*, 1994; Siddiqi *et al.*, 1995; Kwak *et al.*, 1995). Studies with cloned PLD purified from Sf9 cells indicate that RhoA interacts directly with the enzyme and that Rac1 and Cdc42 are also active (Hammond *et al.*, 1997). Interestingly, like the case for RalA and Arf-1 (Kim *et al.*, 1998) a combination of RhoA and ARF results in synergistic activation of homogeneous or partially purified PLD (Hammond *et al.*, 1997; Singer *et al.*, 1996; Kuribara *et al.*, 1995). This suggests the presence of separate but interacting sites for Rho and ARF on PLD. In agreement it has recently been shown that RhoA interacts with a unique c-terminus site of hPLD1 (Yamazaki *et al.*, 1999). As in the case of ARF and RalA, there is evidence that RhoA action on PLD is enhanced by other as yet unidentified cytosolic proteins (Shimooku *et al.*, 1996; Kwak *et al.*, 1995).

There is abundant evidence that PLD is regulated by PKC in most mammalian cells. This comes from studies of the effects of phorbol esters, PKC inhibitors, down-regulation of the enzyme, and overexpression and deletion of

specific PKC isozymes (Lu *et al.*, 2000; Hornia *et al.*, 1999). Although a role for PKC in the actions of many agonists on PLD in many cells has been indicated, there are also instances where the enzyme does not seem to be involved, as in the case of v-Src and v-Ras (Exton JH. 1997; Song *et al.*, 1993; See below).

Physiological Roles of PLD

The involvement of small GTPases like Ras (Jiang *et al.*, 1995a), Rho (Ohguchi *et al.*, 1995), and Arf (Hammond *et al.*, 1995) in the activation of PLD suggest that PLD activation might play a role in cytoskeleton reorganization and intracellular protein trafficking. PLD has been implicated in the regulation of vesicle trafficking by ARF in Golgi. The formation of coated vesicles is also inhibited by ethanol, which reduces PA formation due to the production of phosphatidylethanol, and treatment of Golgi membranes with bacterial PLD promotes coatamer binding (Ktistakis *et al.*, 1996). Studies with ethanol and exogenous PLD in other cell types have also provided evidence that PLD is involved in vesicle transport (Cockcroft S. 1996). There are at least two potential mechanisms for how PLD activation might influence vesicle formation (Figure 6).

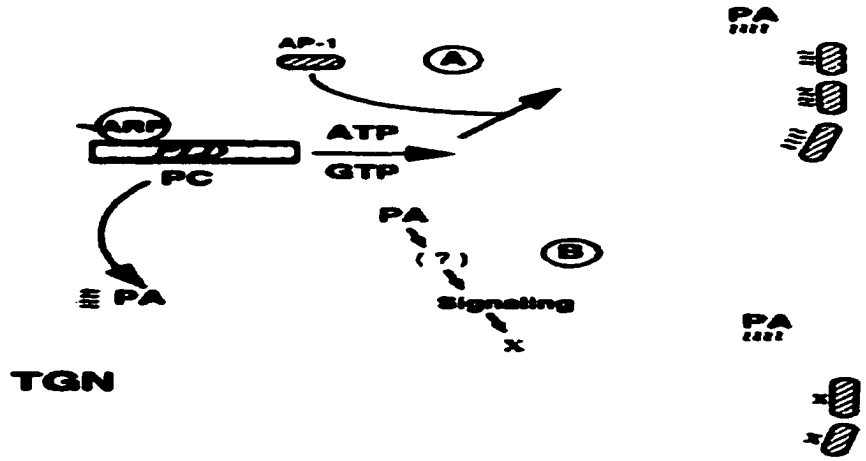


Figure 6. Possible effects of PLD activation in nascent vesicle formation. **(A)** PLD converts phosphatidylcholine to phosphatidic acid resulting in changes in both charge and pH at the membrane, which is hypothesized to alter membrane topology and facilitate vesicle formation. **(B)** PA is rapidly hydrolyzed to a number of possible intermediates which may be converted to other metabolites that could initiate a signaling cascade, the end product of which (X) mediates coat recruitment. (Modified from Chen *et al.*, 1997).

One potential mechanism is changing the properties of cellular membranes by altering their lipid composition. Thus, by causing local changes in PC and PA the physical properties of the membranes could be substantially changed. A second mechanism is by generating PA. This lipid would probably remain in the membrane but could interact with proteins located in the membrane or cytosol (Figure 6). Many proteins have been shown to have their activities changed by PA *In vitro* (Exton JH. 1997), but evidence that they are targets of the lipid *In vivo* is largely lacking.

In addition to its role in vesicle formation PLD activation plays a major role in mitogenic signaling. As mentioned above, activation of PLD results in the production of PA, which is rapidly converted to DG in most cells through the action of phosphatidate phosphohydrolase (Figure 7). Thus the late phase of activation of PKC produced by agonists in many cells is mainly attributable to DG derived from PLD action (Jiang *et al.*, 1994; Jiang *et al.*, 1996; Ha *et al.*, 1993).

Lastly activation of PLD results in the generation of LPA through the action of a specific Phospholipase A2 on PA (Figure 7). LPA has been to shown to be mitogenic via it's binding to the LPA receptor (Moolenaar & van Corven, 1990). This receptor is a classic serpentine receptor, which couples to the heterotrimeric family of g-proteins (Moolenaar *et al.*, 1997). Interestingly, it has been recently reported that upon LPA stimulation, dynamin a small GTPase that facilitates the release of endocytic vesicles (Vieira *et al.*, 1996) becomes tyrosine phosphorylated and associated with the LPA receptor (Kranenburg *et al.*, 1999).

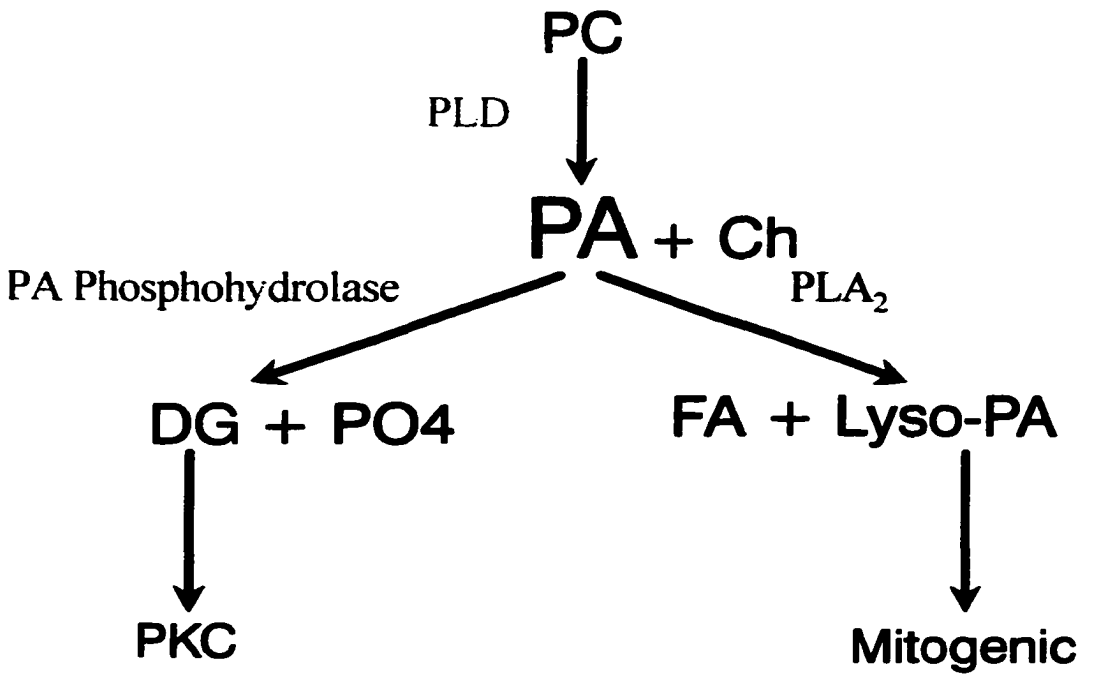


Figure 7. Effects of PLD activation on mitogenic signaling pathways.

PA generated by PLD can be further metabolized to DG and free phosphate by the action of a PA- phosphohydrolase. DG can activate DG responsive PKC Isoforms. Alternatively PA can be converted to Lyso-PA and free fatty acid (FA). Lyso-PA can bind its classic serpentine receptor thereby leading to mitogenic signaling.

The potential function of PLD in the regulation of cell proliferation remains controversial. PA, LPA and bacterial PLD are mitogenic in several cell lines (Exton JH. 1997; van Dijk *et al.*, 1998), however there is a lack of correlation between PLD activity and mitogenesis in some cell lines (Paul *et al.*, 1994). Other signaling pathways are undoubtedly involved in growth control. The mechanisms by which PLD could control the cell cycle are unknown. However, Raf-1 kinase, which is involved in signal transduction from several receptors, has a binding site for PA and is translocated to membranes under conditions where PLD is activated (Ghosh *et al.*, 1996).

CHAPTER 2

Phospholipase D1 and 2 activity cooperates with c-Src Tyrosine kinase to transform 3Y1 cells

INTRODUCTION

c-Src was the first cellular homologue of a viral oncoprotein to be discovered. (Stehelin *et al.*, 1976). It is important for many mitogenic signaling from many RPTKs, and has been implicated in many cancers (Bjorge *et al.*, 2000). There is a tight control of c-Src kinase activity through intramolecular interactions. In the inactive c-Src, a C-terminal tyrosine residue (527 in mouse, 530 in human) lacking in deregulated v-Src, is phosphorylated and interacts with the c-Src SH2 domain, while the c-Src SH3 domain interacts with the linker region between the SH2 domain and the N-terminal kinase lobe. The SH2 and SH3 intramolecular interactions repress kinase activity by displacing the Cα helix in the N-terminal lobe and by positioning the activation loop to block access to the active site. C-Src can be de-repressed not only upon dephosphorylation of phosphorylated Tyr 527, but also by binding through its SH2 to specific tyrosine autophosphorylation sites in ligand stimulated RPTKs, resulting in SH2 displacement from phosphorylated Tyr527, or by binding of the SH3 domain to Pro-X-X-Pro motifs in target proteins (Sicheri *et al.*, 1997). This results in autophosphorylation in trans of the conserved activation loop Tyr 416 and stabilization of the active conformation. This activation mechanism is similar to that of RPTKs, in that the activation event (ligand binding and dimerization for RPTKs and SH2 or SH3 domain ligand engagement for c-Src) results in removal of inhibitory constraints on the kinase domain.

Several PTPs, are implicated in regulating c-Src through dephosphorylation of the c-terminal c-Src kinase (CSK) phosphorylation sir, Tyr527, including receptor-like PTPα, PTPγ, and RPTKε, and the cytoplasmic PTB1B, SHP-1, and SHP-2. Elevated activity or the expression of several of these PTPs correlate with enhanced levels of c-Src kinase activity in a number of transformed cells. (Bjorge *et al.*,2000). The most direct demonstration that c-Src is involved in human cancer was the identification of a mutant c-Src with a truncation of the C terminus, ending with Tyr530 in human colon cancer (Irby *et al.*, 1999) This mutant has deregulated kinase activity because the lack of residues C-terminal to the phosphorylated Tyr530 prevents SH2 association and establishment of the inactive conformation. Again, oncogenic perturbation results from relief of the tightly controlled constraints on kinase activity. It is not clear which signaling pathways are important for c-Src transformation, but a dominant –negative mutant of signal transducer and activator of transcription (STAT)-3 blocks v-Src transformation and c-Myc induction, indicating that STATs might be involved.

Overexpression of a tyrosine kinase is a common genetic defect in a variety of human tumors (Hunter *et al.*, 1998). The epidermal growth factor (EGF) receptor, which has an intrinsic tyrosine kinase that is activated in response to EGF, is frequently overexpressed in human breast and ovarian cancer (Reese *et al.*, 1997). However, overexpression of a tyrosine kinase such as the EGF receptor is not sufficient for a fully transformed or cancerous phenotype. We recently demonstrated that downregulation of protein kinase δ (PKCδ) transforms 3Y1 rat fibroblasts overexpressing either c-Src (Lu *et al.*, 1997) or the EGF receptor

(Hornia *et al.*, 1999). The EGF receptor –overexpressing (EGFR cells) could also be transformed when treated with EGF, suggesting that EGF could accomplish what PKC δ downregulation also accomplished. Downregulation of PKD δ also caused an increased in Phospholipase D activity (Hornia *et al.*, 1999, Song *et al.*, 1993) which is commonly elevated in response to oncongenic and mitogenic stimuli (Feig *et al.*, 1988, Urano *et al.*, 1996).

The Phospholipase D (PLD) activity is elevated in response to most mitogenic signals. Two mammalian PLD genes (PLD1 and PLD2) have been cloned and their gene products characterized. PLD1 is a downstream target of the Ras/RalA GTPase cascade implicated in mitogenic and oncogenic signaling. Consistent with a role in mitogenic signaling, elevated expression of PLD1 resulted in the transformation of cells overexpressing the epidermal growth factor (EGF) receptor (EGFR). However, PLD2 co-localizes with the EGFR in caveolin-enriched light membrane microdomains where many signaling molecule co-localize. We therefore investigated whether PLD2 could also contribute to the transformation of cells overexpressing a tyrosine kinase. We report here that elevated expression of PLD2 transforms 3Y1 rat fibroblasts overexpressing either the EGF receptor or the non-receptor tyrosine kinase c-Src. We also examined the effect of PLD1 under controlled expression and we report that both PLD1 and PLD2 transform cells overexpressing a tyrosine kinase with high efficiency. Since overexpression of a tyrosine kinase is a common genetic alteration in several human cancers, these data suggest that elevated PLD activity may be an

important factor in progression to a malignant phenotype in cells with elevated tyrosine kinase activity.

Phospholipase D (PLD) converts phosphatidylcholine to phosphatidic acid and choline and has been implicated in several aspect of cell physiology from vesicle transport to signal transduction (Exton *et al.*, 1998). PLD activity has also been implicated in mitotic signaling and transformation (Aguirre-Ghiso *et al.*, 1999; Urano *et al.*, 1996; Frankel *et al.*, 1999; Hornia *et al.*, 1999; Lu *et al.*, 2000). Two PLD isoforms have been identified, PLD1 (Hammond *et al.*, 1995) and PLD2 (Colley *et al.*, 1997). We recently reported that an overexpressed PLD1 cooperated with epidermal growth factor (EGF) receptor (EGFR) overexpression to transform rat fibroblasts (Lu *et al.*, 2000). PLD1 has been implicated previously in mitogenic signaling by virtue of an association with RalA (Jiang *et al.*, 1995; Luo *et al.*, 1997; 1998), a downstream target of Ras oncogenic signals (Feig *et al.*, 1999). However, PLD1 has also been implicated in vesicle budding in the Golgi (Ktistakis *et al.*, 1996; Chen *et al.*, 1997; Bi *et al.*, 1997), and localization studies reveal predominantly a peri-nuclear localization for PLD1 (Hammond *et al.*, 1995; Colley *et al.*, 1997), consistent with a role in the Golgi. In contrast, PLD2 has been reported to localize predominantly in caveolin-enriched light membrane fractions where many molecules involved in mitogenic signaling are localized (Colley *et al.*, 1997; Czarny *et al.*, 2000; Xu *et al.*, 2000). Consistent with a role for PLD2 in mitogenic signaling, PLD activity elevated in response to EGF or v-Src was shown to be largely restricted to light membrane fractions (Xu *et al.*, 2000). Thus, circumstantial evidence implicates

both PLD1 and PLD2 as the PLD activated in response to mitogenic signals: PLD1 by virtue of its connection to the Ras/Ral GTPase cascade; and PLD2 by virtue of being localized to membrane microdomains where elevated PLD activity is elevated in response to mitogenic signaling. With regard to localization, PLD1 has also been reported to localize to light membrane fractions along with PLD2, although to a much lesser extent (Kim *et al.*, 1999; Xu *et al.*, 2000). Thus, at the present, relative contributions of PLD1 and PLD2 to mitogenic signaling are confusing.

We recently reported that PLD activity is required for endocytosis of the EGF receptor (Shen *et al.*, 2001). Surprisingly, both PLD1 and PLD2 were apparently required. Endocytosis of the EGF receptor is required for some of the intracellular signals generated by EGF (Vieira *et al.*, 1996; Kranenburg *et al.*, 1999; Shen *et al.*, 2001) including the activation of MAP kinase. Therefore, these data suggest the possibility that mitogenic signals initiated by EGF might also involve both PLD isoforms. In this report, we have investigated the effect of both PLD1 and PLD2 on mitogenic signaling. We present evidence here that elevated expression of either PLD1 or PLD2 transforms cells with an overexpressed tyrosine kinase.

Results

A plasmid vector that expresses Flu-tagged hPLD1 was cotransfected into EGFR cells along with pCEP4 (Invitrogen), which expresses a hygromycin resistance marker. Transfections were attempted in both the EGFR cells and parental 3Y1 cells. Interestingly, hygromycin-resistance colonies were detected after 10 days only in the EGFR cells (58 hygromycin resistant colonies were found), no hygromycin –resistant colonies was detected in the parental cell 3Y1 cells. This was not due to differences in transfection efficiency between the two cell lines, because transfection with pCEP4 alone gave very similar numbers of hygromycin-resistant colonies in both EGFR cells and parental 3Y1 cells (97 and 110 hygromycin resistant colonies respectively). Thus, expression of PLD1 in the parental 3Y1 cells is apparently toxic to the parental 3Y1 cells, which is consistent with previous reports suggesting that a high level of PLD expression is difficult to obtain (Hornia *et al.*, 1999) The EGFR cells are tolerant of higher levels of PLD1 expression. As shown in figure 8, several of the clones displayed an increased colony forming efficiency, and, importantly, the ability to form colonies correlated with the levels of hPLD1 expression.

Stable overexpression of PLD1 in EGFR cells-overexpressing 3Y1 cells increases colony formation efficiency in the absence of EGF.

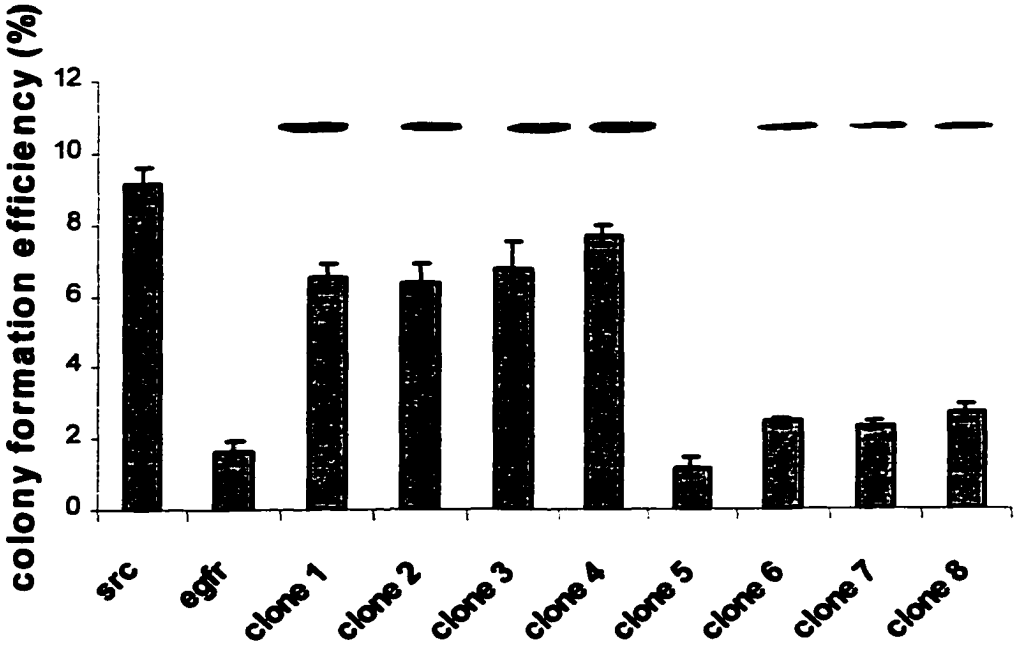
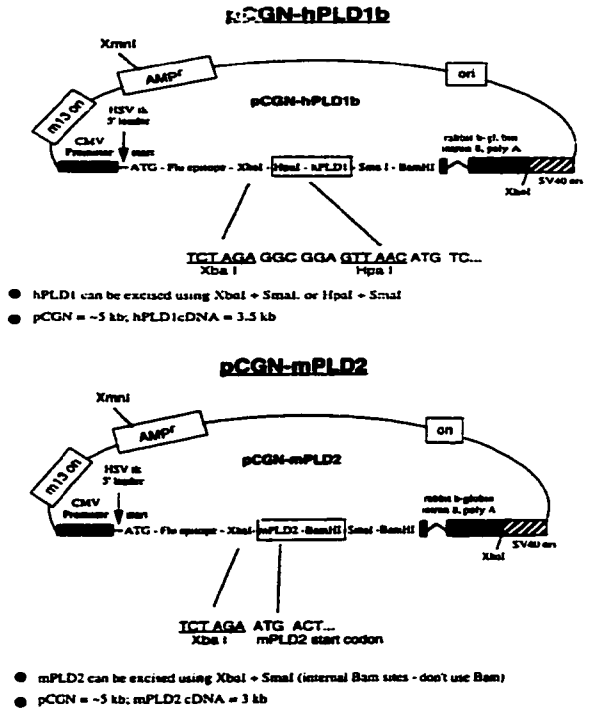


Figure. 8 EGFR cells transfected with pCGN-hPLD1, which expresses Flu-tagged hPLD1 were suspended in soft agar and the ability to form colonies in soft agar was determined.

Conditional expression of PLD1 and PLD2 in c-Src- and EGFR-overexpressing 3Y1 cells

As described previously (Lu *et al.*, 2000), overexpression of PLD is frequently toxic to cells. We therefore developed an inducible expression system for PLD1 and PLD2 as described in Materials and Methods and previously (Joseph *et al.*, submitted). (Figure 8B)

PLD expression could then be regulated by the addition of ponasterone A (PonA). $3Y1^{c\text{-}Src}$ (Lu *et al.*, 1997) and 3Y1-EGFR (Hornia *et al.*, 1999) cells were stably transfected with the PLD1 and PLD2 constructs to generate cell lines that conditionally express PLD1 and PLD2. The cell lines were designated $3Y1^{c\text{-}Src}$-P1, $3Y1^{c\text{-}Src}$-P2, 3Y1-EGFR-P1, and 3Y1-EGFR-P2 with P1 indicating PLD1 expression and P2 indicating PLD2 expression. Upon induction with PonA, increased PLD1 and PLD2 protein could be detected by Western blot analysis using antibodies raised against the unique amino termini of PLD1 and PLD2. (Fig.9A). Similarly increased PLD activity was detected in these cells upon treatment with kinetics that paralleled the appearance of PLD proteins (Fig.9B). The levels of c-Src and EGFR were unaffected by either PLD1 or PLD2 expression (Fig. 9C).



Ecdysone Inducible system

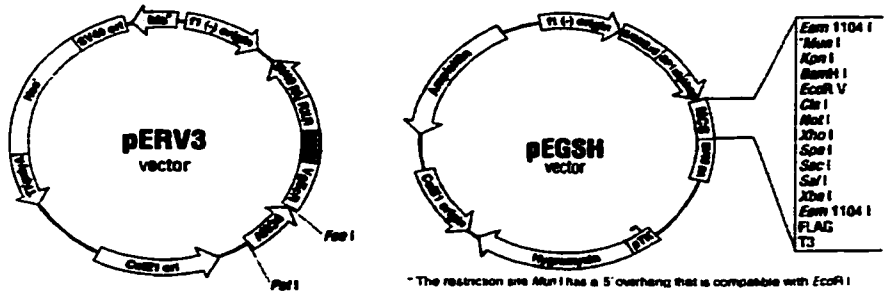


Figure 8B. PLD1 and PLD2 gene were excised from the following pCGN plasmids and cloned into the pEGSH plasmid.

Figure 9A,B, C. Conditional expression of PLD1 and PLD2 in parental, c-Src-, and EGFR-overexpressing 3Y1 cells.

(A) Construction of $3Y1^{c\text{-}Src}$ and 3Y1-EGFR cells that conditionally express PLD1 and PLD2 is described in Experimental Procedures. PonA (10 μM) was added to cells that had been placed in media with 0.5% serum for 20 hr. PLD1 and PLD2 protein levels were then determined at the indicated times by Western blot analysis. (B) Aliquots from cells used in (A) were taken and analyzed for PLD activity. The values were normalized to untreated (0-time values) for the 3Y1-EGFR (upper panel) and $3Y1^{c\text{-}Src}$ (lower panel) cells. Error bars represent the standard deviation for triplicate samples from a representative experiment that was repeated two times. (C) The levels of EGFR and c-Src was determined by Western blot analysis in the 3Y1-EGFR (upper panel) and $3Y1^{c\text{-}Src}$ (lower panel) cells and these cells with PLD1 or PLD2 expression. The cells had been treated with PonA for 20 hrs to induce PLD expression. The parental cells, which express very low levels of both the EGFR and c-Src, were included as controls.

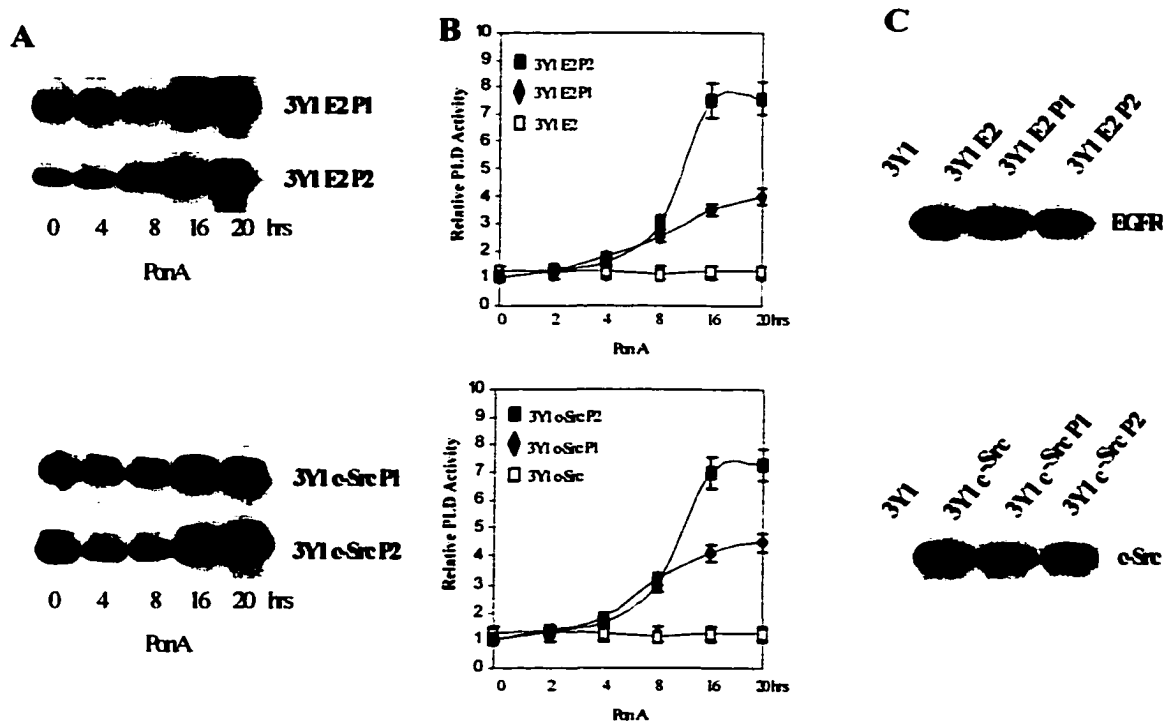


Fig. 9A,B,C

Elevated expression of either PLD1 or PLD2 transforms 3Y1 cells overexpressing the EGF receptor

The efficiency of PLD1 to transform the EGFR-overexpressing cells was relatively low at about 6%. PLD1 expression were found to be poorly tolerated (Lu *et al.*, 2000) and this could have contributed to the low efficiency of transformation. In the present study, we used PLD1-expressing cells selected under non-induced conditions (i.e. in the absence of PonA). Using the 3Y1-EGFR-P1 cells we found that that the colony forming efficiency was slightly elevated in the absence of PonA (Figure 10), which was likely due to some leakiness of PLD1 in the absence of PonA. If PonA was added to elevate PLD1 expression, the level of colony formation efficiency increased by close to five-fold. These data are consistent with our previous report showing that PLD1 cooperated with EGFR overexpression using a constitutive expression system. We next examined the effect of PLD2 on colony formation in the EGFR cells, and as shown in Fig. 21, PLD2 expression stimulated colony formation with an efficiency that was substantially higher than that observed with PLD1. These data indicate that like PLD1, PLD2 also stimulates colony formation in cells overexpressing the EGF receptor.

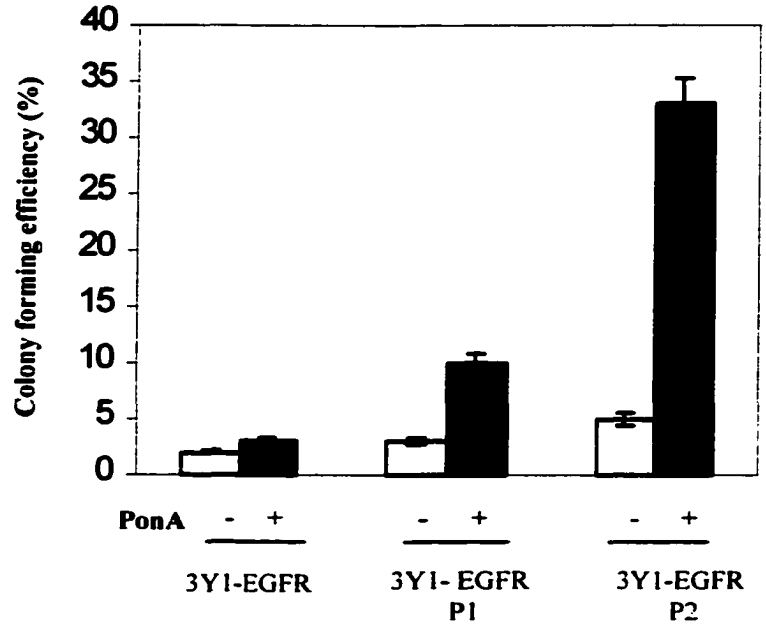


Figure 10. Elevated expression of either PLD1 or PLD2 transforms 3Y1 cells overexpressing the EGF receptor.

Anchorage-independent growth of the 3Y1-EGFR, 3Y1-EGFR-P1, and 3Y1-EGFR-P2 cells was examined in the absence and presence PonA (10 μM). 10^3 cells were suspended in soft agar and the percentage of cells that formed colonies was determined two weeks later. PonA was replenished every four days. Error bars represent the standard deviation for 2 independent experiments performed in triplicate.

PLD1 and PLD2 transform 3Y1 cells overexpressing the non-receptor class tyrosine kinase c-Src

We reported previously that 3Y1 cells overexpressing c-Src were transformed by downregulating PKC δ (Lu *et al.*, 1997). Interestingly, downregulating PKC δ in these cells also led to an increase in PLD activity (Lu *et al.*, 1997; Hornia *et al.*, 1999). We therefore examined the effect of both PLD1 and PLD2 on colony formation in $3Y1^{c\text{-}Src}$ cells. As shown in Figure 11, elevated colony forming efficiency was substantially elevated under non-induced conditions for both the $3Y1^{c\text{-}Src}$-P1 and $3Y1^{c\text{-}Src}$-P2 cells. Upon induction with PonA, the colony forming efficiency went to almost 100% with elevated PLD1 expression and to better than 60% with elevated PLD2 expression (Figure 11). The high efficiency of colony formation indicates that elevated PLD activity cooperates well with elevated c-Src expression to facilitate anchorage-independent growth. The data also indicate the PLD activity cooperates with non-receptor as well as receptor class tyrosine kinases to transform cells.

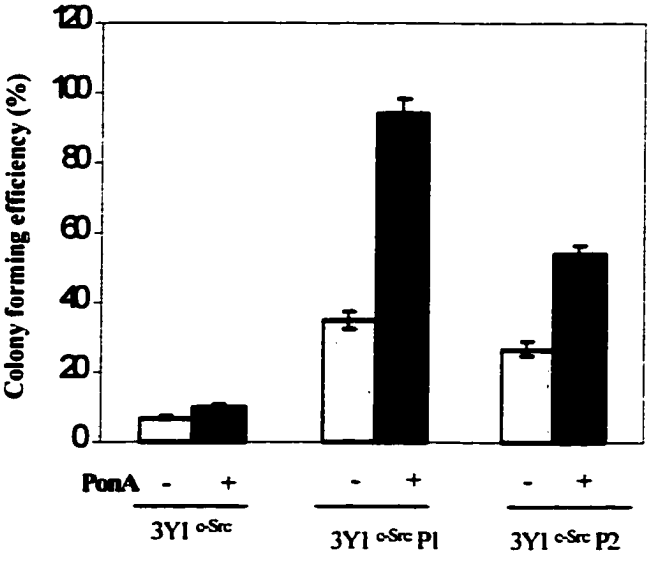


Figure 11. PLD1 and PLD2 transform 3Y1 cells overexpressing the non-receptor class tyrosine kinase c-Src.

Anchorage-independent growth of the 3Y1$^{c\text{-}Src}$, 3Y1 $^{c\text{-}Src}$ -P1, and 3Y1 $^{c\text{-}Src}$ -P2 cells was examined in the absence and presence PonA (10 μM) as in Figure 10.

Elevated expression of either PLD1 or PLD2 stimulates DNA synthesis in 3Y1-EGFR and 3Y1$^{c\text{-}Src}$

We next examined the effect of elevate PLD1 and PLD2 activity on DNA synthesis in the 3Y1-EGFR and 3Y1$^{c\text{-}Src}$ cells. Sub-confluent 3Y1$^{c\text{-}Src}$ and 3Y1 EGFR cells and the corresponding cells with inducible PLD1 and PLD2 were subjected to low-serum (0.5%) conditions for 36 h. The cells were then treated with PonA to elevate PLD expression and DNA synthesis was then assessed 20 hr later by the incorporation of [^{3}H]-thymidine. In Figure 12, it is shown that elevated expression of either PLD1 or PLD2 induced DNA synthesis in both 3Y1-EGFR and 3Y1$^{c\text{-}Src}$ cells. The extent to which PLD activity stimulated DNA synthesis correlated well with the ability to stimulate colony formation. Interestingly, elevated expression of either PLD1 or PLD2 also stimulated DNA synthesis in the parental 3Y1 cells, however these cells did not divide (our unpublished results). These data further establish that both PLD1 and PLD2 stimulate cell proliferation in cells overexpressing a tyrosine kinase.

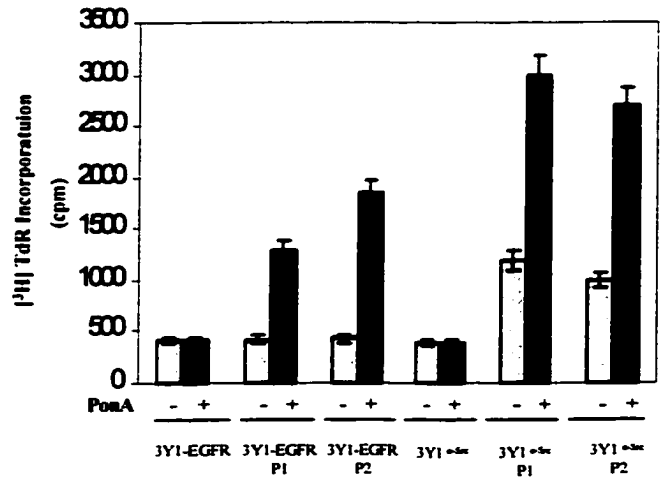


Figure 12. Elevated expression of either PLD1 or PLD2 induces DNA synthesis in 3Y1 cells overexpressing either the EGF receptor or c-Src.

Sub-confluent $3Y1^{c\text{-}Src}$ and 3Y1 EGFR cells as well as the corresponding cells with inducible PLD1 and PLD2 were placed in media containing 0.5% serum for 36 h. The cells were then treated with PonA (10 μM) as shown and DNA synthesis was then determined by a one hr pulse with [^{3}H]-thymidine 20 hr later. The incorporation of [^{3}H]-thymidine (TdR) into TCA-insoluble fractions was determined as reported previously (Lu *et al.*, 1997). Values were from cpm of

[^{3}H]-TdR incorporation values. Error bars represent the standard deviation from two independent experiments done in triplicate.

Discussion

We demonstrated previously that elevated expression of PLD1 cooperated with overexpressed EGFR to generate a transformed phenotype (Lu *et al.*, 2000). In this report, we show that PLD2 can also cooperate with the EGFR to transform rat fibroblasts. Both PLD1 and PLD2 could also transform cells overexpressing the non-receptor class tyrosine kinase, c-Src. These data suggest a role for both PLD1 and PLD2 in mitogenic signaling and some functional redundancy for the two PLD isoforms.

Previous studies on the localization of the PLD isoforms indicated differential cellular distributions for PLD1 and PLD2, which suggested different cellular functions. PLD2 is localized primarily to light membrane fractions containing many signaling molecules including the EGFR. PLD1 on the other hand had a much broader cellular distribution (Xu *et al.*, 2000). The localization of PLD2 to the light membrane fraction suggested that PLD2 would be the major PLD activated by mitogenic signals since mitogenic PLD activity is restricted largely to light membrane fractions. However, PLD1 is functionally associated with Ral GTPase (Jiang *et al.*, 1995; Luo *et al.*, 1997), and a downstream target of Ras, and consistent with a role for PLD1 in mitogenic signaling, EGF-induced PLD activity is dependent upon both Ras and Ral (Lu *et al.*, 2000). The data presented here indicate that both PLD1 and PLD2 are be involved in mitogenic signaling. Consistent with this hypothesis, we recently reported that both PLD1 and PLD2 were required for receptor-mediated endocytosis of the EGF receptor (Shen *et al.*, 2001). Preliminary kinetic studies in cells overexpressing PLD1 and

PLD2 indicate that PLD2 may be activated before PLD1 in response to EGF (our unpublished results) suggesting the possibility that PLD2 activation could contribute to the activation of PLD1. Although a mechanism for PLD1 activation through PLD2 remains to be determined, the data presented here are consistent with a role for both PLD1 and PLD2 in mitogenic signaling.

c-Src was the first cellular homologue of a viral oncoprotein to be discovered. (Stehelin *et al.*, 1976). It is important for many mitogenic signaling from many RPTKs, and has been implicated in many cancers (Bjorge *et al.*, 2000). The ability of PLD activity to cooperate with an overexpressed tyrosine kinase to transform cells has significance for cancer progression. Overexpression of a tyrosine kinase is a common genetic alteration in several human cancers, most notably in breast cancer (Biscardi *et al.*, 1999; Harari and Yarden, 2000). The most direct demonstration that c-Src is involved in human cancer was the identification of a mutant c-Src with a truncation of the C terminus, ending with Tyr530 in human colon cancer (Irby *et al.*, 1999). In cells that have acquired such a mutation, the stimulation of PLD activity could lead to cell proliferation, and with cell proliferation, there is the potential for the accumulation of additional genetic alterations that could lead to a fully malignant phenotype. In this regard, it may be highly significant that PLD activity was found to be elevated in 14 of 17 breast cancer tissues examined (Noh *et al.*, 2000). Overexpression of a tyrosine kinase, with which PLD1 and PLD2 are able to cooperate, is especially prevalent in breast cancer (Biscardi *et al.*, 1999).

The data presented here also lead to the prediction that compounds able to stimulate PLD activity would have a tumor-promoting capability in cells with an overexpressed tyrosine kinase. Consistent with this hypothesis, we recently reported that tamoxifen, which stimulates PLD activity in cells overexpressing c-Src, also transforms these cells (Zhong *et al.*, 2001). Importantly, prolonged exposure to tamoxifen leads to an increase in the incidence of endometrial cancer (Fisher *et al.*, 1994). Thus, the tumor promoting properties of tamoxifen may be due in part to the ability to elevate PLD activity. Whether the elevation of PLD activity by tamoxifen and other environmental agents such as diet remains to be determined, however the data presented here indicate that elevating PLD activity in cells that have elevated tyrosine kinase expression become transformed.

CHAPTER 3

Phospholipase D1 and 2 activities overcome a cell cycle block caused by high intensity Raf kinase signals.

Introduction

The Raf family of serine/threonine is involved in the transmission of cell regulatory signals controlling proliferation and differentiation. The best characterized Raf substrates are MEK1 and MEK2; the activation of MEK1/2 by Raf is required to mediate many of the cellular responses to Raf activation, suggesting that MEK1/2 are the dominant Raf effector molecules. (White *et al.*, 2000) Activated Ras functions as an adaptor that binds to Raf kinases with high affinity and causes their translocation to the cell membrane, where Raf activation takes place. (Moodie *et al.*, 1994) (Kolch *et al.*, 2000) (Figure 13, 14 and 15).

Mammals possess three Raf proteins, Raf-1, A-Raf, and B-Raf. The ubiquitously expressed Raf-1 is the best studied but the least understood isoform. A-Raf and B-Raf exhibit more restricted expression profiles. The very phenotypes of Raf-1, A-Raf, B-Raf make a convincing case foe these proteins being non redundant and serving different functions (Han *et al.*, 1993). Depending on the genetic background the elimination of A-Raf produces intestinal and or neurological defects, but the pups are born alive. In contrast B-Raf knock-out mice have defects in neuroepithelial differentiation and in the maturation and maintenance of endothelial cells, and die in utero due to vascular hemorrhage. Knocking out the raf-1 gene in an inbred background results in death during midgestation. Overall the phenotypes of knockout mice are consistent with the expression data, and indicate that Raf-1 serves a general role in tissue formation, whereas A-Raf and B-Raf fulfill more specialized functions.

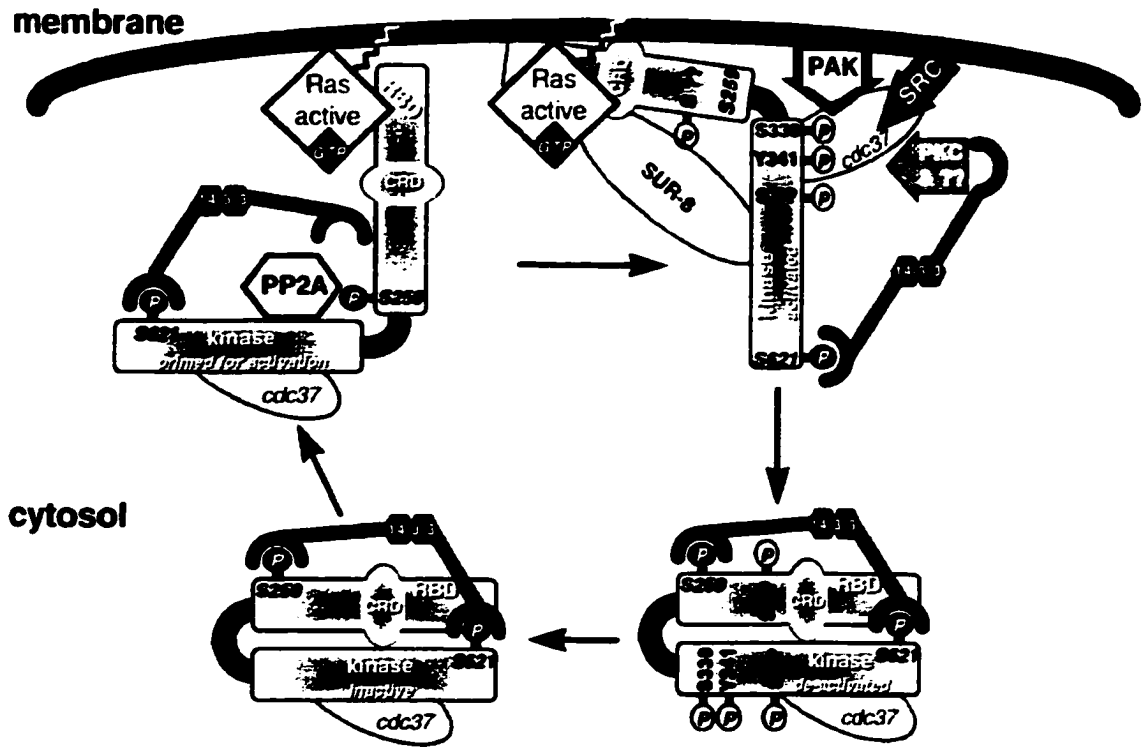


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Figure 13. Model of Raf-1 activation.

Raf-1 is in complex with 14-3-3 protein, when it is recruited by Ras to the plasma membrane it gets phosphorylated and activated. This process relies on the recruitment of GDP/GTP exchange factors to the cell membrane where Ras resides. Activated Raf-1 can now activate Mek1/2, which are downstream effector molecules.

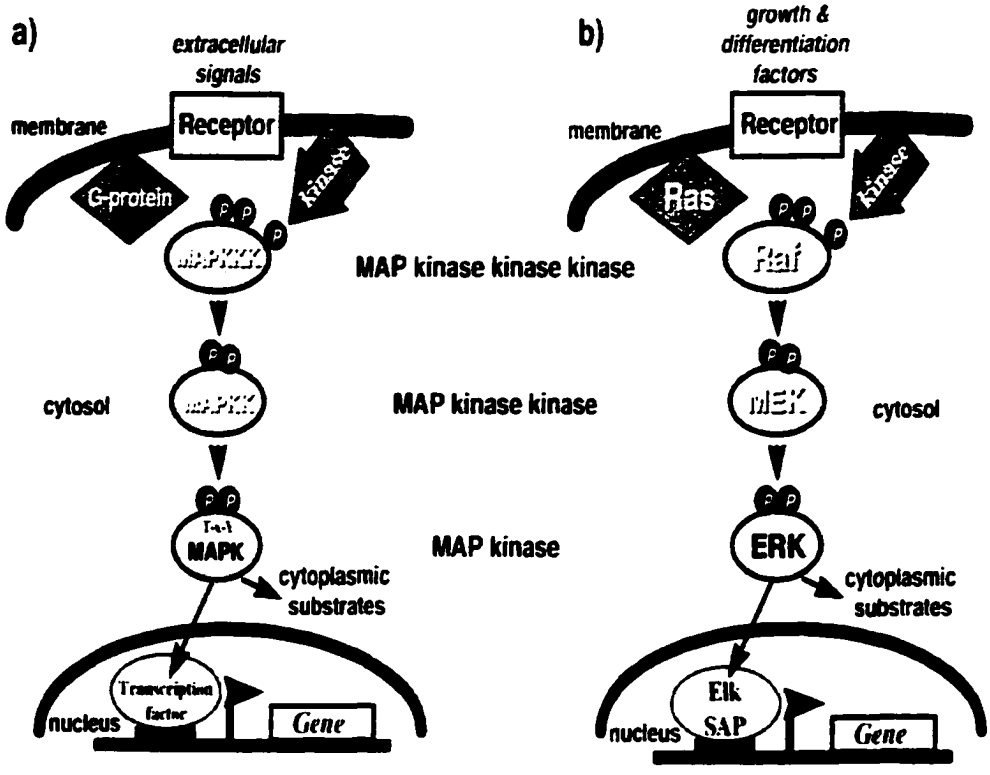

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Figure 14. Schematic representation of the structure of MAPK pathways. **(A)** General set-up of MAPK pathways; **(B)** the ERK pathway in particular. This setup provides not only for signal amplification, but maybe more importantly, for additional regulatory interfaces that allow the kinetics, duration and amplitude of the activity to be precisely tuned.

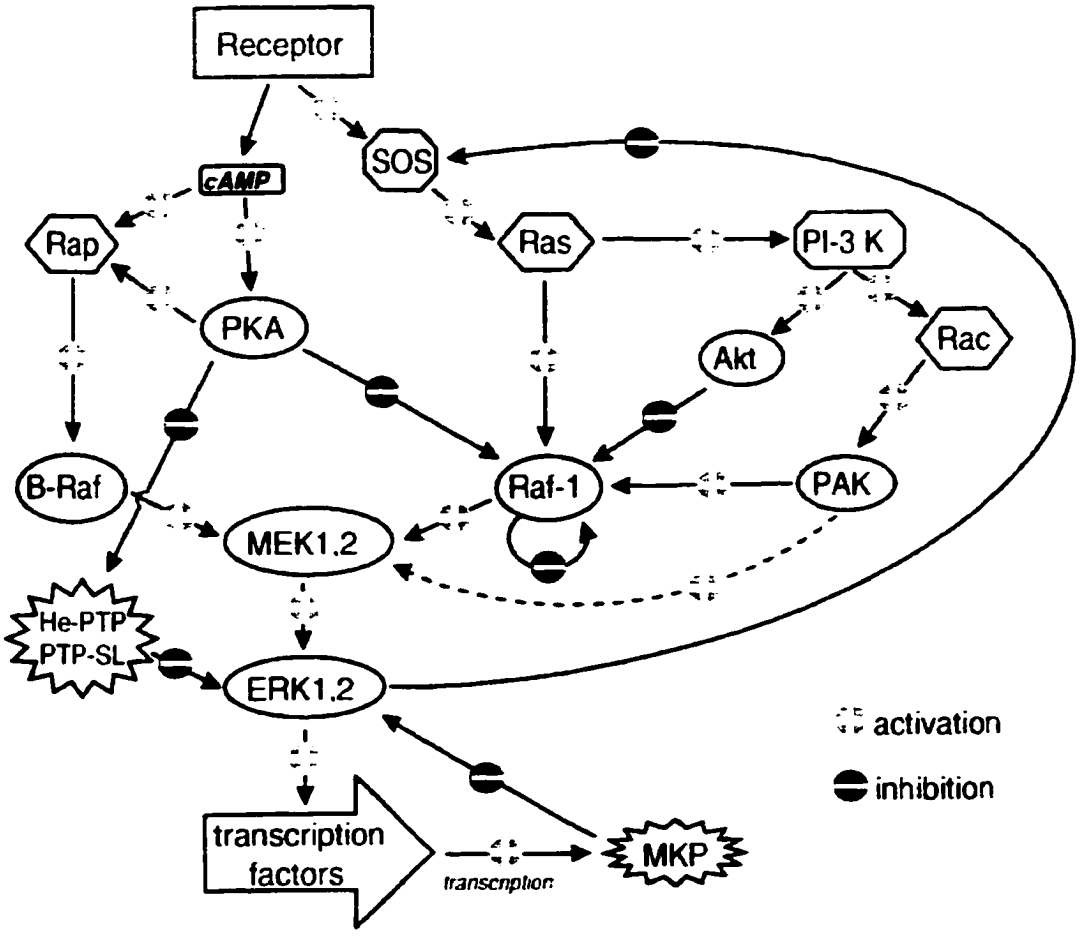

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Figure 15. Regulation of the Ras/Raf/MEK/ERK signaling network. Solid lines represent direct effects on activity. The broken line indicates that PAK phosphorylation enhances the binding of MEK to Raf-1 and hence facilitates MEK activation indirectly.

The ability of Raf to bind Ras in a GTP-dependent manner In vitro and In vivo is the principal biochemical evidence in support of a direct effector role for Raf (Moodie *et al.*, 1993; Vojitek *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993). GTP-bound Ras binds cytoplasmic Raf-1 and translocated it to the plasma membrane where Raf1 kinase becomes activated by a mechanism, which is still poorly understood but is Ras-independent (Stokoe *et al.*, 1994). Activated Raf phosphorylates MAP kinase-extracellular signal-regulated kinase (MEK) (Kyriakis *et al.*, 1992; Cowley *et al.*, 1994), which in turn activates the p42 and p44 MAPK/Erk kinases (Han *et al.*, 1991; Gomez *et al.*, 1991; Kosako *et al.*, 1992; Nakielny *et al.*, 1992). Erk phosphorylation promotes its homodimerization (Khokhlatchev *et al.*, 1998) and results in the translocation of the Erks into the nucleus leading to the activation by direct phosphorylation of transcription factors, such as p62TCF/Elk-1 and Ets-2 (Figure 15). These factors are involved in ternary complex formation at the serum response elements (SRE) (Gille *et al.*, 1992), which regulate the expression of immediate-early genes, such as the c-fos and HB-EGF genes and eventually to cell proliferation (Marshall CJ. 1994; Treisman R. 1996).

The activation of Raf-1 kinase activity requires its translocation from the cytoplasm to the plasma membrane where it is activated through a complex mechanism, which includes the interaction with Ras, and possibly phosphorylation, by PKC, and tyrosine kinases (Avruch *et al.*, 1994; Daum *et al.*, 1994; Morrison *et al.*, 1997) (Figure 16). Whereas, the precise nature of the

events occurring at the plasma membrane remains unresolved, it is clear that the translocation of Raf-1 is crucial. Targeting Raf-1 to the plasma membrane by attaching the Ras membrane localization motif "caax box" to the C terminus of Raf-1 is sufficient for activation of the kinase (Leevers *et al.*, 1994), whereas trapping Raf-1 in the cytoplasm with cytosolic Ras prevents activation (Lerner *et al.*, 1995). However, translocation itself does not bring about the full activation of Raf-1. (Mineo *et al.*, 1997) used a mutant Ras protein that is deficient in binding to wild-type Raf-1-caax, but binds Raf-1 (257L)-caax, to show that the interaction between Ras and Raf-1 stimulates Raf-1 kinase activity 3-fold better than targeting Raf-1 to the membrane alone. Thus, Raf-1 translocation and Raf-1 kinase activation are closely related but distinct phenomena.

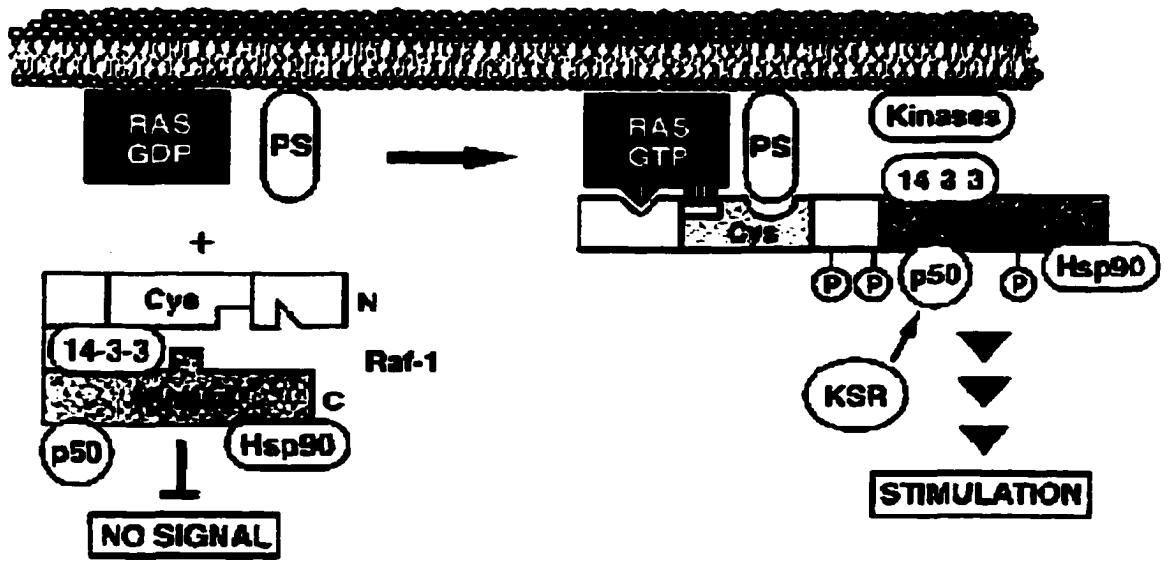

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Figure 16. Proposed events involved in Ras mediated Raf-1 activation. Although Ras mediated Raf activation involves localizing Raf to the plasma membrane, it has been proposed that other events as well are required for full Raf kinase function. These include: 1) Ras interaction with an additional site the cysteine-rich domain (CRD); 2) Interaction with 14-3-3 proteins on multiple sites leading to negative regulation; 3) Additional interaction with several molecules such as kinase suppressor of Ras (KSR), p50, and heat shock protein 90 (Hsp90).

It has been assumed for some time that the interactions of Raf-1 with Ras are the primary mechanism driving the recruitment of Raf-1 to the cell membrane. Recently, other mechanisms that may play an important role in the recruitment of Raf-1 to the membrane have been investigated. For instance, (Ghosh *et al.*, 1996) have explored the interactions of Raf-1 with phosphatidylserine and PA in vitro. Phosphatidylserine appears to bind to the cysteine-rich domain (CRD) of Raf-1 (Figure 12). (Luo *et al.*, 1997) replaced the Raf-1 CRD with the analogous zinc finger domain found on PKC and found that DAG activated this chimera independently of Ras activation, demonstrating that interaction of an effector with the CRD is critical in the activation of Raf-1. Other effectors, such as ceramide (Huwiler *et al.*, 1996) and Rap1A (Hu *et al.*, 1997), interact with Raf-1 at this site and consequently have effects on its activation. The PA-binding site proposed by (Ghosh *et al.*, 1996) does not lie in this crucial lipid binding regulatory domain on Raf-1 but on a second lipid-binding site near the catalytic domain of Raf-1. The influence of effector binding at this site on Raf-1 kinase activity, if any, has not been fully characterized at the present time. (Ghosh *et al.*, 1996) also showed that inhibition of PLD-mediated generation of PA with ethanol inhibited phorbol ester-induced Raf-1 translocation to cell membranes. This suggests that the generation of PA may play an important role in the recruitment and/or activation of Raf-1 kinase. In agreement, (Rizzo *et al.*, 1999) recently showed that stimulation of the MAPK pathway by insulin is dependent on PLD activation, and this effect is mediated through the induction of Raf-1 translocation to the plasma membrane by PA. They found that PA was required for the complete activation of Raf-1 in response to insulin. However, PA alone was not sufficient to activate the Raf kinase in vivo, had no effect on Raf kinase activity in vitro, and could not activate the MAPK cascade. In addition, they showed that PA induced Raf-1 translocation to the plasma membrane and that the generation of PA was essential for the induction of Raf-1 translocation by insulin. Finally, in agreement with a role for PLD and PA in vesicle formation and mitogenic signaling, Raf-1 was found associated with intracellular vesicles containing the insulin receptor and clathrin after stimulation with insulin. Furthermore, Raf-1 association to endocytic vesicles was dependent on the generation of PA, suggesting a model in

which Raf-1 migrates along with endocytic vesicles during receptor-mediated endocytosis via its interaction with PA.

The most recently established Ras effectors are the members of the RalGEF family, which connect Ras to the small GTPase Ral. The activation of Raf-1 kinase activity requires its translocation from the cytoplasm to the plasma membrane where it is activated through a complex mechanism which includes the interaction with Ras, and possibly phosphorylation by PKC, and tyrosine kinases (see fig 9). Whereas, the precise nature of the events occurring at the plasma membrane remains unresolved, it is clear that the translocation of Raf-1 is crucial. Targeting Raf-1 to the plasma membrane by attaching the Ras membrane localization motif "caax box" to the C terminus of Raf-1 is sufficient for activation of the kinase, whereas trapping Raf-1 in the cytoplasm with cytosolic Ras prevents activation (Mineo *et al.*, 1997). However, translocation itself does not bring about the full activation of Raf-1. Mineo *et al.* used a mutant Ras protein that is deficient in binding to wild-type Raf-1, but binds Raf-1 (257L), to show that the interaction between Ras and Raf-1 stimulates Raf-1 kinase activity 3-fold better than targeting Raf-1 to the membrane alone. Furthermore, Roy *et al.*, (91) showed that recruitment of Raf-1 to the plasma membrane by Ras was not sufficient for full activation of Raf-1 and that a second interaction between the cysteine-rich domain (CRD) on Raf-1 and Ras was necessary for full activation. They also showed that deletion of the CRD from membrane-targeted Raf-1 abrogated Raf-1 kinase activity, suggesting that plasma membrane localization of Raf-1 by itself is insufficient for activation of Raf-1 but that a second regulatory event affecting the CRD must occur for Raf-1 activation. Thus, Raf-1 translocation and Raf-1 kinase activation are closely related but distinct phenomena.

Perhaps the most critical aspect of development is the control of cell number, and their many regulatory mechanisms at work to insure that unwanted cell proliferation does not happen (Hanahan *et al.*, 2000, Hirikawa *et al.*, 1988). An important component of maintaining control over cell proliferation is that if a mitogenic signal is either incomplete or inappropriate, the responses are either cell arrest cell senescence, or apoptosis (Evans *et al.*, 1998)

There is a strong correlation between the activation of Phospholipase D (PLD) and mitogenesis (Boarder *et al.*, 1994). In response to most, if not all mitogenic signals, there is an increase in PLD activity. PLD activity is elevated in response to a variety of mitogens including platelet-derived growth factor (Plevin *et al.*, 1991) epidermal growth factor (Kaszkin *et al.*, 1992; Song *et al.*, 1994), and insulin (Karnam *et al.*, 1997; Shome *et al.*, 1997). PLD activity is also elevated in cells transformed by a several transforming oncogenes including v-Src (Song *et al.*, 1991; Wyke *et al.*, 1992), v-Ras (Teegarden *et al.*, 1990; Jiang *et al.*, 1995a; Carnero *et al.*, 1994; Martin *et al.*, 1997), and v-Fps (Jiang *et al.*, 1994). The activation of PLD activity results in the production of phosphatidic acid (PA), which has been implicated as a lipid second messenger that stimulates a variety of signaling molecules (Exton *et al.*, 1994; Exton *et al.*, 1994; Singer *et al.*, 1997) and in the formation of vesicles for membrane trafficking (Ktistakis *et al.*, 1996; Chen *et al.*, 1997; Roth *et al.*, 1997). A role for PLD and PA in the transduction of mitogenic signals is not clear; but the observation that PLD is elevated in

response to most if not all mitogenic stimuli suggests that PLD may be an integral component of mitogenesis.

An important aspect of mitogenic signal transduction is that incomplete or inappropriate signals cause cell cycle arrest, cell senescence, and apoptosis (Evan and Littlewood, 1998). The emerging paradigm is that there is several control mechanisms that insure that unwanted cell proliferation does not happen (Hueber and Evan, 1998; Hanahan and Weinberg, 2000). Weinberg and colleagues demonstrated that primary cells require two cooperating oncogenes to generate a transformed cell in culture (Land *et al.*, 1983). In general, oncogenes that mediate the transduction of mitogenic signals require a second genetic alteration that overcomes restrictions upon cell cycle progression. For example, the signal transducing Ras oncogene cooperates with SV40 large T-antigen, which interferes with inhibition of cell cycle progression through cell cycle checkpoints (Sherr, 1996). Expression of an activated Ras oncogene in the absence of a cooperating oncogene results in cell senescence or apoptosis (Serrano *et al.*, 1997; Joneson and Bar-Sagi, 1999) depending upon the strength of the signal. Similarly Myc, which like SV40 large T antigen, cooperates with Ras to transform primary cell (Land *et al.*, 1983), stimulates apoptosis under growth restrictive conditions (Askew *et al.*, 1991; Evan *et al.*, 1992). Thus, there is a delicate balance between cell division, cell cycle arrest, and cell death that is carefully regulated.

The serine - threonine kinase Raf is a downstream target of Ras signaling that, when activated by mutation, functions as an oncogene (Rapp *et al.*, 1987).

As noted for activated Ras (Franza *et al.*, 1986; Hirakawa and Ruley, 1988), too much Raf signaling leads to cell cycle arrest rather than proliferation (Samuels and McMahon, 1994; Kerkhoff and Rapp, 1998; Woods *et al.*, 1997; Zhu *et al.*, 1998; Sewing *et al.*, 1997). These observations suggest that high intensity Raf signals are recognized as inappropriate, leading to a block in cell cycle progression.

We reported previously that Phospholipase D (PLD) activity is elevated in NIH 3T3 cells transformed by v-Raf (Frankel *et al.*, 1999). PLD has been implicated in cell transformation by v-Src, v-Ras (Aguire Ghiso *et al.*, 1999), and v-Raf (Urano *et al.*, 1996). In addition, PLD was able to cooperate with overexpressed epidermal growth factor receptor to transform rat fibroblasts (Lu *et al.*, 2000). Thus, PLD activity provides a signal that cooperates with overexpressed tyrosine kinase to provide a complete cell proliferation signal. It is not clear how PLD contributes to mitogenic signaling. PLD catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid and choline (Exton, 1998). We recently showed that PLD is required for receptor-mediated endocytosis (Shen *et al.*, 2001). Similarly, the PLD regulator RalA (Jiang *et al.*, 1995; Luo *et al.*, 1998) has recently been implicated in receptor endocytosis (Nakashima *et al.*, 1999; Julien-Flores *et al.*, 2000). Thus, PLD may contribute to mitogenic signaling by facilitating receptor endocytosis, which has been implicated in the transduction of intracellular signals (Vieira *et al.*, 1996; Kranenburg *et al.*, 1999; Shen *et al.*, 2001). Another possible consequence of signaling through PLD is the membrane recruitment of Raf, which has a phosphatidic acid binding site (Ghosh

et al., 1996; Rizzo *et al.*, 1999). Thus, while a precise mechanism for the involvement of PLD in mitogenic signaling is not known, PLD is likely providing a survival signal that, like T antigen, facilitates progression through cell cycle checkpoints. In this report, we have investigated the effect of PLD on the effect of high intensity Raf signals, which causes cell cycle arrest.

Results

High Intensity Raf Signal Reduces PLD Activity

Several studies have shown that a high intensity Raf signal leads to cell cycle arrest and cell senescence (Woods *et al.*, 1997; Sewing *et al.*, 1997; Zhu *et al.*, 1998; Kerkhoff *et al.*, 1998). NIH 3T3 cells expressing estrogen receptor (ER) - Raf fusion proteins inducible by 4-hydroxy-tamoxifen (OHT) (BxB-Raf-ER^{TM} cells) were generated as described previously (Kerkhoff *et al.*, 1997, 1998). Selection of cells expressing the OHT-responsive hybrid Raf-ER protein was done in the absence and presence of OHT. Cells selected in the absence of OHT (inactive Raf kinase) resulted in the expression 20-fold more Raf protein than cells selected in the presence of OHT (active Raf kinase) (Kerkhoff *et al.*, 1998). Thus, addition of OHT to cells with high expression gave a high intensity Raf signal resulting in increased levels of the cyclin-dependent kinase inhibitor p21^{Cip1} and cell cycle arrest (Kerkhoff *et al.*, 1998). We reported previously that PLD activity is elevated in Raf-transformed cells (Frankel *et al.*, 1999). Consistent with this observation, induction of Raf kinase activity with OHT in the low expressing Raf-ER cells results in increased PLD activity (Figure 17A). However, in the high level expressing Raf-ER cells, PLD activity was suppressed by OHT (Figure 17B). The high level Raf-ER expressing cells expressed a higher basal level of PLD activity relative to the parental NIH3T3 cells (Figure17B), presumably due to a higher basal kinase activity of the overexpressed Raf-Raf-ER protein as described previously (Kerkhoff *et al.*, 1998). The time course for

reduced PLD activity was consistent with the time course for induction of Raf kinase activity described previously (Kerkhoff *et al.*, 1998). The dose response for the decreased PLD activity (Figure 17C) correlated with that observed for the increase in Raf kinase activity as determined by increased phosphorylation of mitogen-activated protein kinase (MAPK) kinase (MEK) (Figure 17D). These data indicate that, in contrast to the increase in PLD activity observed in response to a low intensity Raf signal, a high intensity Raf signal reduces PLD activity.

Fig. 17. High intensity Raf signaling reduces PLD activity.

(A) Raf-ER and parental NIH 3T3 cells were placed in media containing 0.5% serum for 16 hr. The cells were then treated with increasing concentrations of OHT as shown and the PLD activity was then determined as described in Materials and Methods 6 hr later. (B) The Raf-ER and NIH 3T3 cells, prepared as in (A), were treated with 200 nM OHT for the times indicated. The PLD activity was then determined as in (A). Values were normalized to the PLD activity in the untreated control NIH 3T3 cells, which was given a value of 100%. Error bars represent the standard deviation for duplicate values from 2 independent experiments. (C) Raf-ER cells were treated with increasing concentrations of OHT for 24 hr as in (A). The cells were then harvested and MEK phosphorylation (p-MEK) and MEK protein levels were then determined by Western blot analysis.

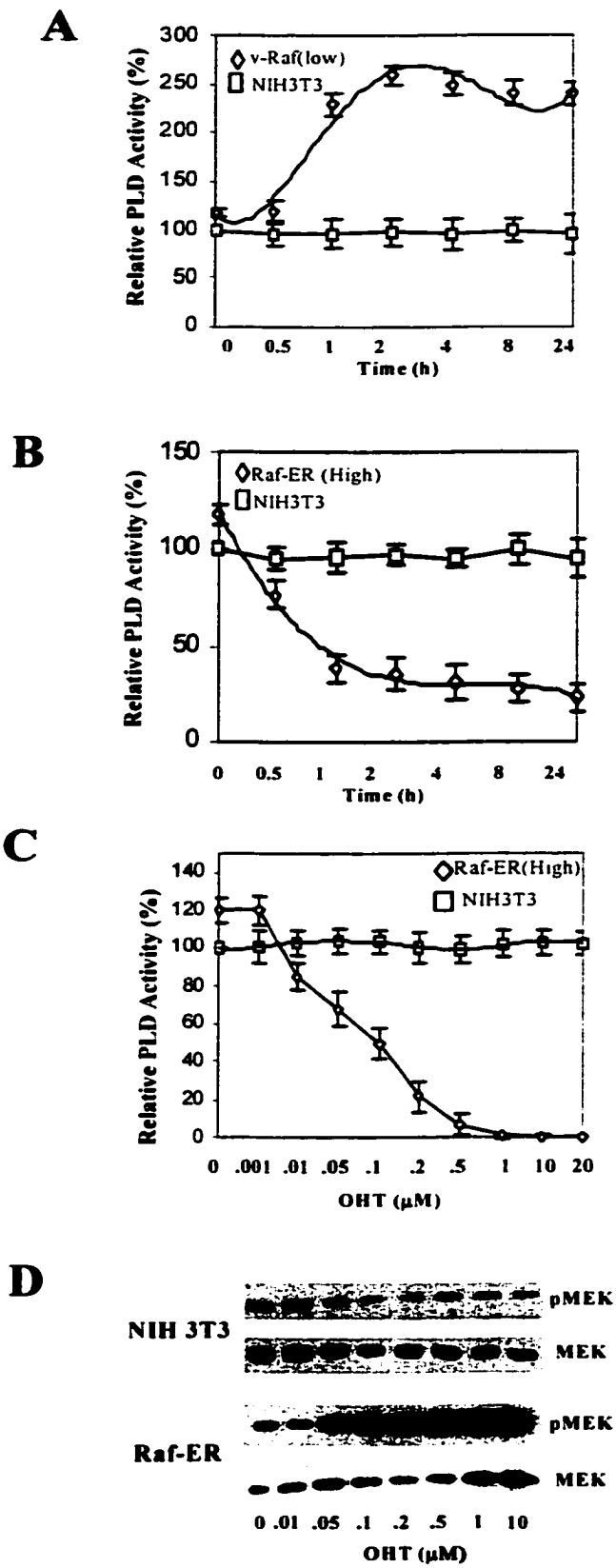


Fig.17A-D

High intensity Raf signals elevate PKC δ and reduce PKC α levels

We reported previously that α and δ isoforms of PKC have antagonistic effects upon both transformation and PLD activity (Hornia *et al.*, 1999). Inhibiting PKC δ transformed cells overexpressing a tyrosine kinase and also led to an increase in PLD activity, suggesting the possibility that PKC δ has an inhibitory effect upon PLD activity and, as a consequence, transformation. In contrast, transformation of cells overexpressing the EGF receptor was dependent upon the α isoform of PKC (Hornia *et al.*, 1999). We therefore examined the effect of high intensity Raf signaling upon PKC α and δ. Before induction with PonA, OHT treatment caused a lowered PLD activity and also an increased in PKCα and an increased PKCδ respectively. As shown in (Figure18A and18B). However, OHT treatment of the Raf-ER cell resulted in an increase in the level of PKC δ that peaked around 8 hr, but could be detected by one hr. The ability to detect an increase by 1 hr correlated with the decrease in PLD activity that was also seen at one hr (Figure 18B). PKC α levels were reduced in response to OHT in Raf-ER cells (Figure 18A). OHT treatment did not have any significant effect upon PKC α or δ levels in parental NIH 3T3 cells. These data reveal that high intensity Raf signals elevate the level of PKC δ and reduce the level of PKC α. These data are consistent with the antagonistic effects of PKC α and δ on transformation and PLD activity (Hornia *et al.*, 1999).

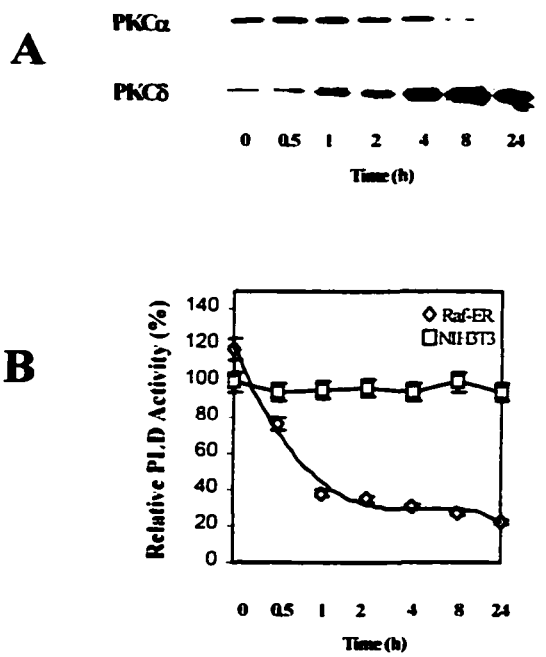
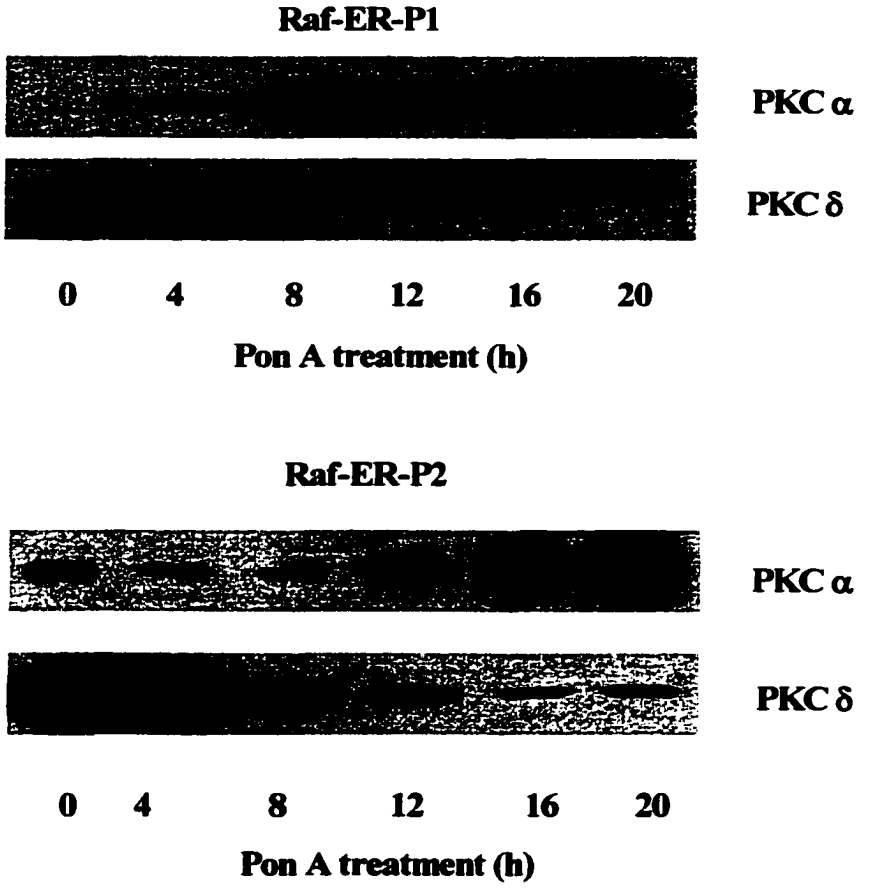


Figure 18A,B. OHT treatment in high intensity Raf kinase cells leads to lowered PLD activity and Changes in PKCα and δ levels. They were then treated with OHT (200 nM) for the times indicated. The levels of PKC α and δ were determined by Western blot analysis as described in Materials and Methods.

Fig. 19A,B. Effect of high intensity Raf signals upon PKC α and δ expression.

Raf-ER cells were subjected to conditions described in Figure 18. They were then treated with OHT (200 nM) for the times indicated. The levels of PKC α and δ were determined by Western blot analysis as described in Materials and Methods. As observed the level of PKC α was increased and the level of PKC δ was decreased.



PLD Expression in Raf-ER Cells

The suppression of PLD activity by high intensity Raf signaling implicates PLD in the cell cycle arrest induced by the high level of Raf kinase. To address this question, we wished to elevate PLD activity in the high level expressing Raf-ER cells. Overexpression of PLD is frequently toxic (Lu *et al.*, 2000). Therefore, an ecdysone inducible expression system was developed for both PLD1 (Hammond *et al.*, 1995) and PLD2 (Colley *et al.*, 1997). PLD1 and 2 genes were excised from the pCGN plasmids and cloned into the pEGSH vector MCS. The expression of the PLD1 and 2 genes are under the inducible system of the ecdysone receptor and the inducer, ecdysone. (Figure 20A,B)

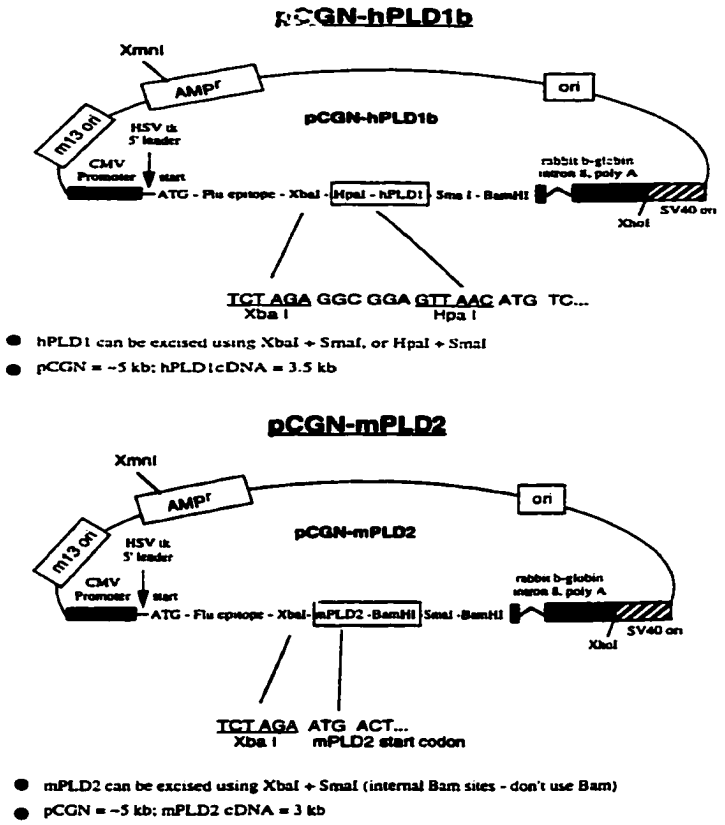


Figure 20A. PLD1 and PLD2 gene were excised from the following pCGN plasmids and cloned into the pEGSH plasmid.

Ecdysone Inducible system

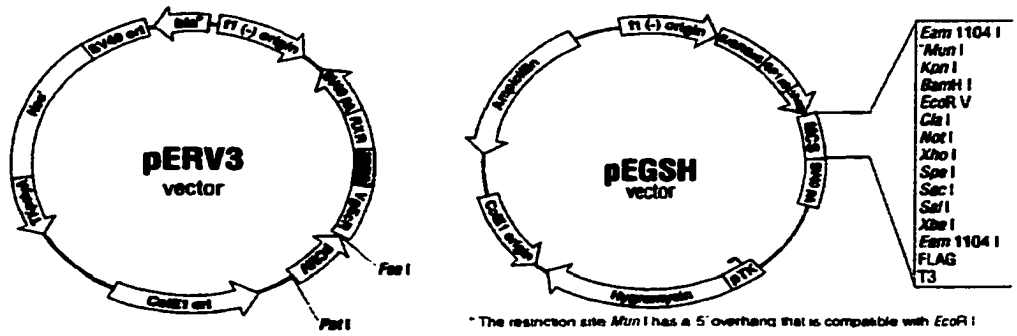
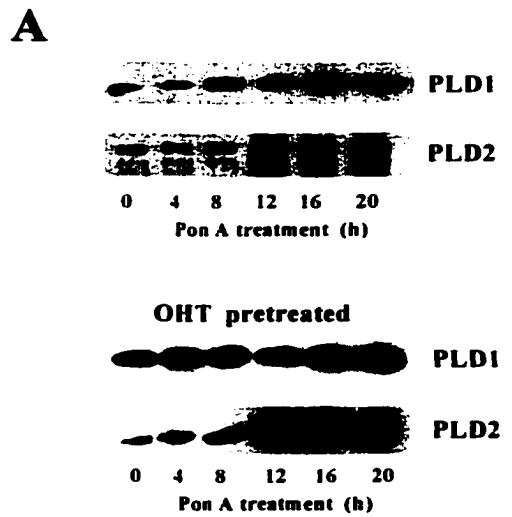


Figure 20B. The ecdysone system consists of two plasmids, the pERV3 plasmid which expresses the ecdysone receptor and the pEGSH plasmid which has an MCS, where the PLD1 and PLD2 gene were cloned in respectively.

PLD expression could then be regulated by addition of ponasterone A (PonA). The high level expressing Raf-ER cells, stably-transfected with PLD1 (Raf-ER-P1) and PLD2 (Raf-ER-P1) constructs, were examined for PLD expression by Western analysis. Upon induction with PonA, increased PLD protein (Figure 21A) and activity (Figure 21B) could be detected in the Raf-ER-P1 and Raf-ER-P2 cells between 4 and 12 hr. The induction of PLD protein and activity were observed in both the absence and presence of OHT (high intensity Raf signals) (Figures 21A and 21B). The induction of PLD2 was more pronounced than the induction of PLD1, consistent with previous observations indicating that PLD1 expression is more toxic to cells than PLD2 expression (our unpublished results). Induction of either PLD1 or PLD2 had no significant effect upon either Raf protein levels or Raf kinase activity as determined by the level of phosphorylated MEK.



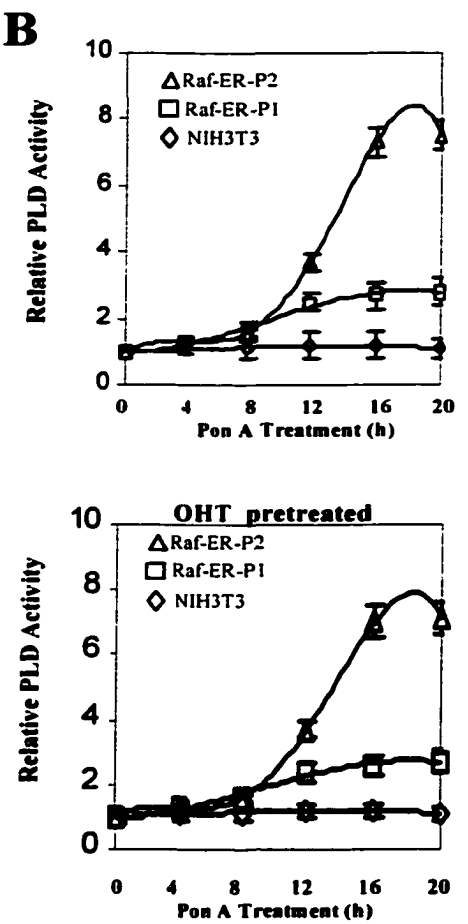


Figure 21 A,B

Figure 21A,B. Conditional expression of PLD1 and PLD2

(A) The construction of Raf-ER cells that conditionally express PLD1 and PLD2 was described in Materials and Methods. PonA (10 μM) was added to cells that had been placed in media with 0.5% serum for 20 hr and PLD1 and PLD2 protein levels were determined at the indicated times by Western blot analysis using anti-PLD1 and PLD2 antibodies respectively. (B) Aliquots from cells used in (A) were taken and analyzed for PLD activity as described in Figure 20

PLD Activity Suppresses p21^{Cip1} Levels Induced by High Intensity Raf Signals

Several reports have shown that the cyclin dependent kinase inhibitor p21^{Cip1} is elevated in response to high intensity Raf signals (Woods *et al.*, 1997; Zhu *et al.*, 1998; Kerkhoff *et al.*, 1998). We therefore investigated the effect of PLD upon p21^{Cip1} levels. As shown in Figure 22A, the Raf-ER-P1 and Raf-ER-P2 cells still responded to OHT with an increase in p21^{Cip1} levels. This was important because, as noted above, there was some leakiness in the expression of PLD1 and PLD2 in the absence of the inducer PonA. If OHT-treated Raf-ER-P1 and Raf-ER-P2 cells were treated with PonA, p21^{Cip1} levels were substantially reduced in the cells expressing either PLD1 or PLD2 (Figure 22B). The time course for reduced p21^{Cip1} levels was consistent with time course for elevated PLD activity seen in Figure 22B. These data demonstrate that elevating either PLD1 or PLD2 activity reverses the increase in p21^{Cip1} levels induced by high intensity Raf signaling.

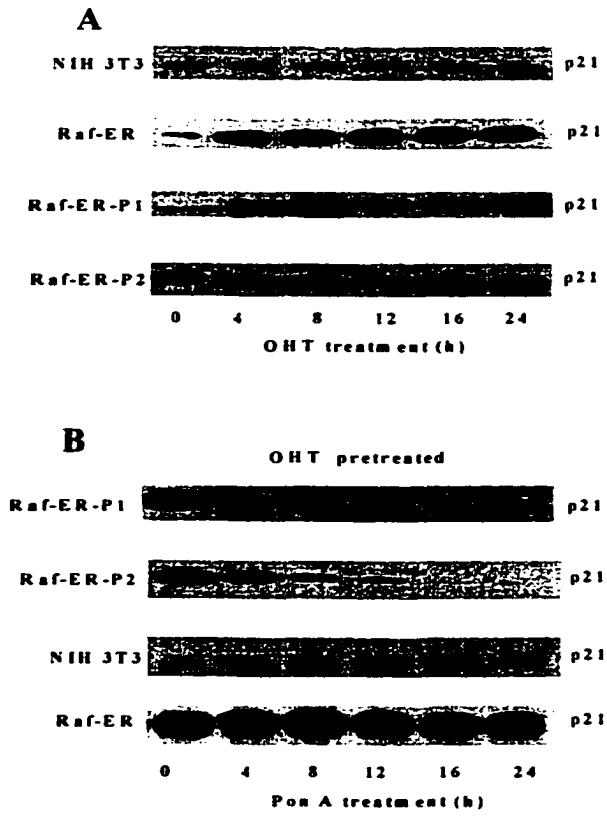


Fig. 22A,B. PLD expression overcomes Raf-induced increases in $p21^{Cip1}$ levels. **(A)** Raf-ER-P1 and Raf-ER-P2 cells were treated with OHT and expression of $p21^{Cip1}$ was determined by Western blot analysis at the indicated times after addition of OHT. **(B)** Raf-ER-P1 and Raf-ER-P2 cells were pre-treated with OHT for 24 hr and then PonA for the indicated times. Cells were then collected and $p21^{Cip1}$ levels were determined as in **(A)**.

PLD Restores Proliferative Capacity To Cells Subjected To High Intensity Raf Signals.

We next examined the effects of elevated PLD expression on cell proliferation in OHT-treated Raf-ER cells. Raf-ER-P1 and Raf-ER-P2 cells in culture were allowed to reach confluence, at which time they were treated with either OHT or OHT and PonA. As shown in Figure 23A, both Raf-ER-P1 and Raf-ER-P2 cells continued to proliferate in the presence of PonA, but not in the absence of PonA. We also examined the ability of the Raf-ER-P1 and Raf-ER-P2 cells to proliferate in low serum. Interestingly, withdrawal of serum from these cells resulted in cell death and reduced cell number. However, in the presence of PonA, the cells not only survived, they continued to proliferate as well (Figure 23B). These data indicate that PLD not only overrides the inhibition of cell cycle progression induced by high intensity Raf signals, but also provides a survival signal that prevents cell death.

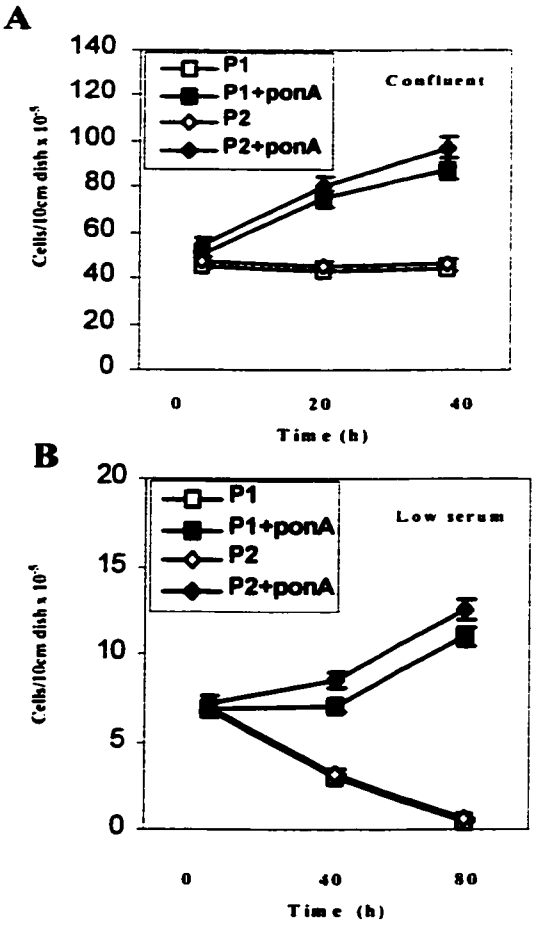


Figure 23A,B. PLD expression restores cell proliferation to cells with high intensity Raf signals.

Raf-ER-P1 (P1) and Raf-ER-P2 (P2) cells were plated at 5 x 10^5 cells / 100 mm dish. The cells were either (A) grown to confluence, or (B) placed in media containing 0.5% serum (low serum). After 24 hr at confluence or in low serum,

OHT (200 nM) and PonA (10 μM) were added and cell number was determined at the indicated times.

We next examined whether PLD expression would facilitate anchorage independent growth in presence of a high intensity Raf signal. As shown in Figure 24A, both PLD1 and PLD2 stimulated colony formation in the Raf-ER cells in both the presence and absence of OHT. The ability to stimulate anchorage independent growth in the absence of OHT indicates that PLD expression cooperates with the leaky Raf signal in the Raf-ER cells to stimulate anchorage independent growth. More significantly however, PLD was able to overcome the cell cycle arrest induced by high intensity Raf signals to allow anchorage independent growth. Note also that in the presence of OHT there are no background colonies as seen in the absence of OHT. We also examined DNA synthesis in confluent Raf-ER-P1 and Raf-ER-P2 cells, and consistent with the increased cell number and colony formation observed with PonA treatment, PonA also stimulated an increase in DNA synthesis in both the Raf-ER-P1 and Raf-ER-P2 cells (Figure 24B). As observed for colony formation, PonA induced an increase in DNA synthesis in the absence of OHT (Figure 24B), further indicating cooperation between PLD activity and the leaky Raf signal to stimulate cell cycle progression. The data in Figure 24 demonstrate that elevated expression of either PLD1 or PLD2 provides a survival signal that overcomes the inhibitory effects of high intensity Raf signals upon cell proliferation. Figure 25 shows the cells growing in the presence of OHT when PLD1 and PLD2 are activated.

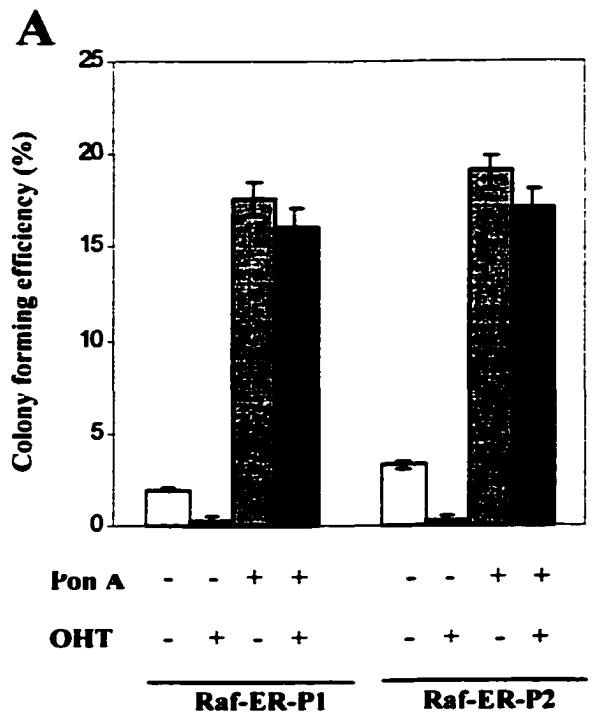
Fig. 24A. PLD expression stimulates anchorage independent growth in Raf-ER cells.

10^3 Raf-ER-P1 or Raf-ER-P2 cells were suspended in soft agar as described in Materials and Methods. OHT (200 nM) and PonA (10 μM) were added as indicated. Fresh media including OHT and PonA were added every 4 days. Colonies were counted 14 days after suspension and the percentage of cells that form colonies was determined. Error bars represent the standard error for triplicate samples.

Fig. 24B. PLD expression stimulates DNA synthesis.

PLD expression stimulates DNA synthesis in Raf-ER cells. Raf-ER-P1 and Raf-ER-P2 cells were allowed to reach confluence in 24 well plates and DNA synthesis was determined by measuring the incorporation of [^{3}H] thymidine (TdR) during a one hr pulse 24 hr after the addition of OHT (200 nM) and PonA (10 μM) as indicated. Error bars represent the standard error for triplicate samples from a representative experiment that was repeated 2 times.

In Raf-ER cells, Raf-ER-P1 and Raf-ER-P2 cells were allowed to reach confluence in 24 well plates and DNA synthesis was determined by measuring the incorporation of [^{3}H] thymidine (TdR) during a one hr pulse 24 hr after the addition of OHT (200 nM) and PonA (10 μM) as indicated. Error bars represent the standard error for triplicate representative experiment that was repeated 2 times.



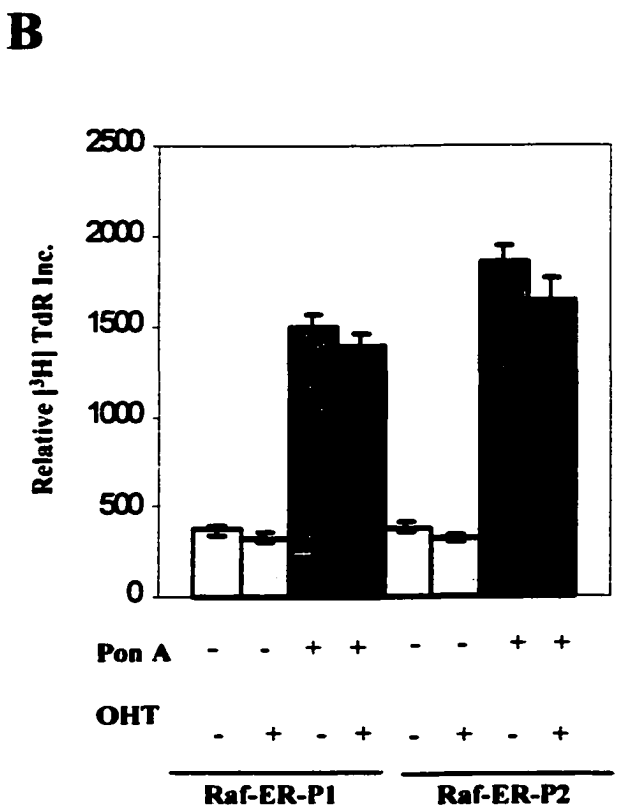
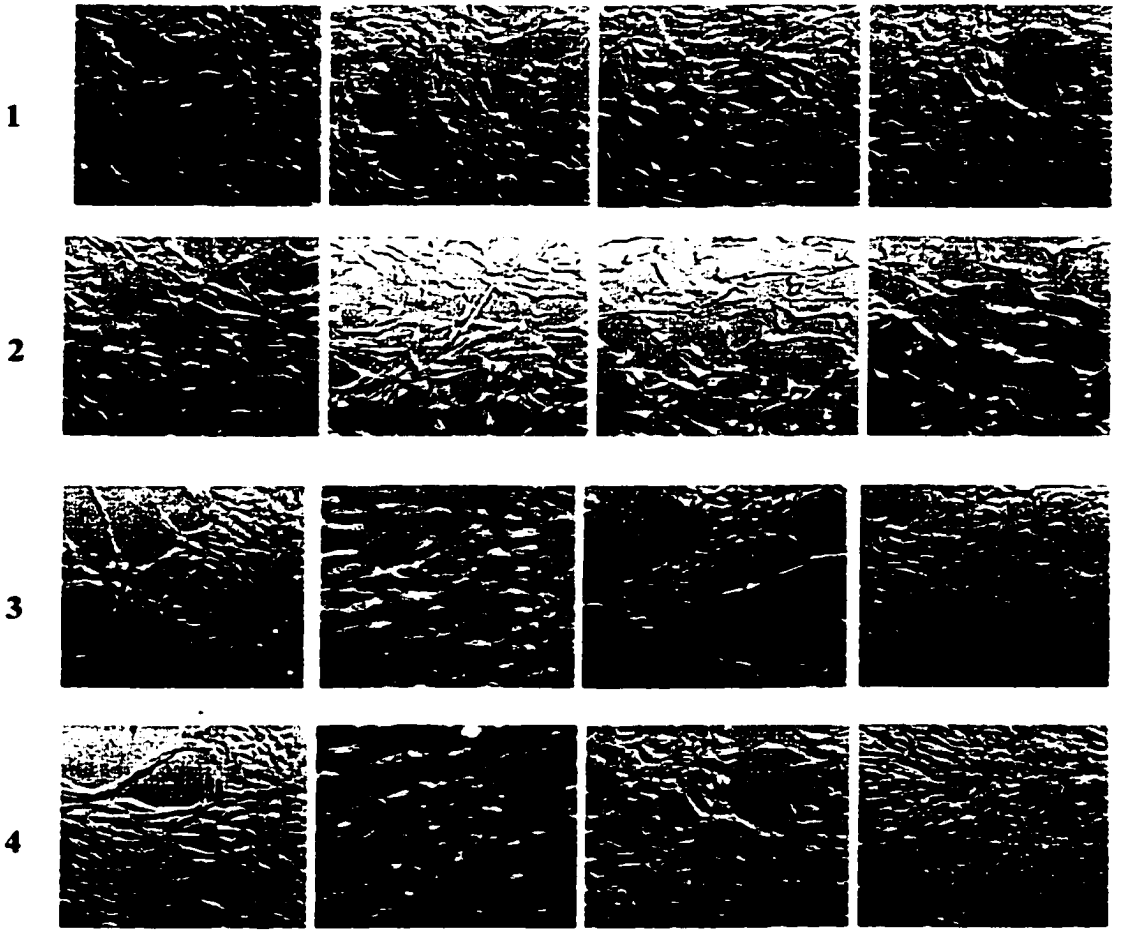


Figure 24 A,B



1-NIH3T3
2-Raf:ER
3-Raf:ERP1
4-Raf:ERP2

Fig.25

Discussion

In this report we have shown that PLD activity can overcome cell cycle arrest induced by high intensity Raf kinase signaling. NIH 3T3 cells transformed by activated Raf have an elevated PLD activity that is dependent upon both Rho and Ral (Frankel *et al.*, 1999). The transformation of NIH 3T3 cells by activated Raf selects for cells that express modest or low levels of activated Raf kinase (Kerkhoff *et al.*, 1998). Using an inducible Raf kinase selected under non-induced conditions, much higher levels of Raf expression were obtained; however, induction of Raf kinase with OHT in the high expression Raf-ER cells resulted in cell cycle arrest rather than transformation (Kerkhoff *et al.*, 1998). In this report, we showed that a high intensity Raf signal does not lead to the increased PLD activity seen with the low expression Raf-ER cells and in Raf-transformed cells (Frankel *et al.*, 1999). In contrast, the high intensity Raf signal repressed PLD activity. Overexpression of either PLD1 or PLD2 in the background of the high intensity Raf signal overcame the Raf-induced cell cycle arrest. These data indicate that PLD activity provides signal that overcomes the cell cycle arrest signals generated by the high intensity Raf signal.

We previously reported that overexpression of PLD1 cooperated with overexpression of the EGF receptor to transform rat fibroblasts. Models for the cooperation between oncogenic signals in the transformation of primary signals have been proposed (Land *et al.*, 1983; Hirakawa *et al.*, 1988; Rapp *et al.*, 1987; Troppmair *et al.*, 1992). According to the models, signaling oncogenes like Ras,

Src and Raf cooperate with oncogenes like T-antigen and Myc, which facilitate passage through cell cycle checkpoints. The ability of PLD1 to cooperate with the EGF receptor (Lu *et al.*, 2000) would suggest a role for PLD in facilitating passage through cell cycle checkpoints. The data presented here are consistent with such a model whereby PLD overcomes activation of cell cycle checkpoint controls by the high intensity Raf signals. Many proteins that regulate cell cycle progression such as p53 also regulate apoptosis (Evan *et al.*, 1998), and in this regard, it is of interest that overexpression of PLD apparently prevented apoptosis in Raf-ER cells subjected to serum withdrawal (see Figure 23B). Thus, it is possible that in addition to preventing cell cycle arrest, PLD also generates a survival signal that prevents apoptosis.

A role for PLD in regulating cell cycle progression, and possibly apoptosis, suggests that upregulating PLD might contribute to cancer progression. Consistent with this idea, elevated PLD activity was reported to be elevated in 14 of 17 human breast cancer tissues (Noh *et al.*, 2000). Agonists that elevate PLD activity might also contribute to cancer by generating signals that stimulate the proliferation of cells that have acquired a mutation that would ordinarily undergo apoptosis or cell cycle arrest. In this context, compounds with the ability to elevate PLD activity would have tumor promoting effects in cells that have acquired an initiating mutation to a signaling oncogene such as Raf. In this regard, it is of interest that tamoxifen, which has been implicated as a tumor promoter in human endometrial cancer (Fisher *et al.*, 1994), stimulates PLD activity in cells overexpressing the tyrosine kinase c-Src (Zhong *et al.*, 2001).

It was somewhat surprising that both PLD1 and PLD2 were able to overcome the inhibitory effects of high intensity Raf signals. However, our previous studies indicated that both PLD1 and PLD2 increased receptor endocytosis (Shen *et al.*, 2001). Endocytosis of the EGF receptor is required for the generation of some intracellular signals mediated by the EGF receptor (Vieira *et al.*, 1996; Kranenburg *et al.*, 1999; Shen *et al.*, 2001). Although PLD1 is largely localized to internal cell membranes (Xu *et al.*, 2000), PLD1 interacts directly with the small GTPase RalA (Luo *et al.*, 1997), which has also been implicated in receptor endocytosis (Nakashima *et al.*, 1999; Jullien-Flores, 2000). PLD2 co-localizes almost exclusively to light membrane fractions from the plasma membrane along with the EGF receptor (Xu *et al.*, 2000). Thus, both PLD1 and PLD2 may work together to facilitate receptor endocytosis and the generation of what we have referred to as a signaling vesicle (Luo *et al.*, 1998). We postulate that the generation of this putative signaling vesicle provides a survival signal that overcomes the inhibitory effects of high intensity Raf signals on cell cycle progression.

CHAPTER 5

MATERIALS AND METHODS

Cells and Cell Culture Conditions.

Parental NIH 3T3 and Raf-Raf-ER^{TM} (Raf-ER) cells used in this study were described previously (23). Unless otherwise indicated, cells were maintained in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% bovine calf serum (Life Technologies). V-Raf transformed NIH3T3 cells were generated by amplification of a transformed focus induced by transfection of the p3611-MSV-v-Raf expression plasmid (Rapp *et al.*, 1983) into NIH3T3. The generation of cells expressing the BxB-ER^{TM} Raf-1 was described previously (Kerkhoff *et al.*, 1997). For induction of BxB-ER^{TM} Raf-1 with 4 hydrotamoxifen (4-OHT) a solution of 1mg/ml in ethanol was added to the medium to a final concentration of 200 nM (5,000 fold dilution). Cell cultures were made quiescent by growing to confluence and then replacing with fresh media containing 0.5% bovine calf serum for one day. For growth of cells in soft agar, 1 x 10^3 cells were suspended in top agar (DMEM, 20% calf serum, 0.38% agar) and overlaid onto hardened bottom agar (DMEM, 20% calf serum, 0.7% agar) as described previously (Hornia *et al.*, 1999, Luo *et al.*, 2000).

Materials

PLD1 and PLD 2 rabbit polyclonal antibodies raised against the N-terminal regions of PLD1 and PLD2 were obtained from QCB, Biosource. A monoclonal antibody for PKC α was obtained from Transduction Laboratories and a polyclonal antibody for PKC δ was obtained from Santa Cruz Biotechnology. The anti-phospho-MEK1/2 antibody was from New England Biolabs. The

antibody raised against $p21^{Cip1}$ was from Santa Cruz Biotechnology. The HL-7 antibody raised against the mouse estrogen receptor was a gift from A. Sewing and H. Land (Sewing *et al.*, 1997). The secondary antibodies to rabbit or mouse immunoglobulin (IgG) conjugated with horseradish peroxidase were from Santa Cruz Biotechnology. G418 and Dulbecco's modified Eagle medium (DMEM) were obtained from GIBCO/BRL Laboratories. Protease inhibitors cocktail was obtained from Calbiochem. Laboratory. Trypsin and amiloride were purchased from Sigma. Biotinylated anti-rabbit IgG polyclonal antibody and streptavidin-alkaline-phosphatase were purchased from GIBCO/BRL. Nitrocellulose membranes were purchased from BioRad (USA). [^{3}H]-thymidine [^{3}H]-Myristate (NET-830) was obtained from New England Nuclear. Phosphatidylbutanol (PBt), PA, and diacylglycerol (DG) standards were obtained from Avanti Polar Lipids. Precoated silica 60A thin layer chromatography (TLC) plates were from Scientific Products.

Generation of Raf-ER cell lines that conditionally express PLD1 and PLD2.

Inducible expression vectors for PLD1 and PLD2 were generated as follows. pBluescript-SK-mPLD1 and pBluescript-SK-hPLD2 (5, 15) were obtained from Dr. Michael Frohman (SUNY-Stony Brook). The mPLD1 gene was excised with Sal1 and Xba1; the hPLD2 gene was excised with Not1 and Sal1 restriction endonucleases, respectively. These genes were ligated into the polylinker region of the PEGSH expression plasmid (Stratagene). The plasmids were amplified in E. Coli (strain XL-1 Blue host strain[b]) (Stratagene). The mPLD1-PEGSH and the hPLD2-PEGSH plasmids were then transfected separately into Raf-ER cells.

Clones were selected with hygromycin as described below. Clones were transfected with a second plasmid, the PERV3 plasmid (Stratagene) using G418 selection. Clones containing both the PERV3 and mPLD1-PEGSH or hPLD2-PEGSH were induced with 10 uM ponasterone A (PonA) (Stratagene) and analyzed for PLD expression as described in the text.

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 reagent (GIBCO) according to the vendors' instructions. Transfected cultures were selected with either G418 (400 μg/ml) or hygromycin (200 μg/ml) for 10-14 days at 37°C. At that time antibiotic-resistant colonies were picked and expanded for further analysis under selective conditions.

Western Analysis

Extraction of proteins from cultured cells was performed as previously described (Lu *et al.*, 1997, Lu *et al.*, 2000). Equal amounts of protein were subjected to SDS-PAGE using an 12% acrylamide separating gel, transferred to nitrocellulose and blocked overnight at 4°C with 5% non-fat dry milk isotonic phosphate buffered saline (PBS: 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4). The nitrocellulose filters were washed three times for five minutes in PBS and then incubated with antibodies as described in the text. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG was used for detection using the Pierce Supersignal chemilluminescent system (Pierce).

Phospholipase D Assays

Prelabeling of phospholipids

Unless otherwise indicated, cells in 60 mm culture dishes were prelabeled for 4 to 6 h with 3 μCi of [3H]-Myristate in 2 ml of Dulbecco's modified Eagle media containing 0.5% newborn calf serum.

Extraction of lipids

Extraction of lipids was performed according to procedures described by Song *et al.*, (Song *et al.*, 1991,1993) with minor modifications. Cells were washed with isotonic tris-saline buffer and rapidly treated with 0.5 ml of MeOH:6N HCl (50:2). Lipids were extracted by the addition of 0.5 ml of CHCl3. Phase separation was obtained by adding 155 μl of 1M NaCl. The organic phase was reextracted with 0.12 ml of 1M NaCl, 0.35 ml of H_2O and 155 μl of MeOH and recovered. An aliquot (10 μl) of the organic phase was removed and total cpm incorporated into cellular lipid/sample was determined. Samples were normalized for total cpm, dried under N_2 and redissolved in $CHCl_3$:MeOH (9:1).

Characterization of phospholipids metabolites by TLC

Extracts of phospholipids metabolites were characterized by TLC as described previously (Song *et al.*, 1993). The following solvent systems were used: For DG, hexane:diethylether:MeOH: glacial acetic acid (90:20:3:2); for PBt, the organic phase of ethylacetate:trimethylpentane:acetic acid:H20 (100:50:20:100). Lipid

standards were visualized by treating TLC plates with iodine vapor. TLC plates were sprayed with EN^{3}HANCE (Dupont) and exposed to Kodak XAR-5 film at -70^{0}C for 2-3 days. Relative levels of PLD activity was then determined by measuring the intensity of the corresponding PBt band in the autoradiogram using a Molecular Dynamics scanning densitometer and ImageQuant software.

DNA Synthesis

Confluent cells were placed in 0.5% serum for 24 h in 24 well tissue culture dishes. DNA synthesis was measured by a one hour pulse with [^{3}H]-thymidine (1 μCi/ml; 20 Ci/mmole). After the one hour pulse, the cells were collected and trichloroacetic acid precipitatable counts were determined by scintillation counting.

Preparation of cell lysates

Medium was removed and 60mm plates were washed with 1x PBS buffer. 0.5 ml of lysis buffer was added to the cell culture plate. The samples are placed on a rocker at 4°C and shaked for 30 mins. The lysates are scraped and the samples are placed into 1.5 ml Eppendorf tubes. The samples are spun at 10,000xg for 20 mins. The supernantant was collected. This is the whole cell lysate.

CHAPTER 5

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